ACINETOBACTER CELL BIOMASS, GROWTH STAGE AND PHOSPHORUS UPTAKE FROM ACTIVATED SLUDGE MIXED LIQUOR

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INTRODUCTION

South Africa has limited water resources and it is therefore essential to prevent pollution so that the available water can be utilized optimally. Large concentrations of nutrients, such as phosphorus and nitrogen, in aquatic environments may cause excessive growth of photosynthetic plants and organisms, giving rise to a situation known as eutrophication. Since many algae can fix nitrogen, phosphorus is considered the most important growth limiting nutrient regarding eutrophication (Toerien *el al.,* 1975). Due to the excessive phosphorus concentrations found in wastewaters, resulting from industrial effluents and domestic detergents, efficient phosphorus removal is essential. In South Africa the Water Act (Act no. 54, 1956) was therefore amended in 1980 to limit the orthophosphate concentration in effluents from wastewater treatment plants to <1 mg $P.1^{-1}$ (Slim, 1987). Biological phosphorus removal, which is gaining support worldwide, is an alternative to chemical phosphorus precipitation. Activated sludge plants have been developed for biological phosphorus removal (Barnard, 1976), but the prevention of eutrophication however remains a problem due to inadequate biological phosphorus removal. The phosphorus concentrations of effluents are currently being reduced to <1 mg P. 1^{-1} by additional chemical precipitation with $FeS0_4$. Chemical precipitation however increases the operational cost of water treatment plants, not only as a result of the chemical cost but also the increased cost of sludge disposal. Another problem resulting from chemical precipitation is the increased salt concentration of the effluent which increases the mineralization of our aquatic environment. The need therefore exists to optimize biological phosphorus removal.

Acinetobacter has become the model organism for biological phosphorus removal since it was first isolated from a phosphorus removing activated sludge plant (Fuhs and Chen, 1975). *Acinetobacter* species can accumulate polyphosphates and, although not the only organism with this ability, they have been found to dominate in enhanced phosphorus removing activated sludge plants (Dienema *et al.,* 1980; Buchan, 1983; Lötter, 1985; Streichan *et al.,* 1990). Cloete and Steyn (1988b) found the average bacterial cell volume of volutin containing cells to be 1.0 μm^3, while the largest percentage of volutin containing cells only had a cell volume of between 0.50 and 0.59 μrn^3. Du Preez (1980) found that the cell volume and mass of A *calcoaceticus* increased with the growth rate, indicating that smaller cells had a reduced growth rate. From these results it would appear that phosphorus was accumulated mostly by the smaller cells, indicating that phosphorus removal by *Acinetobacter* could be influenced by the growth rate of the cells (Cloete and Steyn, 1988b).

The aim of this study was therefore to determine the relationship between cell biomass, phosphorus uptake and growth stage of different *Acinetobacter* isolates.

MATERIALS AND METHODS

Bacterial strains and culture conditions: Acinetobacter strains were obtained from culture collections and isolated from activated sludge according to the method of Bosch and Cloete (1993). The Acinetobacter strains received and isolated were identified further by numerical analysis of their total soluble cell protein profiles using sodium dodecyl sulphate gel electrophoresis (SDS-PAGE) of total soluble cell proteins and numerical analysis (Bosch and Cloete, 1993). The phosphorus uptake ability of the strains in mixed liquor was determined (Bosch and Cloete, 1993) and five of the strains exhibiting substantial phosphorus uptake were used in this study (Table 1). All strains were maintained on Nutrient agar (Biolab) slants at 40°C and subcultured monthly.

TABLE 1. *Acinetobacter* Strains Used in Growth and Phosphorus Removal Studies

Strain	SDS-PAGE identification[a]	Origin
ø5	*Acinetobacter johnsonnii*	Dam
AS60	*Acinetobacter lwoffii*	Activated sludge
AS78	*Acinetobacter colcoaceticus* subsp. *baumannii*	Activated sludge
AS93	*Acinetobacter Iwoffii*	Activated sludge
ATCC[b] 17908[T]	*Acinetobacter junii*	Urine

[a] Identified by Bosch and Cloete (1993)
[b] American type culture collection (ATCC, Rockville, Maryland 20852, U.S.A.); Type strain of *A. junii.*

