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Immobilized Cells [and Discussion]

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Immobilized cells

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Cells of microbes and of higher organisms may be immobilized by a number of methods for a variety of purposes. Although the study of cell immobilization is comparatively novel, the methods that have been developed are very effective and there are few indications that further, greatly superior techniques are likely to evolve. Cells are best immobilized by aggregation, by adsorption onto a support material or by entrapment within gels, of which the natural polysaccharides κ-carrageenan and calcium alginate have proved the most useful.

Aggregation of cells usually involves heat treatment or chemical cross-linkage and causes total loss of cell viability. It is most suitable for the immobilization of cells so as to contain a single enzymic activity: many of the commercial preparations of glucose isomerase consist of aggregated cells. Adsorption of cells to surfaces is a gentle and simple technique: efficient immobilization is aided by the correct choice of pore size in the support material. Cell viabilities and activities are retained but adsorbed cells may be removed from supports fairly readily. Entrapment within gels allows the retention of cell viability and activity and by supplying full growth media, cells can be made to multiply within the beads of gel, giving very high cell densities. Polyacrylamide has been used satisfactorily for cell entrapment but has been superseded in industrial processes by κ-carrageenan or calcium alginate. Industrial processes known to employ immobilized cells include the production of L-malic acid and L-aspartic acid and various steroid conversions.

Very many other processes using immobilized cells have been studied at the academic level. Immobilization in polysaccharide gels, in particular, sometimes gives unexpected properties of longevity or activity to cells. These phenomena are largely unexplained but may be due to the gel material's influencing the chemical composition of the immediate environment of the cells.

The use of immobilized cells has not yet made a large impact on the fermentation industries but these are early days in the development of new technology and it is to be expected that processes involving immobilized cells will find wider industrial use in the near future.

Introduction: why immobilize cells?

Immobilization implies the prevention of free movement of a material of one phase within another. By this criterion individual cells of most higher organisms are immobilized among their fellows and many microorganisms normally exist immobilized as films on the surfaces of solid materials and other, larger organisms, entrapped within slimes or gels of their own making. Thus the deliberate immobilization of cells by man for his own convenience does not necessarily place them in an environment foreign and strange to them.

Surprisingly, cell immobilization is a technique encountered much less frequently than enzyme immobilization, the heyday of research into techniques of cell immobilization being in the late 1970s, a decade later than the immobilization of single enzymes. However, cell immobilization is a very simple, cheap technique, easier to do than most enzyme immobilization processes, yet capable of producing materials suitable for use in large-scale industrial reactors.

Cells are immobilized for a variety of reasons of varying complexity:

(*a*) to present a single enzyme produced by and contained within the cells in a convenient form for commercial use;

(*b*) to make available for use a sequence of enzymes produced by the cell;

(*c*) to provide a highly concentrated source of viable cells for use as they are for the biotransformation of substrates or simply as a large inoculum for conventional fermentation;

(*d*) to bring together biochemical systems that would not normally coexist;

(*e*) to allow the medium-term storage of cells for commercial use without further growth;

(*f*) to preserve cell lines stored only with difficulty by conventional means.

Criteria for success in cell immobilization

Many methods are available for the immobilization of cells. Their development has been intended presumably for commercial use. To find industrial use an immobilization method must meet various criteria.

1. It must be safe. The process must not harm operators of the plant nor consumers of the products. The huge costs of determining the safety of a product means that novel chemicals will be avoided unless there is no alternative. Materials that are already accepted for use in foodstuffs or in food processing will be preferred even if the product is not intended to be a food ingredient.

2. It must be simple. Eventually a process must be operated by relatively unskilled personnel and it must be assumed that anything that can go wrong will go wrong. The logistics of its use must also be simple. Expensive support materials that have to be returned to the manufacturers for regeneration will be avoided unless they are greatly superior in performance to cheaper materials that can be discarded after a single use.

3. It must be gentle. Except where it is intended that only a single enzymic activity be used, cell viability or at least integrity of membranes should be retained. Therefore extremes of heat and pH and organic solvents will be avoided.

4. It must be long-lived. This is true in two senses, durability in use and in cell activity. An immobilized cell preparation must be resistant to abrasion yet not brittle, it must not compress excessively when used in large reactors, it must not be liable to microbial degradation and in some circumstances it must allow the release of gas bubbles without disintegrating. The cell activity must be maintained as long as possible: running an immobilized-cell reactor is inexpensive, but growing the cells, immobilizing them and filling and emptying the reactor is expensive, particularly in labour costs.

5. It must have a high activity. After immobilization, cells are unlikely to have increased activities: it is important that the activity ex-fermenter is retained, i.e. that the 'effectiveness factor', defined as (activity after immobilization)/(activity before immobilization), should be as high as possible. Thus there should be minimal diffusion restriction on the access of solutes to the cells. The binding of cells should be tight enough to prevent them leaking into the product stream. Durability is a more significant factor in determining the cost of a process, so limited diffusion restrictions will be tolerated if they are a consequence of increasing the half-life of the immobilized cell complex enough to increase its overall productivity.

6. It must be cheap. All of the previous criteria will, if satisfied, lead to a cheap process, but immobilized cells will only be used if they have advantages over alternative processes such as

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conventional batch or continuous fermentation, the use of isolated enzymes, whether soluble or immobilized, chemical processes, or agriculture or horticulture. Table 1 summarizes the advantages and disadvantages of immobilized cell processes.

Means of immobilizing cells

The various means by which cells may be immobilized have been reviewed thoroughly by Cheetham (1980). Plainly not all the methods available are suitable for all the uses mentioned above. Where a single enzyme activity is required, comparatively brutal techniques may be employed, if the enzyme will withstand them.

Table 1. Comparison of immobilized cell processes with alternatives

	immobilized cell process	
alternative process	advantages	disadvantages
batch fermentation	continuous process; longer life of cell activity; reduced fermenter volume; may be cleaner, i.e. fewer medium components	greater inconvenience if microbial contamination occurs
continuous fermentation	fewer cells produced, cleaner stream; smaller 'reactor' required	greater inconvenience if microbial contamination occurs
enzyme process	probably cheaper; the enzyme system required may be unstable when isolated	other cell contents may contaminate stream; larger reactor
chemical process	usually reaction is much more specific, there are fewer by-products and higher yields; lower temperatures and pressures	done in dilute, usually aqueous, solution: high recovery costs
agricultural or horticultural process	product available at all times, process indoors; releases land for other purposes	—

1. *Aggregated cells*

Bacterial cells containing glucose isomerase have been immobilized simply by heating the flocculated mass of cells harvested from the fermenter (Bungard *et al.* 1979) or by cross-linking with bifunctional reagents such as glutaraldehyde (Hemmingsen 1979). Fungal mycelium, not strictly cells, may simply be dried and used as immobilized enzyme, for example, α-galactosidase as used as an aid in refining beet sugar (see, for example, Narita *et al.* 1976). The ability of various fungi to grow as mycelial pellets is of advantage in producing commercially useful immobilized cell–enzyme preparations (see, for example, Kobayashi & Suzuki 1976).

An elegant method of aggregating cells is that of Kennedy *et al.* (1976) in which the cells are treated with Ti^{4+} and Zr^{4+} chlorides, which produce a polymeric metal hydroxide precipitate including the cells. The authors presumed that there was some covalent coupling of the cells to the inorganic material, which may differentiate this method from entrapment methods described below.

