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PHOSPHORUS UPTAKE KINETICS OF ACINETOBACTER IN ACTIVATED SLUDGE MIXED LIQUOR

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PHOSPHORUS UPTAKE KINETICS OF *ACINETOBACTER* IN ACTIVATED SLUDGE MIXED LIQUOR

by

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DECLARATION

I, the undersigned, certify the thesis hereby submitted to the University of Pretoria for the degree of M.Sc. and the work contained herein is my own original work and has not previously, in its entirety or in part, been submitted at any university for a degree.

Signature: ....

Date: ....

PHOSPHORUS UPTAKE KINETICS OF *ACINETOBACTER* IN ACTIVATED SLUDGE MIXED LIQUOR

by

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Promoter: Prof. T.E. Cloete
Department: Microbiology and Plant Pathology
Degree: M.Sc. (Microbiology)

SUMMARY

Phosphorus is one of the most important growth limiting nutrients in aquatic environments. Due to inadequate biological phosphorus removal in activated sludge systems, research is needed to optimize biological phosphorus removal.

Acinetobacter strains were obtained from culture collections and isolated from activated sludge. The strains were identified and classified using SDS-PAGE and numeric analysis. *A. baumannii* and *A. haemolyticus* are proposed as subspecies of *A. calcoaceticus* rather than as seperate species.

Acinetobacter is a very heterogenous genus exhibiting variations in phosphorus uptake. No spesific proteins could be attributed to the variation in phosphorus uptake due to the heterogeneity within a species and phosphorus uptake was thus found to be strain spesific rather than specie spesific.

Acinetobacter has the ability to grow and accumulate phosphorus in mixed liquor in both the free and immobilized state. Phosphorus was accumulated mostly in the lag phase of growth by relatively small cells.

DIE KINETIKA VAN FOSFOR OPNAME DEUR *ACINETOBACTER* IN MENGELVLOEISTOF

deur

MARLENE BOSCH

Promotor:	Prof. T.E. Cloete
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OPSOMMING

Fosfor is een van die belangrikste groei beperkende nutriënte in water sisteme. As gevolg van onvoldoende biologiese fosfor verwydering in geaktiveerde slyk sisteme bestaan die behoefte vir navorsing om die proses te verbeter.

Acinetobacter rasse was vanaf kultuurversamelings verkry en 'n aantal was vanuit geaktiveerde slyk geïsoleer. Die rasse was geïdentifiseer en geklassifiseer met behulp van SDS-PAGE en numeriese analiese. *A. baumannii* en *A. haemolyticus* word voorgestel as subspesies van *A. calcoaceticus* eerder as afsonderlike spesies.

Acinetobacter is 'n heterogene genus wat variasies in hul fosfor opname tentoonstel. Die variasie in fosfor opname kon nie aan spesifieke proteïene toegeskryf word nie en

fosfor opname was dus ’n ras spesifieke eienskap en nie ’n spesie spesifieke eienskap nie.

Acinetobacter het gegroei en fosfor opgeneem in beide die geïmmobiliseerde en ongeïmmobiliseerde toestand. Fosfor was in die sloerfase opgeneem deur relatief klein selle.

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CHAPTER 1

INTRODUCTION

The editorial style of the Journal of Bacteriology was followed in this chapter.

In South Africa water supplies are limited and droughts are a constant threat, making it essential to protect the aquatic environment from pollution. Nutrient removal from wastewater is thus important to prevent eutrophication. Phosphorus removal from wastewater is seen as one of the best methods for eutrophication control (Toerien *et al.*, 1975). The orthophosphate concentration of effluents has been limited in 1980 to 1 $mg.l^{-1}$ by an amendment of the Water Act, Act no. 54, 1956 (Slim, 1987). Phosphates can be removed from wastewater by chemical precipitation or biological accumulation. Biological phosphate removal, as an alternative to chemical removal, has gained support and activated sludge processes have been designed and operated for excess phosphate removal (Barnard, 1976).

Acinetobacter strains have often been isolated from and found to dominate in activated sludge plants exhibiting phosphate removal (Dienema *et al.*, 1980; Buchan, 1983; Lötter, 1985; Streichan *et al.*, 1990). For this reason *Acinetobacter* has been used as the model polyphosphate accumulating organism for studying biological phosphate removal. In recent years the enumeration and identification methods, which have resulted in *Acinetobacter* being found to be the dominant polyphosphate organism in activated sludge, have been questioned (Cloete and Steyn, 1988; Hiraishi *et al.*, 1989). However, *Acinetobacter* was recently again found to be the dominant microorganism in activated sludge by Streichan *et al.* (1990) and Auling *et al.* (1991). Although

Acinetobacter may possibly not be the dominant polyphosphate accumulating microorganism in activated sludge, it does have a role to play in phosphate removal. Many of the discrepancies of the past may have resulted from incorrect identification of isolates. The API 20E system, often used for identification, has been reported to be unable to distinguish between *Acinetobacter*, *Pseudomonas* and *Moraxella* spp. (Venter *et al.*, 1989). Although *Acinetobacter* has been used as the model polyphosphate organism in activated sludge systems, the taxonomy remains unclear and therefore the identification of isolates remains a difficult task.

Activated sludge plants have different rates of phosphorus removal even when *Acinetobacter* is present (Buchan, 1981). Certain environmental factors, such as nutrient imbalance (Smith *et al.*,1954), the presence of certain substrates (Lötter, 1985) or any stress producing situation *i.e.* oxygen or phosphate limitation (Nicholls and Osborn, 1979) are needed to induce excess phosphorus uptake, indicating that uptake may be an inducible process with inducible protein systems.

The study of the organisms responsible for biological phosphorus removal, in activated sludge, has been restricted by the lack of suitable techniques for *in situ* studies (Cloete and Steyn, 1988). Since the manipulation of a full scale activated sludge plant is not feasible, many investigations have been conducted on pilot plants (Ramadori, 1987; Streichan *et al.*, 1990; Kuba *et al.*, 1992; Tam *et al.*, 1992). Due to the difficulty in simulating full scale plant conditions, the information gained from these studies has been inconclusive. Cell immobilization allows the study of pure cultures *in situ*, which will make it possible to determine the growth, survival, phosphorus uptake and optimal retention time of the bacterial cells in each zone of the activated sludge process. Different conditions could therefore be simulated in a full scale plant and the effect on the bacterial cells determined, without interfering with the operation of the system. The technique may even be employed for the optimization of phosphate removal by adding immobilized pure cultures of polyphosphate accumulating bacteria (*e.g. Acinetobacter*) to activated sludge systems.

The aims of this study were therefore:

- to isolate a number of *Acinetobacter* strains from activated sludge and to identify and classify the strains using SDS-PAGE and numeric analysis;
- to determine the ability of these strains to grow and accumulate phosphate in mixed liquor;
- to determine if any variation in phosphate uptake could be ascribed to the different species;
- to determine if the variation in phosphate uptake could be ascribed to the presence or absence of certain proteins; using the SDS-PAGE data;
- to investigate the effect of growth phase on phosphate uptake;
- and to determine the ability of *Acinetobacter* strains, immobilized in sodium alginate, to grow and accumulate phosphate in mixed liquor.

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CHAPTER 2

LITERATURE REVIEW

The editorial style of the Journal of Bacteriology was followed in this chapter.

2.1 Introduction.

An inadequate water supply is a major threat to a country's future. The water supplies in South Africa are limited and it is predicted that the water demand will exceed the water supply by the year 2000 (Toerien *et al.*, 1975). Severe droughts experienced in recent years has emphasized the necessity to safeguard our water resourses from pollution. An increase in nutrients such as nitrogen and phosphorus in aquatic environments causes excessive growth of aquatic photosynthetic plants and organisms giving rise to a situation known as eutrophication. The eutrophication of aquatic environments by large populations of plants and algae results in the depletetion of the oxygen supply and in severe cases may cause the death of all other aquatic life. The restriction of nitrogen entering aquatic environments has limited value in the prevention of eutrophication, due to the fact that many algae can fix nitrogen, thereby obtaining both their carbon (CO_2) and nitrogen requirements from the atmosphere. The limitation of phosphorus to aquatic environments is thus seen as the best long term solution to control eutrophication (Toerien *et al.*, 1975; Slim, 1987; Gleisberg, 1992).

Efficient phosphate removal, from industrial and domestic wastewaters, containing excessive phosphate concentrations, is essential. Chemical removal of phosphates, applied in most countries, is expensive and increases the salt and mineral concentration of the effluent (Slim, 1987). Biological phosphate removal is an alternative to chemical phosphate removal and is gaining support worldwide. However, before biological phosphate removal can be used to its full potential, research is needed to determine which microorganisms are involved, how they interact and which mechanisms are involved in enhanced phosphate uptake, particularly in activated sludge.

2.2 Phosphorus removal: Chemical or Biological?

Phosphate removal from wastewater has received considerable attention due to the effect on the eutrophication of natural waters. Chemicals containing cations that form phosphate precipitates (*e.g.* lime, ferric and aluminium salts) have been and are still being used to remove phosphates from water treatment systems. In activated sludge wastewater treatment systems, high removal rates for phosphate have been reported (Vacker *et al.*, 1967; Barnard, 1976; Streichan *et al.*, 1990). Two theories exist to explain the excessive removal of phosphate. The first theory supports biological removal (Levin and Shapiro, 1965), while the second theory, as proposed by Menar and Jenkins (1970), supports a chemical removal mechanism in which calcium phosphate precipitates form.

Menar and Jenkins (1970) put forward a number of arguments in support of phosphate removal by chemical precipitation. They proposed that as the mixed liquor moves down the aeration basin the organic matter and CO_2 content will decrease while the dissolved oxygen content will increase, causing a rise in pH which will cause calcium phosphate precipitates to form in hard water sludges. The precipitate becomes physically entrapped in the sludge floc and is removed with the waste activated sludge. Release of phosphate under anoxic conditions was postulated to be as a result of the decrease in pH under these conditions which would cause the dissolution of the precipitates. In pilot plant activated sludge experiments, they found that decreases in dissolved phosphate were accompanied by decreases in dissolved calcium and for a given set of operating conditions the higher dissolved oxygen concentrations in the aerobic zone caused an increase in the pH of the mixed liquor. They also found that as the phosphate content of the sludge increased, its volatile fraction decreased. Finally, an empirical correlation was indicated between the calcium-phosphate product and the pH. These results all pointed to a pH-dependent chemical precipitation of calcium phosphate. Jenkins *et al.* (1971) reviewed the chemical processes which could bring about phosphate removal and proposed chemical treatments suitable to various wastewater and operational conditions. Chemical removal of phosphates, as proposed by Jenkins *et al.* (1971), has inherent disadvantages, namely the increased cost of sludge disposal, the high metal ion (*e.g.* Al^{3+}, Fe^{3+}, Ca^{2+}, Mg^{2+}) concentration of the sludge, the high pH of the effluent and increased equipment and maintenance costs (Shoda *et al.*, 1980).

On the other hand there are many factors that support a biological mechanism as being responsible for enhanced phosphate removal from activated sludge. High aeration rates strip CO_2 from the system causing an increase in pH which would cause calcium phosphate to precipitate. Levin and Shapiro (1965) however found that large quantities of phosphates were removed at pH 7-8 with a decrease in uptake at pH 9. If a chemical precipitation mechanism was operative an increase in uptake at pH 9 should occur, thus suggesting that the mechanism of uptake was biological. Similar experiments were conducted by other workers, indicating similar results (Carberry and Tenney, 1973; Dienema *et al.*, 1980).

Oxidative phosphorylation is inhibited by 2,4 dinitrophenol (2,4 DNP). Many experiments have been conducted in which the uptake of phosphate was found to be inhibited upon the addition of 2,4 DNP and in many cases phosphate was released. These results suggested that the uptake was biological and aerobic in nature since it ceased when the Krebs cycle was inhibited (Srinath *et al.*, 1959; Levin and Shapiro, 1965; Shapiro, 1967; Yall *et al.*, 1970; Carberry and Tenney, 1973). Heating of activated sludge to 40 and 50^0C before addition to sewage also prevented phosphate uptake, suggesting that phosphate uptake was dependant on the viability of the sludge. The quantity of phosphate removed was also dependant on the percentage activated sludge present. Srinath *et al.* (1959) found a linear relationship between the quantity of activated sludge present and the quantity of phosphorus removed from sewage. Phosphate uptake in sludge took place upon aeration in the presence of a carbon energy source, but with prolonged aeration phosphate was released. Phosphate uptake was therefore associated with the active fraction of sludge (Levin and Shapiro, 1965). Aeration experiments in the absence of microorganisms (0.45μm filtered mixed liquor) resulted in no phosphate removal, suggesting that the microorganisms in the activated sludge were responsible for the phosphate taken up by the sludge (Carberry and Tenney, 1973).

