

# Immobilisation of *Acinetobacter johnsonii* cells within alginate beads

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## Abstract

The growth and distribution of *A. johnsonii* cells, immobilised within alginate beads suspended in an aerated activated sludge mixed liquor medium, were assessed by viable cell counts on nutrient agar and scanning electron microscope (SEM). Both techniques indicated that *A. johnsonii* cells did survive and grow within alginate beads. *A. johnsonii* immobilised cells were metabolically active as they removed phosphate from the activated sludge mixed liquor medium. While cells were expected to occur preferably in the outer layer after a few hours of incubation, beads entrapping bacterial cells showed a random distribution of cell colonies 24 h and 2 weeks after incubation. This constant random distribution might be attributed to constant aeration that would have facilitated mass transfer added to extracellular substances which maintained daughter cells in the colonies close to one another, thus preventing them from moving to the outer layer.

## Introduction

Several polyphosphate (polyP)-accumulating bacteria, especially from the genus *Acinetobacter*, have been isolated from activated sludge in which enhanced biological phosphate removal (EBPR) has been observed (Fuhs and Chen, 1975; Streichan et al., 1990). Currently attention is being drawn to immobilisation of these bacterial cells in order to get an insight into the mechanism of biological phosphate removal. One of these bacteria, namely *A. johnsonii* has been reported to be an efficient polyP-accumulating species (Van Groenestijn et al., 1989; Van Veen et al., 1993), therefore an ideal model organism to illustrate the possible use of the alginate immobilisation technique for EBPR. The efficiency of an immobilised system for EBPR will depend on a number of factors e.g. the bacterial strains being used, the properties of the support material, and the immobilisation method itself. The extent of phosphate-accumulation depends on growth rate of the polyP-accumulating bacteria (Van Groenestijn et al., 1989) which in turn is restricted by the spatial distribution of the cells within the gel bead.

Some electron microscopic observations of different bacteria entrapped in gels have been reported by different investigators (Garde et al., 1981). Structural details about organelles or cellular integrity, matrix architecture and cell distribution within the matrix have been enlightened by electron microscopic observations. Electron microscopic studies have shown alginate to have a sponge-like structure consisting of filaments and cavities and also bulges on the surface and inside the beads (Garde et al., 1981; Bashan 1986). Bashan (1986), using scanning electron microscopy, has indicated that alginate beads at various stages of production revealed a rounded structure with shallow grooves every 10 to 15 µm and a few cavities on its rough surface.

Growth within the matrix has been reported to be limited to the outer 50 µm layer of gel beads. Just before the start of incubation, a homogeneously distributed bacterial population is observed throughout the gel lattice. After a few hours of incubation, the cells in the central part of the bead disappear, while the cells in the layer near to the gel surface multiply (Wada et al., 1980; Shinmyo

et al., 1982).

The aim of this study was to determine the growth and distribution of *A. johnsonii* immobilised cells within alginate beads. P-uptake ability of the immobilised cells was also determined as an example of how the alginate immobilisation technique could be used.

## Material and methods

### Bacterial culture and inoculum used in this study

A culture of *A. johnsonii* strain 105 was obtained from the culture collection at the Environmental Biotechnology Laboratory, Department of Microbiology and Plant Pathology, University of Pretoria. An aliquot of the bacterial stock culture was incubated in 100 ml Biolab nutrient broth on an Edmund Bühler TH 10 rotary shaker, 160 r/min at 28°C for 72 h.

### Activated sludge mixed liquor medium for growth and distribution studies

Activated sludge mixed liquor was collected from the anaerobic tank at the Daspoort activated sludge plant, Pretoria. It was filtered twice on Whatman filter paper (φ 185 mm) and enriched with 5 mg/l CH<sub>3</sub>COONa, 0.18 g/l KNO<sub>3</sub> and 0.5 g/l MgSO<sub>4</sub>·7H<sub>2</sub>O, respectively before autoclaving. The nutrient additions were adapted from Bosch and Cloete (1993).

