A range of ceramic biosupports

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THE USE OF batch reactor techniques for large scale bioprocessing has many drawbacks, the most important of which is probably the loss of expensive, and possibly genetically engineered, biomass during the processing of the reactor contents to extract the saleable product. In addition, use of the biocatalysts is often inefficient and the capital costs associated with their production can be high.[1]

A widespread and continuing interest exists in an alternative approach, that of immobilization of the biological catalysts, whether they be enzymes or cells. The many potential attractions of this approach include short reaction times, high volumetric efficiency, continuous processing, ready separation of product and low capital costs.[2] The technology offers economical methods of recovery and purification: scale-up can be relatively straightforward.

To date, only a small number of immobilized biocatalyst systems have been commercialized, but the potential is obvious. Indeed, the production of high fructose syrups for the food industry, using immobilized glucose isomerase, is one of the major biotechnological success stories.[3]

Most industrial immobilization techniques are based on a combination of two or more of the five conventional primary binding methods:[4] adsorption onto, or within, a carrier; crosslinking of enzymes or cells onto the surface of a support, or within it; crosslinking of enzymes or cells without a carrier; covalent bonding to a carrier; and encapsulation or entrapment within a carrier.

A distinction is usually drawn between methods that rely on adsorption to supports and those that involve chemical bonding or encapsulation. Adsorption alone is only rarely satisfactory, since the intermolecular forces involved are generally inadequate to inhibit sloughing of excess cells or elution of enzyme, as the case may be.

Each enzyme has a different surface chemistry and operational stability. Consequently, no one immobilization method is generally applicable. As a result, a large number of different methods have been developed over the last 10-15 years, mostly involving covalent bonding to the carrier or support.[3] Few immobilized enzyme systems, however, have yet been applied on a commercial scale[5] (*Table 1*), possibly because of the lack of suitable low cost supports.

Specific binding is, perhaps, less relevant for immobilized cells than it is for enzymes. Thus, entrapment, particularly within the calcium alginate matrix, appears to have become the method of choice for those working with cells,[6] since only nontoxic calcium ions need to be added to the sodium alginate/cell mixture to effect entrapment. Yet even this method suffers from a number of disadvantages: 1) the process is undesirably complex and does not lend itself easily to scale-up, since the cells must first be harvested from their growth medium; 2) the gels have poor mechanical properties; 3) the beads degrade in the presence of chelating agents such as phosphate; and 4) there is restricted transport of materials and products through the polymer to and from the biocatalyst. Of these drawbacks, it is the lack of mechanical strength that precludes the use of these materials in large column reactors.

The ideal immobilization matrix would be economically priced, yet display the following properties:[7]

1) The loss of activity of the biocatalyst on immobilization should be low;
2) High biocatalyst loadings should be possible;
3) Substrate and products should be able to diffuse freely through the biosupport;
4) The biosupport should have high mechanical strength and show negligible abrasion in use;
5) The support should be thermally and chemically stable;
6) Regeneration of the support should be possible;
7) The biosupport should offer a high contact area with the surrounding medium.

Many of the requirements listed above could be met by the use of a porous inorganic support that combines high strength with a structure containing pores of appropriate dimensions. If necessary, the surface of such a material could be chemically activated by use of suitable coupling agents to enhance uptake and stability of the support-catalyst bond.

Ceramic biosupports

A range of biosupports, which meets many of the criteria set out above, have recently been developed. The Biofix range (English China Clays International) consists of four materials (*Table 2*), two for cell support (C1 and C2) and two for immobilization of enzymes (E1 and E2). All four products are derived from the clay mineral kaolinite, though the methods of manufacture differ from grade to grade.

As high temperature ceramics, Biofix granules are extremely strong. Moreover, they are extremely resis-

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Table 1

Comparison of four major immobilized enzyme systems[13]

Enzyme	Bioreactor	Enzyme form	Substrate	Operating temperature (°C)	Operating life (hours)
Amyloglucosidase	Packed bed column	Granules	Liquified starch (clarified)	53-60°C	500
Glucose isomerase	Packed bed column	Granules	Corn syrup	58-65°C	1000
Lactase (Fungal systems)	Packed bed column	Porous glass beads	Milk whey	30-40°C	Not reliably known
Penicillin acylase	Bead column	Sephadex beads	Penicillins	35-40°C	3000

