

# New materials and technology for cell immobilization

Gary J. Salter and Douglas B. Kell

University College of Wales, Aberystwyth, Dyfed, UK

The choice of support materials for immobilizing cells is rapidly expanding. The literature that has appeared over the past year suggests that hydrogels will remain the first choice for the foreseeable future, even though they are associated with many widely recognized problems. There is increasing interest in the use of tougher polymeric materials, and especially of inorganic ceramic supports. However, the most suitable cell support can be selected only after the process or form of reactor in which it is to be used has been assessed.

Current Opinion in Biotechnology 1991, 2:385–389

## Introduction

The advantages of immobilized or heterogeneous biocatalysts over those that are 'free' in solution are well known [1], and include stability, reusability, convenience in continuous operation, and volumetric productivity. Some organisms or consortia naturally form self-immobilizing aggregates [2,3]. However, this type of behaviour is very much the exception and for the past decade or more, a large variety of supports and methods of immobilizing biocatalysts have been developed. The field has been dominated by the use of 'hydrogels' such as calcium alginate and  $\kappa$ -carrageenan, particularly for the immobilization of intact cells. This may largely be ascribed to their general ease of preparation and use and their compatibility with a large range of biocatalysts.

A number of disadvantages are associated with the use of hydrogels, however. Firstly, such systems do not conveniently lend themselves to scale-up, as cells must first be harvested from the growth medium, and the production of gel beads is a rather time-consuming and intricate process. Secondly, the nature of the particles used can affect the performance of a continuous reactor. For instance, small particles are associated with pressure drops, and large particles with substrate/product diffusion limitations [4]. The mechanical stability of reactors is typically low because tall packed-bed reactors result in compression and sometimes disintegration of particles, and fluidized-bed or stirred-tank reactors may result in abrasion [4]. Furthermore, if cell growth is required then the particles will have a limited life before they are full [5] or internally disrupted. In some cases the production of gas may disrupt gel particles [5] or result in bubbles that exclude the substrate [4].

A third problem is that, in the case of calcium alginate, substances with even a modest affinity for  $\text{Ca}^{2+}$  will sequester the crosslinking  $\text{Ca}^{2+}$  ions, destabilizing the gel [6]. Additionally, some alginates contain small amounts of polyphenols which may harm sensitive cells [6]. A further limitation is that hydrogels (although not calcium

alginate [7]) are generally unstable when used with organic solvents [6]. Finally, the limited diffusion afforded by gels makes this method unsuitable for use with very high molecular weight or insoluble substrates or products [5].

## Future of hydrogels

Despite the limitations described above, hydrogels look set to remain the major method used for cell immobilization in the foreseeable future. Various methods have been used to tackle the problems associated with hydrogels. Smidsrød and Skjåk-Braek [6] have suggested that high mechanical and chemical stability, controllable swelling properties, low content of toxic, pyrogenic and immunogenic contaminants, defined pore size and a narrow pore size distribution may be achieved by careful selection and purification of alginates, selection and control of the gelling process, and addition of co-polymers. In a study of the correlation between mechanical gel strength and the affinity of cations, it was shown that the rigidity of alginates can be improved significantly by the use of  $\text{Zn}^{2+}$ ,  $\text{Sr}^{2+}$ ,  $\text{Ba}^{2+}$ ,  $\text{Al}^{3+}$  and  $\text{Fe}^{3+}$  [6,8]. Indeed, Kim *et al.* [9] have found that cells entrapped in strontium alginate are superior to those in calcium alginate, in terms of relative activity.

The rates of glucose uptake and ethanol and glycerol production by *Saccharomyces cerevisiae* immobilized in calcium alginate, have been shown to be up to twice those exhibited by planktonic cells, as estimated from *in vivo*  $^{31}\text{P}$ -nuclear magnetic resonance measurements. Together with enzyme kinetic parameters, this information has permitted a proper metabolic control analysis to be carried out, producing a quantitative description of the factors controlling the flux of carbon towards ethanol production [10]. This increased productivity of ethanol appears to be a frequent occurrence when cells are immobilized. Hilge-Roumann and Rehm [11] suggest that, rather than being an effect of cell immobilization *per se*,

## Abbreviation

PVA—poly-(vinyl alcohol)

this observation is a result of the physiological changes that accompany cell aggregation.

