GROWTH AND PHOSPHATE UPTAKE OF IMMOBILIZED *ACINETOBACTER* CELLS SUSPENDED IN ACTIVATED SLUDGE MIXED LIQUOR

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INTRODUCTION

Enhanced biological phosphorus removal is an important alternative to chemical phosphorus precipitation. Although other bacteria have shown phosphate uptake abilities (Brodisch and Joyner, 1982), *Acinetobacter* has become the model organism in the biological process of phosphorus removal from activated sludge (AS) plants (Stephenson, 1987; Toerien *et al.,* 1990). However, a lack of understanding of the mechanisms involved, mainly due to the lack of suitable techniques for studying these bacteria *in situ* prevails.

Immobilization of pure cultures of *Acinetobacter* could facilitate the *in situ* study of these organisms, to possibly explain the mechanism of phosphorus uptake by these bacteria. Immobilization of phosphate accumulating bacteria could also possibly provide an efficient phosphate removal process from wastewater compared to conventional suspended growth reactors (Gersberg and Allen, 1984). However, the behaviour of phosphorus accumulating bacterial cells (like *Acinetobacter)* in the immobilized state must be understood before any further development in this regard can take place.

Observations on immobilized cells indicated that these cells behave differently from cells free in solution (Mattiasson and Hahn-Hägerdal, 1982). Immobilization could affect microbial cell viability and/or growth (Doran and Bailey, 1986). Higher specific rates of substrate utilization for immobilized cells have been demonstrated (Navarro and Durand, 1977; Vijayalakshmi *et al.,* 1979) while, conditions for optimal growth have been reported to differ from those for suspended cells (Doran and Bailey, 1986).

Although various immobilization matrices e.g. kappa-carrageenan, iota-carrageenan, polyacrylamide (Takata *et al.,* 1977) have been used, combined factors such as the cost, the stability at low concentration of the cross-linker and the ease of formulation have resulted in alginate remaining the preferred material for immobilization studies (Poncelet *et al.,* 1992).

The objectives of this study were therefore to assess the effects of immobilization on viability, leakage rate, growth and phosphate uptake ability of immobilized *Acinetobacter* cells suspended in AS mixed liquor using alginate as the immobilization matrix.

MATERIALS AND METHODS

Bacterial cultures used in this study

Cultures of *Acinetobacter johnsonii* strain 105 obtained from the Department of Microbiology and Plant Pathology, University of Pretoria and *Acinetobacter calcocaeticus* strain ATCC23055^T were used.

Inocula were prepared by incubation of an aliquot of the respective bacterial stock cultures in 100 ml Biolab nutrient broth on an Edmund Bühler TH10 rotary shaker, 160rpm at 28 C for 12 h and 72 h.

Culture medium for phosphorus removal studies

Activated sludge mixed liquor was collected from the anaerobic tank of the Daspoort activated sludge plant at Pretoria. It was filtered on Whatman filter paper (ø 185 mm) and enriched with 5 mg/1 CH_3COONa, 0.18 g/1 KNO_3 and 0.5 g/1 $MgS0_4.7H_20$, before autoclaving. The nutrient additions were adapted from Bosch and Cloete (1993).

Immobilization technique

Entrapment of bacteria within alginate beads was carried out using a modification of the method of Bashan (1986). 3% and 3.5% sodium alginate (BDH) solutions were used. 4 ml of the 72 h bacterial culture (for viability and leakage studies) and 0.1 ml of the 12 h bacterial culture (for growth study) respectively were mixed with 20 ml sodium alginate at room temperature on a Heidolph MR2002 shaker at 600 rpm. The matrix and bacteria mixture was then added dropwise using a sterile 20 ml syringe, 26G needle into 150 ml of sterile $CaCl_2$ solution (1.1% m/v) at room temperature. Beads were immediately formed in the $CaCl_2$ solution entrapping *Acinetobacter* cells. Beads maintained for an

additional 1 h in the $CaCl_2$ solution were then washed three times with sterile distilled water. 3% and 3.5% alginate beads entrapping a similar volume of sterile Biolab nutrient broth were used as controls.

Viability determination of the immobilized Acinetobacter cells

Approximately 15 g alginate beads containing entrapped *Acinetobacter* cells, were placed into 100 ml sterile AS mixed liquor and incubated on a rotary shaker, 160 rpm at 28 *C*. Every hour, 0.1 g alginate beads were removed from the reaction vial and washed in sterile distilled water. To recover bacteria for viable counts, the beads were immersed in 1 ml phosphate buffer (1 M, pH 7) and dissolved by vigorous mixing on a Heidolph 54111 shaker for approx. 1 min. Serial dilutions and viable cell counts were performed on Biolab nutrient agar plates incubated at 28 C for 24 h.