Certain microorganisms contain volutin granules consisting largely of metaphosphate (Smith *et al.*, 1954). From this it was concluded that microorganisms with the ability to form volutin can store phosphorus in this form and would therefore be capable of phosphate uptake in excess of their metabolic requirements (Levin and Shapiro, 1965). Bacteria isolated from activated sludge plants exhibiting luxury phosphate uptake (*e.g. Acinetobacter*) have since been shown to contain polyphosphate granules with the aid of light microscopy, electron microscopy and metachromatic staining techniques (Buchan, 1980; Dienema, 1980; Lawson and Tonhazy, 1980; Buchan 1981; Cloete, 1984; Murphy and Lötter 1986; Streichan, 1990; Auling *et al.*, 1991). With the aid of scanning transmission electron microscope X-ray analysis (STEM-EDX), Buchan

(1981) found that the electron dense bodies (volutin) within the cell were composed almost entirely of phosphorus and calcium with traces of magnesium, chlorine and potassium ions. Labelled isotope studies (Yall *et al.*, 1970), direct measurements (Carberry and Tenney, 1973) and STEM-EDX analyses (Buchan, 1981) have indicated that there was no relationship between phosphorus and calcium uptake and that the calcium:phosphorus ratios did not conform to that of a calcium phosphate precipitate. Electron microscope studies indicated that the phosphorus released under anaerobic conditions originated from the polyphosphate inclusions (volutin) within the cell (Buchan, 1981; Murphy and Lötter, 1986).

Phosphate uptake followed a sigmoidal curve indicating that active transport, against a concentration gradient, took place with the co-transport of monovalent cations (*e.g.* Na^+, K^+). Active transport is a biological phenomenon whereas if the mechanism of uptake was physio-chemical, diffusion and precipitation would be involved (Carberry and Tenney, 1973; Comeau *et al.*, 1986).

There has been widespread support of the biological removal theory and although chemical precipitation of inorganic phosphorus salts may occur, it is not believed to be responsible for excess phosphate removal. The activated sludge process has therefore been designed and operated for biological phosphate and nitrogen removal.

2.3 The activated sludge process for enhanced phosphate removal.

The activated sludge process has become the most commonly used method of liquid waste treatment. The term "activated sludge" is generally used to donate an aerobic slurry of microorganisms which remove organic matter from wastewater and are then removed themselves, usually by sedimentation (Grady and Lim, 1980). Biologically degradable waste of domestic or industrial origin is passed through a series of anaerobic, anoxic and aerobic treatments for the removal of organic matter. In the activated sludge system anaerobic refers to the exclusion of both oxygen and nitrates whereas anoxic refers to the exclusion of oxygen, but the presence of nitrates. All activated sludge systems have the following characteristics in common:

- The utilization of a flocculent slurry of microorganisms to remove soluble organic matter from wastewater.
- Microorganisms are removed by sedimentation prior to discharge, thereby producing an effluent low in microbial solids.
- A concentrated slurry of microorganisms is recycled from the clarifier underflow to the primary reactor.
- Performance is primarily dependent on the mean cell residence time (MCRT) of the organisms in the system (Grady and Lim, 1980).

The evolution of the design and working of the activated sludge process for phosphate removal has been reviewed in depth and will only receive a brief explanation here (Barnard, 1976; Grady and Lim, 1980; Toerien *et al.*, 1990). The Phoredox system, also known as the modified Bardenpho system or the 5-Stage Bardenpho system has evolved for carbon, nitrogen and phosphorus removal (Fig.1). The system consists of an anaerobic zone followed by a primary anoxic zone, a primary aerobic zone, a secondary anoxic zone, a secondary aerobic zone and lastly a clarifier (settler). In this system sludge is returned from the clarifier to re-enter the anaerobic zone with the influent. Mixed liquor is returned from the primary aerobic zone to the primary anoxic zone.

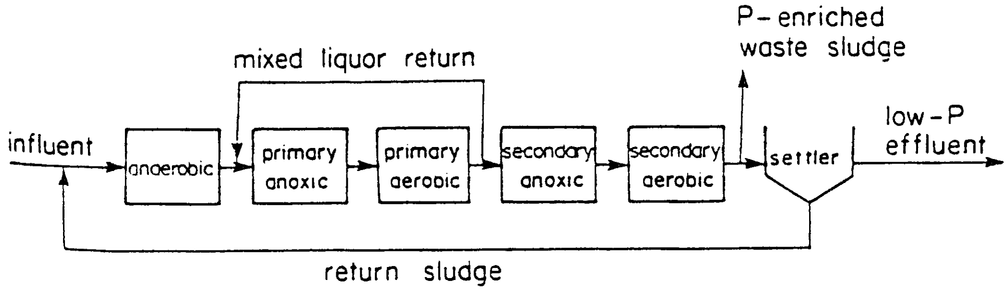

Fig. 1 A schematic representation of a five-stage Bardenpho activated sludge system for carbon, nitrogen and phosphorus removal (after Toerien *et al.*, 1990).

2.3.1 The primary anaerobic zone.

The influent wastewater flows into the primary anaerobic zone together with the return sludge from the clarifier. The anaerobic zone will lead to the enrichment of organisms capable of fermentation. *Enterobacter, Klebsiella, Citrobacter, Pasteurella, Proteus, Serratia* and *Aeromonas* spp. are examples of fermentative bacteria naturally found in water (Buchan, 1984; Lötter and Murphy, 1985). Fermentation however is an inefficient mode of energy production resulting in a low cell yield and thus limiting the population increase of these microorganisms. Fermentation products such as lactic acid, succinic acid, propionic acid, butyric acid, acetic acid and ethanol produced under anaerobic conditions cannot be utilized further under anaerobic conditions and are secreted, thereby becoming available as carbon sources for microorganisms in the forthcoming anoxic and aerobic zones (Fuhs and Chen, 1975; Buchan, 1984). Certain aerobic microorganisms can accumulate the organic substances secreted in the anaerobic zone and store these compounds as internal carbon and energy reserves in the form of poly-β-hydroxybutyrate (Nicholls and Osborn, 1979; Dienema *et al.*, 1980; Buchan, 1983). An increase in the orthophosphate concentration occurs in the anaerobic zone. It has been suggested that this is as a result of phosphate released by the microorganisms capable of accumulating polyphosphate under aerobic conditions (Shapiro, 1967; Fuhs and Chen. 1975; Buchan, 1983; Lötter, 1985; Suresh *et al.*, 1985).

2.3.2 The primary anoxic zone.

The mixed liquor moves from the anaerobic zone to the primary anoxic zone. In the anoxic zone denitrification takes place; this is the reduction of nitrates to molecular nitrogen (equation 1). Organisms capable of denitrification such as *Paracoccus denitrificans, Thiobacillus denitrificans* and *Pseudomonas* spp. proliferate in the anoxic zone (Buchan, 1984).

$$NO_3^- \longrightarrow NO_2^- \longrightarrow NO \longrightarrow N_2O \longrightarrow N_2 \qquad \text{(Equation 1)}$$

2.3.3 The primary aerobic zone.

In the aerobic zone nitrification and phosphate removal takes place. During nitrification ammonia or ammonia ions are oxidized to nitrite ions (equation 2) and then to nitrate ions (equation 3).

$$NH_4 + 2O_2 \longrightarrow NO_2 + 2H_2O \qquad \text{(Equation 2)}$$

$$2NO_2 + O_2 \longrightarrow 2NO_3 \qquad \text{(Equation 3)}$$

The microorganisms responsible for the oxidation of ammonia to nitrite belong to the genera *Nitrosomonas, Nitrosospira, Nitrosococcus* and *Nitrosolobus*. Nitrite oxidation to nitrates is accomplished by *Nitrobacter, Nitrospira* and *Nitrococcus* spp. The dominant aerobic bacteria present in the activated sludge system appear to be Gram-negative rods belonging to the genera *Pseudomonas, Aeromonas, Moraxella, Achromobacter, Alcaligenes, Flavobacterium* and *Acinetobacter* (Buchan, 1984; Lötter and Murphy; 1985). Certain bacteria are capable of storing large quantities of polyphosphates, in the form of intracellular volutin granules. These bacteria are believed to be responsible for the enhanced phosphate removal observed in activated sludge plants. Controversy exists as to whether or not *Acinetobacter* is the dominant phosphate removing organism, since other bacteria (*e.g. Aerobacter, Aeromonas, Pseudomonas,* and *Arthrobacter*) have also been found to accumulate polyphosphates, (Harold, 1966; Shoda *et al.*, 1980; Brodisch and Joyner, 1983; Cloete *et al.*, 1992).

Protozoa, fungi and filamentous microorganisms are also found in sludge. The filamentous microorganisms are important as they promote the formation of bacterial flocs. Floc formation is very important to the sedimentation ability of the sludge. Without the presence of the filamentous organisms a condition known as pin-floc occurs in which weak flocs are formed which settle poorly, whereas proliferation of the filamentous population in sludge may lead to problems such as bulking and foaming (Jenkins *et al.*, 1986). Algae and fungi do not play a very important role in nutrient removal in the activated sludge process. A return flow exists from the primary aerobic basin to the primary anoxic basin to promote complete denitrification and nitrification as well as phosphate removal.

2.3.4 The secondary anoxic and the secondary aerobic zones.

The mixed liquor flows from the primary aerobic zone to the secondary anoxic zone for the denitrification of any nitrates still present in the system (*e.g.* nitrates formed by nitrification in the primary aerobic zone). From the secondary anoxic zone the mixed liquor flows into the secondary aerobic zone for further removal of any residual organic material, ammonia (nitrification) and in particular any phosphate which might be released in the secondary anoxic zone.

2.3.5 The clarifier.

Lastly the mixed liquor flows into the clarifier where the flocs of microorganisms are allowed to sediment out and the effluent ("clean water") is removed from the system. Part of the sedimented activated sludge is returned via the clarifier underflow to the influent sludge and serves as an innoculum of microorganisms, the rest is treated further for use as fertilizer. If the phosphate concentration of the effluent is not below 1.0 $mg.l^{-1}$ as specified by legislation, the residual phosphate in the effluent may be precipitated out chemically before discharge into rivers or dams.

2.4 The Microbiology of enhanced phosphate uptake.

Since enhanced phosphate removal was first postulated to be mediated by a biological mechanism, work has centered around identifying the microorganisms responsible and the parameters that influence and control the process. Two terms are commonly used in biological phosphate uptake, the first is "luxury uptake" which refers to phosphate uptake in excess of metabolic requirements when growth is inhibited by some nutrient other than phosphate (Harold, 1966; Fuhs and Chen, 1975; Dienema *et al.*, 1980) while the second namely "polyphosphate overplus" refers to excessive uptake when a phosphate starved organism is placed in an environment with sufficient phosphate (Levin and Shapiro, 1965; Fuhs and Chen, 1975; Nicholls and Osborn, 1979; Dienema *et al.*, 1980). A certain quantity of phosphate is removed for normal metabolic requirements by all the microorganisms in activated sludge. This removal, as pointed out by Menar and Jenkins (1970), can only account for a maximum of 20-30% of the phosphorus present and not the enhanced removal rates (>90%) observed. Although they made no attempt to identify the microorganisms involved, Levin and Shapiro (1965) did postulate that the microorganisms that are responsible for enhanced phosphate uptake would have to be able to accumulate phosphate in excess of their normal metabolic requirements. On the basis of the findings by Smith *et al.* (1954), that the volutin granules found in microorganisms consist mainly of polyphosphates, they further postulated that this excess phosphate accumulated is stored in volutin granules. Since then electron microscope studies combined with electron dispersive micro-analysis of X-rays (EDX) have been used not only to confirm the presence of the granules within microorganisms, but also to determine that they do contain mostly polyphosphates with some calcium and traces of Mg^{2+}, K^+ and Cl^- ions (Dienema *et al.*, 1980; Buchan, 1981; Buchan, 1983; Cloete and Steyn, 1988b).

Fuhs and Chen (1975) were the first to isolate a bacterium which contained polyphosphate granules, from a phosphate removing sludge. The bacterium was identified as belonging to the genus *Acinetobacter.* Since then *Acinetobacter* strains have often been isolated from and often even found to dominate in activated sludge plants exhibiting enhanced phosphate uptake (Dienema *et al.*, 1980; Buchan, 1983; Lötter, 1985; Streichan *et al.*, 1990). Juni (1984) described the Genus *Acinetobacter* as consisting of aerobic cocco-bacilli which occur in pairs and chains and are found naturally in soil, water and sewage. *Acinetobacter* cells containing polyphosphate granules form large cells present in clusters (Buchan, 1983) and are pleomorphic to the extent of forming long filamentous cells when cultured in conditions of oxygen deficiency (Du Preez, 1980; Lawson and Tonhazy, 1980). Du Preez found that oxygen deficiency alone could not induce pleomorphism and although no other explanation was given for this phenomenon, the author did determine that growth rate did not play a role. The suggestion was made that the pleomorphic elongated cells may impart an advantage in that they become more easily entrapped in the recycled sludge floc than single cells, thus preventing the washout of *Acinetobacter* in activated sludge reactors (Du Preez, 1980). Contrary to the description of Juni (1984) many *Acinetobacter* strains were capable of nitrate reduction and could therefore be responsible for the limited phosphate uptake in the anoxic zone of activated sludge plants (Lötter, 1985).