Cell aggregation is a cheap process and easily done. A drawback is that some enzymes are

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labile to cross-linking agents such as glutaraldehyde and that the cells may require lysis to allow access of substrate to the enzyme. The latter point is true of all immobilized cell processes.

2. *Adsorption*

Adsorption of cells to surfaces is a mild, non-specific process involving the binding of the cell surface to support materials by ionic or less powerful bonds. Ion-exchange materials such as Dowex-1 (Hattori & Fursaka 1961) and DEAE-cellulose (Fujii *et al.* 1973) have been used

TABLE 2. EXTENT OF ADSORPTION OF *SACCHAROMYCES CEREVISIAE* CELLS TO VARIOUS SUPPORT MATERIALS (DAUGULIS *ET AL.* 1981)

support material	cell dry mass/support dry mass / mg g^{-1}
gel-type ion-exchange resins	
strongly basic, anionic (IRA 400)	1
strongly basic, anionic (IRA 458)	3.0
weakly basic, anionic (IRA 68)	10.9
macroreticular-type ion-exchange resins	
strongly basic, anionic (IRA 900)	1
strongly basic, anionic (IRA 904)	4.0
strongly basic, anionic (IRA 938)	46.8
strongly basic, anionic (IRA 958)	5.3
strongly basic, anionic (XE 352)	128.0
activated carbon	1
ceramic chips	1

successfully. Daugulis *et al.* (1981) compared a wide range of ion-exchange resins as immobilization supports for *Saccharomyces cerevisiae* cells producing ethanol (table 2). Clearly the chemical nature of the resin rather than simply its charge is important in allowing efficient cell adsorption.

There are a great many examples of organisms growing naturally on surfaces, to both the advantage and the detriment of the human condition. A prominent example of the latter is dental plaque formation, the necessary precursor of dental caries. Vinegar production relies on bacteria immobilized on suitable surfaces, many sewage-treatment processes similarly involve microorganisms growing on solid surfaces. Considerable thought and ingenuity has gone into the selection and design of materials with surfaces excellent for the adsorption of cells. This subject has been reviewed recently by Kolot (1981). Inorganic materials employed range from brick, straw and sand to carefully designed glasses with controlled pore sizes. Excellent information on the latter subject is presented in a patent by Messing & Oppermann (1979) assigned to Corning Glass Works. Their studies were based on the theory that the optimum pore diameter in an inorganic support material for cell growth by fission is five times the largest dimension of the cell, i.e. it was assumed that cells would double in size before fission, pores would house two cells that would divide simultaneously and a 'gap' of one cell diameter would be required for daughter cells to escape from the pore. For yeasts, which reproduce by budding and increase in size rather less than bacteria before division, the minimum useful pore diameter would be four times the maximum cell dimension. These theories were confirmed in practice by using Corning's materials with controlled pore sizes.

Where secondary fungal products were required it was considered that a pore diameter of

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16 times that of the spore would be optimal to allow the growth of useful amounts of mycelium within the pore.

An alternative approach to the design of a support for cell immobilization was taken by Hollo *et al.* (1979), using plastic materials. Two types were chosen, polyvinyl chloride (PVC) fibres and polypropylene (PP) webs. Softeners in the PVC films were used as a carbon source for microbial growth, the microbes burrowing their way into the plastic. The PP webs were composed of woven fine filaments and therefore had a very large surface area. Their efficiency for bacterial attachment was greatly improved by treating the PP surface with O_2 plasma to allow it to be wetted by water. Cells (of *Pseudomonas aeruginosa*) were immobilized simply by immersing the plastics in a growing culture for 72 h. Biodegradable plastics prepared by using starch as a filler, several of which are described in outline by Doane (1981), appear to be excellent, if perhaps short-lived, materials for cell immobilization.

A combination of organic and inorganic materials (PVC–silica) was described by Goldberg *et al.* (1979). Here microporous silica was embedded in PVC and formed into sheets. Substrate solution was made to pass through the tortuous pores of the silica rather than simply across the surface. The authors described work with immobilized enzymes only, but this approach appears to be very suitable for cell immobilization.

One of the most effective means of growing animal cells in culture is as monolayers on suitable surfaces, which must have positive charges for efficacy. Van Wezel (1982) describes the various materials that have been employed. Probably the most widely used animal cell support is the microcarrier Cytodex, which consists of very small beads of positively charged agarose. These may be used at 5000–25000 cm^{-3} (Van Wezel 1982) in suspension, so that immobilized cells may be cultivated with all the advantages of homogeneous culture.

Volumes have been written on the basic science of cell adhesion to solid surfaces and the subject has been reviewed recently by Gerson & Zajic (1979). Whatever the nature of the cell–support binding, adsorption, is a temporary phenomenon and the cells may be released by changes of pH, ionic strength or even by fluid flow. Thus adsorption is not a suitable cell immobilization method if cell-free product streams are required, particularly where cells are allowed to multiply. Cell adsorption is particularly useful where what is required is a large inoculum in circumstances where cells might be washed out of a conventional continuous culture, for example during a temporary fall in nutrient concentration. Such a use of immobilized cells has become known as 'semi-immobilization' (see, for example, Takahashi *et al.* 1981).

A recent advance of some elegance, but currently applicable only to very high-value processes, is biospecific adsorption. Natural materials, lectins or antibodies may be bound to suitable supports and then used to make very specific tight bonds with cell surfaces. This approach was employed by Horisberger (1976), who bound Concanavalin A to magnetite and used the magnetic immobilized lectin to remove yeast cells from suspension.

3. *Covalent coupling*

Compared with adsorption, covalent coupling of cells (to surfaces) is a brutal technique, often similar to the aggregation techniques described above. Cell viability is usually lost, which is of no consequence where single enzyme conversions are required and where that enzyme is intracellular and not contacted by the cross-linking agent. Jack & Zajic (1977) activated carboxymethyl cellulose by using carbodiimide and covalently linked to this cells of *Micrococcus luteus*. These retained 75% of their histidine ammonia lyase activity and were able to convert histidine

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to urocanic acid over an extended period. However, the dry mass of cells bound per unit mass of support by this method may be significantly less than that bound (to other supports) by adsorption and entrapment. Navarro & Durand (1977) activated glass beads by using glutaraldehyde and bound *Saccharomyces carlsbergensis* to these. Some adsorption into pores also occurred.

A patent (Compere & Griffith 1981) describes the immobilization of cells onto supports, such as pellets, spheres and raschig rings of the types used in distillation columns and which may be constructed of metals, porcelain, glass, nylon, polyethylene, polypropylene or polystyrene. The method involves mixing cells with polyelectrolytes such as gelatin, contacting the surface with the mixture and cross-linking with a suitable bifunctional agent, glutaraldehyde being preferred. A mixture of organisms from a kefir culture (lactobacilli and yeasts) was immobilized in this manner and used to produce lactic acid from cottage cheese whey. This is one of the few examples of the deliberate coimmobilization of more than one organism.

4. *Entrapment*

Compere & Griffith's (1981) method involves both covalent coupling and entrapment but is an unnecessarily vigorous technique for entrapment. Entrapment within a gel and absorption to a surface are the cell immobilization techniques that most approximate to circumstances in which cells might find themselves in Nature. Indeed many organisms are adsorbed to surfaces while entrapped within a gel or slime of their own making. Slimes are of little use in industrial processes but entrapment within gels is proving to be probably the most successful means of immobilizing cells.

