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Immobilization of Cells

With 24 Figures

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1 Introduction

That living microorganisms can attach to each other and to solid surfaces in the form of films is well known [1]. Such attachment occurs in dental plaque, algal and fungal slimes, bacterial film on soil particles [2], on plant or animal tissues such as intestine and rumen [3] and in chicken crop epithelia [4]. The same effect is also observed in the tendency of many bacteria to assume chain structure. Cell adherence, a phenomenon of great importance in monolayer cell culture because it allows easy manipulation of growing cells, has recently been reviewed [5, 6].

Bacterial films, a consequence of bacterial growth and adherence, are so ubiquitous in nature that it may be safe to conclude that wherever there is a solid substrate there is likely to be a bacterial film involved. Examples of early utilization of bacterial films include the production of vinegar by the trickling method, the history of which is reviewed by CONNER and ALLGEIER [7], and the leaching of mineral ores by sulphur oxidizing bacteria [8].

From as early as 1670, vinegar making processes were known in which packings of various plant materials such as grape vine, rape, corncobs, wood, as well as inorganic materials such as pumice, ceramics and charcoal, were used as support for the slime film of the *Acetobacter* microorganisms. Substrate, air, and nutrient solutions were trickled through layers of those packings and converted to the product. This process is inherently adaptable to continuous flow fermentation, and packings of beech wood shavings have been in continuous use in some plants for 50 years [7].

In the field of hydrometallurgy, heap leaching of copper ores [8] was practised in Spain in 1725. There is a Chinese record [9] in the twelfth century of extracting copper from copper sulphate dissolved in natural waters, probably as a result of subterranean leaching, although there was no conscious effort to utilize bacterial action in leaching, nor recognition of its presence. The original dissolution is due to the action of various strains of sulphur oxidizing bacteria, such as those in the genus *Thiobacillus*, on sulphide minerals to produce soluble sulphates. Members in this genus are diverse, including aerobic, anaerobic and faculative anaerobic bacteria growing under different conditions in solution as well as on solid surfaces. On the other hand, sulphate reducing bacteria are generally considered to be responsible for the formation and deposition of sulphide ores in nature.

Processes in which localized bacterial films are active include the aging of meat to improve its texture and flavour, the production of Rochefort cheese and similar solid fermented food, composting, the treatment of waste, whether intentional or not, by trickling it over bacterial slime, the fixation of nitrogen by bacteria attached to roots of leguminous plants, and decomposition and rotting in nature.

Although bacterial films occur often in nature and have found limited application in vinegar production, ore leaching and the preparation of some fermented food, they were not widely utilized in industrial fermentation reactions until the 1970's, when the demand for viable alternatives to enzyme fermentation became increasingly urgent. The theoretical and morphological basis for cell attachment behaviour, however, has commanded much more interest, more with respect to fundamental understanding than to application. Current theories of cell adhesion have been extensively reviewed [5, 6].

The technique of cell immobilization is an outgrowth of enzyme fermentation. Because of their specific catalytic activity and their high performance under mild physiological conditions, enzymes have become increasingly important in fermentation. Enzymes can be used in the free form or in an insoluble form in aqueous systems. In these two forms, enzymes can also be bound to a solid carrier which, in many instances, results in increased operational stability, enhanced activity, and the possibility of a continuous process in which better control of substrates and product flow can be maintained. Nevertheless, enzyme processes suffer from certain disadvantages, the most serious of which are the cost of pure enzymes, the difficulty of recycling them by extraction, and problems of product contamination by leaking, in the case of some immobilized enzymes. To circumvent these disadvantages, the more readily available microbial enzymes, together with the cells containing them, are bound to carriers by various methods, often with remarkable improvements in enzyme activity and half-life.

Although the presence of various reagents and the procedures of drying, freezing, crosslinking, etc. inherent in immobilization may kill the cells, their enzyme activity may not be impaired. In fact, in fermentations requiring single intracellular enzymes, immobilization processes usually increase the permeability of the cell walls, and allow nutrients, substrates and products to diffuse more readily through the cell membranes and thus facilitate the fermentation. The dead cells in this sense act also as a carrier. More complex immobilizations may involve living cells containing multiple enzymes and subcellular components having particular functions in a fermentation reaction.