Acinetobacter cells accumulated polyphosphate to *ca.* 60% of their cell volume which corresponds to 18% ($^{m}/_{v}$) polyphosphate or 10-20% polyphosphate on a dry weight basis by Buchan (1983) and Dienema *et al.* (1980) respectively. Even though these results show that *Acinetobacter* cells have the ability to store polyphosphate, by virtue of their numbers they can only account for a maximum of 34% of the phosphorus removed in activated sludge plants exhibiting enhanced phosphate removal (Cloete and Steyn, 1988b). Other microorganisms or mechanisms must therefore also be involved to effect the removal rates observed.

Pseudomonas, Aeromonas, Aerobacter, Alcaligenes, Arthrobacter, Moraxella, Proteobacteria and *Xanthobacter* spp. as well as the filamentous *Microthrix* and *Norcardia* spp. can also accumulate polyphosphates (Harold, 1966; Shoda *et al.*, 1980; Brodisch and Joyner, 1983; Lötter, 1985; Suresh *et al.*, 1985; Venter *et al.*, 1989 Streichan *et al.*, 1990; Auling *et al.*, 1991). Brodisch and Joyner (1983) found that activated sludge plants had a variety of populations without any microorganism dominating. However the systems studied by these authors had anaerobic retention times of up to 16h which could detrimentally affect the aerobic population and cannot be compared to the finding by Buchan (1981) that *Acinetobacter* was dominant, since the system he studied only had an anaerobic retention time of 30 min. Auling *et al.* (1991) found the polyphosphate bacterial population to be very heterogeneous and that *Acinetobacter* was dominant in nitrification/denitrification systems with a low organic loading while other microorganisms (*e.g. Pseudomonas*) dominated in systems without nitrification/denitrification steps and a high organic loading. Many bacteria besides *Acinetobacter* could thus be involved in phosphate removal, but care should be exercised in population studies as there are many factors which differ between various systems which may cause certain organisms to dominate. These factors, which include sewage composition, retention times at various stages in the process and the detection or identification methods employed need to be outlined if comparisons and generalized statements are to be made. This is supported by the finding of Streichan *et al.* (1990) that the microbial population differed according to the process used and the sewage composition.

The methods employed for the enumeration of polyphosphate bacteria has led to the assumption that *Acinetobacter* is the main polyphosphate bacteria in enhanced phosphate removing activated sludge systems (Toerien *et al.*, 1990). Most enumeration methods have involved viable counts coupled to the use of the API 20E system for rapid identification. These techniques provide questionable results due to the fact that activated sludge bacteria are found in clusters of hundreds of cells which are not easily dispersed and may not all be viable (Buchan, 1980; Toerien *et al.*, 1990). Venter *et al.* (1989) also questioned the use of the API 20E system as an identification tool since the system may give an inaccurate oxidase test, possibly due to the small quantity of growth being tested, and does not therefore always differentiate between *Acinetobacter*, *Moraxella* and *Pseudomonas* species. Using the API-20E system Buchan (1983) and Lötter *et al.* (1986) found that *Acinetobacter* represented 48-66% and 40-90% respectively, of the viable population in various activated sludge systems. However, viable, metabolically active bacterial populations do exist that do not form colonies on agar and therefore do not produce a viable count (Rosak and Colwell, 1987; Byrd *et al.*, 1991). Due to the possible inaccuracies that can occur using viable counts, many workers have attempted to find alternative enumeration methods for activated sludge organisms. Fluorescent antibodies against *Acinetobacter* (Cloete and Steyn, 1988a; Lötter and Murphy, 1985), total counts using acridine orange (Cloete and Steyn, 1988a), Diaminopropane (DAP),(Auling *et al.*, 1991), and quinone profiles (Hiraishi *et al.*, 1989) as a biomarkers for *Acinetobacter* (Auling *et al.*, 1991), have all been used successfully, singularly or in combination, to try and give more accurate population structures. Using a combination of the acridine orange count and the fluorescent antibody count, Cloete and Steyn (1988a) found *Acinetobacter* to constitute less than 10% of the total microbial population in activated sludge. In contrast Lötter and Murphy (1985) found *Acinetobacter* to form *ca.* 55% of the population using a fluorescent antibody technique. The isoprenoid quinones (respiratory quinones), found in the plasma membranes of bacteria, have great value in both taxonomy and ecological studies due to their wide distribution and the structural variation exhibited between different taxonomic groups (Collins and Jones, 1981; Hiraishi, 1989). There are two main groups of quinones, namely the menaquinones and ubiquinones. Gram positive bacteria and anaerobic bacteria have only menaquinones, Gram negative aerobes have only ubiquinones, while facultative bacteria have both menaquinone and ubiquinone. Hiraishi (1988) used quinone profiles to identify the different bacterial populations in twelve different activated sludges. In all the sludges tested both menaquinones and ubiquinones were present with Q-8 being the most predominant ubiquinone. From these results it appears that *Alcaligenes, Comamonas* and certain *Pseudomonas* spp., which contain Q-8 were the dominant bacteria in the activated sludge systems studied. Since *Acinetobacter*, a Q-9 containing bacterium, has often been reported to dominate in activated sludge, Hiraishi *et al.* (1989) used ubiquinone as a biomarker to determine the distribution of *Acinetobacter* in activated sludge. *Acinetobacter* and other Q-9 containing bacteria only formed 3-6% of the total ubiquinone bacteria in activated sludge. These results support the findings of Cloete and Steyn (1988a) and suggest that the role of *Acinetobacter* in activated sludge may have been overestimated in the past. The quinone concentration however differs between taxa and the ratio of ubiquinones to menaquinones in facultative organisms is not constant. This questions the ability of the technique to quantitively determine the microbial populations present in a given environment (Hiraishi, 1989). The use of quinone profiles is however a promising tool for environmental studies, especially if used in combination with other techniques such as direct counts or fluorescent antibodies when quantitive determinations are required.

2.5 Factors affecting phosphate uptake and release.

Acinetobacter strains have an inherent ability to accumulate polyphosphate and also polyhydroxybutyrate (PHB), but they require specific environmental conditions to induce the accumulation of these substances (Buchan, 1983; Lötter *et al.*, 1986; Beacham *et al.*, 1992). If this were true, it can be assumed that other polyphosphate accumulating bacteria also have specific requirements for polyphosphate accumulation. However, most of the experimental work to date uses *Acinetobacter* as a model organism for polyphosphate accumulation.

2.5.1 Aerobic and anaerobic conditions.

Polyphosphate accumulation occurs mostly in the aerobic zone of activated sludge systems indicating that most of the microorganisms involved are aerobes. Increased aeration rates have resulted in increased phosphate uptake (Carberry and Tenney, 1973). Aeration is therefore an important pre-requisite for phosphate removal. In batch tests, Levin and Shapiro (1965) noted that aerated mixed liquor took up phosphate while unaerated mixed liquor released phosphate. Shapiro (1967) further investigated phosphate release and found it to be influenced by temperature and requiring a redox potential of -150mV. Phosphate release was reversible upon reaeration and the release originated firstly from the acid extractable fraction, then the nucleic acid fraction. No significant quantity originated from the phospholipids or phosphoproteins. The release did not originate from cell lysis since it was reversible upon reaeration indicating that the microorganisms were still viable (Ohtake *et al.*, 1985). The phosphate released originated from the polyphosphate granules as indicated by electron microscopy, NMR analysis and phosphorus fractionation (Buchan, 1981; Buchan 1983; Lötter, 1985; Suresh *et al.*, 1985). Many theories exist which attempt to explain the release phenomenon. Shapiro (1967) suggested that release occurred as a direct result of anaerobiosis; Fuhs and Chen (1975) suggested that it was the increase in CO_2 and the decrease in pH caused by anaerobiosis that influenced phosphate release rather than the anaerobiosis itself; Ohtake *et al.* (1985) suggested that release was caused by the depletion of the energy required to maintain the high intracellular levels of polyphosphate as polyphosphate is accumulated against a high concentration gradient and an energy source must therefore be required for its maintenance while Buchan (1980), Gerber *et al.* (1986), Murphy and Lötter (1986) and Tam *et al.* (1992) all suggested that phosphate release was induced by certain substrates. Although no exact explanation exists to explain phosphate release it is believed that the release of phosphate is essential to bring about enhanced phosphate removal in the aerobic zone.

There are also a number of theories explaining the need for an anaerobic zone to enhance phosphate removal. Fuhs and Chen (1975), Buchan (1984), Lötter (1985) and Ohtake *et al.* (1985) suggest that the anaerobic zone is required to establish facultative microorganisms which produce fermentation products such as ethanol, acetate or succinate to serve as carbon source for polyphosphate bacteria, such as *Acinetobacter*, in the aerobic zone. Lötter (1985) suggested that the phosphate release that takes place in the anaerobic zone, results in phosphate starvation which preconditions the bacteria for enhanced phosphate uptake in the aerobic zone ("phosphate overplus"). Nicholls and Osborn (1979) suggested that the anaerobic zone serves to induce a stress situation for aerobic microorganisms in which those aerobic microorganisms with polyphosphate and carbon reserves (*e.g.* PHB) would be selected for, thereby enriching the polyphosphate population. An anaerobic pretreatment would thus result in excessive phosphate removal. It appears that all of these theories have some merit and probably work in combination to bring about effective phosphate uptake.

2.5.2 Anoxic conditions (nitrate).

Nitrate in the anaerobic zone reduces phosphate uptake. Nitrate probably creates an environment in which facultative microorganisms are able to metabolize substrate via oxidative pathways instead of the fermentative pathways which supply carbon sources for *Acinetobacter* and other polyphosphate bacteria, thus not enriching for these organisms (Barnard, 1976; Lötter, 1985; Nicholls and Osborn, 1979). Some *Acinetobacter* strains can reduce nitrates which could explain the limited phosphate uptake in the anoxic zone (Lötter, 1985). Comeau *et al.* (1986) observed phosphate uptake until all the oxidized nitrogen was removed and the PHB reserves had been consumed while Kuba *et al.* (1992) demonstrated that efficient phosphate removal occurred in an anaerobic-anoxic sequencing batch reactor.

2.5.3 Substrate.

Certain substrates induce phosphate release while other substrates promote phosphate uptake. Buchan (1980) tested *Acinetobacter* strains for their ability to grow and accumulate phosphate in the presence of a variety of substrates which could naturally be present in the activated sludge process. Growth was found to decrease with each substrate in the following order: butyrate, propionate, acetate, isobutyrate, ethanol. The substrates that produced the slowest growth, namely isobutyrate and ethanol also produced the largest polyphosphate inclusions. 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. Cloete and Steyn (1988b) found that mostly the smaller *Acinetobacter* cells contained polyphosphate granules thus indicating that polyphosphate is mostly accumulated by slow growing cells. Acetate cultured cells had large polyphosphate granules but only in a small percentage of the cells, whereas butyrate cultured cells had many very small polyphosphate granules. Propionate did not induce phosphate accumulation and no granules were observed. *Acinetobacter* grew well on lower fatty acids, lower alcohols and lactic acid, all compounds which may be formed in the anaerobic zone by facultative microorganisms (Dienema *et al.*, 1980). Acetate, formate and propionate, all induced phosphate release even in the presence of nitrate whereas butyrate, lactate, citrate, glucose, ethanol, methanol, 2,3 butandiol and succinate only induced release under anaerobic conditions (Gerber *et al.*, 1986). Phosphate release upon acetate addition has also been observed by Comeau *et al.* (1986), Murphy and Lötter (1986) and Tam *et al.* (1992). Phosphate release is therefore postulated to be directly linked to the substrates present and not anaerobiosis as such, because release could take place in anoxic and even aerobic conditions. Gerber *et al.* (1986) indicated that even though release occurred once the substrate reached negligible levels, phosphate uptake occurred and the best net phosphate removal was found with acetate, butyrate, propionate and lactate. Electron microscopy indicated that the intracellular phosphate granules disappeared during acetate treatment and was therefore seen as the source of the phosphate released into the external medium (Murphy and Lötter, 1986). Lötter (1985) found acetate to cause release but not succinate. The reason postulated for this was that acetate can readily diffuse into the cell and has the ability to dissipate the proton motive force (pmf) which controls the movement of metabolites through the membrane. Once the pmf has been dissipated, phosphate can diffuse out of the cell. Succinate does not trigger release since it is taken into the cell by active transport and does not affect the pmf.