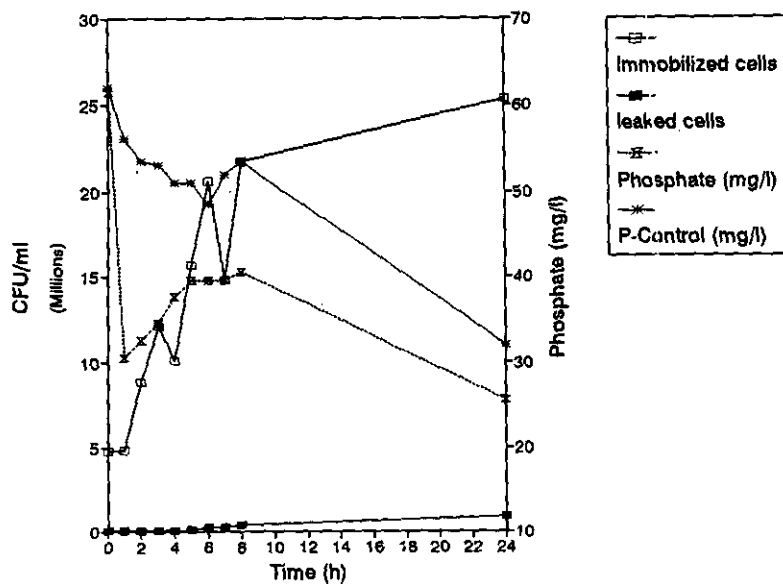
### Immobilisation technique

Entrapment of bacteria within alginate beads was performed according to a procedure of Bashan (1986) modified by Cloete et al. (1994). A sterile 20 ml syringe using a 26G needle was used to add the alginate-bacterial cell mixture dropwise into 1.1% CaCl<sub>2</sub> solution for cross-linking. Beads of approximately 2 mm in diameter were immediately formed in the CaCl<sub>2</sub> solution. 3.5% and 4% sodium alginate (BDH) bead concentrations were used.

Alginate beads entrapping bacterial cells were washed with sterile distilled water and suspended in activated sludge mixed liquor medium and incubated on a rotary shaker, 160 r/min at 28°C. The above cultural conditions, aeration by shaking and temperature were maintained throughout the experimental procedure.

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Received 15 September 1994; accepted in revised form 22 February 1995.



**Figure 1**  
Growth, leakage and phosphate uptake by *A. johnsonii* immobilised cells

### Growth, leakage and P-uptake by *A. johnsonii* immobilised cells

In order to assess the growth of immobilised cells, ca. 15 g alginate beads entrapping *A. johnsonii* cells were incubated in 100 ml activated sludge mixed liquor medium on a rotary shaker, 160 r/min at 28°C. Every hour, viable cell counts were performed on Biolab nutrient agar plates incubated at 28°C for 24 h. To recover bacterial cells for viable cell counts, 0.1 g alginate beads were hourly removed from the experimental system, washed with sterile distilled water, dissolved in 1 ml phosphate buffer (1M, pH 7) and dispersed by vigorous shaking on a vortex mixer prior to serial dilutions.

Simultaneously to the growth determination of immobilised cells, viable cell counts were also performed on the activated sludge mixed liquor in order to determine the leakage rate of *A. johnsonii* immobilised cells.

For phosphate analysis, two replicates of 1 ml activated sludge mixed liquor each were removed hourly from the experimental system and filtered through a Millex-GS 0.22µm filter unit in order to remove all bacterial cells. Phosphate concentrations (i.e. orthophosphate) were determined on the filtrate with the P(VM) 14842 test kit (Merck) using the Merck SQ118 photometer.

### Scanning electron microscopic studies

The distribution of *A. johnsonii* immobilised cells within alginate beads was determined using a Hitachi S-450 scanning electron microscope (SEM). For each observation with SEM, approximately 10 beads were removed from the aerated activated sludge mixed liquor system and fixed for 24 h in 2% glutaraldehyde solution in 0.1M sodium cacodylate buffer. The beads were rinsed three times for 15 min in sodium cacodylate buffer and fixed for 1 h in 1% osmium tetroxide, and rinsed again three times for 15 min in sodium cacodylate. The beads were thereafter dehydrated with increasing ethanol concentrations as follows: 50% for 15 min, 70% for 15 min, 90% for 15 min, 100% three times for 15 min. Half of fixed beads were cut in half and dried in a Hitachi HCP-2 Critical Point Dryer. The dried samples were mounted on a support, coated with gold in a Giko IB-3 ion coater, and examined under SEM.