Problems that arise when using gels, particularly within fixed-bed reactors, have led to a substantial interest in the development of methods for strengthening gels. Thus, Chamy *et al.* [12••] have used  $\text{Al}(\text{NO}_3)_3$  as an agent for hardening  $\kappa$ -carrageenan beads used in a packed bed. They found that hardening decreased bead compaction significantly, leading to a reduction in the pressure drop through the column, and thus reducing the risk of mechanical stress. Cell retention within the column was also improved, resulting in a 20 % increase in column productivity. De Alteriis *et al.* [13••] state that gelatin gels insolubilized by crosslinking with formaldehyde have mechanical properties superior to gels composed of calcium alginate,  $\kappa$ -carrageenan and agar. However, as is often the case, the cost of this increase in mechanical strength is a reduction in mass transfer properties.

Gels containing immobilized cells are now being applied to a wide variety of problems. Of the numerous papers describing the more conventional uses of such immobilized cells for the production of commercially interesting materials, we will only mention that by Vandamme [14••] on antibiotics. Svoboda and Ourednicek [15•] have used sheets of calcium alginate gel to promote cell wall regeneration of yeast protoplasts. This can be achieved because the gel prevents the more soluble cell wall components, such as glycoproteins, from diffusing away from the cell. This technique has important implications for cytology, genetics and biotechnology. In addition, protoplasts may represent the only suitable form of some organisms for immobilization, for example mycelial, clump-forming, non-asexual, spore-forming fungi such as *Sclerotium rolfsii* [16]. Meanwhile, Hocknull and Lilly [7•] have shown that calcium alginate is a suitable support for use in the presence of a range of organic solvents with widely different log P values, reporting a 7.5-fold increase in enzyme (steroid  $\Delta_1$ -dehydrogenase) stability compared with that of free cells exposed to the same concentration of solvent.

## Other polymers

A large number of polymers have been used to aid or to effect cell immobilization. Such polymers may be used alone as the cell support, in a bead or cube, membrane, fibre, or a larger scale 'native matrix' form, or in combination with another support material, to increase the overall strength or possible biomass loading of a support.

### Beads

Many polymers have been used in bead form, including: polymers to which a cell suspension can be added prior to polymerization, resulting in cell entrapment; solid beads with a functional surface to which cells will attach; and inert beads that can be surface-modified to allow cell attachment, typically by crosslinking.

Varfolomeyev *et al.* [17•] have successfully used poly(vinyl alcohol) (PVA) cryogels to entrap thermophilic microorganisms. These carriers are highly porous,

mechanically stable and biologically inert. They can be used at temperatures of up to 65°C and at pH values of 1–11, and are formed by a simple freeze-thaw procedure. Surface immobilization of *E. coli* has been achieved on crosslinked poly(N-benzyl-4-vinylpyridinium bromide) containing styrene (BVPS) resin by Kawabata *et al.* [18•]. Kiremitci and co-workers [19•] have also demonstrated that inert polymeric microcarriers of poly-hydroxyethylmethacrylate and polystyrene used with various surface modifications are suitable for the growth of anchorage-dependent baby hamster kidney cells.

### Cubes

The use of cubes is typically confined to those polymers that require cutting to a suitable size and shape. These include pre-formed polymers such as sponges, or those that are polymerized into large blocks and later cut into smaller ones, for example urethane prepolymer (PU-3) [20]. In a detailed study, Kautola and Linko [21•] found that fumaric acid production from xylose by *Rhizopus arrhizus* mycelium immobilized in polyurethane foam cubes gave a stoichiometric yield 3.5 times higher than that of free cells.

### Membranes

Membranes are used in many forms and arrangements for cell immobilization. Yoshikawa *et al.* [22] have used a membrane of polyvinylidene fluoride for the production of *cis,cis*-muconic acid using a mutant strain of an *Arthrobacter* species. However, as is often the case with membrane reactors utilizing growing cells, the membrane was blocked by excess cells after 100 h. This problem of cell overgrowth has been solved by Kniebusch *et al.* [23••] who used an asymmetric, porous gas-permeable membrane of polyetherimide separating an oxygen-containing 'gas' compartment from a liquid compartment; this allowed a stable biofilm to be maintained.