Substrate obviously has some effect on both phosphate uptake and release but the literature reports some conflicting results which makes it difficult to make definite conclusions. For example, Buchan (1980) obtained no phosphate uptake when cells were cultured on propionate, whereas Gerber *et al.* (1986) found propionate to be one of the best substrates for net phosphate uptake. The proton motive force theory of Lötter (1985) is based on the finding that succinate does not induce release while acetate does and the different mechanisms these substrates use to enter the cell. Gerber *et al.* (1986) did however find succinate to induce phosphate release. Although some conflicting results are presented in the literature, it would appear that the substrate utilized induced phosphate release and not anaerobiosis as such. The biochemical pathways and enzymes which the various organisms possess will influence their ability to utilize certain substrates and will therefore affect their growth and phosphate accumulation ability under various conditions. The role of acetate in phosphate release is explained by the biochemical model of Wentzel *et al.* (1986) for phosphate uptake and release (*cf.* 2.6.1).

2.5.4 Poly-β-hydroxybutyrate (PHB).

In activated sludge carbon is the limiting nutrient and the ability to accumulate and store the available carbon source as a reserve will give that microorganism a selective advantage (Buchan, 1983). The presence of PHB in sludge organisms has often been noted (Nicholls and Osborn, 1979; Dienema *et al.*, 1980; Lawson and Tonhazy, 1980; Buchan, 1983; Comeau *et al.*, 1986). Dienema *et al.* (1980) and Comeau *et al.* (1986) observed that PHB was formed concurrently with acetate uptake. PHB reserves were consumed concurrently with phosphate accumulation (Comeau *et al.*, 1986) and the more nutritionally diverse *Acinetobacter* strains, *i.e.* those with the ability to accumulate PHB, were found to have an increased capacity for polyphosphate accumulation (Lawson and Tonhazy, 1980). The ability of microorganisms to store carbon sources (*e.g.* PHB) thus appears to be a pre-requisite for phosphate accumulation in the activated sludge system. The polyphosphate bacteria are aerobic and can only proliferate once they reach the aerobic zone. However external carbon sources are limited and an internal carbon source will allow the formation of adenosine triphosphate (ATP) which can be used for the active transport of phosphate into the cell (*cf.* 2.6.1).

2.6 Proposed biochemical mechanisms for phosphate uptake and release.

Ever since the presence of volutin granules rich in polyphosphates were observed in numerous microorganisms, attempts have been made to elucidate their metabolic function. Numerous hypotheses and biochemical models for polyphosphate metabolism have been proposed and will be briefly outlined here.

The hypotheses and discoveries governing polyphosphate metabolism prior to 1966 were reviewed by Harold (1966). Phosphate was accumulated under conditions unfavorable to growth and was stored within the cell as a polymer of orthophosphate with phosphoanhydride linkages thermodynamically equivalent to the "energy-rich" phosphate of ATP. Two characteristic patterns of polyphosphate accumulation occurred. The first involved a slow accumulation of polyphosphate after the cessation of nucleic acid synthesis due to the exhaustion of an essential nutrient, while the second involved a very rapid accumulation of polyphosphate after the addition of phosphate to phosphate starved cells, also known as "polyphosphate overplus". Enzymes catalyzing the biosynthesis and degradation of polyphosphate had also been isolated and characterized by that time. Polyphosphate kinase was the only enzyme known to catalyze the biosynthesis of polyphosphate by the following reaction (1): $ATP + (P_i)_n \rightleftharpoons ADP + (P_i)_{n+1}$. The enzyme is reversible and could therefore also degrade polyphosphate. Polyphosphate-AMP-phosphotransferase, polyphosphate glucokinase, polyphosphate fructokinase and polyphosphatase are all polyphosphate degrading enzymes, but polyphosphatase which causes the hydrolysis of polyphosphate to phosphate was believed to be the main degrading enzyme. Mutants of *Aerobacter aerogenes*, which had lost the ability to accumulate polyphosphate, did not exhibit any growth defects and it was therefore concluded that polyphosphate was a dispensable constituent of the cell. Such a widespread substance as polyphosphate must however give a selective advantage to the microorganisms that contain it or it would have been eliminated in the process of evolution. With this in mind, a number of hypotheses were made to explain the function of polyphosphate. Firstly it was hypothesized that polyphosphate acts as a 'phosphagen', meaning that it acts as an energy store in the form of high energy phosphate bonds which may be transferred to adenosine diphosphate (ADP) to form ATP. The validity of the hypothesis was questioned due to the fact that, if energy supplies are limited, polyphosphate did not break down and in *A. aerogenes* polyphosphate was degraded hydrolytically resulting in the loss of the energy rich bond. The second hypothesis suggested that polyphosphate served as a phosphate reserve for the biosynthesis of nucleic acids and/or phospholipids. Support for this hypothesis came from the fact that the enzymes involved in polyphosphate accumulation were derepressed during conditions of phosphate starvation. The hypothesis that a relationship existed between polyphosphate accumulation and cell division was not substantiated as no abnormalities in cell division were found in mutants unable to accumulate polyphosphate. The last hypothesis suggested that polyphosphate served as a regulator of phosphate, ADP and ATP levels in the cell thereby acting as a metabolic phosphate buffer. Polyphosphate metabolism, however accounts for a very small quantity of ATP generated. The hypothesis was then amended to exclude ADP and ATP and suggested that the function of polyphosphate was to control the intracellular phosphate balance with the formation of polyphosphate only once a sufficient quantity of phosphate was present (Harold, 1966).

In a review on polyphosphate metabolism Kulaev and Vagabov (1983) came to the conclusion that although the main function of polyphosphate may be the maintenance of an intracellular homeostasis regarding the concentrations of both monomeric phosphate and free cations, polyphosphate also acts as a donor of phosphate and energy for ATP formation. During antibiotic production polyphosphate was used as energy source and not ATP. Polyphosphate granules contain metal ions and therefore may also have a function in regulating the concentration of metal ions in the cytoplasm which may have adverse effects on the intracellular osmotic pressure and pH.

A number of biochemical models have been proposed to explain the phenomenon of phosphate uptake and release in activated sludge (Nicholls and Osborn, 1979; Marais *et al.*, 1983; Comeau *et al.*, 1986; Wentzel *et al.*, 1986). According to Marais *et al.* (1983) the full potential of the processes designed for phosphate removal from wastewater will not be attained until a biochemical model is put forward that explains the behavioral patterns observed in phosphate removal plants. The biochemical models all propose that polyphosphate is a phosphate and energy source, contrary to the proposal of Harold (1966). Each model proposed has improved on the model preceding it and the model of Wentzel *et al.* (1986) has thus emerged as the most comprehensive to date.

Wentzel *et al.* (1986) used the genus *Acinetobacter* as a typical polyphosphate organism and formulated their model according to the environmental conditions of anaerobic/aerobic sequencing that results in excess phosphate removal with acetate as substrate. Since *Acinetobacter* spp. are obligate aerobes, it was proposed that in the anaerobic zone of an activated sludge plant these microorganisms have no external electron acceptor present and are in an environment rich in organic substrate. If acetate is the substrate present in high concentrations, it will diffuse passively into the cell. Acetate diffuses into the cell in the form of acetic acid causing dissipation of the pmf by the removal of a hydrogen ion during transport into the cell. The lack of an external electron acceptor leads to an increase in the reduced nicotinamide adenine dinucleotide (NADH)/ nicotinamide adenine dinucleotide (NAD) ratio and the lack of oxidative phosphorylation to a decrease in the ATP/ADP ratio, thereby causing inhibition of the tricarboxylic acid (TCA) cycle. Once inside the cell acetate is converted to acetyl-CoA using energy obtained from ATP, thus further decreasing the ATP/ADP ratio. This results in polyphosphate degradation and the transfer of the phosphate and energy to ADP for ATP synthesis. Acetyl-CoA is converted to acetoacetyl-CoA which is then reduced to β-hydroxybutyryl-CoA using the protons and electrons obtained by the oxidation of NADH to NAD. Reduction in the NADH/NAD ratio will remove the inhibition of the TCA cycle for further generation of NADH. By this model a fraction of the acetate entering the cell will be reduced to PHB and a fraction will be oxidised via the TCA cycle to supply the electrons and protons via NADH formation for PHB synthesis. The formation of PHB will decrease the intracellular acetate concentration allowing further diffusion of acetate into the cell to take place. This continued diffusion will however only take place if the pmf is restored. It is proposed that this will be accomplished by the release of phosphate, obtained from the polyphosphate degraded for ATP formation which subsequently released the phosphate again when converting acetate to acetyl-CoA. The phosphate release occurs via a hydroxyl mediated antiport protein carrier and the cations, released with polyphosphate degradation, are also released from the cell via a proton mediated antiport protein. The pmf will thus be maintained in this manner and phosphate will be released in the anaerobic zone.

In the aerobic zone, oxygen is available as electron acceptor, but substrate will now be limited. The polyphosphate bacteria will have their stored PHB as carbon reserve. In aerobic conditions the TCA cycle and oxidative phosphorylation are active resulting in a decrease in the NADH/NAD ratio and an increase in the ATP/ADP ratio. The decreased NADH concentration will stimulate the degradation of PHB to acetate as a carbon and energy source for cell function. The increased ATP concentration will stimulate polyphosphate synthesis with phosphate uptake occurring via a hydroxyl mediated antiport and cation uptake via a proton mediated antiport and the pmf being maintained by ATP. In completely aerobic environments the ATP/ADP ratio will be high, therefore stimulating polyphosphate accumulation and if sufficient substrate is available PHB synthesis will also be stimulated with the protons and electrons for the reduction of acetate to PHB being supplied by the operation of the TCA cycle.

Under secondary aerobic conditions, assuming that very little or no carbon source is available and the cells have depleted their PHB reserves in the primary aerobic zone, but an external electron acceptor is available, namely oxygen, then all synthetic pathways will cease and ATP will be required solely for cell maintenance. It is proposed that a fraction of the protoplasm is used for this ATP production (*i.e.* endogenous respiration) and that under prolonged starvation conditions, that this protoplasm is not obtained from the cell itself but from the lysis of weaker cells. The concomitant release of phosphate originated from this cell lysis and not polyphosphate cleavage. The generation of ATP from protoplasm will maintain sufficiently high levels of ATP to prevent polyphosphate degradation. The polyphosphate released with cell lysis will not be reaccumulated and thus explains the phenomenon of secondary release.

Under anoxic conditions nitrate is available as electron acceptor, the extracellular carbon concentration is low and the cells at this stage contain PHB but little polyphosphate. *Acinetobacter* strains unable to reduce nitrates will behave as if under anaerobic conditions and will degrade any residual polyphosphate for ATP formation for cell maintenance and PHB accumulation with the concomitant release of phosphate. *Acinetobacter* strains able to reduce nitrates will thus produce ATP via oxidative phosphorylation by the reduction of nitrate using PHB as substrate. The energy yield from nitrate reduction is less than from oxygen and little phosphate will therefore be accumulated in this zone.

In the secondary anoxic zone, conditions of low intracellular and extracellular carbon sources exist, similarly to the secondary aerobic zone. If sufficient nitrate were still present in the system to provide an electron acceptor for those *Acinetobacter* strains capable of nitrate reduction they will also survive by endogenous respiration. If however there was no nitrate available or the *Acinetobacter* strain was unable to reduce nitrates, then cell maintenance requirements will decrease the ATP levels to such an extent that polyphosphate degradation takes place for energy production, also seen as secondary release.

Although based to a large extent on the model of Comeau *et al.* (1986), the model of Wentzel *et al.* (1986) explains the cellular mechanism of polyphosphate uptake and release in all sectors of the activated sludge process designed for both phosphate and nitrogen removal. The intracellular ATP/ADP and NADH/NAD ratios are proposed to be the main regulators of the metabolic behavior resulting in excess phosphate accumulation and PHB synthesis, which the model also proposes to be a prerequisite for polyphosphate accumulation. The model of Wentzel *et al.* (1986) was extended by Toerien *et al.* (1990) to explain the concomitant release of sulphate with phosphate under anaerobic conditions.

The most recent work in the field of phosphate metabolism involves an investigation into the transport of inorganic phosphate in an *Acinetobacter lwoffii* strain isolated from activated sludge (Yasaphe *et al.*, 1992). In this study it was proposed that *A. lwoffii* contains two transport systems similar to those found in *Escherichia coli* and *Pseudomonas aeruginosa*. A low affinity system that takes up phosphate constitutively is present and also a high affinity system, associated with the cell membrane, which is only active in phosphate starved cells. It appears that phosphate uptake in *A. lwoffii* is also regulated by a Pho regulon as in *E. coli*. These studies concur with the model of Wentzel *et al.* (1986) in that both the systems require the co-transport of cations. It would appear that the high affinity transport system would be active in the secondary aerobic and anoxic zones where phosphate is limiting, but the exact working of these systems regarding the various environmental conditions imposed on the organism in an activated sludge system still needs to be elucidated. The control mechanisms of phosphate transport whether it is mediated by a Pho regulon, ATP/ADP or NADH/NAD ratios and the interaction between these factors still needs to be clarified.

