New materials and technology for cell immobilization

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The choice of the most effective manner in which to operate an immobilized cell system is both complicated and, to some extent, a matter of guesswork. There is increasing awareness of the factors affecting reactor choice, and present work is aimed at making reactor performance more predictable.

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Introduction

Immobilized cell technology has traditionally been focused around three main areas of interest: first, the development of new or improved support materials; second, new applications and products; and third, the engineering of new or improved immobilized cell reactors. Whilst many interesting and important advances continue to be made in these areas, it should be noted that the basic understanding and control of the systems currently in use is often poor. For example, if we simply consider the biomass immobilized within a reactor, it would be unusual for the total amount of biomass immobilized to be known, whilst the numbers of viable cells would almost certainly be unknown; what then of the more difficult variables such as the physiological state of the cells or the microenvironment around them? If such a basic understanding is missing, how are we to exploit this technology to its full?

A cursory glance at the current journals would reveal that an improved understanding of the basic principles of immobilized cell technology is rapidly becoming the main area of interest. In a recent article by Spalding [1•], it was acknowledged that many technical problems with bioreactors have yet to be solved, that reactors used in the laboratory often cannot be scaled up for industrial use, and that each reactor gives different results, depending on the cell-line and the product the cell is making. Thus, it is now readily accepted that an understanding of the basic principles is needed before immobilized cell technology can be used on a major scale in industry.

To this end, this review will discuss both the traditional areas of reactor and cell support development, and the rapidly growing fields of immobilized cell physiology and reactor kinetics, with somewhat more stress being laid on the latter.

Cell supports and reactors

Immobilization methods

The immobilization of microbial cells often relies simply on the adsorption of cells onto the support. An excellent review of this has been written by Klein and Ziehr [2•] discussing the adsorption process, the strength of adhesion, the activity of the biocatalyst and the operational stability of immobilization techniques presently in use. In a study of adsorption rates of *Pseudomonas putida* onto various supports, Shreve *et al.* [3••] found that supports that have a surface charge (either negative or positive) developed the highest biomasses, typically 2–3 times that found on hydrophobic surfaces, whilst hydrophilic (but uncharged) supports tested were essentially ineffective at adsorbing cells.

A common alternative to simple cell adsorption is the use of a suitable chemical to bind the cells to a support. Of the many methods available, the use of poly(ethylene imine) is becoming especially popular; this is clearly demonstrated in a review on the subject by Bahulekar *et al.* [4••]. D'Souza and Melo [5•] have successfully co-immobilized glucose oxidase and *Saccharomyces cerevisiae* onto cotton thread using poly(ethylene imine). The use of poly(ethylene imine) within the immobilized cell industry could assume great importance, because unlike most chemical agents used, such as glutaraldehyde, it has been cleared by the US Food and Drug Administration as a 'secondary direct food additive' under the Federal Food, Drug and Cosmetic Act [4••].

Supports

New support materials are constantly being developed; recent examples of interest include 'aphrocell' [6•], which is a new type of ceramic carrier that has a

Abbreviation

MSFB — magnetically-stabilized fluidized bed.

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continuous pore structure, and aerenchyma cells [P1•] from a plant tissue composed of unthickened, irregularly shaped cells. Fungal cell wall particles containing hexosamine [P2•] have also been used to promote cell flocculation and aggregation.

Reactors

Although several formats have been scaled-up to pilot or industrial sizes, many problems remain. Thus, there is a continuing need to explore the engineering, and possible control methods, of established bioreactor types, as well as to assess new formats. Whereas many of these new ideas are not suitable for scale-up, or are impractical for 'normal' applications, there still remains a need for specialist reactors and control methods, typically for producing low volumes of high cost chemicals.