Determination of leakage of immobilized Acinetobacter cells

Every hour, simultaneously with immobilized cells viability monitoring, serial dilutions and viable cell counts were also performed using the AS mixed liquor to determine whether any bacteria cells had leaked out of the alginate beads into the AS mixed liquor.

Growth of immobilized A cinetobaeter cells

Immobilized *Acinetobacter* (0.1 ml - 12 h culture) cells were washed with sterile distilled water before determining the initial number of viable cells. Thereafter, 14.5 g beads were suspended in 100 ml AS mixed liquor and incubated on a rotary shaker, 160 rpm at 28 C. 0.1 g beads were removed hourly from the mixed liquor and washed with sterile distilled water. Bacterial cells were recovered for colony counts. Alginate beads without bacteria were used as control.

Growth of Acinetobacter free cells

0.5 ml inoculum of free cells were inoculated into 100 ml AS mixed liquor (this provided the same ratio as for immobilized cells) and the same cultural conditions, as for the immobilized cells were used. Viable cell counts were performed hourly on Biolab nutrient agar plates, incubated at 28 C for 24 h.

The growth rate *(R)*—the number of generations per hour was determined using the following formula:

$$R = \frac{n}{t} = \frac{2.303(\log_{10}N_1 - \log_{10}N_0)}{(t_1 - t_0)}$$

where, R means the growth rate, n the number of generations, N_1 the total population at time one, and N_0 the number of bacteria at time zero.

Phosphate uptake determinations

Two replicates of 1 ml AS mixed liquor each were removed hourly from the experimental vial and filtered through a Millex-GS 0.22 μm filter unit to remove all cells. Phosphate determinations as orthophosphate were performed on the filtrate with the P(VM) 14842 test kit (Merck) using the Merck SQ118 photometer. Phosphate uptake (mg/1) and phosphate uptake per cell were calculated as follows:

[P uptake (mg/1)] = [P uninoculated control (mg/1)] — [P sample (mg/1)]

[P uptake/cell] = [P uptake (mg/1)] ÷ [cfu/ml after incubation x 1000].

RESULTS AND DISCUSSION

The effect of alginate concentration on the viability of immobilized Acinetobacter strains

Immobilized *A. johnsonii* and *A. calcoaceticus* cells remained viable over a 24 h monitoring period, although no growth occurred when 10^7-10^8 cfu/ml of initial cell concentrations were used (Figs 1-4*)*. Space, oxygen, water activity and nutrient transfer could be limiting growth factors at such high cell densities (Mattiasson and Hahn-Hägerdal, 1982) and these factors could contribute to the limited growth which occurred.

A. johnsonii showed a typical stationary growth phase pattern with slight fluctuations within viable cell numbers comparing between 3% and 3.5% alginate (Figs 1 and 3). Although, *A. calcoaceticus* remained in the stationary phase, it showed rather high fluctuations within viable cell numbers (Figs 2 and 4).

The biomass ratio (cfu/ml after 24 h ÷ cfu/ml initial density) of *A. calcoaceticus* was higher than that for *A. johnsonii* whilst 3.5% alginate immobilized cells presented a higher biomass ratio compared to 3% alginate (Table 1). 3.5% aginate therefore had a more pronounced stressed effect on the viability of both strains, than 3% whilst *A. calcoaceticus* was more affected than *A. johnsonii*

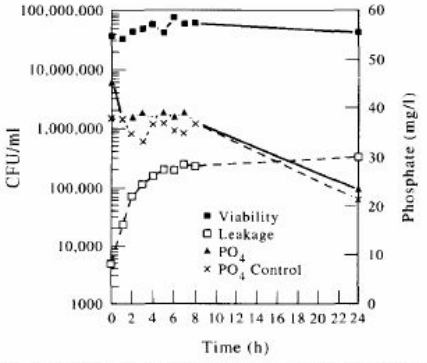

Fig. 1. Viability, leakage and phosphate uptake relationship of immobilized *A. johnsonii* cells within 3% alginate beads suspended in AS mixed liquor.