### Fibres

To date, fibres have been used very little in cell immobilization, despite the obvious advantages that they have over some other systems, with respect to their long exploitation in the textile industry. Ichijo and co-workers [24••] review the studies they have carried out over a number of years, of the uses and effectiveness of PVA 'superfine fibres' and photocrosslinkable PVA. They conclude that these materials make excellent immobilization supports and can be surface-modified in a number of ways to allow maximal binding of the biocatalyst. These supports can also be produced in a number of other shapes such as particles or films, and the fibres can also be combined to make more complex forms of supports such as filter paper or knitted fabric. A bioreactor using braided fibres was found to have a high activity and was free of substantive pressure drops, despite cell proliferation. Kang *et al.* [25•] have used commercially available hollow fibres of hydrophobic and hydrophilic nature, to produce ethanol using yeast. In addition, Bunch *et al.* [26•] have compared hollow fibres of polysulphone with batch culture for lactic acid production.

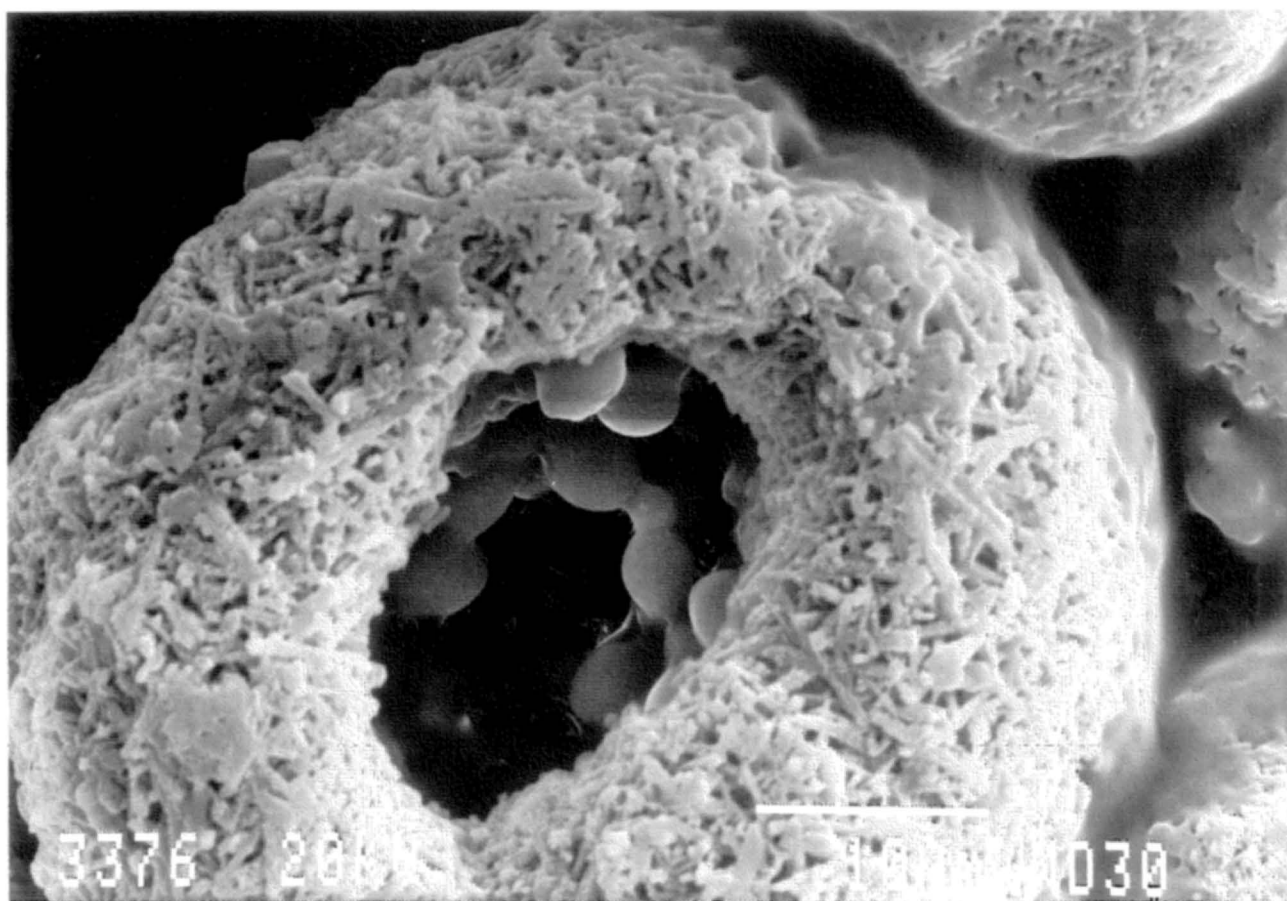


Fig 1. A porous, ceramic microsphere containing cells of *Saccharomyces cerevisiae*, as described in [30••].

#### Native matrices

Many polymers can be formed into a loose matrix suitable for cell immobilization, although care must be taken to select the ideal form, especially if growing cells are to be used. Loose or open matrices tend to retain low levels of biomass, whereas tighter matrices tend to suffer from cell accumulation causing clogging of the reactors. Lin *et al.* [27•] have found that reactors containing *Zymomonas mobilis* immobilized within a natural sponge develop problems with cell clogging after only 4 days of use. By contrast, Amin and Doelle [28•] have demonstrated that long-term, continuous production of ethanol can be achieved by *Z. mobilis* immobilized within polyurethane foam in a novel 'vertical rotating immobilized cell reactor'. In this configuration, foam disks are held between two perforated metal sheets to form an immobilized biomass unit, with 10 of these units being fixed to a rotating shaft within the bioreactor.

#### Miscellaneous polymers

The use of polymers in association with other materials is becoming a common method for the production of cell supports. Flanagan *et al.* [29•] have immobilized *Penicillium chrysogenum* using polyvinyl acetate/acrylic copolymer latex coatings applied to