The immobilization of plant cells has proved to be problematical in many cases. Productivity may also be compromised by the fact that most of the products of interest are retained within the cell vacuole. Thus, many novel reactors aimed specifically at plant cells continue to be developed. Kargi *et al.* [7] have explored the potential of a biofilm reactor using *Catharanthus roseus* cells to produce indole alkaloids, whereas Lang *et al.* [8•] have used a flat sheet membrane reactor, where cells inoculated onto the polypropylene membrane grow to form an even, callus-like layer. Lang *et al.* [8•] claim that *Coffea arabica* cells immobilized using this method produce a ninefold increase in purine alkaloids per gram dry weight of cells per day, compared with suspension cultures.

Facchini and DiCosmo [9•,10] have immobilized *Thalictrum rugosum* and *C. roseus* cells by adsorption onto glass fibres, the reactor containing 10 glass fibre mats held 2 cm apart on a central rod within a glass cylinder. Unfortunately both *T. rugosum* (producing protoberberine alkaloids) and *C. roseus* (producing indole alkaloids) showed reduced productivities compared with suspension cultures. By contrast, Chiou *et al.* [11••] used glass fibers within an unusual packed-bed reactor to culture anchorage-dependent animal cells very successfully over a period of 66 days.

Bramble *et al.* [12••] have cultured cells of *C. arabica* within a magnetically-stabilized fluidized bed (MSFB). Cells were immobilized within alginate beads, the beads being magnetized by the inclusion of magnetite (Fe_3O_4). Although in this instance the production of theobromine and caffeine was less than that from cell suspensions, it is believed that MSFBs have advantages in terms of mass transfer and controlled cell residence time for the continuous cultivation of immobilized plant cells. Terranova and Burns [13•] have successfully used MSFB technology with nickel spheres to filter yeast cells with high efficiency.

Silbiger and Freeman [14••] reported increased conversion of hydrocortisone to prednisolone (using *Arthrobacter simplex*) by the use of a water-miscible co-solvent (5% (v/v) triethyleneglycol). The use of a co-solvent resulted in a five fold increase in solubility of the substrate. The product, also poorly soluble in aqueous media, was recovered by the use of microcrystalline cellulose powder suspended within the reactor as precipitation nuclei. This resulted in a decreased product inhibition and reduced the number of purification steps.

Cell physiology and reactor kinetics

Very few studies have attempted to provide anything approaching a complete understanding of the processes that occur within a reactor. The vast majority of articles have addressed but a single parameter or variable, such as mass transfer or the alterations in some aspect of cell physiology that accompanies immobilization. By combining and correlating such studies, however, it is possible to build up an understanding of what is occurring within an immobilized cell reactor.

Cell physiology and diffusion studies

It has often been stated that immobilized yeast cells demonstrate altered ethanol productivities and tolerances relative to free cells. In an excellent paper by Hilge-Rotmann and Rehm [15••] this was correlated with an increase in the ratio of saturated to unsaturated fatty acids in the plasma membrane. Hannoun and Stephanopoulos [16•] attributed the increase in specific glucose uptake and specific ethanol production to reduced specific growth rates of immobilized cells. Cell-growth rate was strongly linked to the concentration of dissolved oxygen, because fatty acids and sterols essential for growth are synthesized only in the presence of oxygen. In a detailed study of polyol production and enzyme activity by *Pichia farinosa*, Hootmann *et al.* [17•] similarly found an increase in the products glycerol and arabitol per gram of glucose consumed when cells were immobilized. This effect was attributed to the enhanced water stress (accompanying immobilization) that is known to stimulate polyol production in yeasts.

Chun and Agathos [18•] found that the growth rate of the fungus *Tolypocladium inflatum* was increased by immobilization onto celite. This resulted in a higher specific production of cyclosporin A, which is normally growth-associated. This could be further increased by the addition of L-valine to the medium during late exponential growth. Interestingly, if L-valine was added early in the fermentation, increased cell growth was stimulated at the expense of product formation, demonstrating that the relationship between rates of growth and product formation can be rather variable.