Growth studies: Mixed liquor obtained from the anaerobic tank of a five stage Bardenpho activated sludge plant was centrifuged in a Sorval RC-5B centrifuge at 5000 g for 20 min. Mixed liquor medium (ML medium) was prepared according to a modified version of the acetate enrichment medium of Fuhs and Chen (1975) as follows: the supernatant from the centrifuged mixed liquor was prefiltered through Watman No. 1 filter paper and either 200 $mg.l^{-1}$ or $5g.l^{-1}$ sodium acetate (BDH), $0.5\ g.l^{-1}$ $MgS0_4.7H_20$ (Merck) and $0.18\ g.l^{-1}$ KNO_3 (Merck) were added and the pH adjusted to pH7 with 2N HCL, before autoclaving (121°C, 15 min). This ML medium was used for all growth and phosphorus uptake studies. For each isolate tested, two erlenmeyer flasks containing 96 ml sterile ML medium was inoculated with 4 ml of a culture, cultured in Nutrient broth, incubated for 48h at 28°C with shaking (80 rpm) and placed in a shaking waterbath (80 rpm) at 28°C. Growth was monitored by using one flask for absorbance determinations at 550 nm, while the second was used for viable count determinations.

Phosphorus uptake studies: Phosphorus accumulation was monitored by analysis of the phosphorus content of the medium and by determining whether polyphosphate granules were present in the cells, using transmission electron microscopy (TEM). The phosphorus content of the medium was analyzed by removing 1.0 ml samples from the flasks and filtering the samples through 0.22 μm filters (Millipore) to remove all cells. Uninoculated ML medium was used as control and treated in the same manner as the inoculated ML medium. The phosphorus content of the filtered medium was then determined with the P(VM) 14842 test kit (Merck), using the Merck SQ 118 Photometer.

TEM studies: Separate flasks were inoculated and treated in the same manner as the flasks used for the growth and phosphorus uptake studies. The contents of the flasks were centrifuged at 9000 g, for 10 min, to obtain a cell pellet which was then fixed, overnight at 4°C, in a solution containing 2.0% glutaraldehyde in 0.1M sodium cacodylate buffer. The cells were then washed in 0.1M sodium cacodylate buffer for 15 min. The washing process was repeated for three changes of buffer before post fixation in 0.25% osmium tetroxide for 60 min. The cells were washed again (15 min) with three changes of 0.1M sodium cacodylate buffer and then dehydrated for 15 min at each concentration in a graded alcohol series (50, 70, 90 and 3x100% ethanol). Infiltration with 33% Quetol resin took place for 60 min followed by 66% Quetol resin for another 60 min and finally 100% Quetol resin for 4h. The suspensions were then transferred to Beem capsules, centrifuged to obtain a pellet and the resin removed. Quetol resin (100%) added to each Beem capsule and allowed to infiltrate for 18h before being placed in an oven at 65°C for at least 48h to allow the resin to polymerize. Silver-gold sections were obtained using a Reichett-Jung ultramicrotome with a diamond knife. Staining of the ultrathin sections was accomplished by placing them on copper grids and floating the grids on lead citrate for 10 min and 6% aqueous uranyl acetate for 3 min. The stained sections were examined on a Hitachi H600 transmission electron microscope at 50 KV.

Cell size determinations. The cell volumes were determined directly from the electron micrographs, taking into account the magnification factor, according to the method of Cloete and Steyn (1988b).

RESULTS AND DISCUSSION

Growth and phosphorus accumulation of *Adnetobacter* strains in activated sludge mixed liquor. Although strains 5 and AS93 were capable of limited growth in the mixed liquor medium,

containing 200 mg.l^{-1} sodium acetate, very small quantities of phosphorus were removed from the medium (Figs 1-2).

Acinetobacter strains AS 60, AS 78, AS 93, ATCC 17908 and 5 all had the ability to grow and remove phosphorus in the mixed liquor medium containing 5g.H sodium acetate (Figs 3-7).

A lag phase of ca. 5 h was observed for all strains investigated and the stationary phase was reached after 10 and 14 h of growth. The absorbance readings were supported by the viable count trends (Table 2). The phosphorus removed from the medium was accumulated as intracellular polyphosphate inclusions, in the cells of strain AS93 and 5 after 4 and 7 h respectively (Fig. 8).