Some other beads were fixed only for 1 h in 2% glutaraldehyde solution, washed with sterile distilled water, kept in a freezer for

48 h, then freeze-dried for 5 h using an Edwards Freeze Dryer Modulyo. The same procedure as above was then followed.

### Results

*A. johnsonii* immobilised cells displayed a short lag phase of ca. 1 h whereafter logarithmic growth occurred. Phosphate was removed from the activated sludge mixed liquor during the lag phase and when cells reached the stationary phase. The phosphate concentration of the activated sludge mixed liquor decreased from 61 mg/l to 25 mg/l, 24 h after incubation. Between time 1 h and 9 h, *A. johnsonii* displayed a decline in P-uptake (i.e. ascendent P-removal curve). Alginate beads (3.5%) without bacteria (i.e. control) also showed phosphate adsorption which was much lower than that of the immobilised cells over the experimental period. The immobilised cells also leaked out of the alginate beads (Fig. 1).

The existence of single cells and cell colonies observed within alginate beads is shown in the micrographs in Figs. 2 to 6. The distribution of the cells inside the bead was obtained by scanning the whole surface of the bead cross-sections.

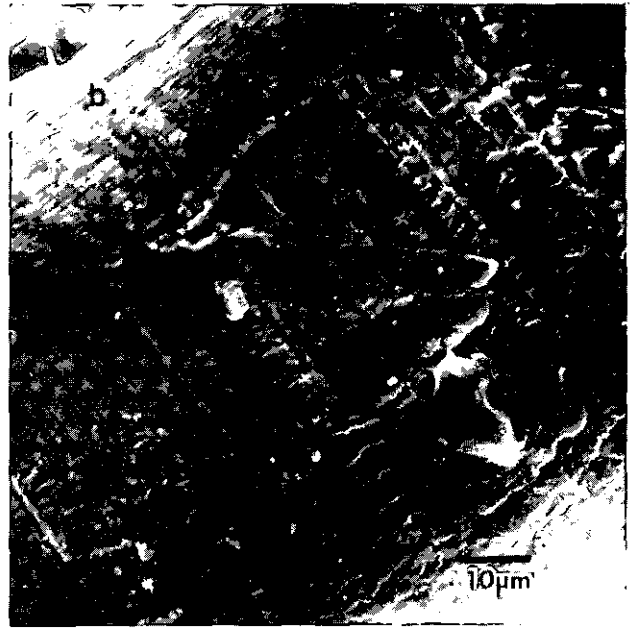
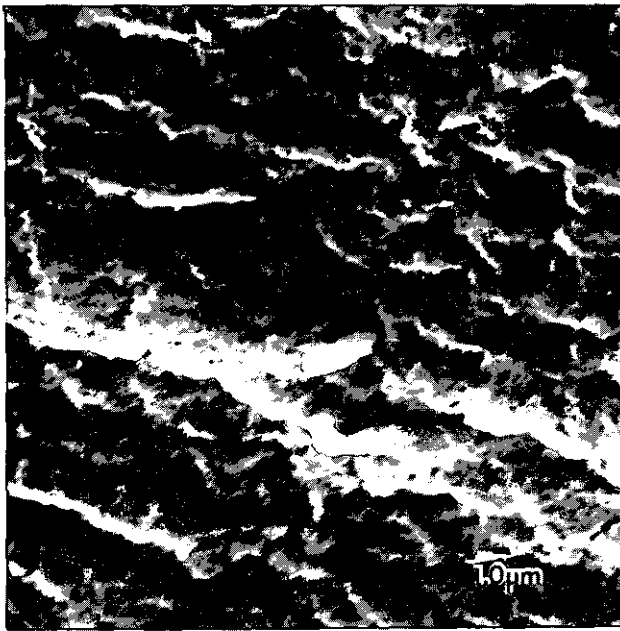
Figure 2 shows a fixed bead without bacterial cells, bead surface, and cross-section, while Fig. 3 shows a freeze-dried bead, showing grooves on the surface and a cross-section showing cavities inside the bead.