A very early example of cell immobilization was used to preserve cultures of *Lactobacillus* species. Skimmed milk was inoculated with a pure culture of the organism to be preserved. As the cells grew the pH fell and the casein coagulated, entrapping them within 'junket' sufficiently firmly that cells could be preserved for lengthy periods in culture collections (R. H. Tilbury, personal communication). More practical methods (for the industrial use of immobilized cells) require entrapment with rather more durable materials.

(*a*) *Polyacrylamide*

It is unclear why polyacrylamide was the first gel to be used to entrap living microbial cells: perhaps the production of such gels was familiar to biochemists and microbiologists from its use in the electrophoresis of proteins. The entrapment of cells is readily achieved by stirring cell preparations into solutions of the appropriate monomers and initiating polymerization. The resultant complex is a block of gel, which may be pelleted as required. Gel strengths and pore sizes may be varied simply by altering the concentration of acrylamide monomer and cross-linking agent: the mechanical strength of the gel increases in proportion to the square root of the acrylamide concentration, but the pore size decreases (B.D.H. pamphlet, *Cyanogum*). Thus the chosen properties of a gel must be a compromise between the mechanical strength given by small pore sizes and efficient mass transfer within the gels, which is favoured by large pores. Satisfactory combinations of strength and activity have been achieved but a very significant loss of cell activity may be caused by the denaturing effect of monomers and the free radicals generated during polymerization.

Nevertheless cells entrapped in polyacrylamide have been used in a wide variety of systems since Mosbach & Mosbach's (1966) immobilization of the lichen *Umbilicaria pustulata*. Industrial

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uses of cells immobilized in polyacrylamide will be discussed below. Once immobilized, cells may be used as they are, as sources of single enzymes, e.g. in the production of aspartic acid from acid by using the aspartase of *Escherichia coli* (Tosa *et al.* 1974) or provided with a full nutrient medium that allows them to multiply within, and out of, the gel. In the latter case the inactivation of many cells during immobilization assumes minor importance.

Very recently Rosevear (1982*a*, *b*) has disclosed an imaginative use of polyacrylamide to immobilize whole cells of many different organisms in sheets. A tough but thin paper sheet is coated with a layer of cells in acrylamide monomers, made viscous by the use of xanthan gum, sodium alginate or a similar material, and polymerization initiated. The resulting sheets of immobilized cells may be rolled into a Swiss roll configuration for ready use within a tubular reactor.

A very wide variety of other artificial polymers have been used to entrap cells of many kinds (reviewed in Cheetham 1980), but none of these has obvious advantages over polyacrylamide and will probably be more expensive; they are therefore of little commercial significance.

(*b*) *Polysaccharides*

Entrapment or enclosure within polysaccharide matrices is the natural state for cells of a very wide variety of microorganisms and thus it is to be expected that deliberate immobilization with polysaccharides will be a successful method. This has proved to be so.

The polysaccharide readily to hand in most microbiology laboratories is agar, and this has been used in attempts to entrap cells, for example by Kawabata & Demain (1979). However, agar gels have poor mechanical strength, and the need to contact the cells with fairly hot agar solution is undesirable. The former fault is more significant than the latter.

Japanese workers have used κ-carrageenan extensively for the immobilization of cells and in several cases have abandoned polyacrylamide entrapment for κ-carrageenan in industrial processes (Chibata 1979). This polysaccharide is a copolymer of β-D-galactose sulphate and 3,6-anhydro-α-D-galactose and is isolated from red seaweeds. It is readily available as a food additive. Gels of κ-carrageenan are formed, as with agar, on cooling but, unlike agar, metal ions are required for gelation to occur. The conditions required for gelation and entrapment of cells are very mild: cell suspension and κ-carrageenan solution are warmed to 37–60 °C and mixed, with the addition of potassium or ammonium salts. The resultant gel may be granulated to a suitable particle size for use in columns or other reactors. Chibata (1979) describes the treatment of κ-carrageenan gels with hardening agents, such as glutaraldehyde and hexamethylene diamine to stabilize them for industrial use.

Another algal polysaccharide that has found widespread use in the immobilization of cells of many types is alginic acid. This is a copolymer of D-mannuronic and L-guluronic acids with the detailed structure varying from source to source, even within different parts of the same organism. A wide range of alginic acids of varying compositions and molecular masses are available commercially. The most important property of alginate acid for cell immobilization is its gel strength: gels are formed with divalent cations which cross-link guluronic acid units of different molecules. Thus alginic acids with high guluronic acid contents are to preferred for cell entrapment unless some other gel property than strength, e.g. large pore size, is required, Alginic acids from *Laminaria* species have high guluronic acid contents: those from *Macrocystis* and *Ascophyllum* species are 'high-mannuronic' materials.

To entrap cells within calcium alginate could hardly be a simpler or gentler process (Kierstan

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& Bucke 1977; Cheetham *et al.* 1979). Cell preparations are stirred into solutions of sodium alginate and the mixture extruded into a solution of a calcium salt. Gelation is instant but it is wise to leave gels in the calcium salt solution for at least 20 min to allow complete gelation of the alginate to occur. Protecting materials, such as osmotica, and substrates may be included in the alginate and calcium salt solutions. No heat treatment whatsoever is required, and immobilized cells retain very high effectiveness factors (Kierstan & Bucke 1977; Bucke & Cheetham 1981). A surprising feature of calcium alginate cell complexes is the longevity of biochemical activities: *Saccharomyces cerevisiae* cells retained the ability to produce ethanol for several months when supplied only with glucose or sucrose (Kierstan & Bucke 1977) and the half-life of the enzyme system of *Erwinia rhapontici* cells that converts sucrose to isomaltulose is over 300 days (Bucke & Cheetham 1981).

In Nature, alginate provides mechanical support for brown seaweeds, which are able to withstand the force of ocean storms, and it is not surprising that calcium alginate beads should provide excellent resistance to hydrostatic pressures and abrasion. Cheetham (1979) demonstrated the feasibility of the use of calcium alginate beads on a large scale in packed-bed reactors, where there were only low intrinsic pressure drops. No channelling, abrasion of pellets or compression was observed.

Calcium alginate is not without its drawbacks in use: the calcium may be removed by chelating agents such as phosphates, (including ATP) and EDTA and displaced by cations such as Mg^{2+} and K^{+}. The ready dissolution of the alginate gel is valuable in that it allows the study of cell division within the gels (Kierstan & Bucke 1977). Strontium and barium alginates are more stable to chelating agents and have been used successfully to entrap cells (Paul & Vignais 1980).

Alginate has been used successfully to immobilize cells into more complex composites. Rosevear (1982*a*, *b*) suspended yeast cells in sodium alginate solution and smeared the mixture onto nylon mesh which was then immersed in calcium chloride solution. The resulting film was supported on stainless steel mesh and rolled into a 'Swiss roll' module for fitting into a cylindrical reactor. Somewhat similarly Lindsey & Yeoman (1982) entrapped plant cells in calcium alginate supported by nylon 'pan scrubber' material.