porous silica particles. We, together with our colleagues [30••], have demonstrated that *Saccharomyces cerevisiae* can be bound to hollow ceramic microspheres using surface treatments with cationic polymers. Minor stability problems were encountered with this system, but these have now been solved (GJ Salter and DB Kell, unpublished data). Carenza *et al.* [31••] have reviewed the use of low-temperature, radiation-induced polymerization of glass-forming monomers for cell immobilization. Many biocatalysts have been immobilized successfully by this method, including chloroplasts, yeasts, bacteria and erythrocytes.

#### Inorganic supports

Inorganic supports generally have one major advantage over other materials, namely, their toughness. Most inorganic supports are totally inert, immune to temperature, pH, chemicals, microbial degradation, and highly resistant to crushing or abrasion. Given that they do not normally have to be produced by the end-user and may well be naturally occurring (e.g. sand used in methanogenic fluidized beds) [32], the inorganic supports also lend themselves more conveniently to scale-up.

### Porous glass

Sintered and porous glass in many forms, including controlled-pore glass, is available from many commercial sources. Using sintered glass in the form of 'Raschig rings', Hecker *et al.* [33•] have carried out a glycerol fermentation of high volumetric productivity using yeast, achieving a constant rate of production for nearly 9 months. Controlled-pore glass beads have also been used to advantage in the culture of animal cells [34•].

### Ceramics

Ceramics can be produced in a variety of forms, from membranes to microspheres, and have been used with all forms of biocatalysts. Bunch *et al.* [26•] have used ceramic hollow fibres to produce lactic acid, and Cornwell *et al.* [35] have used a commercial form of porous ceramic (alumina) spheres, normally used as a support for heterogeneous chemical catalysts, as a reusable support to immobilize *Phanerochaete chrysosporium* expressing lignin peroxidase. In our laboratory, we have demonstrated a novel method of cell immobilization, using highly microporous ceramic microbeads containing a single, large mesopore, where the shape of the particle results in hydrodynamic forces depositing cells within the mesopore of the rigid carrier [30••]. A column of such particles (Fig. 1) was used to produce ethanol from *S. cerevisiae* in high stoichiometric and volumetric yield. This paper also describes a novel, electronic method for the on-line, real-time estimation of biomass accretion in immobilized cell cultures. Manville Corporation [P1•] disclose a related type of immobilization matrix (but not method).