Table 2

Typical technical properties

Grade	C1	C2	E1	E2
Product form	Hollow porous microspheres	Hollow porous microspheres	Porous microspheres	Porous granules
Particle size	20 - 50 μm	50 - 75 μm	20 - 50 μm	0.5 - 1.5 mm
Cavity entrance	10 μm	20 μm	—	—
Mean pore size	900 Å	900 Å	600 Å	300 Å
Surface area/m^2g^{-1}	5.0	9.5	4.0	25
Bulk density/g cm^{-1}	0.55	0.50	1.0	0.72
% Void volume	22 wall 65 cavity	22 wall 70 cavity	50	50
pH stability	1 - 14	1 - 14	1 - 14	1 - 14
Thermal stability	1000°C	1000°C	1000°C	1000°C
Crush strength	>55 MPa	>55 MPa	>55 MPa	>55 MPa

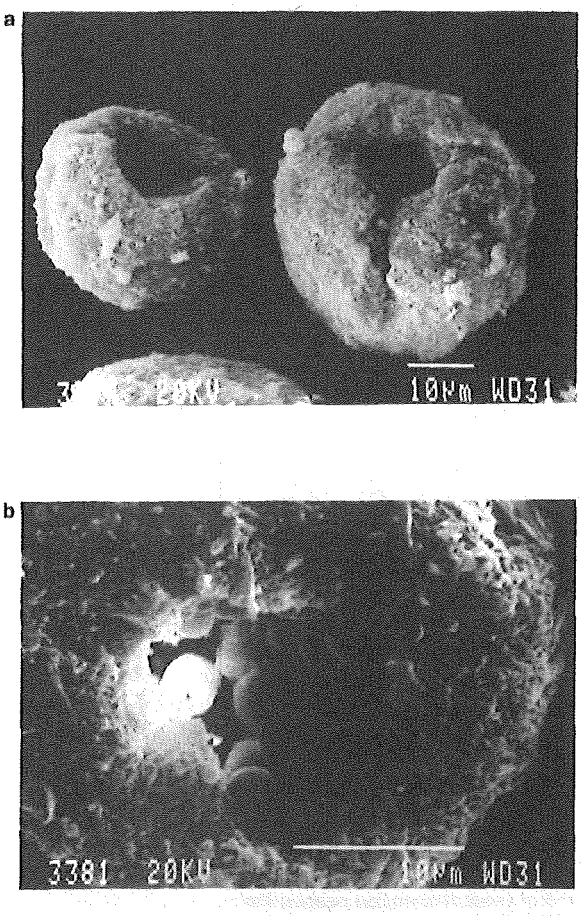

Figure 1 *a) "Bird's nest" form of Biofix C-grade cell supports. b) Yeast cells immobilized on Biofix C1.*

tant to chemical attack, whether by chelating agents, acids, or alkalis. In addition, the thermal stability of the supports means that they can be heated at temperatures up to 1000°C without loss of surface area or porosity. Because of their inert ceramic nature, the materials are nontoxic.

Cell supports

Biofix C1 and C2 consist of a specially treated slurry of a highly purified kaolinite formed into hollow microspheres. The supports have the form of a "birds nest" (*Figure 1*) with porous walls.[8]

Enzyme supports

Biofix E1 and E2 are synthesized by completely different routes, although they are still derived from kaolinite. E1 particles are spherical and of diameter 20 to 50 μm, while E2 consists of millimeter-size granules. The pores in both materials are large enough to accept typical enzymes, 400 to 800 Å for E1 and 200 to 500 Å for E2 (*Figure 2*), the fundamental differences being that the E1 material is somewhat stronger than E2, but has a smaller surface area, typically 5 m^2g^{-1}.

Whole cell Immobilization

Biofix C1 and C2 were specifically designed for use as intact-cell biosupports. The shape of the particles makes them ideal for holding whole cells (the diameter of the entrance to the central hole is ~10 μm for C1 and 20 μm for C2), while the porous walls enable nutrients and products to diffuse relatively freely.

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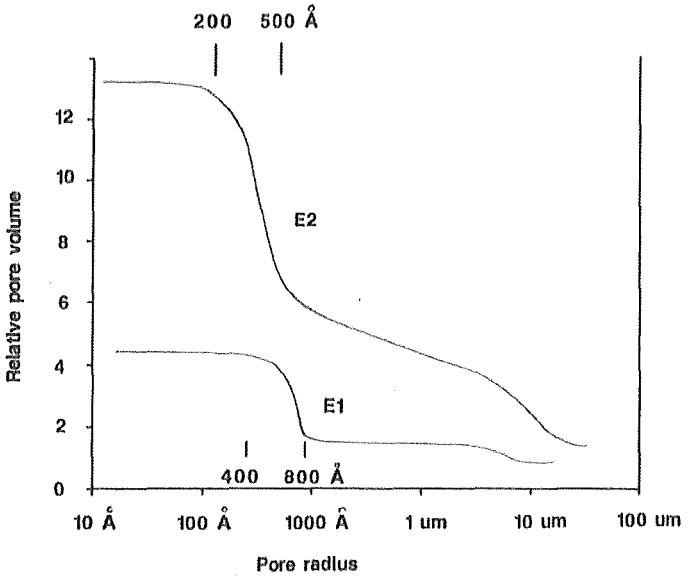