From the literature it appears that *Acinetobacter* has been used as the model organism for polyphosphate accumulation since it was first isolated from an enhanced phosphate removing sludge by Fuhs and Chen (1975). Since then the belief that *Acinetobacter* is the main organism responsible for phosphate removal has been strengthened due to the fact that it has been repeatedly isolated as the dominant organism in such sludges. However the accuracy of such findings using conventional enumeration techniques (*e.g.* viable counts) has been questioned and it has been proposed that other microorganisms are also involved in enhanced phosphate uptake (Cloete and Steyn, 1988a; Hiraishi, 1989). There is still no certainty as to the exact role of *Acinetobacter* in phosphate removal since the most recent research once again reports *Acinetobacter* as being the dominant polyphosphate microorganism in activated sludge (Streichan *et al.*, 1990; Auling *et al.*, 1991). In an activated sludge system many factors influence the bacterial population (*e.g.* sewage composition) and it may be possible that although *Acinetobacter* may dominate in one system it may form a negligible part of another system. Although *Acinetobacter* has been studied extensively in relation to phosphate uptake in activated sludge, very little progress has been made in the taxonomic classification of the organism which may have contributed to incorrect identifications in the past. Excess phosphorus uptake by *Acinetobacter* may be induced by certain environmental conditions (*e.g.* nutrient imbalances, certain substrates or stress factors) and since *Acinetobacter* strains vary in their ability to accumulate phosphorus, uptake may be an inducible process with inducible enzyme (protein) systems. For these reasons a taxonomic study of the genus *Acinetobacter* was necessary.

2.7 The taxonomy of *Acinetobacter*.

Due to its apparent role in phosphate uptake, the taxonomic status of the genus *Acinetobacter* is important, especially for isolation and identification purposes when studying the role of the genus in phosphate uptake in activated sludge systems or when doing population studies.

A group of Gram negative, aerobic, chemoorganotrophic, non-flagellated, non-pigmented cocci and rods exhibiting 'twitching' motility was studied by Baumann *et al.* (1968a) and referred to as the Moraxella group. The Moraxella group vary considerably in their growth requirements, but possess one common characteristic: their failure to utilize polysaccharides, disaccharides, polyalcohols or glucose as carbon sources for growth. The Moraxella group was divided into two distinct subgroups by their oxidase reaction which is an indication of the presence of cytochrome c. The oxidase-positive moraxellas possess cytochromes b and c while the oxidase-negative moraxellas possess cytochromes a and b. The oxidase-negative moraxellas can be distinguished from the oxidase-positive moraxellas by their greater resistance to penicillin (Baumann *et al.*, 1968a; Baumann *et al.*, 1968b; Juni, 1984), the broad spectrum of carbon sources utilized, including the utilization of glucose via the Entner-Doudoroff pathway by a small number of strains, and the absence of transformation between the two groups (Baumann *et al.*, 1968a; Juni, 1972; Juni, 1984). The oxidase-positive moraxellas were placed in the genus *Moraxella* by Baumann *et al.* (1968a). The oxidase-negative moraxellas, being common inhabitants of soil, water and sewage (Juni, 1984) and being able to utilize a variety of carbon sources, have in the past been placed in a large variety of genera of which *Bacterium*, *Neisseria*, *Micrococcus*, *Diplococcus*, *Alcaligenes*, *Achromobacter*, *Acinetobacter* and *Pseudomonas* are examples (Baumann *et al.*, 1968a). On the basis of various nutritional and biochemical characteristics Baumann *et al.* (1968b) divided 106 oxidase-negative moraxellas into two distinct groups, A and B, within which there were seven subdivisions with a similarity coefficient above 0.7 (A1-A3, B1-B4). The oxidase-negative moraxellas were placed in the Genus *Acinetobacter*, a genus originally established by Brisou and Prevot (1954) to accommodate the non-motile members of the Genus *Achromobacter*. Group A was given the epithet *calcoaceticus* with subgroup A1 considered the main biotype and A2 and A3 additional biotypes. The epithet *lwoffii* was given to subgroup B2 and *hemolysans* to B3 with B4 considered as a subspecies of B3 with the sub-specific name *haemolyticus*. Subgroup B1 was considered to contain atypical strains of groups A and B and was not given any specific name. The work done by Baumann *et al.* (1968b) was supported by DNA-DNA homology studies (Johnson *et al.*, 1970) in which five groups emerged with greater than 50% homology to their respective reference strains, corresponding to the groupings made by Baumann *et al.* (1968b). No DNA homology was found between oxidase-positive and oxidase-negative strains, further supporting the division of the two groups (Johnson *et al.*, 1970). Significant rRNA homology however indicates that a distant relationship does exist between the two groups (Johnson *et al.*, 1970). Interspecies transformation of *Acinetobacter* strains confirms that all the strains tested belong to the same genus and the lack of transformation with oxidase-positive moraxellas also supports the division of the two groups into separate genera (Juni, 1972).

Identification of *Acinetobacter* to the species level has been difficult, especially as only one strain, *A.calcoaceticus*, is described in Bergy's Manual of Systematic Bacteriology (Juni, 1984) and only two species , *A.calcoaceticus* and *A. lwoffii*, are on the Approved List of Bacterial Names (Skerman *et al.*, 1989). Twelve hybridization groups (genospecies) were however recently described by Bouvet and Grimont (1986) and four of these genospecies were given new names (*Acinetobacter baumannii*, *A. junii*, *A.*

johnsonii and *A. haemolyticus*) while the existing species *A.calcoaceticus* and *A. lwoffii* were redescribed. The four new species were all of clinical origin. Three radiation resistant *Acinetobacter* strains have also been isolated from cotton (Kairiyama *et al.*, 1979) and soil (Nishimura *et al.*, 1988b). These isolates, resistant to gama-radiation, were placed in a new species, namely *A. radioresistens* on the basis of phenotypic characteristics, outer-membrane protein patterns, DNA-DNA hybridization and electrophoretic analysis of enzymes (Ino and Nishimura, 1989; Nishimura *et al.*, 1986; Nishimura *et al.*, 1987; Nishimura *et al.*, 1988a; Nishimura et al., 1988b).

While trying to establish whether there was any DNA homology between *A. radioresistens* and other *Acinetobacter* strains Nishimura *et al.* (1987) found high DNA homologies between strains of *A. baumannii* and the type strain of *A. calcoaceticus*, 81.8%-96.5% homology versus the 37%-39% homology found by Bouvet and Grimont (1986). They subsequently proposed that the type strain of *A. baumannii* (Bouvet and Grimont, 1986) be classified as a subspecies of *A. calcoaceticus* and not as a species. This suggestion was supported by enzyme electrophoretic analysis where *A. baumannii* formed part of a subcluster of *A. calcoaceticus* (Nishimura *et al.*, 1988b). The classification of *A. baumannii* as a subspecie of *A. calcoaceticus* can be justified further by the fact that both these species form part of group A of Baumann *et al.* (1968b) and hybridization group 1 of Johnson *et al.* (1970). For most of their phenotypic studies Bouvet and Grimont (1986) grouped genospecies 1 (*A. calcoaceticus*) and genospecies 2 (*A. baumannii*) together since the only differences between the two species were that *A. baumannii* strains could grow at 44^0C, produce β-xylosidase and utilize D-malate. Due to the discrepancies in percentage homology found, no definite proposals can be made until further work is done.

Electrophoresis is one of the more popular and versatile analytical techniques used for taxonomic studies. The technique can also be successfully applied as a mechanism of identification. Electrophoresis was therefore suited to this study where activated sludge isolates needed to be both identified and classified taxonomically and also to determine whether their capability take up phosphorus could be correlated to the presence or absence of specific proteins.

2.8 Electrophoresis.

The electrophoresis technique has been reviewed in detail (Kersters and De Ley, 1980) and some of the main principles, advantages and disadvantages will be outlined briefly.

The technique entails placing an electrically charged molecule (*e.g.* proteins or nucleic acids) in an electric field and thereby making it move through a 'solid phase' to an oppositely charged electrode. The solid phase serves as a molecular sieve separating the molecules according to size. The solid phase used depends on the application and can be polyacrylamide, agarose, agar, starch, paper or cellulose acetate.

The electrophoresis of proteins only, will be considered further. The discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is the most commonly used electrophoretic technique for proteins (Laemmli, 1970). The technique entails pre-treating the proteins with SDS in conjunction with a heat treatment. The SDS forms a SDS-peptide complex, thereby masking the normal electric charges of the molecule, while the heat denatures the proteins so that peptide chains with no secondary structure are obtained, thus allowing only size to play a role in separation.

A bacterial strain growing in standardized conditions will always produce the same set of proteins. These proteins are an expression of the bacterial genes or nucleotide sequences of the bacterial DNA. The quantity of each protein produced is also genetically determined. Protein electrophoresis can therefore be considered as a genomic 'fingerprint'. It allows accurate clustering and identification of strains within a species that corresponds to results obtained by DNA-DNA hybridizations and even %G+C values (Kersters and De Ley, 1975). The technique is much faster than phenotypic and biochemical analysis and by including internal reference strains and by making use of numerical analysis reproducible and objective comparisons can be obtained.

The technique like any other has a few inherent limitations. Small variations in band positions may occur in different electrophoretic runs of the same sample. Strictly standardized procedures must therefore be followed and each sample repeated in triplicate with internal reference proteins. A complex banding pattern is obtained in which each band consists of a number of structurally different proteins having the same electrophoretic mobility. Another limitation is the chance of human error due to the large number of experimental steps required.

PAGE has been applied extensively as a taxonomic and identification tool in a large number of genera (Gottlieb *et al.*, 1966; Ames, 1974; Kersters and De Ley, 1975; Dicks and Van Vuuren, 1987; Dijkshoorn *et al.*, 1987; Kersters and De Ley, 1980; Kiredjian *et al.*, 1986; Van Zyl and Steyn, 1990) on both soluble whole cell and soluble membrane proteins. Besides bacterial proteins, the technique has also been applied to bacteriophage structural proteins (Laemmli, 1970). Cellular proteins (Alexander *et al.*,1984) and cell envelope proteins (Dijkshoorn *et al.*, 1987) of *Acinetobacter* strains from clinical origins have been analyzed by SDS-PAGE and found to be more useful in subdividing the genus into ‘varieties’ or ‘biotypes’ than available biochemical identification kits. A certain degree of heterogeneity was found in the protein patterns confirming findings by other workers that *Acinetobacter* is a heterogeneous genus (Dijkshoorn *et al.*, 1987). The analysis of enzymes and outer membrane proteins of radiation resistant *Acinetobacter* strains by SDS-PAGE has led to the classification of a new species namely *A. radioresistens* (Nishimura *et al.*, 1986; Nishimura *et al.*, 1988b; Ino and Nishimura, 1989) and the SDS-PAGE groupings also corresponded to DNA homology studies (Ino and Nishimura, 1989).

SDS-PAGE is a valuable technique which can be utilized to its full potential especially in a heterogeneous genus such as *Acinetobacter*, to identify the different strains and possibly even to find explanations for some of its relatively unique characteristics such as luxury phosphate uptake in activated sludge.

Another valuable technique which can be very useful in the study of the phosphate uptake ability of *Acinetobacter* isolates, is immobilization. Cell immobilization makes it possible to study pure cultures in their natural habitat. Immobilized pure cultures of *Acinetobacter* placed in an activated sludge system will enable one to determine the survival of the culture in the various zones, the optimal mean cell retention time in each zone and the ability of the culture to grow and accumulate phosphate in each zone. In this way the optimal operating conditions for the activated sludge system can be determined without any disruption of the operation of the system.

2.9 Immobilization.

Immobilization refers to the localization or physical confinement of a biological catalyst on or within a solid matrix that will allow the catalyst to remain fixed relative to substrate and product and therefore available for continual reuse (Inloes *et al.*, 1983).

Natural and synthetic polymers have been used to immobilize enzymes, subcellular organelles, whole microbial, plant and animal cells, spores and multicomponent systems. Immobilization techniques have gained attention mainly for their uses in biochemical reactors, but many potential uses exist which are constantly being discovered and explored.

Klein (1988) summarized the basic immobilization methods as seen in Fig. 2, with entrapment being the most widely used method.

