# Immobilized Living Fungal Mycelia for the Growth-Dissociated Synthesis of Chemicals

By Clem Kuek, Soil Science and Plant Nutrition Group,
School of Agriculture, University of Western Australia,
Nedlands, Western Australia, AUSTRALIA 6009

*ARTICLE 56:6:4/5*

**Immobilized microbial cells**

The study of immobilized cells to produce chemicals has expanded rapidly in the last decade (see reviews[1,2,3,4,5,6]). The interest in immobilized cells is undoubtedly associated with the potential advantages of the system for biological processing which are discussed in the reviews mentioned.

Immobilized microbial cells can be defined as cells which are physically confined or localized in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously.[7] The state of development of the technology is such that the word "catalytic" should now be replaced with "metabolic". Physical confinement is achieved by attachment to an inert immobilizing material. Reuse is made simple because the aggregations of cells and immobilizing material is much denser than the liquid phase (medium) thus allowing either easy recovery by gravitational sedimentation in batch culture, or retention in continuous culture.

The technology for immobilizing microbial cells is derived from that for immobilizing enzymes. Cells were first immobilized as an extension of the rationale for the catalysis of single-step reactions by immobilized enzymes. Immobilization of cells meant that isolation and purification of enzymes became unnecessary. For this purpose, cell viability is not a concern but multi-step reactions which were investigated later, require the mediation of viable cells[12] because they were metabolically more versatile. This meant that techniques which retained cell viability after immobilization had to be developed. There are two types of immobilized living cells. The type developed first was of cells which were immobilized after growth in free culture. A later development was the type where propagules were immobilized and then cultured to allow growth. The resultant biomass remains immobilized. Such technology was initially applied in multi-step reactions such as the conversion of glucose into ethanol using *Saccharomyces cerevisiae*[8] and into hydrogen using Clostridium butyricum.[9] More recently, interest in the application of immobilized living cells to the synthesis of biologically active macromolecules such as antibiotics and enzymes has increased.

The initial and main interest has been in the immobilization of unicellular microorganisms. Only recently has this been extended to fungi. Immobilized fungi were first investigated for a role in biotransformations such as that of cortexolone to hydrocortisone by *Curvularia lunata*[10], and of glucose to itaconic acid by *Aspergillus terreus*.[11] Immobilized fungi are increasingly studied for application in processes which have traditionally only used free mycelia.

Immobilization of microorganisms would be an excellent approach to fully exploit the phenomenon of "growth-dissociated" synthesis of many chemicals which are synthesized in that manner. This is now recognized and recent investigations include the production of solvents by *Clostridium acetobutylicum*[12] and alpha-amylase by *Bacillus amyloliquifaciens*.[13] Examples of studies with fungi are the production of cellulase by *Trichoderma reesei*,[14] and antibiotics such as penicillin G from *Penicillium chrysogenum*[15] and patulin from *Penicillium urticae*.[16]

**Growth-dissociated synthesis**

When maximal synthesis of a microbial product only occurs after completion of the growth phase of the producing microorganism, the latter can be said to exhibit "growth-dissociated"[1] synthesis. For example, studies on Aspergillus niger have revealed that the specific rate of glucoamylase synthesis is inversely proportional to the specific rate of growth.[18,19,20] The physiology of glucoamylase synthesis is therefore biphasic. Similarly, it is well-known that the physicochemical requirements for optimum growth and for maximal synthesis of cellulase by Trichoderma spp. are different[21] and that a growth rate close to zero is preferred for maximal cellulase synthesis.[22] Secondary metabolites, for example antibiotics, are classical examples of growth-dissociated synthesis. The physiology growth-dissociated synthesis cannot be fully exploited using current fermentation practice.