It seems that calcium alginate and κ-carrageenan are much the best polysaccharides for cell entrapment, but other polysaccharides have been investigated. Rosevear (1982*a*, *b*) used sodium polygalacturonate in an analogous manner to his use of sodium alginate described above, but included xanthan gum in the mixture. Combinations of polysaccharides such as xanthan gum and locust bean gum form stable gels, but as with κ-carrageenan heat treatment is required for gel formation and there seems to be no advantage over alternatives (see, for example, Bucke & Cheetham 1981). Chitosan is also capable of forming gels but has not found wide usage in cell immobilization (see, for example, Muzzarelli 1980).

Cellulose acetate has been used successfully to immobilize enzymes and whole cells (Marconi & Morisi 1979). Aqueous suspensions of cells are emulsified with a solution of cellulose acetate in an immiscible organic solvent and the whole emulsion is extruded into a coagulation bath. The resulting fibres contain the cells in aqueous suspension entrapped in microscopic cavities. De Rosa *et al.* (1980) prepared membranes of cellulose acetate containing cells of *Caldariella acidophila*, which were used in ultrafiltration reactors. The cellulose-acetate–cell complex is very stable and long half-lives of activities are recorded (Marconi & Morisi 1979), but the contact of cells and organic solvents is undesirable.

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A very simple and apparently gentle method of cell entrapment is described in a patent by Sakimae & Onishi (1981). Suspensions of cells in water containing cryoprotectants such as glycerol, polyethylene glycol and sugars are sprayed into water-immiscible solvents such as *n*-hexane cooled to very low temperatures (-50 to -70 °C). The beads of frozen cells are separated from the freezing solvent and added to solutions of polymeric materials such as cellulose acetate, polystyrene, polyvinyl acetate and polyacrylic ester, still at very low temperatures. The solvent and the ice are then removed by freeze-drying, leaving a preparation of cells entrapped in sheets or beads of polymer.

(*c*) *Proteins*

A cell immobilization technique that probably falls between entrapment and covalent coupling is the use of reconstituted collagen (Venkatasubramanian & Vieth 1979). Cells are mixed with dispersed collagen fibres at an appropriate pH, the mixture is dried into a film, which is strengthened by cross-linking with glutaraldehyde. The resulting collagen–whole-cell complex is very similar in general terms to whole mammalian tissues in which cell masses are connected and supported by collagen. Venkatasubramanian & Vieth (1979) consider that microfibrillar collagen is capable of forming a variety of non-covalent bonds with cell wall materials, which are sufficient to entrap the cells. Glutaraldehyde cross-linking strengthens the matrix, protexts against microbial attack and increases hydrothermal stability. The last is a particularly valuable effect as it permits the use of the cell complex in industrial reactors at somewhat elevated temperatures. The cast, tanned films are readily rolled up to provide large surface areas within reactors.

Industrial uses of immobilized cells

1. *Cells used as sources of single enzymes*

(*a*) *Sugar transformations*

The most commercially significant use of an immobilized enzyme normally employs that enzyme entrapped within the cells or mycelium that produced it. Glucose isomerase (EC 5.3.1.5) is used to produce at least 3.5 Mt of high-fructose syrups from glucose annually. Glucose may be produced very inexpensively indeed from corn starch, its production being 'subsidized' by the protein and oil by-products. Glucose is significantly less sweet than sucrose but fructose is more sweet: thus the conversion of glucose to fructose is desirable to make a more acceptable food ingredient. This conversion is possible in alkaline conditions, but the isomerization is non-specific, the unwanted sugars psicose and mannose being produced as well. In the early 1960s it was found that xylose isomerase was capable of isomerizing glucose very specifically to fructose. This observation formed the basis of an industry that has deeply altered the carbohydrate sweetener economy of the U.S.A. The subject has been reviewed thoroughly by Bucke (1977, 1982) and by Antrim *et al.* (1979). Table 4 summarizes types of cells used as sources of glucose isomerase (GI) and the immobilization methods employed.

All these methods have found large-scale commercial use because the cell complexes are cheap (i.e. have long half-lives), easy to use, and non-toxic.

There are no obvious competitors with the various immobilized cell processes even though the activities per unit of reactor volume could be improved. Although GI is produced by highly developed strains of organism that give relatively high enzyme yields the specific activity of the

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enzyme (on a protein basis) is low and may be further lowered very significantly on immobilization (Poulsen & Zittan (1977) quote only a 20–30% retention of measurable enzyme activity). Thus a soluble, purified GI immobilized on to a solid support material should have a far higher activity per unit of reactor volume. If the productivity (i.e. activity × lifetime) of such a preparation were equal to and the overall cost the same as that of an immobilized cell preparation, the former would be preferred in the interests of reducing capital costs or increasing throughput.

TABLE 3. ADVANTAGES AND DISADVANTAGES OF CELL IMMOBILIZATION METHODS

method	advantages	disadvantages
1. aggregation	cheap, permanent	not applicable if cell viability required; heat or chemical treatment needed
2. adsorption	very gentle; applicable where cell viability required; supports may be designed to adsorb cells of particular sizes	temporary immobilization; cells will enter product streams; support materials may be costly
3. entrapment		
(*a*) polyacrylamide and other artificial polymers entrapping during polymerization	very wide range of gel properties readily obtained to control substrate, product, inhibitor ingress and egress; cell division possible within beads	essential enzymes may be destroyed on immobilization; monomers are hazardous in use
(*b*) κ-carrageenan and agar	food grade material; cheap in use; some cell viability retained, cell division occurs readily; lengthy activities obtained	cells will experience elevated temperatures; agar gives weak gels
(*c*) calcium alginate	food grade material; cheap in use; cell viability retained; cell division occurs readily; gas bubbles released without damage to gel; activities survive for extended periods	gels destroyed by chelating agents
(*d*) cellulose acetate	very good longevity of activities	cells must contact organic solvents: limited possibility of cell division
(*e*) collagen	good for use as membranes	cross-linking (tanning) needed for stability

The carbohydrate sweeteners industry provides another simple but effective use of immobilized cells. The trisaccharide raffinose is a nuisance; in the juice of sugar beets it inhibits sucrose crystallization and exhibits a 'molassogenic effect', causing the retention of sucrose in the molasses: together with stachyose and verbascose, it produces the well known but undesirable consequences of eating beans. To combat the former problem it is necessary to produce α-galactosidase completely free of invertase. This was achieved by Narita *et al.* (1976) who isolated strains of moulds, principally *Absidia* species, that produced high activities of α-galactosidase but no invertase. The dried mycelium was used batchwise in stirred tank reactors, as was a similar preparation of *Mortierella vinacea* var. *raffinosutiliser* (Obara & Hashimoto 1977). Arnaud & Bush (1976) produced a similar preparation of α-galactosidase by cultivating a strain of *Penicillium duponti* on the liquor used to blanch haricot beans, which contain raffinose and stachyose.

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Another single-step conversion of a sugar with the use of whole cells as the source of enzyme activity is sucrose inversion (Linko *et al.* 1980) by *Saccharomyces cerevisiae* entrapped in calcium alginate beads and stabilized by glutaraldehyde treatment. This preparation had a half-life (calculated, presumably) of 5–7 years! It could completely convert the sucrose in cane molasses (33% dry matter, 16.3% sucrose) at a flow rate of 0.45 bed volumes per hour at 40 °C. Suitably immobilized invertase is extremely stable: Marconi & Morisi (1979) described a preparation of yeast invertase trapped in cellulose acetate that had a half-life of 15 years!