Early Work on Enzyme Immobilization

The history of cell immobilization is closely associated with that of enzymes [10–12]. Empirical studies of enzyme immobilization, mainly by adsorption onto inert solids, follow a pattern of experimental work in the early 1900's leading to the formulation of the Michaelis-Menten hypothesis of enzyme catalysis (Table 1.1). Thus as early as 1908, MICHAELIS and EHRENREICH studied the adsorption of a range of enzymes on various solid adsorbents as a function of pH, and found that adsorption is pH dependent [13]. Adsorption was found to be irreversible in the case of peptone on animal charcoal [14] and also dependent on the ionic character of the adsorbent [15]. From these early observations followed a series of studies on the electrophoresis of enzymes [16–18]. The dependence of adsorption on the concentration of the enzyme in solution was studied by JACOBY in 1916 [19]. The 1920's heralded a period of application of the principles of enzyme adsorption, mainly in the area of enzyme purification. The status of enzyme purification by adsorption techniques was reviewed by FABRE in 1923 [20].

Table 1.1. Early examples in adsorption of biological materials enzymes and polypeptides

Materials adsorbed	Carrier	Description	Ref.
Diastase, invertase, pepsin, trypsin, rennin	Kaolin, talc, animal charcoal, Al_2O_3, Fe_2O_3	Extent of adsorption	[13]
Peptone, albumose	Animal charcoal	Reversibility of adsorption	[14]
Zymose		Adsorption, affinity	[15]
Invertin		Electrophoresis	[16]
Pepsin		Electrophoresis	[17]
Malt diastase	Kaolin	Electrophoresis	[18]
Pepsin	Al_2O_3		[63]
Albumin-peptone	Al_2O_3		[64]
Invertase	Animal charcoal, aluminum hydroxide		[65]
Urease	Fibrin flakes	Activity and contraction of adsorbed enzyme	[19]
Proteins, enzymes, toxins, and sera	Aluminum hydroxide	Purification studies	[21]
Sucrase, amylase	Aluminum hydroxide		[22]
Sucrase	Kaolin, aluminum hydroxide	Absorption affinity	[23]
Amylase	Aluminum hydroxide	Function of ethanol as co-absorbents	[24]
Sucrase	Aluminum hydroxide	Adsorption isotherms	[66]
Invertin		Purification by adsorption	[67]
Pepsin	Animal charcoal	Selective elution	[25]
Diastase	Animal charcoal	Temperature effects, irreversibility	[26]
Invertin	Kaolin, aluminum hydroxide		[23]
Amylase	Alumina gel	Enzyme purification	[29]
Papain	Aluminum hydroxide	Procedures of purification	[30]
Serum enzyme	Various adsorbents	Deactivating power of adsorbents	[31]
Pepsin, rennin, catalase, peroxidase	Cellulose	Strength of adsorption	[33]

Aluminum hydroxide was generally selected as the adsorbent in the purification of various biological substances such as proteins, toxins, enzymes, and antitoxins [21]. Adsorption studies of various enzymes on $Al(OH)_3$ and animal bone charcoal were undertaken to examine the physical and chemical behaviour of adsorption, for example, the adsorption isotherm [22, 23], effects of pH, solvent and inhibitors [24] and the reversibility of adsorption [25, 26]. Such fundamental studies provide the basis for the use of adsorbents in the purification of enzymes, still an important aspect of enzyme technology.

Early attempts at enzyme purification are reviewed by WILLSTÄTTER [27] in 1926 and later in 1932 [28]. The main subjects of investigations from the 1920's to the 1950's were adsorption efficiency as a function of pH [29], inactivation by adsorbents [30, 31], inhibition and activation [32], and selectivity of adsorption [33]. These topics were reviewed by ZITTLE [34] in 1953.