Single cells within cavities are shown by a cross-section at time 0 h (Fig. 4). Those were randomly distributed within the alginate bead. Figure 5a presents a cross-section of a bead at time 24 h showing a colony of cells inside a cavity. Such colonies were found in the outside layer as well as in the central part of the bead. This also indicated a random distribution of cells. Figure 5b shows the surface of a bead with some linked bacterial cells 24 h after incubation.

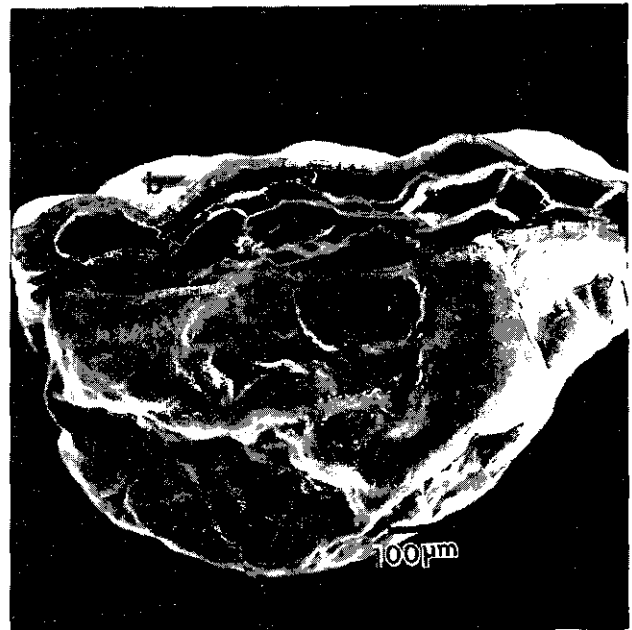
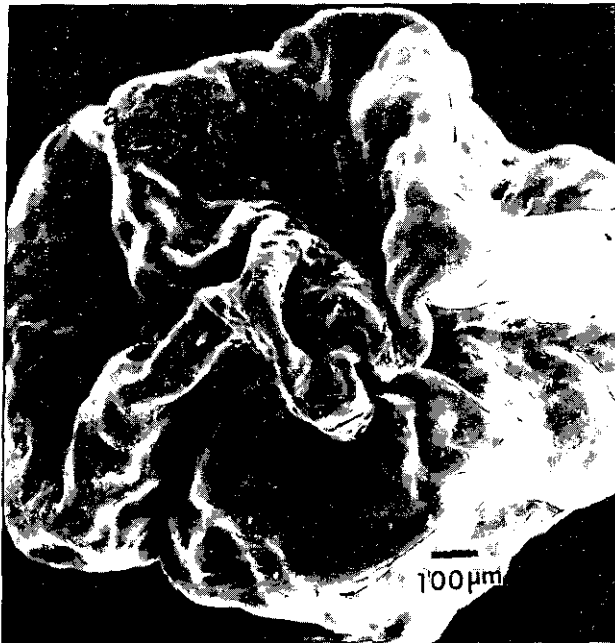
Figure 6 shows a cross-section of a two-week-old bead with a cell colony inside a cavity. Cells were adhering to one another by extracellular substances (Fig. 6a). These cells were found in the central part of the bead. A colony of cells covered by a biofilm was found in the outside layer of the bead (Fig. 6b).