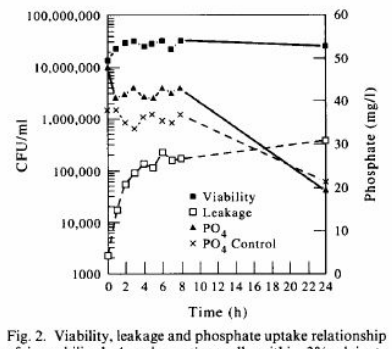

Fig. 2. Viability, leakage and phosphate uptake relationship of immobilized *A. calcoaceticus* cells within 3% alginate beads suspended in AS mixed liquor.

The effect of immobilization on the viability of *Acinetobacter* cells was dependent, both on alginate concentration, the strain under consideration and the cell density.

Leakage rate of immobilized Acinetobacter cells

Both *A. johnsonii* and *A. calcoaceticus* leaked out of the 3% and 3.5% alginate beads (Figs 1-4). A slightly higher leakage rate was observed at 3% than at 3.5% alginate for both *A. johnsonii* and *A. cacoaceticus* (Table 2).

A. johnsonii compared to *A. calcoaceticus* gave a higher leakage rate indicating that leakage rate might be dependent upon the concentration of alginate and the strain under consideration.

Both *A. johnsonii* and *A. calcoaceticus* leaked cells showed typical growth curves indicating the viability of leaked cells (Figs 1-4).

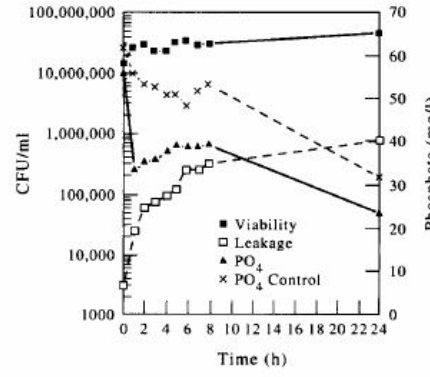

Fig. 3. Viability, leakage and phosphate uptake relationship of immobilized *A. johnsonii* cells within 3.5% alginate beads suspended in AS mixed liquor.

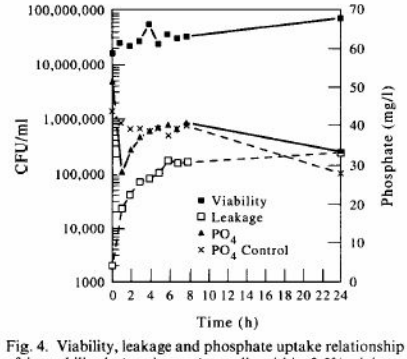

Fig. 4. Viability, leakage and phosphate uptake relationship of immobilized *A. calcoaceticus* cells within 3.5% alginate beads suspended in AS mixed liquor.

Growth of immobilized and free Acinetobacter cells

12 h cultures of both immobilized and free *A. johnsonii* cells presented ascendent growth curves, requiring no time to adapt to their new environmental conditions (Fig. 5). Growth rates determined for immobilized and free *A. johnsonii* cells were 0.91 and 0.84, respectively. Similar growth rates between immobilized and free cells were also reported by Dhulster *et al.* (1984). The stationary growth phase was reached after 9 h (Fig. 5).

A. calcoaceticus immobilized cell numbers declined after inoculation and started growing after 2 h, while free cells started growing immediately. Immobilized *A. calcoaceticus* cells therefore did not adapt immediately to the new environmental conditions probably as a response to stress. Similar observations

were reported by Shinmyo *et al.* (1982). Growth rates calculated for immobilized and free cells were 1.24 and 0.65, respectively. The stationary growth phase was also reached after 9 h (Fig. 6). Increase in microbial cell numbers within gel matrices compared to free cells have been reported for *Azospirilum brasilense* and *Saccharomyces carlsbergensis* cells (Bashan, 1986). This was also observed in this study where immobilized *A. johnsonii* and *A. calcoacelicus* cells yielded more biomass after 24 h than free cells (Table 1).