TABLE 4. IMMOBILIZED CELLS USED INDUSTRIALLY AS SOURCES OF GLUCOSE ISOMERASE

organism	immobilization method	reference
Bacillus coagulans	cell paste cross-linked with glutaraldehyde	Hemmingsen *et al.* (1979) (Novo Industri A/S)
Arthrobacter sp.	cells flocculated by polyelectrolytes, formed into cylinders	Bungard *et al.* (1979) (I.C.I.)
Actinoplanes missouriensis	cells trapped by gelation, cross-linked with gluataraldehyde	Roels & Van Tilberg (1979) (Gist Brocades, N.V.)
Streptomyces olivaceus	cells cross-linked with glutaraldehyde	Borglum (1980) (Miles Labs Inc.)
Streptomyces phaeochromogenes	entrapped in κ-carrageenan, hardened with glutaraldehyde and hexamethylene diamine	Chibata *et al.*, cited in Abbot (1978)
Streptomyces sp.	entrapment within cellulose acetate	Marconi & Morisi (1979)

All of the enzymes described above may be isolated from the cells that produced them and purified before immobilization. An example of a simple carbohydrate conversion that requires the use of whole bacterial cells because the enzyme involved is unstable when isolated is the production of isomaltulose (palatinose) from sucrose. Sucrose finds uses in foodstuffs such as chocolate, as a bulking agent that gives the product its characteristic texture yet makes it excessively sweet. Isomaltulose has many of the desirable properties of sucrose but only 35% of the sweetness and thus has potential as a food ingredient. Various organisms are capable of transforming sucrose to isomaltulose, including *Serratia plymuthica*, *Protaminobacter ruber* (South German Sugar Co. 1976) and *Erwinia rhapontici* (Bucke & Cheetham 1981). The first two organisms have been used to produce commercial quantities of isomaltulose by batchwise fermentation (South German Sugar Co. 1976) or by continuous fermentation (Bayer A.G. 1979). Immobilization of *Erwinia rhapontici* cells in calcium alginate (Bucke & Cheetham 1981) gave many advantages over alternative processes. Table 5 compares the efficacies of several immobilization methods applied to *Erwinia rhapontici* and emphasizes the value of calcium alginate entrapment. An additional advantage was that sucrose (550 g l^{-1}) could be used as feedstock for immobilized *E. rhapontici* cells, greatly reducing the chances of serious microbial contamination occurring and reducing the cost of evaporation later in the process. The process has been scaled up to employ a 25 l reactor operated continuously for several weeks, producing few problems.

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(*b*) *The production of organic and amino acids*

Immobilized bacterial cells hzve been used for some years to produce L-aspartic acid and L-malic acid from fumaric acid, a cheap and readily available substrate (Chibata 1979). The former reaction uses *Escherichia coli* cells as source of aspartase (aspartate ammonia lyase, EC 4.3.1.1). This enzyme is unstable when isolated, but whole cells entrapped in polyacrylamide proved to be very stable. Chibata (1979) describes the discovery that the entrapped

TABLE 5. EFFECTIVENESS OF IMMOBILIZATION TECHNIQUES FOR USE IN BIOTRANSFORMATION OF SUCROSE TO ISOMALTULOSE WITH THE USE OF CELLS OF *ERWINIA RHAPONTICI*

cell immobilization technique	activity†	half-life/h
calcium alginate	0.325	8500
DEAE-cellulose	0.583	400
polyacrylamide	0.13	570
aggregated cells (glutaraldehyde)	0.153	40
K-carrageenan–locust bean gum	0.263	38
adsorption to bone char	0.01	25
agar	0.34	27
xanthan–locust bean gum	0.10	8
free cells	0.60	36

† Measured as grams of isomaltulose formed per gram of wet cells per hour.

E. coli cells lysed during the first 24–48 h after immobilization, causing a tenfold increase in activity. When a 1 M solution of ammonium fumarate, pH 8.5, containing 1 mM $MgCl_2$ was used as feedstock, the half-life of activity was 120 days at 37 °C. The cost of the process was only 60% of the previous process, which used whole cells batchwise. Chibata (1979) attributed the cost saving to lower costs of catalyst, labour, and waste treatment. The process was greatly improved when κ-carrageenan replaced polyacrylamide as the medium for cell entrapment, especially when the preparation was stabilized by cross-linking with glutaraldehyde and hexamethylenediamine (Chibata 1979): of the 15-fold increase in productivity over that of an already commercial process, Chibata (1979) comments modestly, 'This...gives us very satisfactory results'.

Initial attempts to produce L-malic acid from fumaric acid by using polyacrylamide-entrapped cells of *Brevibacterium ammoniagenes* containing fumarase (EC 4.2.1.2) were unsatisfactory because of the production of succinic acid as a side product (Chibata 1979). This was eliminated by treating the immobilized cells with bile extract. A commercial process gave 70% of the theoretical yield of L-malic acid with 1 M sodium fumarate as substrate at pH 7.0. The reaction reaches equilibrium where about 80% of the fumaric acid is converted to L-malic acid. The substitution of κ-carrageenan for polyacrylamide increased the productivity, but much less dramatically than for L-aspartic acid production.

As far as can be ascertained, no other amino or organic acids are produced by methods analogous to those described above. However, it is likely that new commercial sources of L-phenylalanine will be needed to produce the recently approved new high-intensity sweetener aspartame (L-aspartyl-β-L-phenylalaninemethyl ester). A possible route is via phenylalanine ammonia lyase (EC 4.3.1.5) immobilized in cells of yeasts such as *Rhodotorula gracilis*. This had been done by Nelson (1976) using cells bound to a glycidylmethacrylate polymer, which, used batchwise, gave a 90% conversion of ammonium cinnamate to phenylalanine.

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Other potentially useful acids may be produced by using single enzymes entrapped in microbial cells. These include L-alanine (from L-aspartate via aspartate β-decarboxylase of *Pseudomonas dacunhae*), L-citrulline (from L-arginine via L-arginine deiminase of *Pseudomonas putida*) and urocanic acid (from L-histidine via L-histidine ammonia lyase of *Achromobacter liquidum*) (Chibata 1979).

A conversion that is receiving increased attention is that of L-malic acid into L-lactic acid. The Institut Nationale de la Recherche Àgronomique (1979) used cells of *Lactobacillus casei* immobilized in polyacrylamide gels continuously for 10 months, apparently to decarboxylate pure L-malic acid. The preparation is intended for use in lowering the acidity of wines.

(*c*) *Other materials*

One of the earliest commercial uses of immobilized cells was the formation of prednisolone from Reichstein compound S via cortisol, by using the 11-β-hydroxylase and Δ′-dehydrogenase activities of cells immobilized in polyacrylamide gels. The first activity was provided by mycelia of *Curvularia lunata* (Mosbach & Larsson 1970): activity could be regenerated by exposure of the immobilized preparation to nutrients. Later the process was improved by immobilizing *C. lunata* spores in calcium alginate and allowing them to germinate and grow within the polymer matrix (Ohlsen *et al.* 1980) Immobilized cells produced cortisol at the same rate as free cells, the pH range and optima were similar, as were the temperature optima, but immobilized cells were more stable at higher temperatures. It was necessary to dissolve the substrate in organic solvents, which also increases the permeability of the cells. Low levels of methanol (2%) dimethyl sulphoxide (1%) or Tween 80 (1%) were tolerated by immobilized preparations.