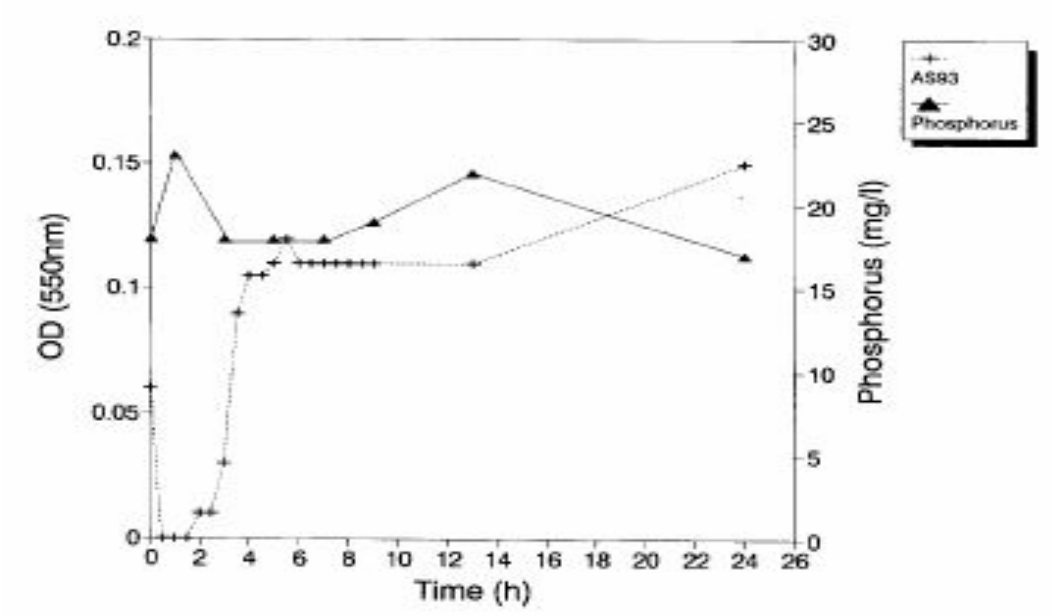

Fig. 1. Growth and phosphorus uptake of A. *lwoffii* strain AS93 in mixed liquor medium (200 mg/1 sodium acetate).

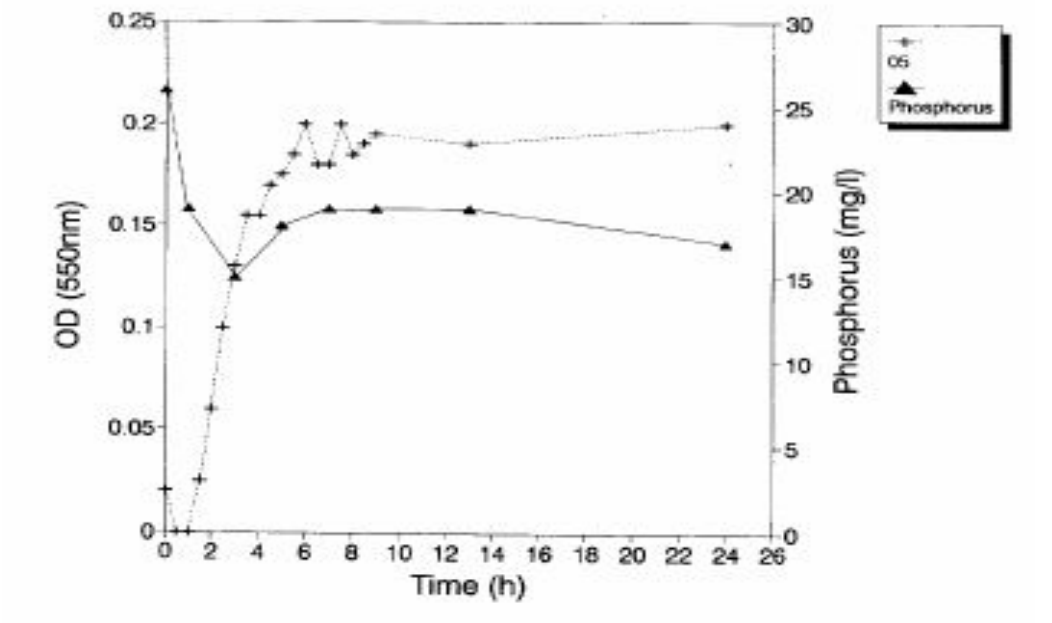

Fig. 2. Growth and phosphorus uptake of *A. jolinsonnii* strain ø5 in mixed liquor medium (200 mg/1 sodium acetate).