Figure 2 *Pore size distribution for Biofix enzyme supports E1 and E2.*

Cell loading

The efficiency of immobilization was assessed by flushing suspensions of *Saccharomyces cerevisiae* through short packed columns of Biofix C2. The suspensions were prepared in 0.2 *M* potassium phosphate buffer (pH 7). After columns were loaded, they were washed with several column volumes of the same buffer.

The initial assumption was that cells might be immobilized within the Biofix particles by simple

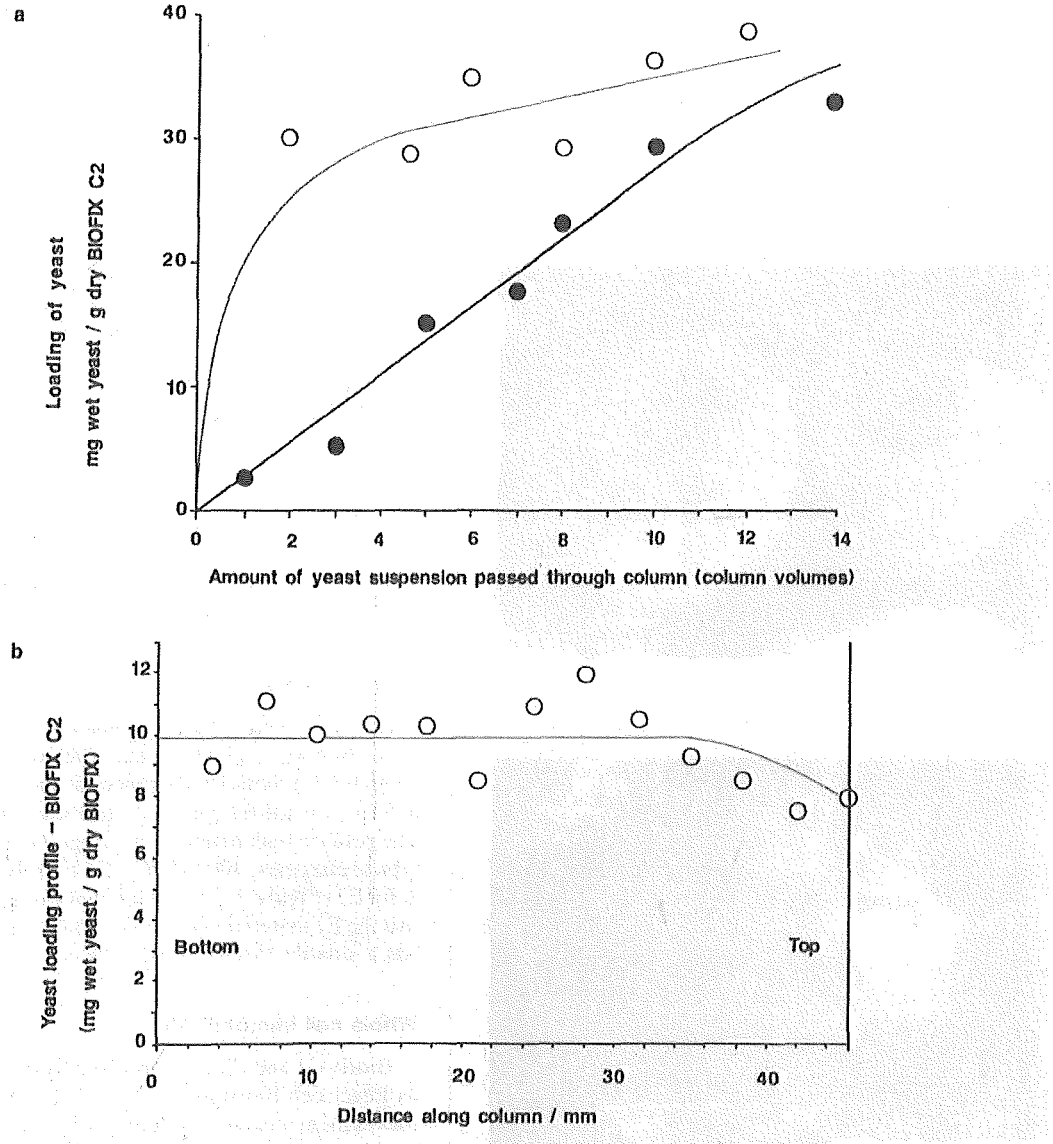

Figure 3 *a) Cell loading as a function of volume of yeast suspension passed the column. Concentration of yeast suspension ○ = 30 mg/mL; ● = 1 mg/mL; b) typical loading profile as a function of distance up the column.*