### Discussion

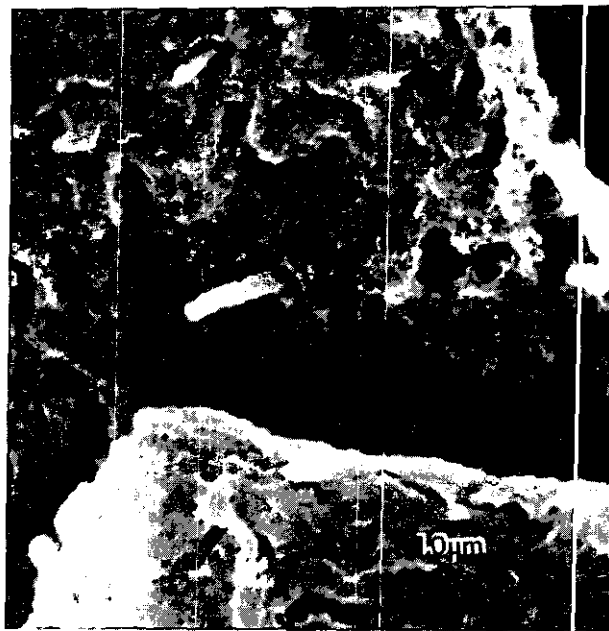
The results in this study not only confirmed the findings of Garde et al. (1981) and Bashan (1986) that the alginate bead structure



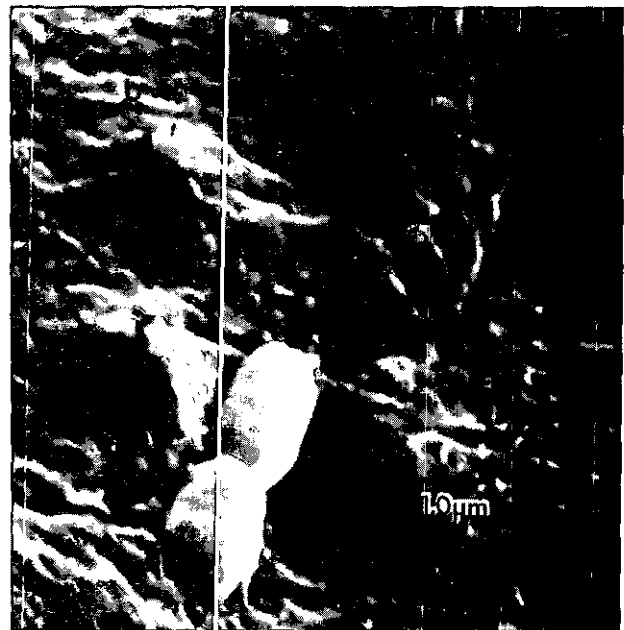
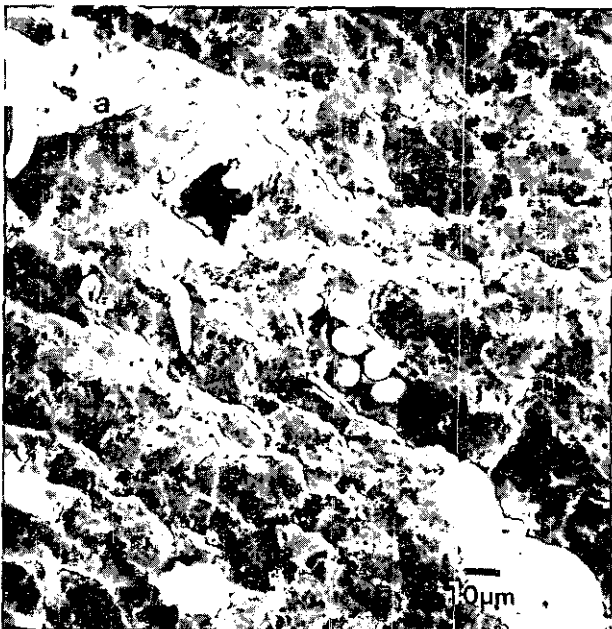
**Figure 2**  
*Beads without bacterial cells:*  
 (a) surface of the bead  
 (b) half cross-section