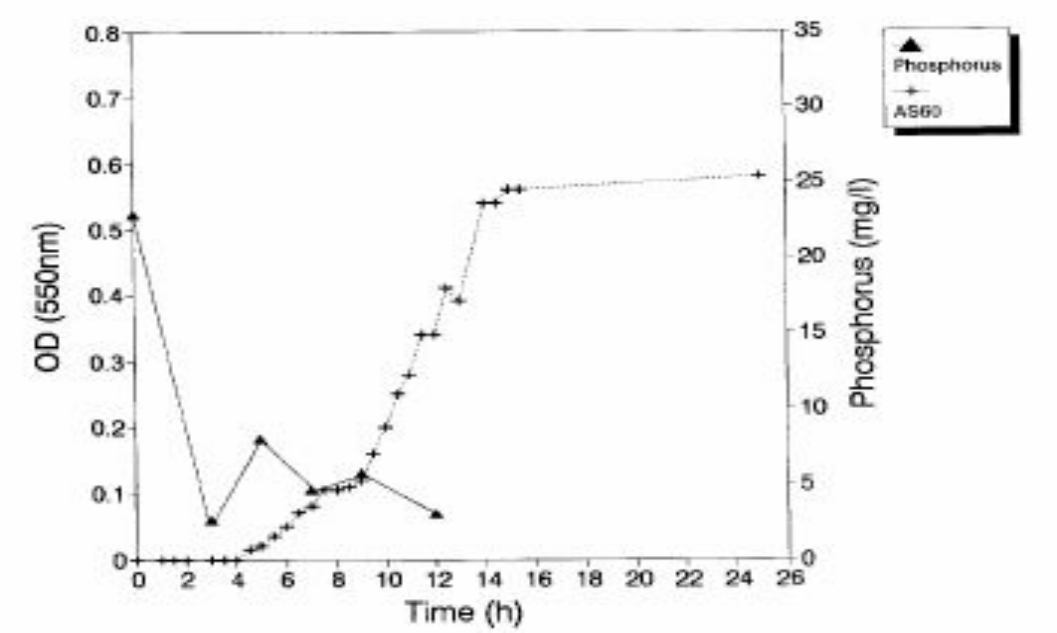

Fig. 3. Growtli and phosphorus uptake or A. *lwoffti* strain AS60 in mixed liquor medium (5 g/1 sodium acetate).

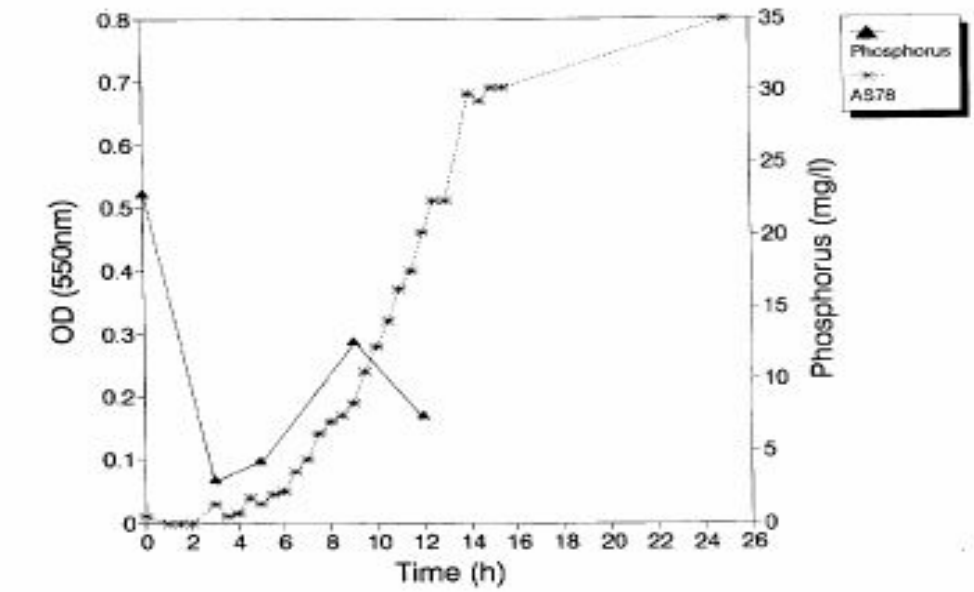

Fig. 4. Growth and phosphorus uptake of *A. calcoaceticus* subsp. *baumannii* strain AS 78 in mixed liquor medium (5 g/l sodium acetate).