### Conclusion

There is much continuing interest in the use of hydrogels, such as alginate, for immobilizing cells. A major thrust is directed towards improving their mechanical properties. The future will also see an increased trend towards the exploitation of the desirable mechanical and other properties of inorganic immobilization matrices.

### References and recommended reading

Papers of special interest, published within the annual period of review, have been highlighted as:

- of interest
- of outstanding interest

1. MATTIASSEN B (ED): Immobilized Cells and Organelles (Vols I and II) [book]. Boca Raton, Florida, USA: CRC Press, 1983.
2. MACLEOD FA, GUIOT SR, COSTERTON JW: Layered Structure of Bacterial Aggregates Produced in an Upflow Anaerobic Sludge Bed and Filter Reactor. *Appl Environ Microbiol* 1990, 56:1598-1607.
3. CHRISTENSEN R, GONG CS, TANG R, CHEN L, RIMEDIO N: A Multi-chamber Tower Fermentor for Continuous Ethanol Fermentation with a Self-Aggregating Yeast Mutant. *Appl Biochem Biotechnol* 1990, 24/25:603-612.

Long-term (460 h) ethanol fermentation is achieved with a self-aggregating yeast mutant. Very few ( $< 10^3 \text{ ml}^{-1}$ ) 'free' cells are observed.

4. D'SOUZA SF: Immobilized Cells: Techniques and Applications. *Indian J Microbiol* 1989, 29:83-117.
5. BIRNBAUM S, LARSSON P-O, MOSBACH K: Immobilized Cells. In *Solid Phase Biochemistry* edited by Scouten WH [book]. New York: John Wiley 1983, pp 679-762.
6. SMIDSRØD O, SKJÅK-BRAEK G: Alginate as Immobilization Matrix for Cells. *Trends Biotechnol* 1990, 8:71-78.
- Useful and comprehensive review of approaches aimed at improving the properties of alginates.
7. HOCKNULL MD, LILLY MD: The Use of Free and Immobilised *Arthrobacter simplex* in Organic Solvent/Aqueous Two-Liquid-Phase Reactors. *Appl Microbiol Biotechnol* 1990, 33:148-153.
- Substantial differences in the effects of solvent polarity on the productivity of free versus immobilized cells are observed. Long-term and high-purity steroid production is demonstrated with immobilized cells.
8. OGBONNA JC, PHAM CB, MATSUMURA M, KATAOKA H: Evaluation of Some Gelling Agents for Immobilization of Aerobic Microbial Cells in Alginate and Carrageenan Gel Beads. *Biotechnol Techniques* 1989, 3:421-424.
- $\text{SrCl}_2$  gelling was found to be the best gelling agent for both alginate and carrageenan, with respect to cell leakage and the diffusion coefficients of glucose and  $\text{O}_2$ .
9. KIM BW, KIM IY, CHANG HN: Bioconversion of Hydrogen Sulphide by Free and Immobilized Cells of *Chlorobium thiosulfatophilum*. *Biotechnol Lett* 1990, 12:381-386.
- Bioconversion of hydrogen sulphide is more greatly enhanced by strontium alginate relative to either calcium alginate or to free cells.
10. GALAZZO J, BAILEY JE: Fermentation Pathway Kinetics and Metabolic Flux Control in Suspended and Immobilized *Saccharomyces cerevisiae*. *Enzyme Microb Technol* 1990, 12:162-172.
- A detailed, quantitative study of factors controlling the flux to ethanol in free and immobilized cells, showing that alginate entrapment increases the rate of glucose uptake and shifts the most rate-limiting step of ethanologenesis to phosphofructokinase availability.
11. HILGE-ROTMANN B, REHM H-J: Comparison of Fermentation Properties and Specific Enzyme Activities of Free and Calcium-Alginate-Entrapped *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* 1990, 33:54-58.
12. CHAMY R, NUNEZ MJ, LEMA JM: Optimization of the Hardening Treatment of *S. cerevisiae* Bioparticles. *Enzyme Microb Technol* 1990, 12:749-754.
- Describes the successful use of  $\text{Al}(\text{NO}_3)_3$  as a hardening agent for  $\alpha$ -carrageenan gels.
13. DE ALTERIIS E, SCARDI V, MASI P, PARASCANDOLA P: Mechanical Stability and Diffusional Resistance of a Polymeric Gel Used for Biocatalyst Immobilization. *Enzyme Microb Technol* 1990, 12:539-545.
- The mechanical properties of gelatin gels are improved by crosslinking with formaldehyde.
14. VANDAMME EJ: Biochemical, Genetic Biotechnical Aspects of Antibiotic Production Via Immobilised Biocatalysts. In *Biochemistry of Peptide Antibiotics* edited by Kleinkauf H, von Doren H, [book]. Berlin: Walter de Gruyter 1990, pp 379-410.
- Everything one might wish to know about the application of immobilized cells for the production of antibiotics.
15. SVOBODA A, OUREDNICEK P: Yeast Protoplasts Immobilized in Alginate: Cell Wall Regeneration and Reversion to Cells. *Curr Microbiol* 1990, 20:335-338.
- Much better reversion to cells is achieved when protoplasts are immobilized.
16. KEIKAR HS, SHANKAR V, DESHPANDE MV: Rapid Isolation and Regeneration of *Sclerotium rolfsii* Protoplasts and their Potential Application for Starch Hydrolysis. *Enzyme Microb Technol* 1990, 12:510-514.
17. VARFOLOMEYEV SD, RAININA EI, LOZINSKY VI, KALYUZHNIN SV, SINTSYN AP, MAKHLIS TA, BACHURINA GP, BOKOVA IG,