Table 1.2. Early examples in adsorption of biological materials

Materials adsorbed	Carrier	Description	Ref.
Bacteria	Animal and vegetable charcoal, Fuller's earth	Differential staining	[44]
Bacteria and Agglutinins	Animal charcoal, barium sulphate, aluminum hydroxide	Bacteria agglutination	[45]
Bacteria	Soil	Adsorption capacity	[52]
Bacteria	Soil		[51]
Bacteria		Adsorption of iodine on bacteria	[46]
Bacteria		Behaviour of gram-positive and gram-negative bacteria on iodine adsorption	[47]
Bacteria		Disinfection by iodine	[48]
Bacillus caryocyaneus		Adsorption of Bordeaux B	[50]
Bacillus caryocyaneus		Phenolized gentian violet staining	[68]
Bacteria, moulds, yeasts	Industrial clay	Identification of micro-organisms	[69]
Azotobacter chroöcoccum	Loams	Influence of adsorbed ionic species	[70]
Various bacteria	Kaolin, $CaCO_3$, $Al(OH)_3$, $BaSO_4$	Selective adsorption	[55]
Azotobacter	Dispersed sand	Study of parameters in adsorption	[56]
Bacteria	Pectin, white clay, active charcoal, lignin	Bacterial growth	[59]
Bacteria	Filter paper	Study of antibiotic sensitivity	[60]
Bacteria	Soil	pH dependence of adsorption	[71]
Bacteria	Soil	Desorption of bacteria from soil	[72]

The period from the 1930's to the 1960's is characterized by intensive work on particular applications, methods of immobilization, and the use of synthetic and natural polymers (Table 1.2). In the 1930's, WHITE in the US and GAUTHERET in France developed completely synthetic media for plant cells in tissue culture. Industrial and research applications of enzymes immobilized on polymer supports were extensively investigated, notably in the field of immunology. MANECKE developed a number of synthetic resins as specific adsorbents for serum proteins [35–37].

By 1953, some enzymes were successfully immobilized on polymer solids, and patents were granted for a number of techniques developed by KATCHALSKY throughout the 60's [38–40]. At this time, several chemical reagents were developed for enzyme immobilization, in addition to the organic and inorganic solid

adsorbents. The new immobilization reagents formed covalent bonds between enzymes and the solid support, and include diazotized polyaminostyrene, resin containing carboxylic acid chloride functional groups and diazotized aminobenzylcellulose. A review of these chemical immobilization methods has been made by BRANDENBERGER [41].

In 1957, BRANDENBERGER [42] reviewed all the current techniques, five of which he developed, for binding physiologically active proteins and enzymes onto solid supports. The amount of carbon dioxide liberated from the hydrolysis of the unreacted isocyanate groups after the immobilization of a protein on a polyisocyanate carrier was used as a criterion for covalent bonding in immobilization.

In the 1960's increasingly frequent application of enzymes was made to fermentation. Since that time, research work on immobilization has multiplied enormously, much of it with an industrial focus. The two processes, enzyme immobilization and cell immobilization, have a common mode of action, that is, heterogeneous enzyme catalysis; it should be noted that lysis of microbial cells after their immobilization was one of the early methods of immobilizing enzymes. However, numerous new procedures have been developed for immobilization of cells so that it now stands by itself as a separate process, often complementary to that of enzyme immobilization. The first successful industrial production using an immobilized enzyme was based on aminoacyclase, and was developed by CHIBATA et al. [43] in 1969. Racemic mixtures of amino acids were optically resolved through selective enzymatic action to yield the D- and L-optical isomers. Since then several other industrial scale production fermentation processes using immobilized biological substances have been developed.

Early Work on Cell Immobilization

Study of the attachment behaviour of microorganisms has always lagged behind that of enzymes. The lag might be attributed to the greater inherent complexity of microbial cells relative to enzymes. As discrete chemical compounds, enzymes are easier to purify, characterize and assay. Initially, the only method of attachment of microbial cells to a solid was by adsorption, which remains the most direct among the modern techniques, although it is not necessarily the most effective or easiest.