The second stage of the production of prednisolone was done by using cells of *Arthrobacter* (*Corynebacterium*) *simplex* immobilized in polyacrylamide (Aktiebolaget Fermenta 1980). The activity of the preparation was increased by incubation with peptone solution, which allowed cell division within the gel pellets. The maximum rate of prednisolone production was 31.7 mg h^{-1} per gram of gel, much superior to the 0.17 mg h^{-1} per gram of catalyst obtained by using *A. simplex* cells entrapped in collagen (Venkatasubramanian & Vieth 1979). In the latter case the cells had not been supplied with a growth medium.

The productivity of the immobilized cell preparations used in predisolone production is low, but that is of little importance because of the very high value of the product. Venkatasubramanian & Vieth (1979) describe a much more productive sterol modification, that of cholesterol oxidation by collagen-entrapped cells of *Mycobacterium rhodochrous*, which oxidized 800 mg substrate h^{-1} per gram of catalyst.

Many other possible sterol transformations that might be done by using immobilized cells are described by Aktiebolaget Fermenta (1980), and attempts to improve sterol conversions by the use of hydrophobic supports to immobilize cells to improve access of hydrophobic substrates to the cells are summarized by Cheetham (1980).

The use of enzymes to produce epoxides from alkenes is becoming increasingly popular. Furuhashi *et al.* (1980) produced epoxides of ethylene, propylene, 1-butane, 1-decene, 1-undecene and 1,15-hexadecadiene by using cells of various organisms entrapped in polyacrylamide. The immobilized cell preparations apparently consisted of 4 ml blocks, which were used batchwise. Epoxidation activity fell with use but could be regenerated by the addition of 0.05% urea to the reaction medium. Although the examples quoted used *Nocardia corallina*, higher

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concentrations of propylene oxide (up to 115 mg l^{-1}) were produced by *N. butanica* and *N. paraffinica*.

The regeneration of cofactors inexpensively is perpetually of interest to enzyme technologists. The use of whole viable cells overcomes the need to do this, but immobilized cells have been used to regenerate several cofactors or even avoid their use. An example of the latter is given by Murata *et al.* (1979). The use of ATP is avoided by producing glucose 6-phosphate from glucose and metaphosphate by using cells of *Achromobacter butyri* entrapped in polyacrylamide gels. This is a process for which calcium alginate could not be used as the entrapping medium. Similarly $NADP^+$ may be produced from NAD^+ by using the polyphosphate NAD kinase of *Brevibacterium ammoniagenes* immobilized in polyacrylamide (Kolot 1981).

Klibanov & Puglisi (1980) used *Alcaligenes eutrophus* cells as sources of hydrogenase, which allowed the reduction of NAD^+, FAD, FMN, methyl viologen, methylene blue, 2,6-dichlorophenolindophenol, phenazine methosulphate, Janus green and potassium ferricyanide. Calcium alginate and K-carrageenan were greatly superior to polyacrylamide as the entrapping medium.

2. *Immobilized cells as sources of multi-enzyme systems*

(*a*) *Ethanol production*

The production of ethanol by yeast cells was one of the first biological processes to be studied in immobilized forms. In perhaps 6 years since the earliest publications there has not been any publicized use of immobilized yeast or bacterial cells to produce ethanol (or alcoholic beverages) on an industrial scale, so it is probably accurate to state that, so far, immobilized cells have no great commercial advantages over conventional fermentation processes with conventional substrates. The conventional processes have been under development for somewhat longer!

Objectives of recent studies on the production of ethanol have concentrated on high reactor productivities, the achievement of high ethanol concentrations, maximization of substrate use, the use of novel substrates, and the use of high fermentation temperatures.

In addition, the production of ethanol by yeasts is an attractive model system for the evaluation of methods of immobilizing cells so as to retain the activity of a multi-enzyme complex. Many workers on this class of study have not aimed at commercial objectives.

One of the earliest publications about the immobilization of cells in calcium alginate gels (Kierstan & Bucke 1977) described the production of ethanol by *Saccharomyces cerevisiae* cells supplied only with glucose or sucrose, without a source of fixed nitrogen. Not surprisingly the productivity of the cells was low but the ability of the immobilized cell complex to produce ethanol survived for several months. Cells of *Kluyveromyces marxianus* similarly immobilized produced ethanol from inulin for only a few days but retained inulase activity for several weeks (Kierstan & Bucke 1977).

Navarro & Durand (1977) immobilized *Saccharomyces carlsbergensis* cells on porous glass beads activated by using γ-amino propyltrimethoxysilan alone or together with glutaraldehyde. Cells were immobilized simply by stirring with the activated support for at least 15 min. Significant numbers of cells were bound only to the glutaraldehyde-activated material. The amount of glutaraldehyde bound to the glass beads increased with the porosity of the beads (i.e. the total surface area of the beads) and it was assumed that the yeast cells were bound to the surface of the beads rather than entrapped within their pores. Immobilization reduced the fermentation rate of the cells but strikingly increased the yield of ethanol from glucose. *S. cerevisiae* cells,

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immobilized on activated pectate cross-linked by using Fe^{3+} ions, in aerobic conditions had an oxygen uptake rate some times that of the initial cell suspension (Vijayalakshmi *et al.* 1979). Marcipar *et al.* (1980) used Biodamine, an aluminosilicate material, to immobilize cells of various yeast strains by simple adsorption within the pores of the support. Immobilized *S. cerevisiae* cells respired at 6–7 times the rate of the initial suspension.

In a study aimed at the continuous production of ethanol, Daugulis *et al.* (1981) adsorbed *S. cerevisiae* cells on to beads of commercially available anionic resins (table 2). The cells were not desorbed significantly by the passage of an undefined medium containing glucose at 120 g l^{-1}. An unexpectedly low extent of conversion of glucose to ethanol could not be explained by the authors. The immobilized cell reactor was operated continuously without trouble for 200 h.

The ability of immobilized cells (of various organisms) to grow within beads of κ-carrageenan was studied by Wada *et al.* (1979). *S. cerevisiae* cells were immobilized at a density of 3×10^6 cells ml^{-1}: after incubation with a growth medium, the cell density reached 5×10^9 cells ml^{-1}, with the new cells forming a layer near the surface of the κ-carrageenan bead. Later such preparations were used to produce ethanol continuously from a medium containing glucose at 100 g l^{-1} (Wada *et al* 1980). A stable steady state was maintained for over 3 months and the yield of ethanol was close to the theoretical maximum. Clear evidence was presented that only growing cells were able to produce ethanol. The same authors (Wada *et al.* 1981) continued their studies by investigating the production of ethanol from media containing glucose at 250 g l^{-1}. A strain of *S. cerevisiae* (IFO 2363) was selected for its ability to produce ethanol from such media and immobilized as before, in κ-carrageenan. Cells immobilized at low densities (less than 10^7 ml^{-1}) did not grow satisfactorily when supplied with media containing glucose at 250 g l^{-1}, but high cell densities (*ca.* 8×10^9 ml^{-1}) were obtained with glucose at 100 g l^{-1}. An abrupt change of glucose concentration from 100 to 250 g l^{-1} did not result in high ethanol concentrations but when there was a stepwise increase of glucose concentration from 100 to 150, 200 and 250 g l^{-1} the 'conditioned' preparations could produce ethanol at 114 mg ml^{-1}, with a conversion rate of glucose to ethanol of 96% of theoretical, continously for over 2 months.