- SKLYANKINA OA, AGAFONOV EB: Application of Polyvinyl Alcohol Cryogels for Immobilization of Mesophilic and Thermophilic Microorganisms. In *Physiology of Immobilized Cells* edited by de Bont JAM, Visser J, Mattiasson B, Tramper J [book]. Amsterdam: Elsevier 1990, pp 325–330.
- Review of the stated approach and the excellent properties of this immobilization matrix.
18. KAWABATA N, NISHIMURA S, YOSHIMURA T: New Method of Immobilization of Microbial Cells by Capture on the Surface of Insoluble Pyridinium-Type Resin. *Biotechnol Bioeng* 1990, 35:1000–1005.
- Describes the production of L-aspartate from ammonium fumarate in a quantitative yield and with high volumetric productivity.
19. KIREMITCI M, OZILGEN M, PISKIN E: Attachment and Growth Kinetics of Anchorage-Dependent BHK Cells on Microcarriers. *Enzyme Microb Technol* 1989, 11:830–836.
- Typical carriers are found to contain only a finite number of attachment sites for anchorage-dependent baby hamster kidney cells.
20. NATH PK, IZUMI Y, YAMADA H: NADH Production From NAD<sup>+</sup> with a Formate Dehydrogenase System Involving Immobilized Cells of a Methylophilic *Arthrobacter* Strain. *Enzyme Microb Technol* 1990, 12:28–32.
21. KAUTOLA H, LINKO Y-Y: Production of Fumaric Acid with Immobilized Biocatalysts. *Appl Biochem Biotechnol* 1990, 24/25:161–170.
- Polyurethane foam cubes are an excellent support for the production of fumaric acid with immobilized biocatalysts.
22. YOSHIKAWA N, MIZUNO S, OHTA K, SUZUKI M: Microbial Production of *Cis,cis*-Muconic Acid. *J Biotechnol* 1990, 14:203–210.
23. KNIEBUSCH MM, WILDERER PA, BEHLING R-D: Immobilization of Cells at Gas-Permeable Membranes. In *Physiology of Immobilized Cells* edited by de Bont JAM, Visser J, Mattiasson B, Tramper J [book]. Amsterdam: Elsevier 1990, pp149–160.
- Micro-organisms of interest are immobilized on an asymmetric, porous, gas-permeable membrane separating an oxygen-containing compartment from a liquid compartment.
24. ICHIO H, NAGASAWA J, YAMAUCHI A: Immobilization of Biocatalysts with Poly(vinyl alcohol) Supports. *J Biotechnol* 1990, 14:169–178.
- Excellent review of the longstanding and pioneering work of this group on a variety of PVA supports for whole-cell immobilization.
25. KANG WK, SHUKLA R, SIKKAR KK: Ethanol Production in a Microporous Hollow-Fibre-Based Extractive Fermenter with Immobilized Yeast. *Biotechnol Bioeng* 1990, 36:826–833.
- Successful use of hollow-fibre reactors with low cell leakage in a yeast/ethanol model system.
26. BUNCH AW, HIGTON G, BALL J, KNOWLES CJ: The Physiological State of Cells Immobilized in Hollow-Fibre and Ceramic Membranes. In *Physiology of Immobilized Cells* edited by de Bont JAM, Visser J, Mattiasson B, Tramper J [book]. Amsterdam, Elsevier 1990, pp 127–135.
- Comparative study of hollow-fibre and ceramic membranes, the latter performing better but the former being easier to work with.
27. LIN JJ, DALE MC, OKOS MR: Ethanol Production by *Zymomonas mobilis* in an Immobilised Cell Reactor Separator. *Process Biochem* 1990, 25:61–66.
