Scanning electron microscopic examination of calcium alginate beads immobilizing growing mycelia of *Aspergillus phoenicus*

C. Kuek* AND T.M. Armitage

Soil Science and Plant Nutrition Group, School of Agriculture, University of Western Australia, Nedlands, Western Australia 6009

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Calcium alginate beads inoculated with conidia of Aspergillus phoenicus were incubated in various culture vessels for 120 hrs. Scanning electron microscopy revealed that the degree of agitation was a factor in surface stability of the beads. Highly significant was the sucessful restriction of mycelial growth to the subsurface, a condition required if the full advantages of immobilized fungi are to be realized.

Keywords: Calcium alginate beads; immobilized fungi; surface abrasion; subsurface mycelium.

Introduction

While interest in immobilized microbial cells has mainly centred on unicellular microorganisms,[1,2] immobilized fungal mycelia is now receiving more attention. Immobilization may be of two types. Firstly there is entrapment of pre-grown mycelia, for example as in the hydroxylation of indolyl-3-acetic acid by Aspergillus niger[3] and for the production of itaconic acid by Aspergillus terreus.[4] Secondly, there is the entrapment of propagules which are allowed to grow in situ . The propagules may be spores as in steroid hydroxylation by Curvularia lunata[5] and hydroxylation of progesterone by Rhizopus stolonifer[6] or hyphae as in alkaloid production by Claviceps purpurea.[7,8]

The spatial distribution of entrapped growing fungal mycelium in the immobilization material has implications for the loss of both the former and the latter through abrasion, and also on the exchange of solutes with the growing mycelium. Despite this, there is relatively little information in this area. An indication of the nature of morphological development of immobilized Aspergillus niger[9] and Penicillium urticae[10] conidia has recently been given. Ideally, the ratio of mycelium to immobilizing material should be as high as possible to ensure correspondingly high productivity. All the mycelium should be confined within the immobilizing material and no growth should occur external to the latter (i.e. in the liquid phase) so as to minimise loss of viable cells and change in flow characteristics of the immobilized particle. This is of primary importance in immobilization. In most published work to date, the nature of growth of entrapped mycelia is either not specified or is shown to include growth external to the immobilizing material. This paper investigates the distribution of mycelia arising from conidia, on the surface and subsurface of calcium alginate beads and the effect of abrasion on bead surfaces during incubation.

*To whom correspondance and reprint requests should be addressed

Materials and methods

Production, enumeration and immobilization of conidia

The fungus used was a N-Methyl-N'-Nitro-N-Nitrosoguanidine induced mutation of Aspergillus phoenicus ATCC 15556, designated R4M5.10 which was improved in glucoamylase productivity. Conidia from a seven day culture incubated at 35°C on potato dextrose agar (Difco Laboratories, Detroit, Michigan) supplemented with a further 10 g L^{-1} agar were suspended in 0.01 % Tween 80 and filtered through a sintered glass funnel (Corning Pyrex, porosity number 1) to remove mycelial fragments. Conidial concentration was then determined by a viable count. During the 24 hour incubation of the viable count plates (PDA plus 0.1 % Triton X-100), the suspension was stored at 4°C. The suspension was diluted to 1.0 X 10^7 conidia mL^{-1}. 1.0 mL of this diluted suspension was then added to 50 mL 2 % alginate (Manugel GMB, kind gift of Kelco A.I.L., Melbourne, Australia) solution which had been autoclaved at 121°C for 15 minutes. Beads were produced by pumping the alginate/conidia mixture through a 26 Gauge hypodermic needle from a height of 10 cm into 0.1 M CaCl2 which was slowly stirred. The number of beads produced was determined by a drop counter. The beads were allowed to harden in the $CaCl_2$ for about 30 minutes after which they were drained and placed in growth medium.