To attempt to explain why, in the stationary growth phase, the number of living cells was 10 times higher in the gel than in the liquid medium, Wada *et al.* (1981) determined the partition coefficients of glucose and ethanol between gel and solution. They were 0.86 and 0.67 respectively: the authors considered that this distribution was responsible for the better growth of yeast cells within the gels.

The production of CO_2 from beads of immobilized yeast cells has an adverse effect in packed-bed reactors. Cho *et al.* (1981) compared the efficiencies of packed-bed and fluidized-bed reactors with the use of cells of *Saccharomyces cerevisiae* entrapped in calcium alginate. Table 6 summarizes the productivities of these reactors in comparison with free-cell, continuous, fermentations with and without cell recycling by using media containing glucose at 100 g l^{-1}.

The primary reason for the improved performance of the fluidized-bed reactor was the increased rate of removal of CO_2 from the reactor: about 65% of the total effective reactor volume of a packed-bed reactor might be occupied by CO_2. Shiotani & Yamane (1981) overcame this problem simply by using a packed-bed reactor horizontally.

Williams & Munnecke (1981) studied the immobilization of yeast cells in calcium alginate in some detail. They used sodium alginate from Sigma Chemical Co. (this is from *Macrocystis* species and has a high mannuronate:guluronate ratio; it therefore has a high viscosity and low

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gel strength compared with the *Laminaria* alginate supplied by B.D.H. Ltd and used by Kierstan & Bucke (1977) and Cheetham *et al.* (1979)). The optimum alginate concentration was 15 g l^{-1}. Immobilized cells showed a very broad pH optimum for ethanol production, the same rate being maintained between pH 3 and 8: a free cell culture had a sharp pH optimum between 4 and 5. Immobilized cells had a lower temperature optimum than free cells. A maximum

TABLE 6. COMPARISON OF ETHANOL BIOREACTORS (CHO *ET AL.* 1981)

(Calculations based on V_t, the total reactor volume)

reactor	productivity of ethanol/(g l^{-1} h^{-1})
continuous free cell	7
continuous with cell recycling	14
packed-bed	10
fluidized-bed	21

TABLE 7. COMPARISON OF PRODUCTIVITIES OF VARIOUS IMMOBILIZED CELL PROCESSES FOR THE PRODUCTION OF ETHANOL (MODIFIED FROM MARGARITIS *ET AL.* 1981)

reference	system	substrate sugar concentration/(g l^{-1})	substrate sugar utilized (%)	dilution rate, D	basis for calculation of D†	productivity for EtOH/(g l^{-1} h^{-1})
Kierstan & Bucke (1977)	*S. cerevisiae* Ca alginate	glucose 100 (no N source)	80	0.1	V_t	4.3
Chibata (1979)	*S. cerevisiae* carrageenan	glucose 100 (full growth medium)	98	1.0	V_b	50
Wada *et al.* (1979)	*S. cerevisiae* carrageenan	glucose 100 (full growth medium)	86	1.0	V_b	43
Grote *et al.* (1980)	*Z. mobilis* carrageenan	glucose 150 (full growth medium)	85	0.8	V_l	53
Grote *et al.* (1980)	*Z. mobilis* Ca alginate	glucose 150 (full growth medium)	75	0.85	V_l	44
Linko & Linko (1981)	*S. cerevisiae* Ca alginate	molasses 175	83	0.3	n.a.	21
Williams & Munnecke (1981)	*S. cerevisiae* Ca alginate	glucose 127 (no N source)	63	4.6	V_l	54
Margaritis *et al.* (1981)	*Z. mobilis* Ca alginate	glucose 100	87 97	2.4 1.6	V_l V_l	102 71

† V_t, total volume of bioreactor; V_b, volume of immobilized cell preparation; V_l, volume of liquid in bioreactor. V_t is larger than V_b or V_l. Productivity calculations based on V_t are the most meaningful in practice. Karanth (1982) agrees.

productivity of ethanol, with the use of glucose (127 g l^{-1}) and 0.05 M $CaCl_2$ with no nitrogen source, of 53.8 g l^{-1} h^{-1} was obtained. In these conditions only 85 % of the glucose was consumed.

Linko & Linko (1981) employed *Laminaria* alginate to entrap *Saccharomyces cerevisiae* cells of various strains. Because of its lower viscosity Linko & Linko (1981) were able to use alginate at 80 g l^{-1}. They used complete media containing cane molasses, which allowed cell concentrations of 2 to 5×10^9 cells ml^{-1} and obtained productivities of ethanol of up to 50 g ethanol l^{-1} h^{-1}.

Veliky & Williams (1981) modified the calcium alginate immobilization method by treating

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the surface of alginate beads with polyethylene imine to stabilize the structure against destruction by chelating agents. This treatment lowered the respiration rate of the entrapped yeast cells but did not reduce the ethanol production rate. Birnbaum *et al.* (1981) used three different methods to stabilize calcium alginate beads and concluded that a combination of polyethyleneimine and glutaraldehyde gave the best results at least expense.

TABLE 8. CHANGES IN CONSTITUENTS OF FREE-LIVING AND IMMOBILIZED *SACCHAROMYCES CEREVISIAE* CELLS (SIESS & DIVIES 1981)

	free-living cells (growth phase)	immobilized cells after 48 h	after 648 h
protein/deoxyribose	1550–1030	734	500
protein/mannan	21–14	13	12
protein/glucan	12–9	9	4
protein/trehalose	7–15	30	85
protein/glycogen	9–6	5	3
protein/alkaline glycogen	33–47	25	10
ribose/deoxyribose	46–76	53	25

Entrapment of yeast cells in polyacrylamide destroys 40–80% of the cells, the survival rate depending on the age of the cells (Siess & Divies 1981), old (stationary phase) cells surviving best. The viable cells remaining are able to divide but those nearest the gel surface both divide and ferment most actively.

One of the fullest studies on the production of ethanol by using immobilized cells (Holcberg & Margalith 1981) compared the performance of *Saccharomyces cerevisiae* ATCC 7754 entrapped in agar, polyacrylamide, K-carrageenan and calcium alginate. In all the gels except calcium alginate, immobilized cells produced higher ethanol concentrations than free cells, this effect being particularly striking at high glucose concentrations. Ethanol production was inhibited somewhat by calcium ions.

Interestingly, the suspension of cells in sodium alginate solutions of different viscosities led to an increase in the rate of ethanol production, the highest ethanol concentration occurring in sodium alginate at 5 g l^{-1}. Cells entrapped in agar gels produced the very high ethanol level of 14.5% (by mass) from glucose at 300 g l^{-1}, at 20 °C. Holcberg & Margalith (1981) attribute the ability of entrapped cells to ferment a very high glucose concentration partly to the establishment of gradients within the gels, the concentration of glucose at the cell surface being significantly lower than in bulk solution. Such gradients did not account for the whole effect, however, simple contact of cells with a polymer allowing higher ethanol contents to be obtained.