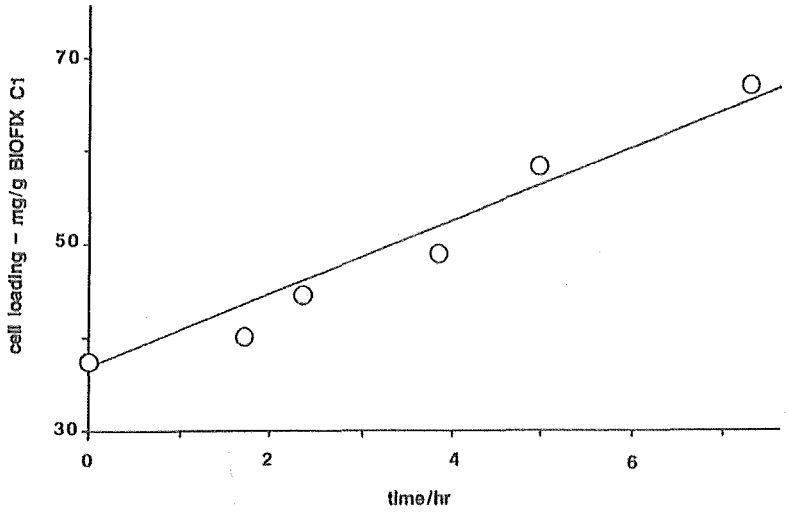

Figure 4 *Growth of* Saccharomyces cerevisiae *on 7 g packed bed Biofix C1. The cells were immobilized from 200 mL of a 30g/L suspension of yeast. Excess cells were flushed from the column and the fixed cells cultured using 10 mL of 0.1 wt % yeast extract and 1 wt % glucose solution per hour.*

filtration. On this basis it would be expected that an absolute maximum of 50% (and probably nearer 10%) of the particles, namely the orifices of which were oriented toward the flowing stream, might entrap cells. However, electron microscopic inspection of particles such as those in Figure 1 indicated that essentially all particles had entrapped cells. This indicated that cells had entered even those Biofix microspheres with orifices oriented away from the flowing stream. Similar experiments with unleached particles (through which filtration was thus impossible) showed an efficiency of immobilization similar to that obtained from the leached support. A mechanism other than simple filtration was indicated, and a consideration of the hydrodynamic forces operating suggested that these alone could be responsible for deposition of cells inside the hollow microspheres. This mechanism has been termed hydrodynamic deposition.[8]

A study was made of how cell loadings varied at various points on the packed column. Use of a protein assay showed that an equilibrium was quickly established between free and immobilized cells down the entire length of the column, i.e., the extent of all immobilization was found to be relatively uniform throughout the length of the packed bed (*Figure 3*). This is important, as simple interparticle filtration

immobilization would lead to a biocatalyst column with a greater activity at the top than at the bottom of the column, leading to problems during scale-up, particularly when two or three columns are used in series.

The yeast cells immobilized in Biofix remained viable. Consequently, it proved possible to enhance the activity of a biocatalyst column by immobilization of the cell of interest and then culturing in situ to the required level of activity (*Figure 4*).

The remaining point of interest concerns the long-term attachment of cells to the support. The method of deposition relies on flow patterns, rather than any chemical bonding. As a result, if the flow through the column is interrupted for some time, then some leakage of cells into the supporting medium is found. It is preferable, if the column has not been used for some time, to drain and rewet it before use. More permanent fixing of the cells can be achieved, if desired, by one of small amounts of standard bifunctional cross-linking agents.[9]

Enzyme immobilization

Enzymes are much smaller than cells: typical diameters are 50 Å compared with perhaps 4 μm for a yeast cell. Consequently, the "bird's nest" morphology of the C-grades of Biofix is of no advantage. Rather, what is required is a surface having pores in the range of 200 to 800 Å, to allow ready ingress of enzymes and substrate.[10] Both E1 and E2 grades (Table 2) are macroporous solids having pore diameters in this range.