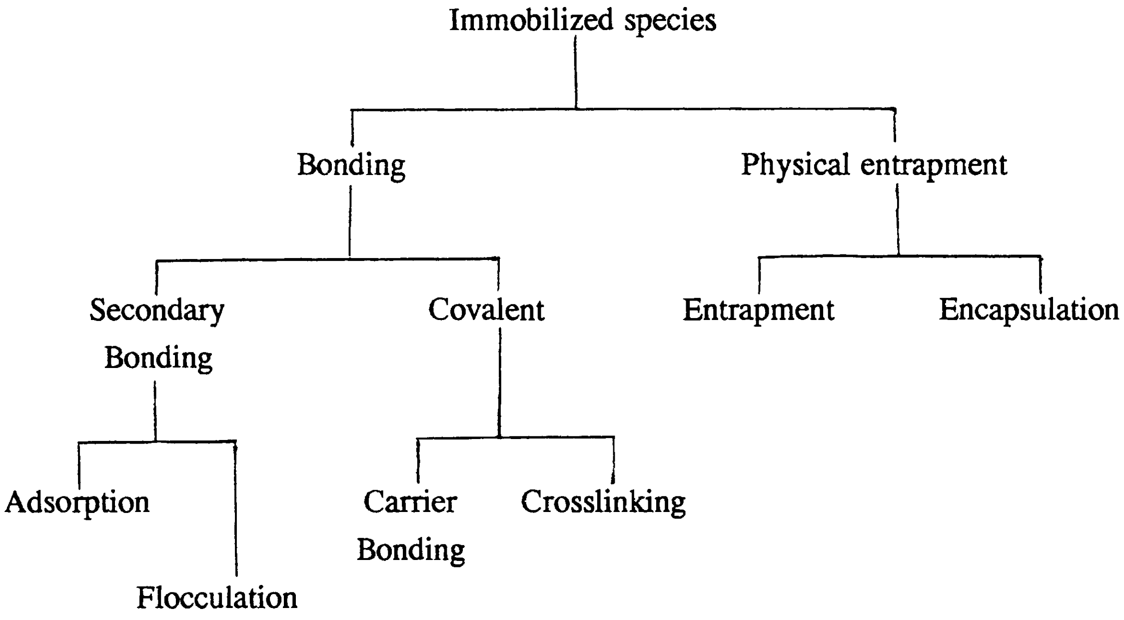

Fig. 2. Methods used for cell immobilization (Klein, 1988).

Numerous polymers have been used for a variety of applications. Takata *et al.* (1977) and Nagashima *et al.* (1984) compared a large number of different natural and synthetic polymers for the immobilization of whole cells and found that kappa-carrageenan and sodium alginate respectively to give the best results. These two polymers have generally been the most popular choice for the immobilization of cells and enzymes (Tosa *et al.*, 1979). Some other immobilization matrixes that have been used include polyacrylamide (Chibata *et al.*, 1974; Nagashima *et al.*,1984; Brodelius, 1988; Vandamme, 1988), resins (Hattori and Furusaka, 1960; Nagashima *et al.*, 1984), gelatin coated glass beads (Doran and Bailey, 1986), agar (Gersberg and Allen, 1984; Nagashima *et al.*, 1984; Brodelius, 1988), agarose (Brodelius, 1988), hollow fibre membranes (Inloes *et al.*, 1983), ceramics (Messing, 1988; Scharer *et al.*, 1988), polyurethane (Brodelius, 1988), iota-carrageenan, furcellan (Takata, *et al.*, 1977), porous polystyrene, porous polyester (Nagashima *et al.*, 1984), polyurea (Chibata *et al.*, 1974) wood chips and charcoal (Scharer *et al.*, 1988).

2.9.1 Matrix characteristics.

Most of the disadvantages of immobilization are linked to the physio-chemical stress exerted by the matrix used. The choice of a suitable matrix can therefore limit the possible disadvantages considerably and is thus one of the most important steps in any immobilization process. A suitable matrix should have the following characteristics (Takata et al., 1977):

- The mixing of enzymes or cells with the polymer should be easy and the mixture should be stable in the liquid state.

- The polymer should easily be induced to gel under mild conditions which will not modify the structure of enzymes or cells.
- Gels must have a reasonable strength that will not be destroyed in enzyme reactions.
- Gels must be stable at high temperatures and over a wide pH range.
- The pore size of the gel should be small enough to prevent high molecular weight compounds from leaking out, while allowing small molecular weight compounds to easily pass through the gel lattice.

Klein (1988) also brings to attention the stability of the matrix and the importance of the microenvironment created by the matrix in relation to diffusional limitations (*i.e.* porosity), concentration gradients, pH gradients, ionic strength and the hydrophilic/ hydrophobic balances between the matrix and the medium. The size, shape, cost, availability and mechanical strength of the matrix also needs to be taken into account (Nagashima *et al.*,1984; Klein, 1988). The specific pore size of the matrix determines the diffusional ability of substances into and out of the matrix. Not only the molecular size, but also the structural configuration and the charges on the molecules will determine their diffusional ability (Tanaka *et al.*, 1984). Kierstan *et al.* (1982) found that the composition of the polymers differed from one supplier to the next which affected the characteristics of the gel formed and thereby its possible applications. Alginate for example consists of D-mannuronic and D-guluronic units and alginate consisting of mostly D-mannuronic units forms a highly porous gel best for cell immobilization while alginate consisting of mostly D-guluronic units forms a gel with slower diffusional characteristics best for use in diffusional chromatography.

When focusing on the immobilization of whole cells, factors such as growth, metabolic activity of cells, diffusion and physical characteristics and limitations need to be taken into account.

2.9.2 Growth within the matrix.

Cells do have the ability to grow within a matrix (Wada *et al.*, 1980; Larreta Garde, 1981; Shinmyo *et al.*, 1982; Inloes *et al.*, 1983; Nagashima *et al.*, 1984; Dhulster *et al.*, 1984; Bashan, 1986). Increases in bacterial cell numbers within gel matrixes have been shown by cell counts and with electron microscope studies. Bashan (1986) immobilized *Azospirillum brasilense* (ATCC 29710) cells in sodium alginate gel beads and found an increase from 1.05×10^2 to 1.04×10^{10} colony forming units (cfu) per 25 beads within 48 h incubation, while Wada *et al.*, (1980) found immobilized *Saccharomyces carlsbergensis* cells to increase in number from 3.5×10^6 to 5.4×10^9 cells.ml^{-1} gel (kappa-carrageenan) within 6 h and cells grown in suspension only increased from 6×10^6 to 4.8×10^8 cells.ml^{-1} medium. Dhulster *et al.* (1984) found that the growth rates of immobilized (kappa-carrageenan) and free *E. coli* cells were similar, but Shinmyo *et al.*, (1982) however found that *Bacillus amyloliquefaciens* cells immobilized in kappa-carrageenan had a growth rate $^1/_5$ - $^1/_{10}$ of that of free cells.

Transmission electron microscopy has shown alginate to have a sponge like structure consisting of filaments and cavities. Alginate containing immobilized *Rhodopseudomonas capsulata* had one cell per cavity directly after immobilization, 2 cells per cavity after 6 h and 8 to 10 cells per cavity after 24 h (Larreta Garde, 1981). After 24 h the cavities were also found to be arranged in groups indicating movement within the matrix. Bacteria have also been seen to shape the matrix. Bashan (1986) demonstrated bulges on the alginate gel surface and inside the beads with scanning electron micrographs. *Escherichia coli* cells immobilized in hollow-fibre membranes reach high cell densities after incubation indicating that growth was not inhibited by cell to cell contact, but the densely packed cells exerted enough pressure in certain areas to deform the polymeric wall structure (Inloes *et al.*, 1983). Growth can be limited however by increasing the gel concentration (Bashan, 1986).

Growth has however been limited to the outer 50μm of gel beads. Directly after immobilization cells are homogeneously distributed throughout the gel lattice, but after only a few hours incubation the cells in the center become less and eventually disappear while cells on the outer edge multiply (Wada *et al.*, 1980; Shinmyo *et al.*, 1982; Dhulster *et al.*, 1984). This limitation of growth to the periphery of the matrix could be due to diffusional limitations of either oxygen or nutrients or both.

2.9.3 Diffusion characteristics of the matrix.

Immobilized cells are densely packed and therefore need more oxygen per unit volume and the oxygen supply should cope with added mass transfer resistance such as liquid-solid interfaces and intra-particle diffusion (Chang and Moo-Young, 1988). Shinmyo *et al.* (1982) found oxygen diffusion in kappa-carrageenan gels with different concentrations of immobilized *B. amyloliquefaciens* cells to be constant. Gels containing higher cell concentrations had lower respiratory activity. Gel beads containing only 1.4 mg cells.g^{-1} wet gel had respiratory activity equal to that of free cells. Respiration is thus limited by oxygen diffusion as the oxygen cannot diffuse fast enough to supply larger cell numbers. Dhulster *et al.*, (1984) found not only that cell growth of immobilized *E. coli* cells in kappa-carrageenan beads to be limited to the outer 50μm, but that the Catechol 2-3 oxygenase enzyme activity was lower than that of free cells by a factor of 20 for the same number of cells and this difference they ascribed to oxygen mass transfer inhibition into the gel. Smaller gel beads or a gel matrix with higher oxygen solubility was suggested as possible ways to improve the oxygen transfer. If the oxygen demand is low, oxygen will penetrate to the center of the support and no cells will be oxygen limited, but when the demand is high only the cells near the surface will have enough oxygen for survival. It is therefore wise to immobilize high oxygen demanding cells on the surface of small particles to avoid oxygen limitations. An increase in the gas velocity will also increase the liquid-solid or gas-liquid mass transfer rate (Chang and Moo-Young, 1988).

Insufficient nutrient diffusion is a growth limiting factor (Wada *et al.*, 1980; Shinmyo *et al.*, 1982; Nagashima *et al.*, 1984). To overcome nutrient diffusion limitations Nagashima *et al.*, (1984) immobilized sterols, unsaturated fatty acids and dissolved oxygen together with the yeast cells in calcium alginate. Investigation of the diffusion characteristics of calcium alginate indicated that substances with a molecular weight of less than 2×10^4 (*e.g.* glucose) have a diffusion coefficient similar to water, indicating free diffusion in and out of the gel and an increase in the alginate or calcium chloride concentrations had no noticeable effect on diffusion rates (Kierstan and Bucke, 1977; Tanaka *et al.*, 1984; Proulx and de la Noue, 1988). The pore size of the gel is critical in that too small a pore size inhibits diffusion, whereas too large a pore size allows leakage of the immobilized species. The molecular weight is mostly used as an indicator of molecular size but in some cases the structure and configuration of the molecule that must diffuse in or out of the gel must also be taken into account. Tanaka *et al.*, (1984) found that certain higher molecular weight substances (albumin, -globulin and fibrinogen) could not diffuse into the matrix but could diffuse out and that the diffusion was limited by increasing alginate and calcium chloride concentrations.

2.9.4 Metabolic activity of immobilized cells.

Changes in the metabolic behavior of cells after immobilization has been manifested in that product yields from bioconversions being much higher than that of cells in suspension. Ethanol production from glucose by *S. cerevisiae* cells was about 20 times greater when immobilized in alginate (Nagashima *et al.*, 1984), *ca.* 45% greater when immobilized on gelatin coated glass beads (Doran and Bailey, 1986) and the yield from *S. carlsbergensis* cells immobilized in kappa-carrageenan was 10 times greater than the yield from cells in suspension (Wada *et al.*, 1980). *Bacillus amyloliquefaciens* cells gave greater yields of α-amylase when immobilized in kappa-carrageenan (Shinmyo *et al.*, 1982). Joubert and Britz (1988) found that the immobilization of a saccharolytic sulphate-reducing bacteria fermenting glucose did not alter the metabolite composition meaningfully. The higher metabolic efficiency found after immobilization was investigated by Mattiasson and Hahn-Hägerdal (1982), who put forward the hypothesis that differences in the water activity (a_w) and oxygen supply caused changes in the metabolic patterns of immobilized cells. The environment within a gel (*e.g.* alginate) has a low a_w and oxygen concentration. The low a_w causes an increase in osmotic pressure, which the cell registers as environmental stress thereby causing a shift to maintenance metabolism. Immobilized cells, due to decreased a_w maintain an increased maintenance metabolism at the expense of cell growth and improved yields from reactions connected to the maintenance metabolism and not demanding oxygen might be expected. High yields of secondary metabolites are thus obtained. In the immobilized state the a_w is decreased, oxygen is limited and yeasts will therefore ferment glucose to ethanol at the expense of growth. Glucose conversion to ethanol by immobilized yeast was 95-100% of the theoretical maximum which is *ca.* 20 times greater than the yield obtained from free cells (Wada *et al.*, 1980; Nagashima *et al.*, 1984). Lactobacilli have reduced lactate production under conditions of stress (low a_w) and increased diacetyl production.

2.9.5 Physical characteristics of immobilization.

The actual shape and size of the immobilization matrix has an influence on diffusion and growth within the matrix. To decrease diffusional limitations, small spherical balls with diameters (ϕ) ranging from 1 to 5mm are mostly used for the immobilization of whole cells (Wada *et al.*, 1980; Schrerer *et al.*, 1981; Shinmyo *et al.*, 1982; Kierstan *et al.*, 1982; Luong and Tseng, 1984; Tanaka *et al.*, 1984; Bashan, 1986). Scherer *et al.*, (1981) however found cells to remain active longer in slightly larger balls (3-3.7mm ϕ vs 1.2-2.5 mm ϕ). The mechanical strength and pore size of the matrix are also of vital importance to the intended application of the immobilized entity, *i.e.* a mechanically weak matrix cannot be used in a very turbulent reactor. Various chemical treatments of matrixes after immobilization to increase the mechanical strength and stability of the matrixes and to decrease biodegradation have been used successfully (Takata *et al.*, 1977; Bashan, 1986). Bacterial growth distorted the shape of some polymers and although a certain degree of flexibility is advantageous a certain minimum strength is needed (Larreta Garde *et al.*, 1981; Inloes *et al.*, 1983). The pore size of the matrix will not only determine the diffusional characteristics of the matrix but also the size of the particle it can immobilize. The leakage of cells or enzymes from immobilization matrixes has repeatedly been documented and is mostly an undesirable occurrence (Shinmyo *et al.*, 1982; Dhulster *et al.*, 1984; Luong and Tseng, 1984).