Growth medium and incubation

The growth medium contained, per litre; soluble starch (BDH Chemicals, Poole, England) 20 g; K_2HPO_4, 70 mg; KH_2PO_4, 130 mg; Mg SO_4, 200 mg; $Na_2SO_4.10\ H_2O$, 200 mg; $FeSO_4.7H_2O$, 200 mg; $MnSO_4.H_2O$, 10 mg; $ZnSO_4.7H_2O$, 2 mg; $CuSO_4.5H_2O$, 2 mg; monosodium glutamate, 550 mg; yeast extract (Difco Laboratories, Detroit, Michigan) 500 mg and 0.025 M $CaCl_2$. The medium was sterilized at 121°C for 15 minutes. Incubation in Gallenkamp orbital incubator (orbit diameter = 32 mm); 350C; 250 r.p.m.; 120 hrs. : (i) 4000 beads and 50 mL of growth medium were placed in a 250 mL Erlenmeyer flask. (ii) 16000 beads and 200 mL of growth medium were placed in a 2.8 L Fernbach flask. Incubation in a bubble column (internal diameter = 3.0 cm; downward blowing sparger with 0.5 mm orifice); 350C; air = 5.0 L min.-1; 120 hrs.: 16000 beads were placed in the column with 200 mL of growth medium. Beads were examined after 120 h with respect to the type of incubation system employed.

Preparation of beads for scanning electron microscopy

Beads were rinsed clean of growth medium with deionised water before fixing. To investigate subsurface mycelium, some of the beads were placed in 10 % sodium hexametaphosphate overnight to dissolve calcium alginate. The resultant mycelial aggregation was washed thoroughly before fixing which involved immersion in the following solutions with a water rinse in between each step:

(a) 2.5 % glutaraldehyde for 2 hours
(b) 1 % osmium tetroxide for 2 hours
(c) 1 % tannic acid in 0.05 M cacodylate buffer, pH 7.4, for 1hour
(d) 2 % uranyl acetate for 1 hour

Following stepwise dehydration using ethanol, the beads were critical-point dried. Before coating with 20 nM platinum some beads were fractured by inserting a razor blade into them and then causing splitting by a lifting motion. The beads were examined using a Phillips SEM 500 scanning electron microscope.