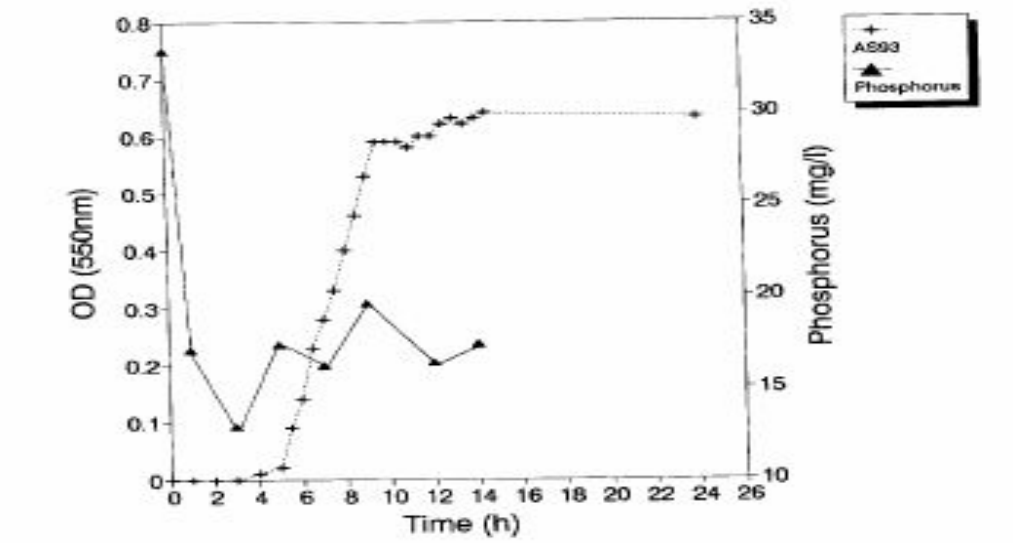

Fig. 5. Growth and phosphorus uptake of *A. lwoffii* strain AS93 in mixed liquor medium (5 g/l sodium acetate).

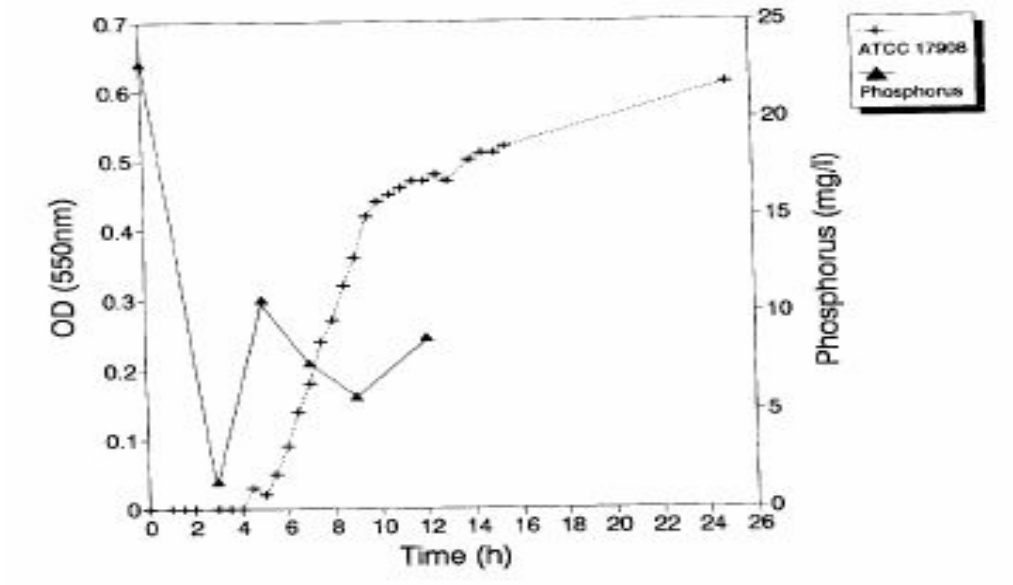

Fig. 6. Growth and phosphorus uptake of *A. junii* strain ATCC 17908 in mixed liquor medium (5 g/1 sodium acetate).

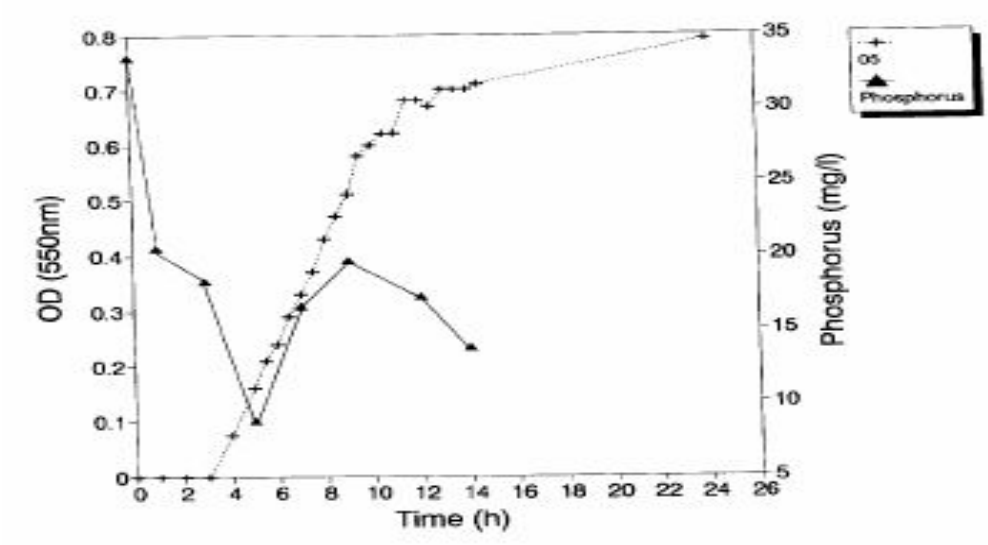

Fig. 7. Growth and phosphorus uptake of A. johnsonnii strain f5 in mixed liquor medium (5 g/1 sodium acetate).