The complex physiology of hybridoma cells makes them difficult to cultivate, especially when they produce toxic metabolites. Hagedorn and Kargi [19•] have

shown that by using the correct molecular weight cut-off membrane within a flat sheet membrane reactor, the continuous removal of toxic metabolites and the addition of nutrients may be achieved. This results in higher cell concentrations and specific antibody productivities.

Mass-transfer

Mass-transfer problems are usually associated with the use of cell entrapment techniques, although in practice they are just as likely to be encountered with suspension cultures in poorly designed reactors, especially if gases (usually oxygen) are required by the cells.

Diffusion within gels has been the subject of considerable interest for some time. In a recent review by Westrin and Axelsson [20••], 11 experimental investigations of diffusion in gels containing immobilized cells were analyzed. Based on this, a procedure for the prediction of effective diffusion coefficients in cell-containing gels was recommended. Similarly, for the estimation of diffusion coefficients within gels, Petersen *et al.* [21•] offered a bead method that was claimed to minimize the errors usually associated with this technique. It was claimed that the use of this comparatively simple method reduced errors that typically ranged from some 29% to over 600%, to those accepted using other methods (3–8%).

Kang *et al.* [22•] addressed the problem of improving oxygen transfer in a three-phase fluidized-bed bioreactor. This was achieved successfully (and modelled) by the addition of floating 'bubble breakers' to the reactor. The results indicated that the liquid-phase volumetric oxygen transfer coefficient (k_La) could be improved by 20–25%. Petersen and Davison [23] have also modelled a three-phase fluidized-bed bioreactor; their model demonstrated that the CO_2 generated by the microbial fermentation of glucose caused a great deal of backmixing in the top of the tapered-bed reactor. Thus reactor yields and productivities would be increased if a plug-flow system could be more closely approximated. Similarly, Livingston [24] has successfully modelled the diffusion and reaction of 3,4-dichloroaniline and oxygen within a draft-tube fluidized-bed reactor containing a mixed culture immobilized on celite.

An interesting study has been performed by Kimmel *et al.* [25•] on the mass-transfer and scale-up parameters of a trickle-bed reactor. The fermentation involved the use of gas-phase substrates (CO, H_2 and CO_2) by three strict anaerobes in a tri-culture to produce methane, the calculated yield achieved being 80% of that theoretically possible.

Conclusion

A great deal of work still remains to be done if we are to understand fully and exploit reactors that employ immobilized cells. It is clear, however, that inroads into the removal of remaining lacunae are being made. Models have now been validated for a large variety of reactor types. Such findings strengthen the confidence with which these models may be employed in the design and operation of such biological reactors.

References and recommended reading

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

- • of special interest
- •• of outstanding interest

1. • SPALDING BJ: **A Growth Market for Bioreactors.** *Bio/technology* 1991, **9**:338–341.
Comments on the state of the bioreactor industry and the problems of scale-up.

2. • KLEIN J, ZIEHR H: **Immobilization of Microbial Cells by Adsorption.** *J Biotechnol* 1990, **16**:1–16.
A mini-review dealing with many aspects of microbial adsorption onto a wide range of inorganic supports.

3. •• SHREVE GS, OLSEN RH, VOGEL TM: **Development of Pure Culture Biofilms of *P. putida* on Solid Supports.** *Biotechnol Bioeng* 1991, **37**:512–518.
Comparison of biofilm development and accumulation on two classes of support materials: charged surfaces and non-charged hydrophobic and hydrophilic surfaces. The effects of surface roughness and porosity were also examined.

4. •• BAHULEKAR R, AYYANGAR NR, PONRATHNAM S: **Polyethyleneimine in Immobilization of Biocatalysts.** *Enzyme Microb Technol* 1991, **13**:858–868.
Comprehensive review of the use of poly(ethylene imine) to immobilize both enzymes and whole cells on or within a variety of supports including hydrogels.

5. • D'SOUZA SF, MELO JS: **A Method for the Preparation of Coimmobilizates by Adhesion using Polyethyleneimine.** *Enzyme Microb Technol* 1991, **13**:508–511.
The use of poly(ethylene imine) to co-immobilize glucose oxidase and *S. cerevisiae* onto cotton thread, to convert sucrose to fructose and gluconic acid.