Table 1. Biomass ratios of immobilized *A. johnsonii* and *A. calcoaceticus* cells within 3% and 3.5% alginate beads

	Biomass ratios (T_{24}/ T_0)	
% Alginate	*A. johnsonii*	*A. calcoaceticus*
3.0	1.1	2.0
3.5	2.8	4.2

Table 2. Leakage rate of immobilized *A. johnsonii* and *A. calcoaceticus* cells within 3% and 3.5% alginate beads

	Leakage rate (cfu/ml)	
% Alginate	*A. johnsonii*	*A. calcoaceticus*
3.0	5.04×10^3	2.26×10^3
3.5	3.17×10^3	1.19×10^3

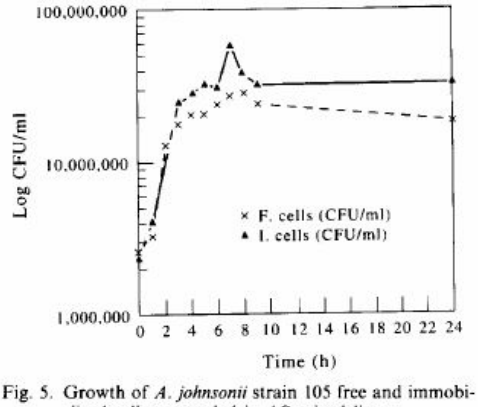

Fig. 5. Growth of *A. johnsonii* strain 105 free and immobilized cells suspended in AS mixed liquor.

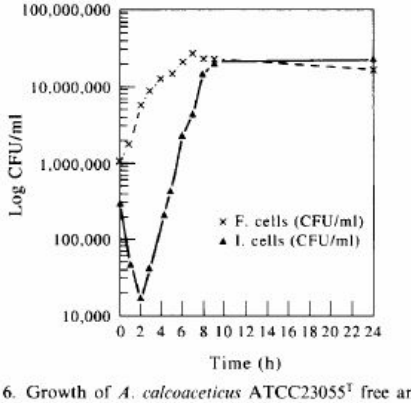

Fig. 6. Growth of *A. calcoaceticus* ATCC23055^T free and immobilized cells suspended in AS mixed liquor.

Phosphate uptake by immobilized growing cell systems

Phosphate in the figures and tables, indicates the rate of orthophosphate expressed as mg per litre (ppm) or per cell. Both *A. johnsonii* and *A. calcoaceticus* growing systems did not remove phosphate within the first hour of incubation. However, *A. johnsonii* showed phosphate uptake after 24 h of incubation (Table 3). *A. calcoaceticus* showed no phosphate uptake whilst growing. These results are in agreement with the findings by Harold (1963), Lawson and Tonhazy (1980), and Bosch (1992) who indicated that polyphosphate accumulation took place only when cells were not actively multiplying i.e. during lag and/or stationary growth stages.

Phosphate uptake by immobilized cells in the stationary phase

In order to keep cells in the stationary phase, cells harvested after 72 h were used as inocula. As control,

3% alginate beads without bacteria absorbed 1.1 x 10^{-3}mg P/l per mg of alginate. *A. johnsonii* and *A. calcoaceticus* immobilized (3% alginate) cells removed 2.4 x $10^{-10}$and 2.9 x 10^{-10}mg P/cell, respectively after 1 h of incubation (Figs 1 and 2, Table 4). After 24 h, *A. calcoaceticus* immobilized cells removed more phosphate per cell than *A. johnsonii* cells (4.5 x 10^{-10} compared to 1.3 x 10^{-10} mg/cell). *A. johnsonii* cells removed more phosphate after 1 h compared to 24 h (2.4 x 10^{-10} compared to 1.3 x 10^{-10} mg/cell) while on the contrary, *A. calcoaceticus* removed more phosphate per cell after 24 h, compared to 1 h (4.5 x 10^{-10} compared to 2.9 x 10^{-10} mg/cell). Between 1 h and 24 h, *A. johnsonii* showed a decrease in phosphate uptake per cell, while *A. calcoaceticus* phosphate uptake per cell gradually increased. From 2 h to 8 h, phosphate release was sporadically observed for both strains.

Table 3. Phosphate uptake of immobilized *Acinetobacter* growing cells within 3% alginate beads

		cfu/ml		Phosphate	
Strain	Time (h)	Before incub.	After incub.	mg/l	mg/cell
johnsonii					
105	After 1 h	9.45 x 10^5	2.57 x 10^6	- 3	—
	From 8 - 24 h	3.20 x 10^7	2.95 x 10^7	0.5	1.7 x 10^{-11}
	After 24 h		2.95 x 10^7	1	3.3 x 10^{-11}
calcoaceticus					
ATCC23055^T	After 1 h	9.40 x 10^4	2.40 x 10^4	- 2	—
	From 8 - 24 h	7.35 x 10^5	4.50 x 10^7	0	0
	24 h		4.50x 10^7	- 0.5	—

Note: — means negative uptake and should be interpreted as phosphate release.