Recently, increased attention has been paid to alternative substrates for ethanol production by using immobilized cells. Linko *et al.* (1981) entrapped *Kluyveromyces fragilis* cells in calcium alginate and produced ethanol continuously for several days from whey preparations. Margaritis & Bajpai (1981) also used *K. fragilis* entrapped in calcium alginate to ferment fructans from Jerusalem artichoke. D-Xylose, derived from hemicelluloses, is an attractive substrate for ethanol production, but very few organisms will do this. Maleszka *et al.* (1981) improved the efficiency of ethanol production from xylose by *Pachysolen tannophilus* by entrapping the cells in calcium alginate.

An approach that employs more fully the flexibility of gel-entrapment of cells to allow the utilization of 'non-fermentable' substrates is to coimmobilize enzymes with the cells. Many

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enzymes, however, are readily leached from gels (Kierstan & Bucke 1977) and it is necessary to immobilize the enzyme in some way. Chiang *et al.* (1982) coimmobilized *S. cerevisiae* cells and glucose isomerase in calcium alginate, and also used glucose isomerase and immobilized yeasts in separate columns, the function of the glucose isomerase being to isomerize xylose to xylulose which is fermentable by the yeast. The latter approach was more successful, perhaps because of the very different optimum conditions of the two enzyme systems.

Hagerdal & Mosbach (1981) covalently bound β-glucosidase to alginate, then entrapped the modified enzyme and yeast cells in calcium alginate to produce ethanol from cellobiose. Similarly Svensson & Ottesen (1981) increased the molecular size of glucoamylase by coupling it to soluble dextran before coimmobilizing it in alginate with yeast cells.

Finally in connection with ethanol production are examples of the immobilization of the bacterium *Zymomonas mobilis*. Grote *et al.* (1980) and Margaritis *et al.* (1981) entrapped cells in calcium alginate and successfully produced ethanol at high yields for extended periods. Amin & Verachtert (1982) compared *Z. mobilis* and *Saccharomyces bayanus* immobilized (separately) in carrageenan gels and concluded that *Z. mobilis* has greater potential for ethanol production than do yeasts.

2. *Acetone and butanol*

The simultaneous production of acetone and butanol from glucose by cells of *Clostridium acetobutylicum* immobilized in calcium alginate was studied by Haggstrom & Molin (1980). Immobilized spores allowed to germinate and grow within the gel gave better results than cells immobilized in the logarithmic or resting stages of growth. Butanol productivity was higher from immobilized cell reactors than from the normal batch fermentation. Similarly, Krouwell *et al.* (1980) showed that *n*-butanol and isobutanol could be produced continuously by using preparations of *Clostridium butylicum* produced by immobilizing spores in calcium alginate.

3. *Macromolecules*

The antibiotic bacitracin may be produced by using washed cells of *Bacillus* species supplied with simple nutrients, but productivity decreases rapidly with time. Immobilization of washed cells in polyacrylamide gel (Suzuki & Karube 1979) lowered the initial activity but the entrapped cell preparations, supplied with a peptone saline medium, gradually attained the activity of the original cells, probably because of cell growth within the gel.

The same authors reported the production of bacterial α-amylase by using cells of *Bacillus subtilis* entrapped in polyacrylamide. As with bacitracin, initial rates of enzyme production were low but after six repeated (batch) incubations with meat and yeast extract media, high production rates were obtained (Kokubu *et al.* 1978).

Later *Bacillus amyloliquefaciens* was immobilized, for the same purpose, in κ-carrageenan (Shinmyo *et al.* 1982). Bacterial α-amylase was produced extracellularly and continuously at rates equalling or exceeding that of the free cells, even though the specific growth and respiration rates were lower.

4. *Plant cells*

Since the observation that plant cells could be immobilized simply and successfully in calcium alginate (Brodelius *et al.* 1979) this has been the most favoured method of entrapping cells of many species. When bioconversions of added materials are required, the particular advantage of immobilization appears to be the increased productivity of the cells, even though reaction

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rates, primarily of hydroxylation, are lowered on cell immobilization (Jones & Veliky 1981; Veliky & Jones 1981; Alfermann *et al.* 1980).

In a review dealing with experimental systems for studying the production of secondary metabolites by plant tissue cultures, Lindsey & Yeoman (1982) review methods of immobilizing plant cells, stressing the importance of allowing the establishment of internal gradients within cultured cells if high yields of secondary products are to be obtained. Thus the internal state, both physical and chemical, of a cultured cell is more similar to that of a cell in a normal plant tissue. Lindsey & Yeoman (1982) immobilized cells of various species in alginate gels or in agar gels, the gels being supported by nylon pan-scrubber material. Growth of cultures was better in alginate than in agar, but immobilized cells reached the stationary phase of growth more rapidly than free cells owing to nutrient limitation. This was no bad thing as stationary phase cells produce secondary metabolites more reliably than growing cells.

Conclusions

It will be clear from this far from comprehensive review that a very large amount of work on immobilized cells has been done in a very short period of time. It is clear also that the use of immobilized cells has not yet revolutionized the fermentation industry. It has led to large changes in the carbohydrate sweetener industry and in the production of amino acids, but the development of glucose isomerase as an industrial-scale catalyst has not been entirely dependent on the use of immobilized cells.

It is probably fair to state that almost all the studies on methods of cell immobilization have been somewhat empirical: methods have been tried, have worked and have been reported. In some cases immobilization has greatly increased the longevity of single enzyme activities or of the viability of whole cells: such increases may be crucial for the commercial viability of a process that would be uneconomical when attempted with conventional fermentation. The use of immobilized cells may revive interest in biological processes shelved as 'slightly too expensive'.

There is room for much fascinating work on the reasons for the preserving effect of entrapment in gels, particularly alginate and carrageenan, on biological activities. There are a few hints, given by Holcberg & Margalith (1981) and Wada *et al.* (1981) that gradients of concentration within the gels and the partition of possibly damaging metabolites between the gel and the bulk liquid may favour the survival of the cells. Lindsey & Yeoman (1982) would, presumably, agree with this: Klibanov & Barta (1981) might argue that oxygen concentration within the cell preparations is crucial.

There is a need for more 'science' to be used in the selection of cell immobilization methods. Calcium alginate is probably the most widely used gel, followed closely by κ-carrageenan, perhaps because it is cheap and very easy to use, but it is perhaps not the ideal gel for use when high oxygen levels are required and is certainly unsuitable for use with chelating materials.

With more knowledge of the effect of the support material on the physiology of immobilized cells and more imagination employed in the choice of immobilization material, cell immobilization may well become a technique of great commercial importance.

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Discussion

D. E. Brown (*Department of Chemical Engineering, U.M.I.S.T., Manchester, U.K.*). A fed-batch fermentation process is a type of immobilized cell system in which perhaps 30 kg dry mass of cells per cubic metre of reactor will be encouraged to produce some given product for an extended period of time. To allow a comparison of reactor capacities, could Dr Bucke give a rough figure for the quantity of cells as kilograms dry mass per cubic metre that we might expect in a reactor containing immobilized cell material?

C. Bucke. In the conversion of sucrose to isomaltulose we employ beads containing 30% wet mass of cells in calcium alginate: the quantity of cells in kilograms dry mass per cubic metre is therefore approximately 36. Higher cell densities are used by other workers, e.g. Kokobu *et al.* (1978) probably used 75 kg m^{-3} after growth of cells within the polyacrylamide gel, and Daugulis *et al.* (1981) adsorbed 128 mg dry mass of cells per gram of anionic resin (probably 100 kg m^{-3}).

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