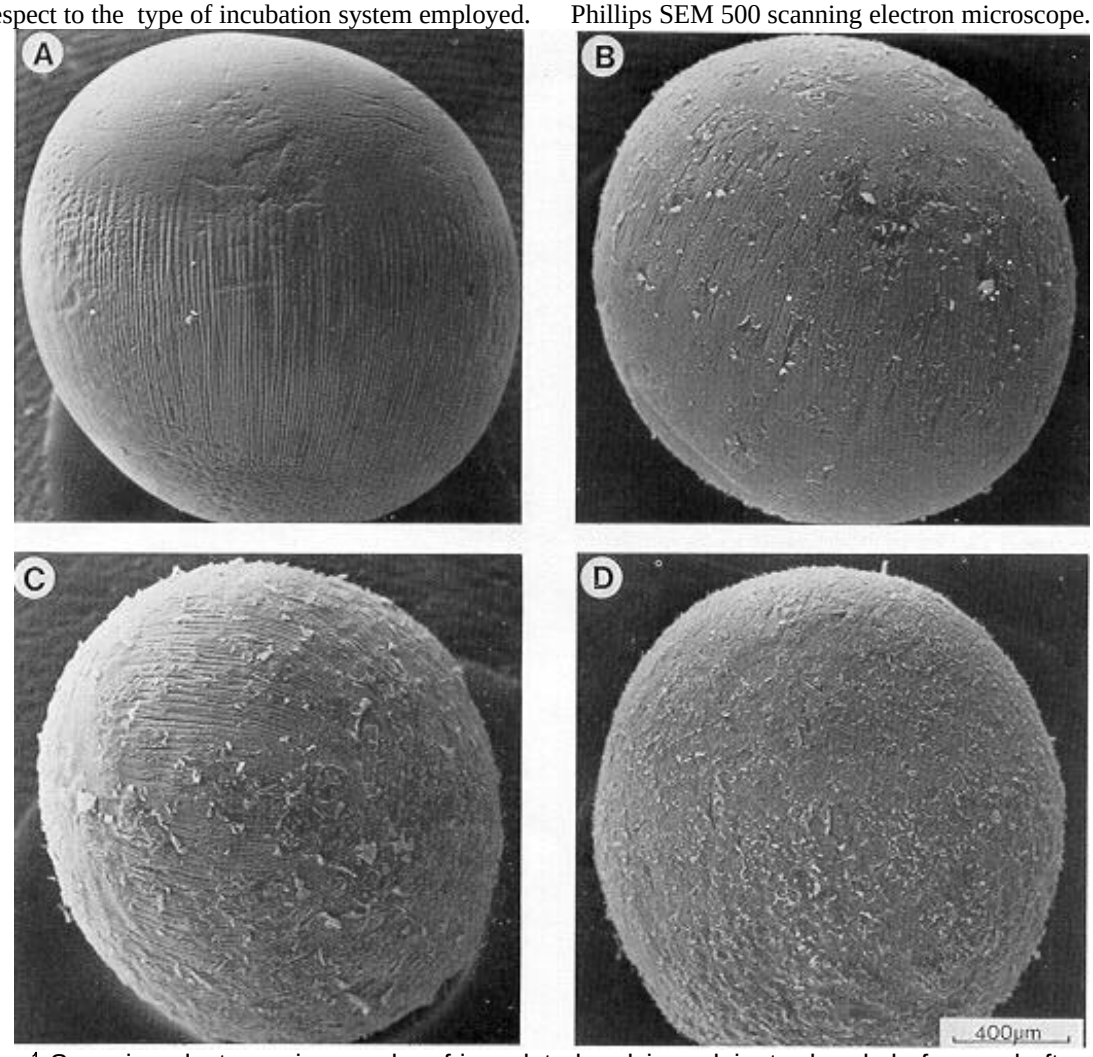

Figure 1 Scanning electron micrographs of inoculated calcium alginate beads before and after 120 hr. incubation in various culture vessels: A, Bead before incubation; B, Fernbach flask; C, Erlenmeyer flask; D, Bubble column

Results

Freshly prepared beads examined after 30 minutes hardening in 0.1 M $CaCl_2$ solution were spheroid in shape and 1.5 - 1.6 mm indiameter. All had characteristic linear indentations caused by the entry of each sodium alginate drop into the calcium chloride solution (Fig. 1A). These markings serve as a convenient reference point for assessment of abrasive damage to the bead surface. The condition of the beads after the various incubation procedures varied markedly (Figs. 1 and 2). Loss of calcium alginate was lowest via Fernbach flasks and highest via the bubble column. This corresponds well with the observation that agitation was most vigourous in the bubble column and least in Fernbach flasks. Calcium alginate appeared to be lost via the peeling off of a thin outer layer (Fig. 2C) which appears to have a different structure from underlying calcium alginate. The presence of this layer is well known amongst some workers (Cheetham, pers. comm.) and may be the same as that which has been observed and described as "a membrane-like coating".[11]

Highly significant is that the bead surfaces were not overgrown with mycelium as has been previously reported.[3,8,9] As far as is known, this is the first time that this has been shown. It appears that the mycelium visible on the surface were exposed and sheared off at the liquid / bead interphase as a result of the loss of calcium alginate. Dissolution of calcium alginate revealed an abundance of subsurface mycelium (Figs. 3A,B) throughout which fruiting structures could be found. Mycelial growth appeared to be equally spread within the bead (Fig. 3C). The structure of calcium