Early study of bacterial adsorption was limited to choice of a solid support and suitable conditions, study in soil systems, study of activity of the adsorbed microbes, and later, after development of the gram positive and gram negative classifications of microorganisms, study of the selectivity of adsorption of bacterial cell surfaces. The adsorbing powders of animal and vegetable charcoal and Fuller's earth were found to be effective in 1918 [44]. The dependence of adsorption on particle size and the "surface development" of the solid support was investigated using basic methylene blue and acid dyestuffs. BLEYER [45] in 1922 studied the adsorption behaviour of the agglutinins on suspensions and colloids and found animal charcoal and fine precipitates such as $BaSO_4$ to be the most effective. The dispersed agglutinin was shown to agglutinate homologous bacteria preferentially over absorbents in colloidal state, even though the adsorption of agglutinin on the colloid was found to be stable with heating and in 0.01 N NaOH.

Later in 1932, a series of investigations was conducted on the adsorption of iodine [46] on gram positive and gram negative bacteria [47] in relation to the action of disinfection [48]. DIETZEL et al. surveyed the then-current theoretical and practical application of the bactericidal properties of various absorbent preparations [49]. The effect of pH on the adsorption of the dye Bordeaux B on the cells of *Bacillus caryocyaneces* was investigated by LASSEUR and DUPAIX-LASSEUR [50].

Adsorption studies of bacteria on soils were undertaken by KHUDIAKOV [51] to determine the flocculating behaviour of the bacteria, the activity of the adsorbed cells and the capacity [52]. In 1936, GLICK [53] found that of all the microorganisms found on samples of industrial clay only the aerobic or facultative bacteria showed an increase in viability after 3 months storage at the optimum temperature of 30 °C. PEELE [54] used CO_2 evolution as a measure of activity of bacteria adsorbed on soils and postulated that adsorption was due to electrical attraction. Other inorganic solid adsorbents investigated include kaolin, $CaCO_3$, $Al(OH)_3$ and $BaSO_4$ [55]. In 1950, the physical parameters for the adsorption of *Azotobacter* on sand were found to include the electrokinetic potential which is a function of pH and which could be used as a criterion for reversible or irreversible adsorption (adhesion) [56]. This work is the forerunner of the study of bacterial adhesion by the zeta potential, a topic extensively reviewed by GERSON and ZAJIC [6], LIPS and JESSUP [57], and ROGERS [58].

Organic adsorbents used in this period include pectin [59] and filter paper impregnated with methyl cellulose [60].

Evaluation of Whole Cell Immobilization as a Process

Evaluation of cell immobilization as a process relative to alternative processes is highly complex, and must take into account a great number of factors, the foremost of which are economic factors, environmental factors, and, in the case of production of food stuff, taste and aesthetics. Economic factors include cost of starting materials, energy and equipment, and required technical skill. Thus comparison of the advantages and disadvantages of these processes can only be made in a very general way, with the assumption that all other factors are constant.

Relative to conventional chemical processes, the most basic difference has to do with the nature of the process, that is, chemical synthesis vs. biological (enzyme) fermentation. In general, enzymatic reactions are much more efficient and specific as to identity and stereochemistry of products and conditions of reaction. Organic synthesis reactions on the other hand, although more various in nature, often require harsh conditions in temperature and pressure, use of organic solvents, extremes of pH and less selective but very reactive reagents. Transformations in organic reactions are particularly sensitive to steric crowding which can effectively preclude the possibility of reaction, no matter how much driving force is used.

Enzyme catalysis has the additional advantages of adaptability to low grade substrates such as recycled waste material or surplus material from other industries, and the availability of a number of metabolic pathways which may lead to different products by careful control of conditions and substrates. Biological catalysis reactions generally cause less pollution problems. In contrast to these advantages, biological catalysis reactions suffer from several disadvantages, the

most serious of which are the instability of enzymes and microbial cells and the need to carefully maintain external conditions and to supply the requisite cofactors, all of which add to the expense of the process. Enzymes in the purified form are usually expensive. Further, because of their high reactivity, biological catalysis by microbial cells often leads to byproducts which add to the problem of purification and extraction. In the free form, the enzyme is difficult to recycle. Neither enzymes nor cells are strong mechanically and their mechanical properties often impose constraints on the design of fermentation equipment. Other constraints include restrictions in the form of physical conditions such as pH, temperature, pressure, the nature of the solvent system, and the presence of inhibitors.