2.9.6 Applications of the immobilization technique.

Agriculture. Bashan (1986) used sodium alginate beads containing immobilized *Azospirillum brasilense* cells as a slow release synthetic inoculant for plants. Although generally regarded as a disadvantage, the fact that alginate is biodegradable was used to effect a slow release of the bacteria without any environmental pollution. *Rhizobium* cells immobilized in polyacrylamide have also been used as an inoculant for legumes and compared well to the peat based carriers commonly used as inoculant (Dommergues *et al.*, 1979). Microorganisms and fungal spores with the potential to be used as biocontrol agents to control plant diseases have been immobilized in an alginate-clay matrix. Although the viability varied according to the organism and the spore type, most remained viable for at least 2-3 weeks. Alginate was used as carrier since it is considered safe, is used as a food additive and was found to be non toxic to non target organisms (Fravel *et al.*, 1985).

Fine chemicals. Immobilized cells have been used for α-amylase production (Shinmyo *et al.*, 1982), methanol conversion to methane (Sherer *et al.*, 1981); organic acid production (Horitzu *et al.*, 1988) ethanol production (Kierstan and Bucke, 1977; Wada *et al.*, 1980; Luong and Tseng, 1984; Nagashima *et al.*, 1984; Doran and Bailey, 1986; Ramakrishna *et al.*, 1988) and the production of protease enzymes used in cheese-making, for predigested dietary products, the fortification of fruit juices and soft drinks and the hydrolysis of milk proteins (Vuillemard and Amiot, 1988). Immobilized plant cells can be used for bioconversions and for the production of biochemicals such as nicotine, caffeine, ubiquinone-10, anthrocyanins and morphine (Brodelius, 1988). Immobilized enzymes have been used for the production of L-aspartic acid (Chibata *et al.*, 1974), the conversion of urea to L-glutamic acid (Gu and Chang, 1988), the production of invert sugar for the food industry by immobilized invertase (Illanes *et* *al.*, 1988), urea hydrolysis (Melnyk, 1988), the hydrolysis of lactose in milk (Park and Pastore, 1988) and a milk clotting enzyme (Chymosin) has been immobilized for continuous milk clotting (Amourache and Vijayalakshmi, 1988).

Wastewater. For waste water treatment immobilized cells have been used in a two-stage reactor using immobilized acid-formers and *Methanobacter* sp. for anaerobic waste treatment with the production of methane (Messing,1988) and immobilized algae for the removal of macronutrients such as phosphorus, ammonium, nitrite and nitrate (Proulx and de la Noue, 1988).

Medicine. In the medical field, immobilized heparinase, a heparin-degrading enzyme needed to prevent blood clotting has been used in extraporeal blood circulation found in kidney dialysis, cardiac surgery and organ transplantation (Yang *et al.*, 1988). Immobilized animal cells have also been used for the production of antibodies (Behie and Gaucher, 1988; Dean *et al.*, 1988; Nilsson, 1988). Antibiotic production (penicillin-G) using immobilized cells has great scope in the pharmaceutical industry (Behie and Gaucher, 1988; Vandamme, 1988).

2.9.7 Advantages.

Immobilization has certain advantages over free cell or enzyme systems which makes it a popular choice in many cases:

- Recovery of the product and catalyst without major separation techniques (Inloes *et al.*, 1983; Dhulster *et al.*, 1984; Hahn-Hägerdal, 1990).
- Immobilization of highly purified enzymes allows for greater reaction specificity and predictability (Tosa *et al.*, 1979; Inloes *et al.*, 1983).

- Increase in enzyme functional stability. (Dhulster *et al.*, 1984).
- Continual reuse of the catalyst.
- Whole cells can be used for multistep conversions requiring different enzymes and permitting *in situ* regeneration of the necessary co-factors as purified co-factors are difficult and expensive to prepare (Kierstan and Bucke, 1977; Inloes *et al.*, 1983). Pure cultures can therefore be immobilized and placed in an activated sludge plant for the investigation of phosphate removal or nitrate reduction.
- The immobilization of whole cells has the added advantage over immobilized enzymes that no isolation or purification steps are necessary which could impair the enzyme activity. The enzymes are thus maintained in their natural and active configurations (Inloes *et al.*, 1983).
- Greater cell densities are found compared to suspension cultures, thereby resulting in higher metabolic activities and production rates (Inloes *et al.*, 1983; Dhulster *et al.*, 1984; Luong and Tseng, 1984; Nagashima *et al.*,1984; Doran and Bailey, 1986). Large cell densities can therefore be maintained to increase the effectivity of phosphate removal as compared to the free cells *in situ*.
- Immobilized cells can be used to conduct biochemical reactions in natural systems without the disruption of the ecosystem and *in situ* studies are thus possible as the microorganisms can be regained for further study (Tosa *et al.*, 1979). The study of phosphate removal in the activated sludge system can therefore be carried out *in situ*. The microorganisms can also be manipulated within the system regarding retention times in various zones and the effects of competition and species interaction can be studied in the absence of predation and without any disruption of the system.
- No special equipment is necessary and therefore the initial investment cost is low (Nagashima *et al.*, 1984). Studies on the activated sludge system have often

resulted in the costly construction of pilot plants which can be replaced to a large degree by immobilization studies.

- Long term use is possible. Nagashima *et al.* (1984) reported using immobilized *Saccharomyces cerevisiae* cells in a continuous ethanol fermentation for up to 6 months.
- Immobilization of genetically engineered organisms increases their potential uses without any danger to the ecosystem.
- Greater experimental and operational ease is possible with immobilized cells, compared to free cells, especially in natural or industrial systems *eg.* activated sludge processes (Inloes *et al.*, 1983).

2.9.8 Disadvantages.

There are also various disadvantages to immobilization which need to be considered:

- Viscosity of the immobilization matrix resulting in gas transfer inhibition *eg.* the viability of strict aerobes such as *Acinetobacter* may be impaired if gas transfer is inhibited (Mattiasson and Hahn-Hägerdal, 1982; Shinmyo *et al.*, 1982; Dhulster *et al.*, 1984; Brodelius, 1988; Chang and Moo-Young, 1988).
- Decrease in water activity (Mattiasson and Hahn-Hägerdal, 1988; Monbouquette and Ollis, 1988).
- Decrease in surface tension due to the presence of polymers.
- Cost effectivity.
- Leakage of cells or enzymes from the matrix (Shinmyo *et al.*, 1982; Dhulster *et al.*, 1984; Luong and Tseng, 1984; Bashan, 1986; Monbouquette and Ollis, 1988). The leaching of cells out of an immobilization matrix is undesirable especially when conducting studies on natural or industrial systems *eg.* the

leaching of a number of bacteria in an activated sludge system would disrupt the natural population structure and thereby possibly the functioning of the system.

- Growth limitations due to mechanical resistance of the matrix (Inloes *et al.*, 1983; Dhulster *et al.*, 1984; Doran and Bailey, 1986; Klein, 1988; Monbouquette and Ollis, 1988).
- Cell-carrier toxicity (Brodelius, 1988; Monbouquette and Ollis, 1988).
- Growth limitations due to nutrient diffusion limitations *eg.* inhibited diffusion of large substrate molecules (Wada *et al.*, 1980; Monbouquette and Ollis, 1988).
- Metabolic changes in immobilized cells (Mattiasson and Hahn- Hägerdal, 1982; Doran and Bailey, 1986).
- Biodegradation of the matrix (Kierstan and Bucke, 1977; Bashan, 1986).

A large number of uses exist for immobilization techniques and although a large number have already been explored there are even more that still need to be investigated. There is no limit to the entities that can be immobilized and the applications of immobilization stretch from industry to agriculture to medicine.

2.10 Conclusions.

Controversy exists in the literature regarding the importance of *Acinetobacter* in phosphate removal, due to its minor proportions in certain sludges investigated. *Acinetobacter* has however recently been found the dominant organism in activated sludge (Streichan *et al.*, 1990; Auling *et al.*, 1991). The possibility that certain sludges could select for different populations could explain these discrepancies. However, population studies are difficult, especially if the aim is to determine the relative importance of *Acinetobacter* in activated sludge. The use of quinone profiles and other biomarkers (*e.g.* DAP) are useful techniques for ecological studies, but need to be combined with other enumeration and identification techniques. However before such studies can be executed the taxonomy of *Acinetobacter*, especially at specie level needs to be clarified. A taxonomic study was therefore undertaken to identify and classify *Acinetobacter* isolates from activated sludge. The aim of this study was therefore to determine which *Acinetobacter* species were present in the activated sludge and if there were any correlation between their phosphate uptake ability and their protein profiles. The growth and phosphate uptake of the isolates in mixed liquor was therefore determined. The immobilization technique has potential in wastewater treatment, especially in relation to phosphate removal. Immobilization of polyphosphate accumulating cells has great potential for *in situ* studies in the activated sludge process. Pure cultures can therefore be studied and conditions manipulated in a full scale activated sludge plant without any interference to the operation of the process. The ability of *Acinetobacter* to grow and accumulate phosphate in the immobilized state was therefore investigated.

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CHAPTER 3

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THE IDENTIFICATION AND CLASSIFICATION OF *ACINETOBACTER* STRAINS EXHIBITING VARIATIONS IN PHOSPHATE ACCUMULATION USING SDS-PAGE AND NUMERICAL ANALYSIS

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* *The editorial style followed in this chapter are in accordance with the requirements of the journal Current Microbiology.*

Abstract. The limitation of phosphate concentrations in effluents is of international concern due to the risk of eutrophication. *Acinetobacter* spp. have often been isolated from activated sludge plants exhibiting enhanced phosphate removal. The ability to remove phosphate from mixed liquor by *Acinetobacter* isolates and reference strains was therefore determined. The ability to remove phosphate was found to vary between the *Acinetobacter* strains. The taxonomic relationships between these various strains were elucidated using SDS-PAGE and numerical analysis, and the correlation between their taxonomy and phosphate uptake ability was determined. The ability to remove large amounts of phosphate was found to be strain specific rather than specie specific. The classification of *A. haemolyticus* and *A. baumannii* as separate species is questioned.

Key words: *Acinetobacter* - SDS-PAGE - Numerical analysis - Taxonomy - Phosphate uptake - Activated sludge.

Introduction

The presence of non-biodegradable organic compounds and phosphorus in detergents and industrial effluents can lead to severe pollution problems. Excessive phosphorus concentrations can enhance the eutrophication process in natural water systems and for this reason the phosphorus concentration of effluents entering natural lakes and rivers has been limited in many countries. In South Africa the Water Act (Act no. 54, 1956) was amended in 1980 to limit the orthophosphate content of effluents to 1.0 mg/l [33]. Biological phosphorus removal as an alternative to chemical removal has gained interest in recent years [8, 10, 13, 27, 36]. In activated sludge processes, which are essentially biological treatment processes, high phosphorus removal rates, often termed "luxury" uptake, have been reported [2, 13, 23]. Although *Acinetobacter* cannot account for all the phosphorus removed [11] and other polyphosphate accumulating bacteria have also been isolated [6, 34, 35], *Acinetobacter* strains have often been found to dominate in enhanced phosphate removal plants [7, 9, 12, 13, 24, 25, 34]. It is however still unclear exactly how important a role these microorganisms play in enhanced phosphorus removal or the exact mechanism by which "luxury" uptake takes place.

Previous studies in this laboratory have indicated that the rate of phosphorus uptake varied between different *Acinetobacter* strains (Prof. T.E. Cloete, University of Pretoria, personal communication). It was suspected that this could be due to genetic variations amongst the different strains. It was therefore important to consider the taxonomic relationships of these organisms in order to elucidate the differences in terms of phosphorus uptake ability amongst the various isolates. *Acinetobacter* strains were therefore isolated from activated sludge and initially identified using the Analytical Profile Index for non-enteric Gram negative rods (API 20NE). This yielded an unsatisfactory result as the type strain of *A. calcoaceticus* was incorrectly identified as *A. baumannii* which questioned the identification of the isolates at specie level. Therefore an alternative method for both identification and studying the potential genomic differences had to be considered. Protein electrophoresis can be considered as an indirect genomic 'fingerprint' due to the fact that a bacterial strain growing in standardized conditions will always produce the same set of proteins which are an expression of the genes or nucleotide sequences of the bacterial DNA [21]. It allows accurate clustering and identification of strains within a species that corresponds to results obtained by DNA-DNA hybridizations and even %G+C values [20]. The technique is much faster than phenotypic and biochemical analysis and the inclusion of internal reference strains and the use of numerical analysis allow reproducible and objective comparisons of the protein patterns. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was therefore used as both identification and taxonomic tool to study the *Acinetobacter* population found in activated sludge. The taxonomic relationships were also correlated to their ability to accumulate phosphate.