From the results it would appear that most of the phosphorus was accumulated in the lag phase, which is a period of adjustment prior to the onset of cell division. During this period the cells synthesize the cellular components, enzymes and metabolic intermediates needed for cell synthesis. If cells are transferred to a medium of different composition, the biosynthetic pathways needed for the production of the metabolites not present, or for the utilization of those different metabolites present in the new medium, will be synthesized during the lag phase (Van Denmark and Batzing, 1987). Although there was no increase in cell numbers during this phase the cells were metabolically active and were capable of phosphorus uptake. Since no active growth occurs in the lag phase the cells will be relatively small and phosphorus uptake in this phase of growth would explain the observation that smaller cells mostly contained polyphosphate granules (Du Preez, 1980; Cloete and Steyn, 198Xb). Just prior to and during the initiation of the logarithmic growth phase a portion of the accumulated phosphorus was released. Some of the phosphorus released was then accumulated again near the end of logarithmic growth and during the stationary phase. These results correspond to results for an *Aerobacter aerogenes* culture in which it was determined that a reciprocal relationship exists between polyphosphate accumulation and nucleic acid synthesis (Harold, 1963). The author postulated that once nucleic acid synthesis began (i.e growth) competition for the available phosphorus would prevent the accumulation of polyphosphate (Harold, 1963). The definition of the 'luxury uptake' of phosphorus, as observed in activated sludge, is that excessive quantities of phosphorus are accumulated once growth has been arrested due to the lack of some nutrient (Fuhs and Chen, 1975). These results not only confirm that polyphosphate accumulation takes place only when cells are not actively multiplying, but also indicate that phosphorus accumulation occurred as part of the natural growth cycle (i.e. lag phase) and stress conditions (e.g lack of nutrients) were not a prerequisite for 'luxury uptake', but rather that the cells have a natural affinity to store polyphosphate.

A lower nutrient environment (i.e. 200 $mg.l^{-1}$ sodium acetate) resulted in less phosphorus removal from the medium, yet the quantity accumulated per cell was comparable to that of the corresponding strain cultured in ML medium containing 5 $g. l^{-1}$ sodium acetate (Table 2).

TABLE 2. Viable Counts and Phosphorus Uptake of *Acinetobacter* Strains in Mixed Liquor Medium*

Strain	Viable count CFU.ml^{-1}		Phosphorus (P) uptake[a] mg.l^{-1}	Phosphorus (P) uptake[b] mg.cell^{-1}
	Before incubation	After incubation[c]		
Free cells [a]	(200 mg.l^{-1} sodium acetate)			
ø5	5.8 x 10^{7}	2.43 x 10^{8}	9.00	3.7 x 10^{-11}
AS93	8.7 x 10^{6}	1.27 x 10^{8}	1.00	7.8 x 10^{-12}
Free cells [b]	(5 g.l^{-1} sodium acetate)			
ø5	2.24 x 10^{7}	1.31 x 10^{9}	20.00	1.51 x 10^{-11}
AS60	1.38 x 10^{6}	8.66 x 10^{9}	20.00	2.29 x 10^{-12}
AS78	2.40 x 10^{7}	7.35 x 10^{8}	15.00	2.09 x 10^{-11}
AS93	1.23 x 10^{7}	9.05 x 10^{8}	16.00	1.78 x 10^{-11}
17908	2.86 x 10^{7}	8.80 x 10^{8}	14.00	1.60 x 10^{-11}

[a] [P uptake (mg.l^{-1})] = [P uninoculated control (mg.l^{-1})] - [P sample (mg.l^{-1})]
[b] [P uptake.cell^{-1}] = [P uptake (mg.l^{-1})] ÷ [CFU.ml^{-1} after incubation x 1000]
[c] Free cells: [a] Strains AS 93 and ø5: 24h
[b] Strains AS60, AS78 and 17908: 13h;
Strain ø5 and AS93: 12h

Similar quantities of phosphorus were removed per cell, i.e. 3.7 x 10^{-11} and 1.51 x 10^{-11} mg.cell^{-1} for strain 5, in ML medium containing 200 mg.l^{-1} and 5 g.l^{-1} sodium acetate respectively. This suggests that cells may be limited to a certain quantity of polyphosphate uptake irrespective of the substrate availability. This polyphosphate limit may however differ between the different strains and would account for the variations in phosphorus accumulated. Higher substrate concentrations would therefore lead to greater phosphorus removal due to the resultant biomass increase (i.e. 9 mg.l^{-1} versus 20 mg.l^{-1} for strain 5) (Table 2).