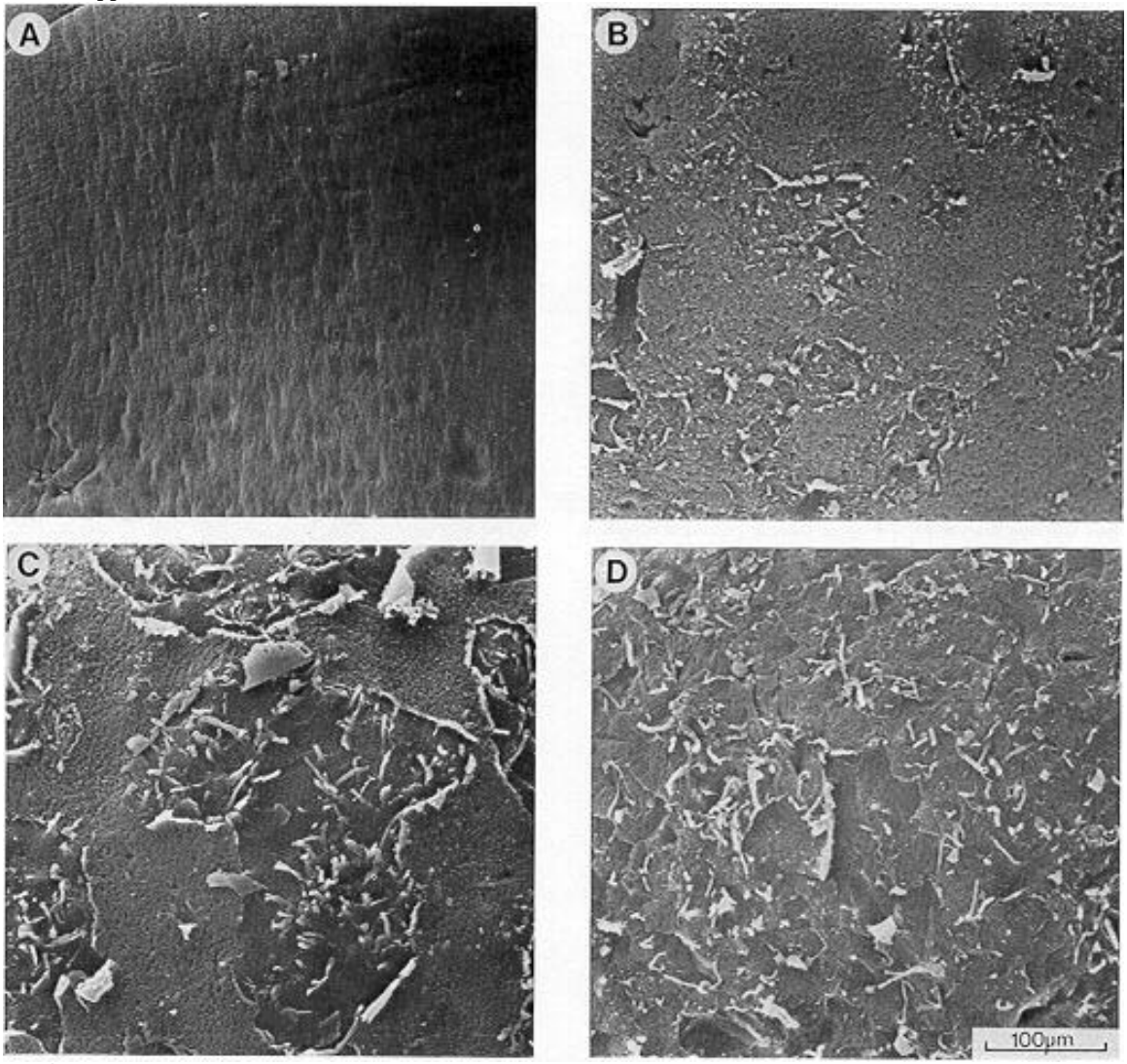

Figure 2 Scanning electron micrographs of the surface detail of inoculated calcium alginate beads before and after 120 hr. incubation in various culture vessels:A, bead before incubation; B, Fernbach flask; C, Erlenmeyer flask; D, Bubble column