Among biocatalyzed processes, the comparison between whole cells (or organelles) and enzymes (or coenzymes), in their free or immobilized forms, is complex. The generally recognized situation is that immobilized enzyme processes are outgrowths of free cell fermentations and immobilized cell processes are outgrowths of immobilized enzyme processes. Thus immobilized systems are looked upon as alternatives to the established free cell fermentations only when they offer distinct advantages over existing processes. Valid comparisons may be possible between processes involving immobilized cells and free cells, and between processes involving immobilized cells and immobilized enzymes.

Immobilized Cells versus Free Cells

If the comparison is limited to preparative fermentation processes, as distinct from such processes as waste disposal and mineral leaching, where the cost of the pure enzyme or the microorganisms is often of secondary importance, the advantages of immobilized cells are mainly with respect to general productivity and operational flexibility.

Firstly, the productivity of immobilized cells is generally as high as, if not higher than the corresponding free cell fermentations. This productivity can be explained by the fact that the microenvironments offered by the carrier are more stabilizing to the organism or its enzymes, which generally show optimal activity only under very narrowly prescribed physical conditions. In by far the great majority of the reported examples of cell immobilization, regardless of whether dead or viable cells are involved, enzyme activity is enhanced, as indicated by a longer half life. For living cells, the rate of growth, indicated by the generation time, is also increased by immobilization. Consequently both the operational stability of the immobilized organisms and the productivity are improved. Thus, taken as a whole, enzymes in immobilized cells, whether viable or not, retain their activity better than in free cells.

Another advantage of immobilized cell systems is that they can be described readily by well developed theoretical and hydrodynamical treatments of heterogeneous catalysis systems, particularly if the system utilizes solid carriers in the form of uniform spherical particles.

With respect to operational flexibility, comparison is not as straightforward. In general, immobilized cells make possible continuous fermentations which do not work very well with free cells. Because of the possibility of higher cell loadings, reaction rates may be higher. Cell immobilization also enables higher dilution without culture washout, and greatly facilitates recycling or reuse of micro-

organisms. A greater degree of control is possible, mainly in the areas of selectivity in maintaining the stage of cell activity at maximum productivity, in reactivation or regeneration of enzyme activity, in selective isolation of inhibitory reaction intermediates or products, in enhancement of productivity by co-immobilization of metabolically complementary microorganisms involving the transfer of a wide range of substances between cells, in product separation and, finally, in waste disposal. Nevertheless, cell immobilization systems do suffer from a number of problems. The initial expense for such a system is usually high and the process usually requires a large reactor. The mechanical properties of the system – microbial cell bound to solid carrier – are more complex than those of free cells and have to be taken into account in order to provide a continuous, recycling process and effective agitation and filtration. System designs must allow for the increased diffusion barrier through the cell and the carrier for substrates, products, and cofactors, so that cells bound to a carrier generally need permeabilizing treatment, especially when high molecular weight substrates or products are encountered.

Reagents such as monomers, crosslinking reagents, and radical initiators used in the polymerization of synthetic organic carriers are often cytotoxic and the organic solvents used in these reactions can lyse microorganisms and denature the enzymes contained in them.

Immobilized Cells versus Immobilized Enzymes

With respect to preparative fermentations, the important determining factors in comparing immobilized cell processes with immobilized enzyme processes are the cost and the nature of the reaction process. It is generally recognized that the use of an enzyme is more costly in terms of initial cost, extraction, purification, recycling, reactivation, decontamination and final disposal. In many processes, these costs will determine the choice between the two alternatives. The nature of the reaction process is important in terms of whether the reaction is single enzyme (intracellular) catalyzed or multiple enzyme catalyzed, involving the whole metabolic system of the cell, and requiring many sequential enzymes, coenzymes and cofactors. Multiple enzyme systems immobilized on a carrier are generally used for investigative studies rather than for production. On the other hand, whole cells that produce a required metabolite have been used co-immobilized with enzymes, although mostly in experimental studies. In general, a natural cellular environment contributes to the stability and activity of the enzyme and the cell membrane offers some protection against detachment of the enzyme. Enzymes so contained can assume their natural structural conformation, both with respect to the carrier and to the substrate. With whole cells there is the further advantage of controlling the growth stage of the cells to maximize product conversion. Balanced against these advantages, immobilized whole cells have the disadvantage of an increased diffusion barrier, especially for high molecular weight substrates and products. Because of the more complex biochemical make-up of the whole cells, side reactions are more likely, and sterile conditions may be required. Some methods of immobilization, for example, covalent attachment, or crosslinking, invariably kill the cells and thus preclude their use in multienzyme fermentations. At present, in industrial production, the two immobilization systems – microbial