6. • SUEKI M, KOBAYASHI N, SUZUKI A: **Continuous Acetic Acid Production by the Bioreactor System Loading a New Ceramic Carrier for Attachment.** *Biotechnol Lett* 1991, **13**:185–190.
Assessment of a new ceramic carrier within a packed column for the production of acetic acid.

7. KARGI F, GANAPATHI B, MARICIC K: **Indole Alkaloid Formation by *Catharanthus roseus* Cells in a Biofilm Reactor.** *Biotechnol Prog* 1990, **6**:243–248.

8. • LANG JA, YOON K-H, PRENOSIL JE: **Effect of Permeate Flux Rate on Alkaloid Production in a Novel Plant Cell Membrane Reactor Using *Coffea arabica* Cells.** *Biotechnol Prog* 1990, **6**:447–451.
The immobilization of cells onto a polypropylene membrane sheet in a uniform callus-like layer, to produce purine alkaloids with high productivity.

9. • FACCHINI PJ, DICOSMO F: **Plant Cell Bioreactor for the Production of Protoberberine Alkaloids from Immobilized *Thalictrum rugosum* Cultures.** *Biotechnol Bioeng* 1991, **37**:397–403.
Adsorption of cells onto glass fibre mats within a novel reactor for the production of alkaloids.

10. FACCHINI PJ, DICOSMO F: **Secondary Metabolite Biosynthesis in Cultured Cells of *Catharanthus roseus* (L.) G. Don Immobilized by Adhesion to Glass Fibers.** *Appl Microbiol Biotechnol* 1991, **35**:382–392.

11. •• CHIOU T-W, MURAKAMI S, WANG DIC, WU W-T: **A Fiber-bed Bioreactor for Anchorage-dependent Animal Cell Cultures: Part 1. Bioreactor Design and Operations.** *Biotechnol Bioeng* 1991, **37**:755–761.

Novel bioreactor system using glass fibres within a packed-bed, incorporating an inner draft tube for gas sparging/transfer. High cell concentrations were achieved when operated continuously for 66 days.

12. •• BRAMBLE JL, GRAVES DJ, BRODELIUS P: **Plant Cell Culture Using a Novel Bioreactor: The Magnetically Stabilized Fluidized Bed.** *Biotechnol Prog* 1990, **6**:453–457.

A feasibility study on the use of MSFBs. This system was judged to offer several advantages over other methods for the production of secondary metabolites using plant cells.

13. • TERRANOVA BE, BURNS MA: **Continuous Cell Suspension Processing Using Magnetically Stabilized Fluidized Beds.** *Biotechnol Bioeng* 1991, **37**:110–120.

The use of an interesting technology to remove cells from suspension; the technology used may be applicable to immobilized cell reactors.

14. •• SILBIGER E, FREEMAN A: **Continuous Δ^1-hydrocortisone Dehydrogenation with *In Situ* Product Recovery.** *Enzyme Microb Technol* 1991, **13**:869–872.

Continuous process using a poorly soluble substrate, exploiting a water-miscible co-solvent. Insoluble product was removed by the use of precipitation nuclei suspended within the reactor.

15. •• HILGE-ROTMANN B, REHM H-J: **Relationship Between Fermentation Capability and Fatty Acid Composition of Free and Immobilized *Saccharomyces cerevisiae.*** *Appl Microbiol Biotechnol* 1991, **34**:502–508.

Ethanol was found to cause a shift towards saturation in the fatty acid composition under aerobic conditions. Immobilized cells contained significantly more saturated fatty acyl residues than free cells did.

16. • HANNOUN BJM, STEPHANOPOULOS G: **Intrinsic Growth Rates of Alginate-entrapped *Saccharomyces cerevisiae.*** *Biotechnol Prog* 1990, **6**:341–348.