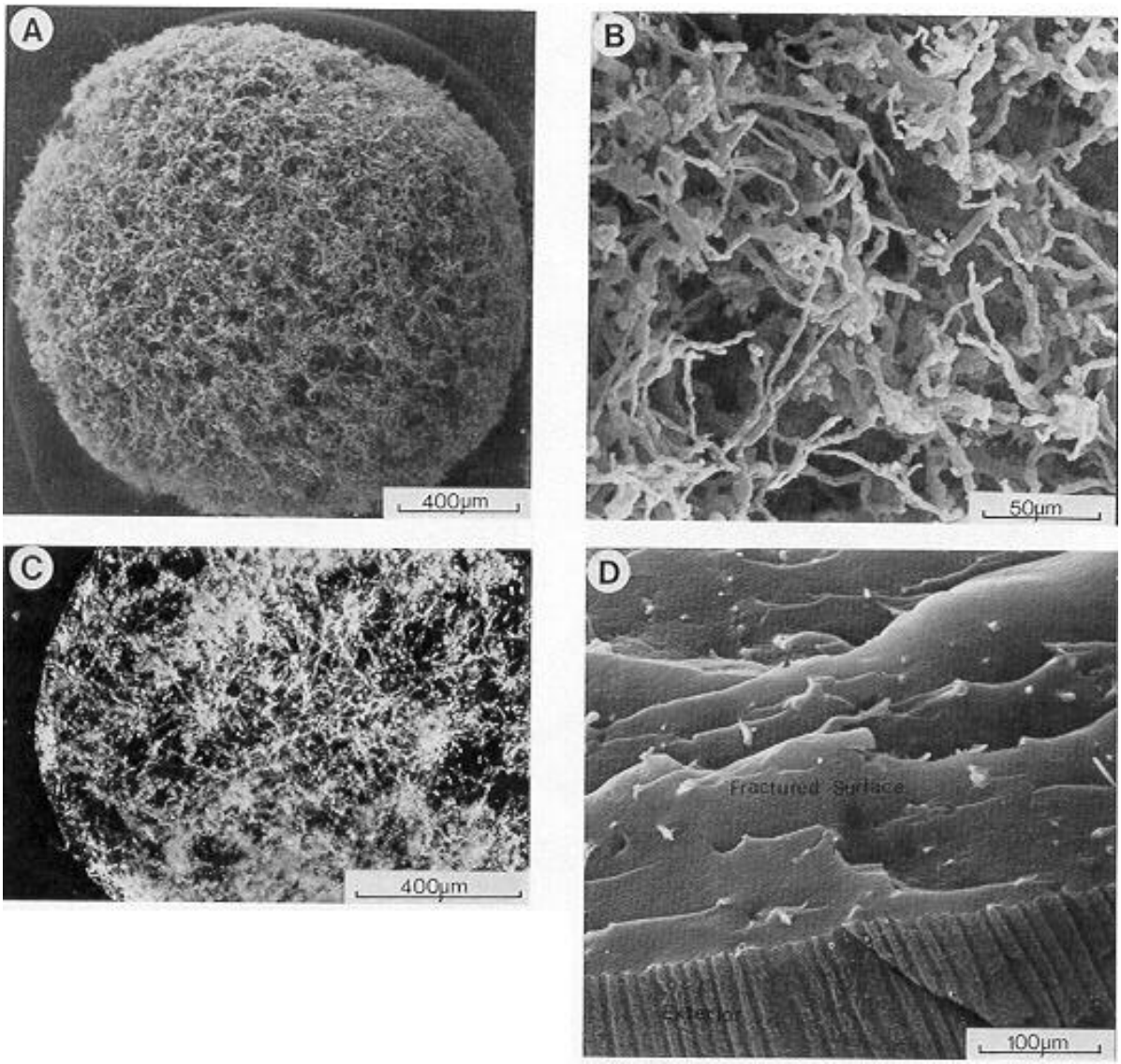

Figure 3 A and B, Subsurface mycelium of Fernbach flaskcultured calcium alginate beads at 120 h revealed by the dissolution of calcium alginate (Compare *Figure 1B* with *Figure 3A* and *Figure 2B* with *Figure 3B*). C, Transverse section of a Fernbach flask cultured bead at 120 h showing the distribution of mycelium. D, Erlenmeyer flask cultured bead at 48 h showing inner bead surface following fracture (Viewed at 45° to the plane of the fracture).

alginate inside the beads was solid (Plate 3D) and not sponge-like as has been reported in some other studies.[11,12]

Discussion

Where mycelial growth is confined to the subsurface, surface stability of the beads becomes an important consideration. In this, the degree of agitation during incubation was found to be a factor. Bead abrasion would be the most logical explanation of loss of immobilizing material which is predisposed to loss by the presence of an outer layer. A membrane-like coating is thought to be the result of contact between sodium alginate and calcium chloride at the beginning of gel formation.[11] The implication of the results is that this outer layer is mechanically weaker than the underlying calcium alginate. However, the peeling observed could be facilitated by the manner in which calcium alginate is broken down in the presence of phosphates in the medium. Once the outer layer is removed peeling appears stop, resulting in the exposure of a surface that is structurally different (Fig. 2D. Compare with Fig. 2B). Therefore in the short term at least, vigourous agitation causes bead deterioration and could potentially cause the release of viable cells into the medium.

All the incubation methods resulted in satisfactory bead conformation in that surface overgrowth of mycelium was avoided although incubation in Fernbach flasks is best because it results in the least abrasion. The containment of mycelium to the subsurface in culture via Fernbach flasks is a significant achievement because it minimizes the loss of biomass which in turn should make possible sustained productivity and the prevention of the growth of free cells in the medium. Some studies have suggested that the structure of calcium alginate inside beads is sponge-like[11,12] while this study reveals that the structure is as would be predicted for a gel. The sponge-like appearance observed elsewhere could be the result of dehydration of incompletely fixed beads. Despite the solid appearance, oxygen and nutrient diffusion through the calcium alginate used is clearly not so restrictive as to force mycelium to grow at the surface although further studies to be made on maximising biomass by increasing the inoculum size may reveal otherwise. However, it is hypothesized that the system is likely to be self-limiting in that biomass levels will be determined by the amount of oxygen available and that at maximal biomass level the mycelium should still remain subsurface. Restriction of mycelial growth to the subsurface as was achieved, allows the beads to be used to full advantage in fluidized beds, airlift fermentors and bubble columns, in the idiophase production of glucoamylase.

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