**Exploiting growth-dissociated synthesis**

Growth-dissociated synthesis makes it advantageous to divide a production process into two separated sequential phases: (i) the accumulation of biomass, (ii) the repeated use of the same biomass for synthesis of the growth-dissociated product. The following potential advantages may be gained by this arrangement : a) The physicochemical requirements of each phase can be optimized separately. On the basis of catabolite repression alone, the chemical requirements are likely to be significantly different in certain fermentations. Further, the only nutrients needed in a medium for the product synthesis phase would be those required for maintenance of the biomass accumulated during the growth phase; b) Since growing biomass is not a requirement for product synthesis, the biomass which is discarded after each batch culture in current industrial practice, may be reused provided it is physiologically maintained. Reuse of biomass means that both production time and costs may be reduced as biomass need not be grown for each production run. The same biomass may be used repeatedly in batch cultures but the full advantage is gained when they are reused in a continuous process ("continuous" is defined here as the continuous inflow and outflow of medium through a reactor vessel and not

"continuous culture" which is characterized by growth of biomass).

The physical separation of the growth and product synthesis phases ("phase separation") in a production process, particularly when applied to fungi, is a novel approach because it is yet to be routinely used in some processes where it would clearly be of advantage viz. in the production of glucoamylase, cellulase and antibiotics. There have been very few studies on production using separated phases. It is most likely that phase separation has not been incorporated in current batch production practice because of the difficulty in recovering mycelia in a state which is fit for reuse. It cannot be applied in continuous culture because the physiology of growth-dissociated synthesis is not compatible with a system where the growth of biomass is required to replace losses due to efflux.

The recent advent of techniques for the immobilization of microbial cells now allows better exploitation of the synthesis of growth-dissociated products because the technique enables the retention or recovery of biomass for reuse. However, a major problem has to be overcome before immobilized fungi can find wide use in the fermentation inductry.

**Mycelial growth on surfaces of immobilized aggregations**

The potential for the use of immobilized fungi in bioreactors is most likely to be limited by the growth of mycelia at the microbial aggregate*-medium interphase (Fig.1) as has often been reported.[14,23,24,25,26] The only published accounts that do not report mycelial growth at the interphase did not specify the morphology at the interphase. Four problems arise from mycelial growth at the aggregate-medium interphase:

1) hyphal fragments are easily broken off with possible subsequent growth into free mycelia. This defeats the purpose of immobilization. The accumulation of free mycelia in the production of citric acid by immobilized Aspergillus niger was a contributory factor to the process being assessed as no better than traditional batch fermentation.[27]

2) free mycelia can cause clogging of outlets in continuous culture vessels. In industrial operations, free mycelia can adhere to surfaces resulting in corrosion, and reduction in the performance of heat exchangers and equipment downstream from the fermenter.[28]

3) because of the known effect of hyphae or mycelia in liquid suspension,[29,30,31] complete confinement of mycelia to the subsurface should result in the reduction of culture viscosity. However, the presence of mycelia at the interphase does not allow this to occur, a situation akin to that with free mycelial pellets. The productivity of an aerobic submerged culture is largely dependent on gas-to-liquid oxygen transfer which is reduced with increasing culture viscosity. Low culture viscosities are therefore preferable. This is recognized in some fungal fermentations where the mycelial contribution to culture viscosity was reduced by growing mycelia in celite microbeads.[32,33]

4) mycelial growth at the interphase effectively decreases the density of the fungal aggregation, thus negating one of the advantages of immobilization. Low densities slow the sedimentation rates of the aggregations during recovery of the biomass but more importantly, the performance of such

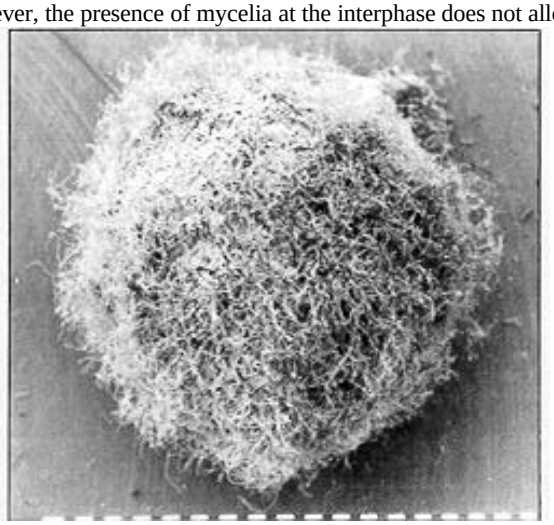

Fig. 1: Scanning electron micrograph of the surface of an inoculated calcium alginate bead which was overgrown with mycelia of *Aspergillus phoenicus* R4M5.10 mycelia during incubation. Cursor = 0.1 mm