- Use of an integrated reactor-separator for ethanol production, with cells immobilized in sponge or in tery cloth.
28. AMIN G, DOELLE HW: Production of High Ethanol Concentrations from Glucose Using *Zymomonas mobilis* Entrapped in a Vertical Rotating Immobilized Cell Reactor. *Enzyme Microb Technol* 1990, 12:443–446.
- A novel configuration with high volumetric productivity is described.
29. FLANAGAN W, KLEI HE, SUNDSTROM DW, LAWTON CW: Optimization of a Pellicular Biocatalyst for Penicillin G Production by *Penicillium chrysogenum*. *Biotechnol Bioeng* 1990, 36:608–616.
- Describes the month-long production of penicillin G in an immobilized cell reactor, with significantly improved productivity over the traditional batch method.
30. SALTER GJ, KEIL DB, ASH IA, ADAMS JM, BROWN AJ, JAMES R: Hydrodynamic Deposition: a Novel Method of Cell Immobilization. *Enzyme Microb Technol* 1990, 12:419–430.
- Passage of dilute cell suspensions through columns of appropriately designed ceramic particles results in cell immobilization. Cell growth was monitored electronically *in situ*. Ethanol production was achieved with high volumetric productivity, using *S. cerevisiae*.
31. CARENZA M, FUJIMURA T, KAETSU I: Radiation Cured Particulates for Cell Culture Supports. *Chimicaoggi* 1990, 8:41–49.
- Reviews cell immobilization by low-temperature radiation-induced polymerization of glass-forming monomers based on acrylic and methacrylic esters.
32. LAUWERS AM, HEINEN W, GORRIS LGM, VAN DER DRIFT C: Variation of Parameters Affecting the Start-up of Methanogenic Fluidized-Bed Reactors. *Biotechnol Lett* 1989, 11:907–912.
33. HECKER D, BISPING B, REHM H-J: Continuous Glycerol Production by the Sulphite Process with Immobilized Cells of *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* 1990, 32:627–632.
- For a given productivity of ethanol the dilution rates obtained were four-fold higher using immobilized *S. cerevisiae* cells than in free cell continuous cultures.
34. LOOBY D, GRIFFITHS B: Immobilization of Animal Cells in Porous Carrier Culture. *Trends Biotechnol* 1990, 8:204–209.
- Useful review, stressing advantages of porous carriers for achieving high cell densities in animal cell systems.
35. CORNWELL KL, TINALD-BUTEZ M-F, TARDONNE PJ, CABASSO I, HANMEL K: Lignin Degradation and Lignin Peroxidase Production in Cultures of *Phanerochaete chrysosporium* Immobilised on Porous Ceramic Supports. *Enzyme Microb Technol* 1990, 12:916–920.

### Annotated patents

- of interest
  - of outstanding interest
- P1. MANVILLE CORPORATION: Preparation of a Catalyst Carrier for Immobilizing Microbial Cells by Forming Pellets Containing Diatomaceous Earth, Binder and Burn-out Material, and Calcining the Pellets. 1989, UK Patent 2185908.
- A method for producing porous inorganic supports for use with whole cells is disclosed.

GJ Salter and DB Kell, Department of Biological Sciences, University College of Wales, Aberystwyth, Dyfed SY23 3DA, UK.