Soil, water and sewage, being the natural habitat of these organisms, are environments subject to large fluctuations in nutrient availability which would explain the evolution of polyphosphate accumulation as a storage mechanism for times when its availability is low, thereby providing the organisms with a selective advantage. In the biochemical model for polyphosphate accumulation as proposed by Wentzel *et al.* (1986), in a completely aerobic environment where the Krebs cycle is active, the ATP/ADP ratio will be sufficiently high to supply the energy for polyphosphate accumulation provided sufficient substrate is available. The protons and electrons required would be supplied by the operation of the Krebs cycle. This was supported by our findings that phosphorus was accumulated in a completely aerobic environment. An average of ca. 17.06 mg l^{-1} of the phosphorus was removed from the medium by the five strains tested, with a maximum of 19.9mg.l^{-1} being removed by strain AS60 and a minimum of 14.1mg.l^{-1} by ATCC 17908 (Table 2). Although strain AS60 removed the largest quantity of phosphorus from the medium, in relation to biomass, it accumulated the least per cell, while strain 5 accumulated the most per cell. The larger quantity of phosphorus removed was therefore a function of the larger cell numbers and not due to a greater affinity of the cells for polyphosphate accumulation. This suggests that biomass is critically important to phosphorus removal. *Acinetobacter* strain 5 therefore effectively accumulated the most phosphorus per cell (Table 2). Nutrient availability would therefore appeal to enhance phosphorus uptake by virtue of the increased biomass and not due to an enhanced accumulation per cell.

The TEM photographs show that the phosphorus removed from the medium was accumulated by the bacteria as intracellular polyphosphate granules (Fig. 8).