*Immobilized microorganisms (i.e. biomass and immobilizing material) are often called "biocatalysts" but the term is not appropriate for situations where they are used for the synthesis of enzymes and other macromolecules. Therefore, the term used here is "microbial" or "fungal aggregations".

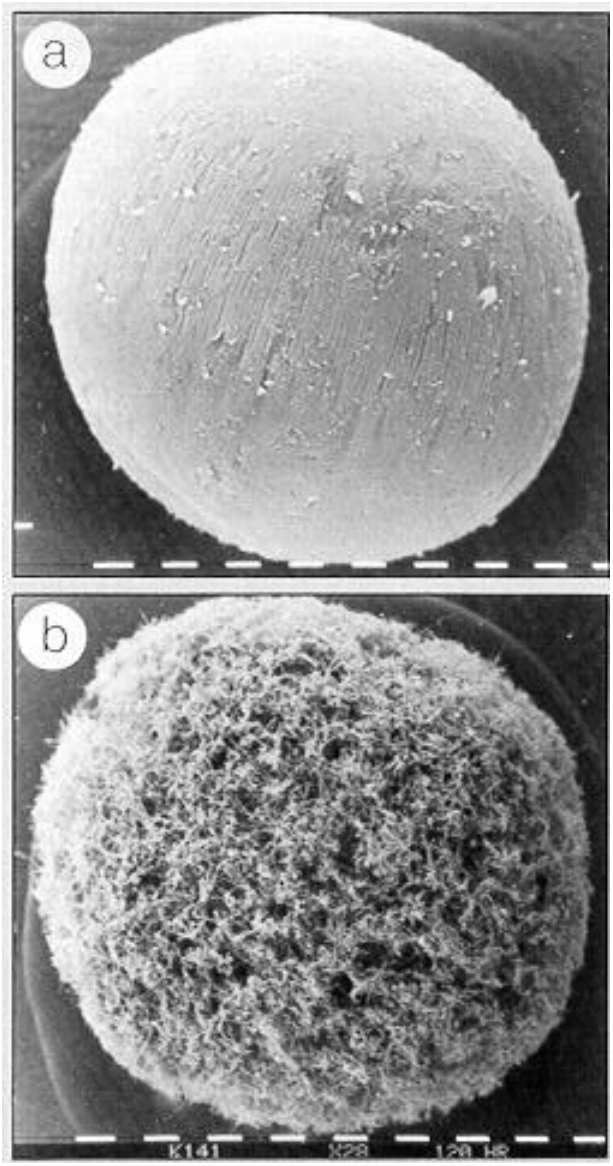


Fig 2: Scanning electron micrographs of a] the surface of a calcium alginate bead containing subsurface mycelium of Aspergillus phoenicus R4M5.10. b] subsurface mycelium of calcium alginate beads revealed by the dissolution of calcium alginate. Cursor = 0.1 mm

aggregations in fluidized bed reactors is limited because the minimum fluidization velocity is reduced. This means that high flow rates of medium through the fluidized bed is impossible without disruption of the fluidized bed.

**Speculation**

The problems associated with mycelial growth at the interphase may be one of the reasons why there are so few reports on the application of immobilized fungi in fermentation processes. This situation is likely to remain until the problem is overcome.

**Prognosis**

It has now been demonstrated that it is possible to grow mycelia within a calcium alginate gel matrix and subsequently use it repeatedly while the immobilized mycelia remains completely confined in the subsurface[34,35] (Fig. 2 a,b). This achievement is currently being applied in the experimental production of glucoamylase. More importantly, this work may spur efforts with other fungi and processes. Therefore there is justification for being more confident about an increasing role for immobilized fungi in the fermentation industry.