Preliminary studies

Unmodified Biofix supports have low surface charge density and are chemically unreactive with enzymes. To achieve uptake levels above 3 mg/g support, activation is required. For example, enzymes such as trypsin and urease were immobilized successfully on E1 (*Figure 5*) after pretreatment with a solution of 2.5 wt % of glutaraldehyde in phosphate buffer at pH 7. Enzyme concentrations of 1% wt/vol were used, and in the case of urease a 38% retention of enzymic activity was found.

Studies using γ-aminopropyl silane treated support[11] and enzymes of differing size—lysozyme (MW 14,000) and lipase (MW 170,000)—showed that uptake levels were insensitive to enzyme size, at least in this range (*Table 3*).

Immobilization of lipase

Of much topical interest is the immobilization of lipases.[12,13] The uptake and reactivity of a lipase from *Candida cylendrecea* (supplied by Biocatalysts, Pontypridd) on Biofix E1 and E2 has been studied. The particular reaction studied was the conversion of DL — citronellol to citronellol butyrate.

The supports were activated before use by treatment within γ-amino propyl silane and coupling to the enzyme was accomplished by use of glutaraldehyde solution. The enzyme was prepared as a 10 mg/mL^{-1} solution in 0.05 M^{-3} Na_2HPO_4 (pH 7) buffer. In general, the amount of enzyme taken up by E2 is greater than that seen with E1, but the differences were not in

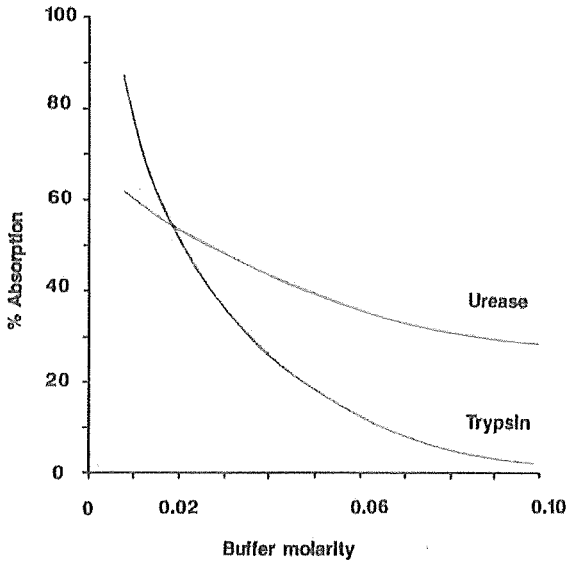

Figure 5 *Immobilization of trypsin and urease on Biofix E1. The support was activated with 25 mL of 2.5 wt % glutaraldehyde per g of support (phosphate buffer, pH 7). After washing, an enzyme concentration of 1 mg per mL of buffer was used and the protein remaining in solution assigned by UV spectrophotometry.*

Table 3

γ-aminopropyl silane treated samples of Biofix E1 and E2

Sample	Bound protein (mg/g dry weight of support) Lysozyme	Lipase
E1	18.4 ± 0.9	13.7 ± 1.7
	16.6 ± 2.0	16.1 ± 1.5
E2	22.4 ± 0.6	23.8 ± 0.4
	19.2 ± 1.8	30.2 ± 2.4

Table 4

Amount of lipase bound to γ-aminopropyl silane-treated samples[a] of Biofix E1 and E2, together with the % conversion of Citronellol to Citronellyl butyrate after 24 hr

Sample	Bound lipase (mg/g dry wt support)	% Conversion
E1	31.6 ± 0.1	69.2 ± 3.7
	32.7 ± 1.0	51.0 ± 2.4
E1 (stored 100 days in refrigerator)		26 ± 2
E2	41.4 ± 0.9	60.8 ± 7.3
	41.3 ± 1.0	51.1 ± 0.1

[a] The treatment method employed here differed from that used to collect the data shown in Table 3.

proportion to the surface areas of the two solids (*Table 4*).

Experiments to test the activity of the immobilized enzyme were carried out soon after preparation of the reaction column and again after the column had been stored in a refrigerator. Conversions achieved with E1 supports were similar to those given by E2, even though E2 bound more enzyme (Table 3). The stored columns showed reasonable long term stability, with retention of 50% of the initial activity after three months.

Overall, the most obvious advantage of using an immobilized enzyme for this type of system is not only the high conversions achieved, but rather that the formation of a thick cream reaction mixture (with the consequent product separation problems) is avoided.

Summary

Immobilized biocatalysts have many advantages. The new range of materials described exhibit high mechanical strength, chemical and thermal resistance, and lack of toxicity. Future applications are seen for these materials in a variety of biotechnological and other applications, such as in selective removal of products from reactor broths and in water purification.

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