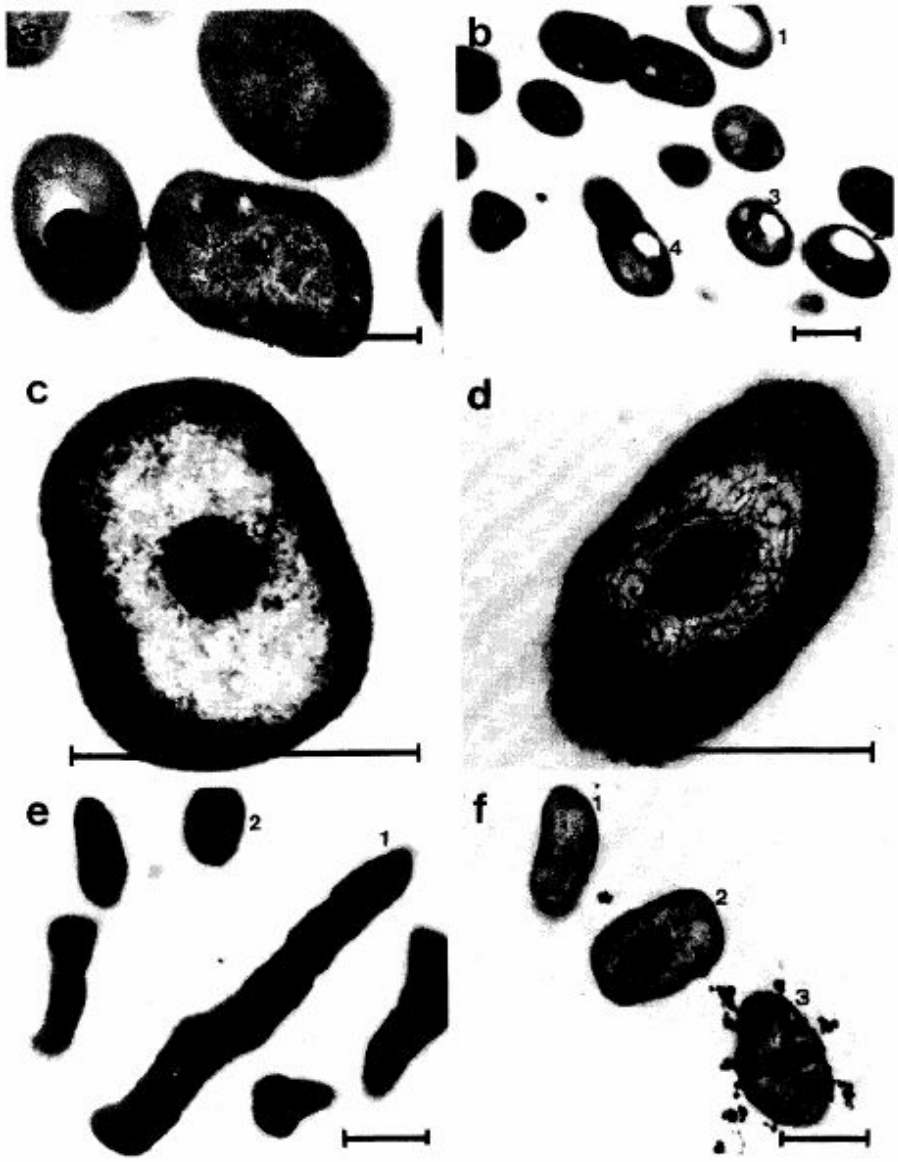

Fig. 8. TEM photographs of Acinetobacter strains f5 and AS93 showing intracellular polyphosphate granules: (a) Strain AS93 after 4h (35 000 x); (b) Strain AS93 after 4h, the translucent boles are where the polyphosphate granules were ripped out during sectioning (15000 x); (c) Strain AS93 after 7h (80 000 x); (d) Strain f5 after 7h (60 000 x); (e) Strain f5 after 7h, showing pleomorphism (20 000 x); (f) Strain f5 after 9h still contained polyphosphate granules (20 000 x). In each electron micrograph the bar represents 1mm.

The phosphorus concentration of the mixed liquor medium decreased dramatically in the first 5h, i.e. the lag phase (Figs 3-7), and polyphosphate granules were already observed at 4h (Fig. 8a,b,c). Although there were slight fluctuations in the phosphorus content of the medium, the cells still contained polyphosphate granules at 7 and 9h which was expected since the system was aerobic and phosphorus release was therefore not expected.

Bergy's manual (Juni, 1984) states that the average length of *Acinetobacter* cells is 1.5-2.5 µm. The cells in this study, containing polyphosphate granules, were ca. 1.0-1.5 µm in length (Fig. 8), indicating that cells with polyphosphate granules were mostly relatively small cells. Due to the cocco-bacilli shape and pleomorphic nature of *Acinetobacter* cell length alone is not a good measure of size, therefore the cell volume was determined (Table 3). Cloete and Steyn (1988b) found that most of the polyphosphate containing cells had a cell volume of 0.5-0.59 μm^3 or less. The frequency distribution of the cell volumes (Table 4) clearly indicates that most of the cells with polyphosphate granules are very small with cell volumes of between 0.1-1.9 μrn^3. It was therefore concluded that small slow growing cells accumulated polyphosphate. Pleomorphic *Acinetobacter* cells, containing polyphosphate granules, were found after 7h (Fig. 8e). The pleomorphic nature *of Acinetobacter* has been noted before as a result of oxygen deficiency (Du Preez, 1980). Du Preez (1980) suggested, that as the cell density increased during growth, the oxygen uptake rate increased and the oxygen transfer rate would eventually become the growth limiting factor. The dissolved oxygen concentration was however not determined in this study. The pleomorphic cells were however noted after 7h incubation at which time the cells were in the logarithmic growth phase and oxygen transfer could have been limiting due to the increased cell density as suggested by Du Preez (1980). Du Preez (1980) also found that oxygen limitation alone could not induce pleomorphism and that the growth rate did not play a role in this phenomenon.

TABLE 3. Cell Volume of *Acinetobacter* Cells Containing Polyphosphate Granules

Electron micrograph*	Strain	Cell volume μm^3
a	AS93	0.65
b(l)	AS93	0.22
b(2)	AS93	0.17
b(3)	AS93	0.12
b(4)	AS93	0.18
c	AS93	3.29
d	ø5	2.53
e(l)	ø5	0.53
e(2)	ø5	0.10
f(l)	ø5	0.14
f(2)	ø5	0.48
f(3)	ø5	0.28

*See Fig. 8.

TABLE 4. Frequency Distribution For The Size Of Polyphosphate Containing *Acinetobacter* Cells

Cell volume (μm^3)	Number of cells	% of total
0.10-0.19	5	41.67
0.20-0.29	2	16.67
0.30-0.39	0	0
0.40-0.40	1	8.34
0.50-0.59	1	8.34
0.60-0.69	1	8.34
0.70-0.79	0	0
0.80-0.89	0	0
0.90-0.99	0	0
>1.0	2	16.67

Growth rate did however influence the cell size. Slow growing cells were smaller than fast growing cells (Du Preez, 1980). The cells containing polyphosphate were relatively small, indicating a slow growth rate, especially since phosphorus was mostly accumulated in the lag phase when the cells were not actively growing.

ACKNOWLEDGEMENTS

The authors would like to acknowledge and thank the Water Research Commission for financial support which made this study possible.

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