**Figure 3**  
*Freeze-dried bead:*  
 (a) whole bead showing grooves  
 (b) cross-section showing cavities



**Figure 4**  
Cross-section at time 0 h showing single cells



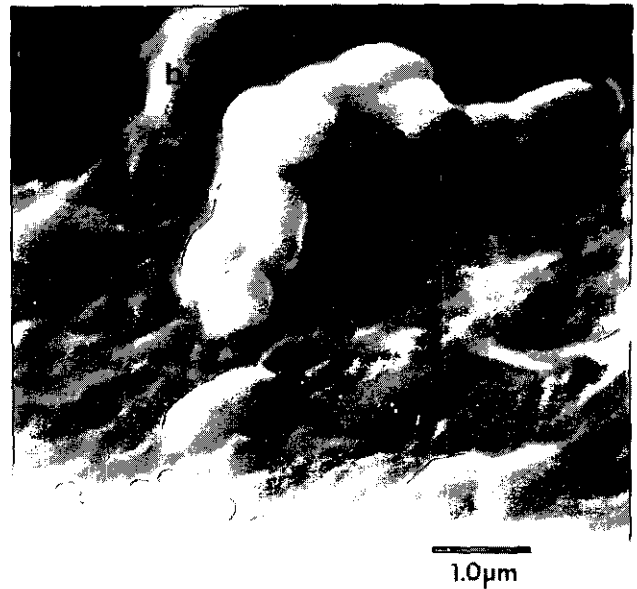
**Figure 5**  
Cross-section 24 h after incubation:  
(a) the inside of the bead  
(b) the surface of the bead.

shows grooves and bulges, but also pointed out the fact that the intensity of those structures may vary according to the fixation and dehydration procedures used (compare freeze-dried bead Fig. 3a, b to glutaraldehyde and osmium tetroxide fixed beads, Figs. 4 to 6).

In terms of growth, both viable cell counts and visual observations (SEM micrographs) indicated that *A. johnsonii* cells did survive and grow within alginate beads. This is illustrated by Figs. 4 to 6 when distinguishing between single cells at time 0h and cell colonies as observed 24 h and two weeks after incubation.

Since no cell colony was initially observed (i.e. time 0 h), cell colonies were the result of growth within the alginate beads. Growth is referred to as a change in the total population, rather than an increase in the size/mass of an individual micro-organism (Pelczar et al., 1986). Although *A. johnsonii* immobilised cells did leak out of the alginate beads, the cell yield inside the alginate beads remained higher than that in the bulk activated sludge mixed liquor.

Unlike Wada et al. (1980) and Shinmyo et al. (1982), who



**Figure 6**  
Cross-section two weeks after incubation:  
(a) extracellular substances  
(b) colony covered by a biofilm

reported growth to be limited to the outer layer of the bead, our observations indicated that growth occurred in the cavities all over the bead showing no remarkable preferences between the outer layer and the central part. Cells were observed in the central part of the alginate beads even two weeks after incubation. Cell colonies in the outer layer, as well as in the central part were bound to one another by extracellular material to form a biofilm (Fig. 5a, b). This might have contributed to preventing cells in the central part to move to the outer layer. According to Chang and Moo-Young (1988) large beads tend to develop anaerobic conditions in most of their interior. However, the results showed that the alginate beads with a diameter of approximately 2 mm, did not develop such anaerobic conditions when incubated in a well-aerated culture medium. The aeration by shaking might have facilitated oxygen transfer within the beads.

In terms of phosphate, high P-removal by the immobilised cells was observed 1 h after incubation which corresponded to the lag phase of growth and again 24 h after incubation during the stationary phase of growth. Our results confirmed the findings of Harold (1963), Lawson and Tonhazy (1980) and Bosch (1992), that polyP accumulation occurs when cells are not actively multiplying.

The observations in this study indicated a constant random distribution of immobilised cells within alginate beads for an extended time period of two weeks. This indicated that the space within the alginate bead was maximally used for growth to obtain high cell densities, thus allowing a higher biocatalytic activity, like enhanced phosphorus removal, in a small volume. The results also suggested that the distribution of cells within alginate beads varied between random distribution and outer layer preference depending on the bead size and the aeration conditions. The alginate immobilisation technique offers many possibilities for *in situ* studies on *Acinetobacter* and other polyP bacterial cells, since no limitation with regard to spacial distribution of cells within the matrix was observed. This technique is ideally suited for the auto-

ecological study of bacteria, since pure cultures can be immobilised, be suspended in their natural habitat and again be recovered for further study.

### Acknowledgements

We are indebted to Eskom and the Water Research Commission for funding this research program. We also thank Miss Weideman and Mr Hall for assistance with freeze-drying and electron microscopy, respectively.

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