whole cells and single enzymes – are at about the same stage of development [61, 62].

Scope of Present Book

The literature on immobilized whole cells has burgeoned since the early 1970's. In addition to a great number of review articles, several books have been written. In the present work, the literature on immobilized cells, organelles and other subcellular materials is reviewed to provide a survey complementary to presently available publications. Topics such as affinity chromatography, dialysis culture, cell culture, and immunology, are not covered because of the limitations of the present work and the availability of extensive existing literature.

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2 Methods of Cell Immobilization

Methods of cell immobilization roughly parallel those of enzyme immobilization and can best be classified by the nature of the mode of attachment, that is, as mechanical, chemical or ionic. In mechanical immobilization, the cells are localized by means of physical barriers. In chemical immobilization, covalent bonds are formed among cells or to a solid phase. In ionic immobilization, electrostatic, van der Waal's or London forces of attraction are present. Cells can also attach themselves to solid supports in the course of natural growth, using a combination of these means. This classification is obviously not clear-cut but does serve the purpose of organizing the diverse methods of immobilization available. In Table 2.1, examples of cell immobilization are classified by mode of attachment.

2.1 Mechanical Immobilization

2.1.1 Mycelial Pellet and Mat

The mycelium of a fungus consists of tubular filaments (hyphae) on the top of which are the spores. Typically the hyphae of a fungus are about 50 microns in length and under active growth will branch out, interweave and fuse with one another to form a net. Occasionally such mycelial mats cause problems in fermentations because overcrowding of mycelial growth will cause inactivation in a fermentation using filamentous cells, such as *Rhizopus nigricans* [1] in the hydroxylation of the steroid progesterone, probably due to reduction of the surface area of contact between the medium and the fungus so that the nutrients and oxygen become less accessible.

The growth and citric acid production of *Saccharomycopsis lipolytica* in a trickle flow fermentor containing wood chips as the solid carrier have been studied [2, 3]. Kinetic data indicate that acid production, which starts after a linear growth phase, follows a constant specific rate for 80 h. Studies with simple models confirm that limitations in oxygen diffusion as well as metabolic modification in the immobilized cell are responsible for the 30% reduction in growth and citric acid production compared with free cell fermentations. However, in at least one instance, namely, the production of citric acid by the organism *Aspergillus niger*, where the supply of oxygen has to be controlled, the formation of mycelial pellets actually ensures that a limited supply of oxygen is maintained for optimum citric acid production [4]. Cells of *Streptomyces* containing glucose isomerase have been heat-treated, mixed with an 8% citrate solution at pH 6.0 for 1.5 h, then air-dried.

Table 2.1. Examples of cell immobilizations

(I) Mechanical Attachment

(a) on mycelium mass

Cell	Microbial enzyme	Solid support	Substrate	End Result	Ref.
Saccharomycopsis lipolytica		wood chips	glucose		3
Morterella vinacea	α-galactosidase		p-nitrophenyl(α-D-galactopyranoside)(PNPG)	hydrolysis	7
Absidia griseola var izuchii				α-galactosidase	8
Streptomyces	glucose isomerase	heat fixing	glucose		157
Rhizopus nigricans	hydroxylase	agar alginate gels	progesterone	11 α-hydroxylation	1
Aspergillus ochraceus		mycelium pellets	N-acetyl-DL-methione	ℓ-methionite	9
Escherichia coli, Salmonella typhimurium, Bacillus sp.			18-crown-6		11
Escherichia coli.		filamentous cells	18-crown-6		12
Saccharomycopsis lipolytica		wood chips	glucose	citric acid	2