Under anaerobic conditions immobilized cells were demonstrated to have a reduced growth rate and increased glucose uptake and ethanol production compared to free cells.

17. • HOOTMANN U, BISPING B, REHM H-J: **Physiology of Polyol Formation by Free and Immobilized Cells of the Osmotolerant Yeast *Pichia farinosa.*** *Appl Microbiol Biotechnol* 1991, **35**:258–263.

Yields of glycerol and arabitol per unit glucose consumed were found to be higher for immobilized cells than for free cells.

18. • CHUN G-T, AGATHOS SN: **Comparative Studies of Physiological and Environmental Effects on the Production of Cyclosporin A in Suspended and Immobilized Cells of *Tolypocladium inflatum.*** *Biotechnol Bioeng* 1991, **37**:256–265.

Spore germination, growth and specific productivity were better in immobilized cells than in suspended cells.

19. • HAGEDORN J, KARGI F: **Production of Monoclonal Antibodies by Hybridoma Cells in a Flat Sheet Membrane Bioreactor.** *Biotechnol Prog* 1990, **6**:220–224.

Membrane pore size was optimized to allow simultaneous supply of nutrients and removal of toxic metabolites, resulting in higher cell concentrations and specific antibody production.

20. •• WESTRIN BA, AXELSSON A: **Diffusion in Gels Containing Immobilized Cells: A Critical Review.** *Biotechnol Bioeng* 1991, **38**:439–446.

A review of 11 experimental investigations of diffusion in gels containing immobilized cells. A procedure for the theoretical prediction of diffusion coefficients was recommended. Evidence is brought forward that the restriction on diffusion may be explained simply in terms of excluded volume effects.

21. • PETERSEN JN, DAVISON BH, SCOTT CD: **Minimizing the Errors Associated with the Determination of Effective Diffusion Coefficients when Using Spherical Cell Immobilization Matrices.** *Biotechnol Bioeng* 1991, **37**:386–388.

A suggested method for significantly reducing the very large errors associated with the bead method to estimate diffusivity.

22. • KANG Y, FAN LT, MIN BT, KIM SD: **Promotion of Oxygen Transfer in Three-phase Fluidized-bed Bioreactors by Floating Bubble Breakers.** *Biotechnol Bioeng* 1991, **37**:580–586.

It is claimed that floating 'bubble breakers' can increase oxygen transfer by up to 25% within the stated reactor system.

23. PETERSEN JN, DAVISON BH: **Modeling of an Immobilized-cell Three-phase Fluidized-bed Bioreactor.** *Appl Biochem Biotechnol* 1991, **28/29**:685–698.

24. LIVINGSTON AG: **Biodegradation of 3,4-dichloroaniline in a Fluidized-bed Bioreactor and a Steady-state Biofilm Kinetic Model.** *Biotechnol Bioeng* 1991, **38**:260–272.

25. • KIMMEL DE, KLASSON KT, CLAUSEN EC, GADDY JL: **Performance of Trickle-bed Bioreactors for Converting Synthesis Gas to Methane.** *Appl Biochem Biotechnol* 1991, **28/29**:457–469.

The conversion of CO, H_2 and CO_2 to CH_4 by a tri-culture, giving very high yields (80%) compared with theoretical yields.

Annotated patents

- • of interest
- •• of outstanding interest

P1. • VEB KOMBINAT SPIRITUOSEN WEIN UND SEKT BERLIN: **A Biocatalysis Module on the Basis of Denatured Plants or Parts of Plants.** 6/2/91 UK Patent 2210057 B.

Cells are immobilized within plant tissues rich in aerenchyma cells. Microorganisms that are immobilized within these cells may be cultivated to fill the available space.

P2. • EDEBO L: **Fungal Cell Wall Material with Flocullant Properties. Method For its Production.** 2/5/91 International Application WO 91/05869.

Fungal cell wall particles containing hexosamine that may be used to remove/recover negatively charged particles or to immobilize microorganisms and enzymes.

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