**References**


1. Abbott, B.J. ( 1977 ) Immobilized cells. In: "Annual Reports on Fermentation Processes 1", Perlman, D. (ed.), Academic Press, London; pp. 205-233.
2. Abbott, B.J. (1978) Immobilized cells. In : "Annual Reports on Fermentation Processes 2", Perlman, D. (ed.), Academic Press, London; pp. 91-123.
3. Messing, R.A. (1980) Immobilized microbes. In: "Annual Reports on Fermentation Processes 4", Tsao, G.T. (ed.), Academic press, London; pp. 105-121.
4. Chibata, I. and Tosa, T. (1981) Use of immobilized cells. Ann. Rev. Biophys. Bioeng. 10: 197-216.
5. Fukui, S. and Tanaka, A. (1982) Immobilized microbial cells. Ann. Rev. Microbiol. 36: 145-172.
6. Bucke, C. (1983) Immobilized cells. Phil. Trans. R. Soc. Lond. B 300: 369-389.
7. Chibata, I. (1978) "Immobilized enzymes. Research and Development", John Wiley and Sons, New York; p.7.
8. Kierstan, M. and Bucke, C. (1977) The immobilization of microbial cells, subcellular organelles, and enzymes in calcium alginate gels. Biotechnol. Bioeng. 19: 387-397.
9. Karube, I.; Matsunaga, T.; Tsura, T.; and Suzuki, S. (1977) Biochemical fuel utilizing immobilized cells of *Clostridium butyricum*. Biotechnol. Bioeng. 19: 1727-1733.
10. Sonomoto, K.; Hoq, M.M.; Tanaka, A.; and Fukui, S. (1983) 11 Beta-hydroxylation of cortexolone (Reichstein compound S) to hydrocortisone by *Curvularia lunata* entrapped in photo-cross-linked resin gels. Appl. Environ. Microbiol. 45: 436-443.
11. Horitsu, H.; Takahashi, Y.; Tsuda, J.; Kawai, K.; and Kawano, Y. (1983) Production of itaconic acid by *Aspergillus terreus* immobilized in polyacrylamide gels. Eur. J. Appl. Microbiol. Biotechnol. 18: 358-360.
12. Haggstrom, L. and Molin, N. (1980) Calcium alginate immobilized cells of *Clostridium acetobutylicum* for solvent production. Biotechnol. Lett. 2: 241-246.
13. Shinmyo, A.; Kimura, H. and Okada, H. (1982) Physiology of alpha-amylase production by immobilized *Bacillus amyloliquefaciens*. Eur. J. Appl. Microbiol. Biotechnol. 14: 7-12.
14. Frein, E.M.; Montenecourt, B.S. and Everleigh, D.E. (1982) Cellulase production by *Trichoderma reesei* immobilized on kappa-carragenan. Biotechnol Lett. 4: 287-292.
15. Morikawa, Y.; Karube, I.; and Suzuki, S. (1979) Penicillin G production by immobilized whole cells of *Penicillium chrysogenum*. Biotechnol. Bioeng. 21: 261-270
16. Deo, Y.M. and Gaucher, G.M. (1983 ) Semi-continuous production of the antibiotic patulin by immobilized cells of *Penicillium urticae*. Biotechnol Lett. 5: 125-130.
17. Enatsu, T. and Shinmyo, A. (1978) In vitro synthesis of enzymes. Physiological aspects of microbial enzyme production. Adv. Biochem. Eng. 9: 111-144.
18. Okazaki, M.; Shinmyo, A.; and Terui, G. (1965) Studies on the physiological age of mycelia in the glucamylase-producing culture of Aspergillus niger and the phenomenon of over-ageing in relation to multi-stage continuous culture. J. Ferment. Technol. 43: 581-589.
19. Okazaki, M. and Terui, G. (1966) An inquiry into the physiology of preferential synthesis of glucamylase in relation to multi-stage continuous culture (II) Main causes of preferential glucamylase synthesis. J. Ferment. Technol. 44: 276-286.