Table 4. Phosphate uptake of immobolized *Acinetobacter* non-growing cells within 3 % alginate beads

		cfu/ml		Phosphate	
Strain	Time (h)	Before incub.	After incub.	mg/l	mg/cell
johnsonii					
105	After 1 h	3.77 x 10^7	3.39 x 10^7	8.12	2.4 x 10^{-10}
	From 8 - 24 h	6.01 x 10^7	4.28 x 10^7	-1.62	—
	After 24 h		4.28 x 10^7	5.75	1.3 x 10^{-10}
calcoaceticus					
ATCC23055^T	After 1 h	1.28 x 10^7	2.19 x 10^7	6.38	2.9 x 10^{-10}
	From 8 - 24 h	3.24 x 10^7	2.50 x 10^7	8.25	3.3 x 10^{-10}
	24 h		2.50 x 10^7	11.25	4.5 x 10^{-10}

Note: — means negative uptake and should be interpreted as phosphate release.

Table 5. Phosphate uptake of immobilised *Acinetobacter* non-growing cells within 3.5 % alginate beads

		cfu/ml		Phosphate	
Strain	Time (h)	Before incub.	After incub.	mg/l	mg/cell
johnsonii					
105	After 1 h	1.55 x 10^7	2.79 x 10^7	16.5	5.9 x 10^{-10}
	From 8 - 24 h	3.04 x 10^7	4.40 x 10^7	-5.7	—
	After 24 h		4.40 x 10^7	2.5	5.7 x 10^{-11}
calcoaceticus					
ATCC23055^T	After 1 h	1.57 x 10^7	2.44 x 10^7	20.5	8.4 x10^{-10}
	From 8 - 24 h	3.11 x 10^7	6.58 x 10^7	-4.7	—
	24 h		6.58 x 10^7	2.2	3.3 x 10^{-11}

Note: — means negative uptake and should be interpreted as phosphate release.

3.5% alginate beads, without bacteria, also showed phosphate adsorption of 1.5 x 10^{-3}mg P/l per mg of alginate (Figs 3 and 4). Comparing the phosphate uptake abilities of *A. johnsonii* and *A. calcoaceticus* after l h (5.9 x 10^{-10} and 8.4 x 10^{-10} mg/cell) and after 24 h of incubation (5 . 7 x 1 0 $^{-11}$ and 3.3 x 10^{-11} mg/cell), no remarkable difference was found (Table 5). However, both strains showed a remarkable difference in their phosphate uptake ability after 1 h compared to 24 h after incubation (i.e. 5.9 x 10^{-10} compared to 5.7 x 10^{-10} mg/cell, and 8.4 xl0^{-10} compared to 3.3 x 10^{-11} mg/cell). The largest quantities of phosphate were removed within the first hour, irrespective of the species under consideration. The results in Figs 3 and 4 also indicated that phosphate was released slowly between 2 h and 8 h, and removed again after 24 h. Excess removal of phosphate has been reported to occur mainly under aerobic conditions, while phosphate uptake and release processes are reversible (Ohkate *et al.,* 1985; Marais *et al.,* 1983). In this study phosphate was released between 2 - 8 h under aerobic conditions. Release of phosphate in *A. calcoaceticus* under aerobic conditions after the cessation of growth has been reported by Ohkate *et al.* (1985). Hopson and Sack (1973) also reported aerobic phosphate release after the cessation of growth in *Escherischia coli.* Osborn and Nicholls (1978) have found that, when the aerobic period exceeded 4h in the AS processes, a slow release of phosphate occurred even during the aerobic phase. The latter could explain the results obtained in this study since the cells were under aerobic conditions and not actively growing.

CONCLUSIONS

This study indicated that immobilization did affect the viability of *Acinetobacter* cells. The effect of immobilization on the viability depended both on the alginate concentration, the strain, and the cell density. The leakage rate, however, depended only on the alginate concentration and the strain. Leaked cells were viable since they displayed a typical growth curve. The growth rate of immobilized *A. calcoaceticus* was twice that of free cells, while *A. johnsonii* immobilized and free cells showed similar growth rates. Immobilized *A. calcoaceticus* and *A. johnsonii* growing cells did not take up phosphate. However, those in the lag and stationary growth phases showed a high phosphate uptake ability. The largest quantities of phosphate were, however, removed within the first hour (lag phase) compared to 24 h after incubation.

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