20. Okazaki, M. and Terui, G. (1966) An inquiry into the physiology of preferential synthesis of glucamylase in relation to multi-stage continuous culture (III) Main causes of preferential glucamylase synthesis with special reference to the longevity of m-RNA specific for the enzyme. J. Ferment. Technol. 44: 287-294.
21. Mandels, M.; Sternberg, D.; and Andreotti, E. (1975) Growth and cellulase production by Trichoderma. In: "Proc. Symp. Enzymatic Hydrolysis of Cellulose", Aulanko, Finland. Bailey, M.; Enari, T.M.; and Linko, M. (eds.); pp. 8 –109.
22. Ryu, D.; Andreotti, E.; Mandels, M.; and Gallo, B. (1979) Studies on quantitative physiology of Trichoderma reesei with two-stage continuous culture for cellulase production. Biotechnol. Bioeng. 21: 1887-1903.
23. Deo, Y.M.; Costerton, J.W.; and Gaucher, G.M. (1983) Examination of immobilized fungal cells by phase-contrast and scanning electron microscopy. Can. J. Microbiol. 29: 1642-1649.
24. Kopp, B. and Rehm, H.J. (1984) Semicontinuous cultivation of immobilized *Claviceps purpurea*. Appl. Microbiol. Biotechnol. 19: 141-145.
25. Baklashova, T.G.; Koshcheenko, K.A.; and Skryabin, G.K. (1984) Hydroxylation of indolyl-3-acetic acid by immobilized mycelium of *Aspergillus niger*. Appl. Microbiol. Biotechnol. 19: 217-223.
26. Eikmeier, H.; Westmeier, F.; and Rehm, H.J. (1984) Morphological development of *Aspergillus niger* immobilized in Ca-alginate and kappa-carrageenan. Appl. Microbiol. Biotechnol. 19: 53-57
27. Borglum, G.B. and Marshall, J. (1984) The potential of immobilized biocatalysts for production of industrial chemicals. Appl. Biochem. Biotechnol. 9: 117-130
28. Ash, S.G. (1979) Adhesion of microorganisms in fermentation processes. In : "Adhesion of microorganisms to surfaces", Ellwood, D.C.; Melling, J.; and Rutter, P. (eds), Academic Press, London; pp. 57-86
29. Chain, E.B.; Gualandi, G.; and Morisi, G. (1966) Aeration studies. IV. Aeration conditions in 3000 litre submerged fermentation with various microorganisms. Biotechnol. Bioeng. 8: 595-619
30. Metz, B.; Kossen, N.W.F.; and Van Suijdam, J.C. (1977) The rheology of mould suspensions. Adv. Biochem. Eng. 11: 103-156
31. Pace, G.W. (1980) Rheology of mycelial fermentation broths. In: "Fungal Biotechnology", Smith, J.E.; Berry, D.R.; and Kristiansen, B. (eds.), Academic Press, London; pp.95 – 110
32. Gbewonyo, K. and Wang, D.I.C. (1983) Confining mycelial growth to porous microbeads : A novel technique to alter the morphology of non-newtonian mycelial cultures. Biotechnol. Bioeng. 25: 967-983
33. Wang, D.I.C.; Meier, J.; and Yokoyama, K. (1984) Penicillin fermentation in a 200-liter tower fermenter using cells confined to microbeads. Appl. Biochem. Biotechnol. 9: 105-116.
34. Kuek, C. and Armitage, T.A. (1985) Scanning electron microscopic examination of calcium alginate beads immobilizing growing mycelia of *Aspergillus phoenicus*. Enz. Microb. Technol. 7: 121-125.
35. Kuek. C. (1991) Batch production of glucoamylase using *Aspergillus phoenicus* immobilized in calcium alginate beads. Appl. Microbil. Biotechnol. 35: 466-470.