Materials and Methods

Bacterial strains and culture conditions. Two *Acinetobacter* reference strains were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM) GmbH (Braunschweig, Federal republic of Germany), 7 from the American Type Culture Collection (ATCC, Rockville, Maryland 20852, United States of America) and 12 *Acinetobacter* isolates were donated by Prof. T.J. Britz (Department of Microbiology, University of the Orange Free State, Bloemfontein, South Africa). *Acinetobacter* strains were isolated from the aerobic zone of a Bardenpho activated sludge plant, using acetate enrichment agar [7] containing $50\mu g.l^{-1}$ cyclohexamide as isolation medium and identified as belonging to the genus *Acinetobacter* using the API 20 NE system.

All cultures received were microscopically checked for purity and with the exception of the cultures obtained from ATCC, the API 20NE system was used to confirm that all the cultures belonged to the genus *Acinetobacter* (Table 1). All strains were maintained on Nutrient agar (Biolab) slants at 4^0C and subcultured monthly.

Determination of phosphate uptake by the *Acinetobacter* strains. Mixed liquor obtained from the anaerobic tank of the Daspoort activated sludge plant was centrifuged in a Sorval RC-5B centrifuge at 5000 g for 20 min. The supernatant was prefiltered through Whatman No.1 filter paper and $5g.l^{-1}$ sodium acetate (BDH), 0.5 $g.l^{-1}$ $MgSO_4.7H_2O$ (Merck) and 0.18 $g.l^{-1}$ KNO_3 (Merck) were added and the pH adjusted to pH 7 with 2N HCl, before autoclaving (121^0C, 15 min). Suspensions (1.0 ml) of the *Acinetobacter* strains were made in duplicate, each containing *ca.* 10^8 cells, in the sterile mixed liquor (ML) medium. The 1.0 ml suspensions were inoculated into 19.0 ml sterile ML medium and shaken in a shaking waterbath (80 rpm) at 28^0C for 24 h. The bacterial suspensions were filtered through $0.22\mu m$ 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 phosphate content of the medium was then determined with the P(VM) 14842 test kit (Merck), using the Merck SQ 118 Photometer. The amount of phosphate ($mg.l^{-1}$) removed from the medium by the bacteria was determined using the following formula:

PO_4^{3-} uptake by *Acinetobacter* strains = [Control PO_4^{3-}] - [PO_4^{3-} after 24h exposure to *Acinetobacter* strains]

SDS-PAGE of the total soluble cell proteins.

Preparation of total soluble cell protein extracts. The proteins were prepared under strictly standardized conditions as follows:

Cells were harvested by centrifugation (11 000 g) from a 48 h culture grown in Nutrient broth at 28^{0}C with shaking in a shaking waterbath (80 rpm). The cell pellet was washed (11 000 g) twice with phosphate buffered saline (pH 7.3) before being resuspended in 850μl sample treatment buffer (0.062M Tris-HCl (Merck); 10% ($^v/_v$) glycerol (Merck); pH 6.8). The bacterial cells were broken by ultrasonication (Ultrasonic Homogenizer 4710 Series, Cole-Palmer Instrument Co., Chicago, Illinois 60648) at 60 watts for 5 x 30 s bursts with 15 s intervals for cooling. SDS was added to a final concentration of 2% ($^w/_v$) before placing the samples in boiling water for 10 min to denature the proteins. Unbroken cells and cell debris were removed by centrifugation (9 000 g, 5 min) in a Hermle 360K centrifuge. The protein concentration of the supernatant was then determined using the method of Lowry *et al.* [26]. Finally 2-β-Mercaptoethanol was added to each sample to a final concentration of 5% ($^v/_v$) and the protein samples were stored at -12^{0}C until required for electrophoresis.

SDS-PAGE. SDS-PAGE was performed using the method of Laemmli [22] with certain modifications. Separation gels containing 12% acrylamide were made from a stock solution of 29.2 g acrylamide (BDH) and 0.8 g N'N'-methylenebisacrylamide (BDH) made up to 100 ml with distilled water. Stacking gels of 5% acrylamide were made from the same stock. Separation gels were polymerized chemically by the addition of 0.035% ($^w/_v$) ammonium persulphate (BDH) and 0.05% ($^w/_v$) N,N,N',N',-Tetramethylethylenediamine (TEMED, Bio-Rad). Polymerizing separation gels were immediately overlaid with pure iso-butanol (Merck). The iso-butanol was washed off with distilled water after 1 h and the gels were overlaid with 0.375M Tris-HCl buffer (pH 8.8) before being left to polymerize overnight at room temperature. The buffer was washed off with distilled water before overlaying the stacking gels which were polymerized chemically by the addition of 0.05% ($^w/_v$) ammonium persulphate and 0.01% ($^w/_v$) TEMED for 30 min. The gels were 0.75 mm thick, 125 mm long and contained 20 sample wells. Each well was filled with electrode buffer and 0.5 μl bromophenolblue tracking dye (0.01 mg.ml^{-1} sample treatment buffer). Protein extracts of *Psychrobacter immobilis* LMG 1125 were used as internal reference proteins, as required by the computer programme used for numerical analysis. Samples were loaded at a concentration of 4 μg protein per well. Electrophoresis was carried out using a HSI vertical slab gel unit SE-600 series (Hoefer Scientific Instruments, San Francisco) at a constant current of 15 mA and 25 mA per stacking and separating gel respectively, at 10^{0}C. Electrophoresis was terminated after *ca.* 210 min when the buffer front had run 100 mm.

Staining and destaining. Gels were stained for 1 h according to the method of Jackman [15], using Coomassie Brilliant Blue R250 (Merck). Gels were destained in a solution containing 25% methanol ($^v/_v$) and 10% acetic acid ($^v/_v$). The destaining solution was replaced 3 times. The destained gels were stored in 3.5% acetic acid [1].

Numerical analysis. Gels were scanned on a Hoefer GS 300 Transmittance/ Reflectance Scanning Densitometer (Hoefer Scientific Instruments, San Francisco). Numerical analysis, based on the correlation coefficient (r) was determined using the unweighted average linkage cluster analysis, was done using the Gel Compar programme version 1.3 (Helix C.V., Gent, Belgium).

Results and Discussion

Identification of strains using the API 20NE system. Only 19 of the 105 round, cream coloured colonies randomly isolated from the acetate enrichment agar plates were identified as belonging to the genus *Acinetobacter* using the API 20NE system. The API 20NE system was however insufficient for identification to species level (Table 1) even though the system does incorporate all 6 species as described by Bouvet and Grimont [5]. More than 50% of the strains tested were identified as

A.baumannii, including the type strain of *A.calcoaceticus* (DSM 30006) and *A. calcoaceticus* (DSM 1139) which were both identified with 99.9% probability as being *A. baumannii* (Table 1).

Identification of strains using SDS-PAGE. The creation of the genus *Acinetobacter*, to include all oxidase negative moraxellas, was supported by biochemical and phenotypic studies [3, 4], DNA-DNA hybridization studies [16] and transformation studies [17]. The genus *Acinetobacter* is classified within the family Neisseriaceae [18], and although classification and identification to the genus level is adequate, the subdivision of species within the genus is not satisfactory. Bergey's Manual of Systematic Bacteriology [18] only mentions one specie (*A. calcoaceticus*) whereas the Approved Lists of Bacterial Names [32] lists the genus as consisting of 2 species, namely *A. calcoaceticus* and *A. lwoffii*. The most recent classification was that of Bouvet and Grimont [5]. They divided the genus into 12 genospecies on the basis of DNA-DNA hybridization studies and proposed specie names and type strains for four of these genospecies, namely *A. baumannii* (ATCC 19606), *A. junii* (ATCC 17908), *A. johnsonii* (ATCC 17909) and *A. haemolyticus* (ATCC 17906). Two other genospecies, containing the two existing species namely *A. calcoaceticus* and *A. lwoffii*, were redescribed. Three *Acinetobacter* strains resistant to gamma-radiation were isolated from cotton [19] and soil [31] and placed in a new specie, namely *A. radioresistens*, on the basis of phenotypic characteristics, outer-membrane protein patterns, DNA-DNA hybridizations and electrophoretic analysis of enzymes [14, 28, 29, 30, 31].

Five of the *Acinetobacter* type strains (Table 2) as proposed by Bouvet and Grimont [6], with the exception of the type strain of *A. lwoffii*, were used as reference strains for clustering and identification to the species level using numerical analysis of SDS-PAGE protein patterns. *A. lwoffii* (ATCC 21130) was used as representative of the specie. The dendrogram obtained by numerical analysis of the electropherograms was divided into 5 main clusters (Fig. 1). The correlation coefficients (r) between these clusters vary between 40 and 50%. The correlation coefficients are relatively low and are indicative of a very heterogeneous genus, a characteristic which has been noted both biochemically and genetically [4, 5, 16].

Cluster 1 was the most heterogeneous and contained 3 subclusters. The type strains of *A. baumannii* (ATCC 19606) *A. haemolyticus* (ATCC 17906) and *A. calcoaceticus* (ATCC 23055 and DSM 30006) were found in subclusters 1a, 1b and 1c respectively. The type strain of *A. calcoaceticus* obtained from both the ATCC and DSM culture collections, are essentially the same strain and were expected to group together, however the % similarity (84%) was not quite as high as was expected. The results agree with those obtained by Nishimura *et al.* [31]. In their studies they obtained 4 main clusters Z-1 to Z-4. Cluster Z-1, the largest and most heterogeneous, consisted of 3 subclusters. The subcluster containing the type strain of *A. calcoaceticus* was considered to be representative of the entire cluster. The remaining two subclusters, containing the type strains of *A. baumannii* and *A. haemolyticus* respectively, were considered as subspecies of *A. calcoaceticus*. Previously, in DNA homology studies between strains of *A. baumannii* and the type strain of *A. calcoaceticus*, Nishimura *et al.* [30] found homologies in the region of 81.8%-96.5% versus the 37%-39% homology originally obtained by Bouvet and Grimont [5]. The grouping of these three species within one cluster supports the suggestions of Nishimura *et al.* [31], that *A. baumannii* and *A. haemolyticus* be considered as subspecies of A. calcoaceticus rather than as species in their own right.

Cluster 2 has two distinct subclusters with the type strain of *A. johnsonii* clustering at a similarity value of 76% with subcluster 2b. All the organisms in subcluster 2b were therefore considered to belong to the specie *A. johnsonii*. Since the two subclusters also clustered at 70%, subcluster 2a was considered to be a subspecie of

A. johnsonii. Nishimura *et al.* [31] also found *A. johnsonii* to form a distinct and separate cluster (Z-2) with a similarity value of 70%.

Cluster 3 has two subclusters, with a similarity value of 69%. Subcluster 3a contains the type strain of *A. junii* (17908) while subcluster 3b contains the reference strain of *A. lwoffii* (ATCC 21130). Genospecies 5, designated as *A. junii*, resulting from the homology studies of Bouvet and Grimont [5] only contained 4 strains which were all originally classified as *A. lwoffii*, indicating that the two species were closely related. Nishimura *et al.* [31] found *A. lwoffii* to form a distinct and separate cluster with a 50% correlation to the other clusters, but they did however not include any representative strains of *A. junii* in their studies. More work needs to be done to determine the exact relationship between *A. lwoffii* and *A. junii*.

Clusters 4 and 5 were considered as atypical *Acinetobacter* strains. These bacteria did not cluster with any of the reference strains and it was impossible to place them into a known specie. It is possible that they may represent new species but more work, such as DNA homology studies will have to be done before any proposals can be made.

Phosphate uptake ability of the various *Acinetobacter* strains. All the *Acinetobacter* strains tested accumulated phosphate to varying degrees. Clusters 1, 3 and 4 were very heterogeneous, with standard deviations (σ_n) of 13.25, 7.42 and 10.25 respectively, regarding their phosphate uptake abilities. Clusters 2 and 5 were more homogeneous with σ_n=3.15 and σ_n=3.08, respectively. Clusters 1, 3 and 4 were taxonomically very heterogeneous and this was also expressed in their phosphate uptake abilities, whereas cluster 2 formed a homogeneous cluster both taxonomically and with regard to the phosphate uptake ability of the strains. The large variation in phosphate uptake between the various strains within a cluster (e.g. clusters 1, 3, and 4) indicated that the phosphate uptake ability was strain specific rather than specie or even sub-specie related. No correlation was found between the abilities of the various strains to accumulate phosphate and their origin, since the strains of medical origin and environmental origin had standard deviations of $\sigma_n = 7.65$ and $\sigma_n = 7.88$ respectively. The strains that did not fall into either of these groups were more heterogeneous with $\sigma_n = 13.06$. Since all the flasks were innoculated with the same cell density (*ca.* 10^8) and incubated for the same period of time (24h), the higher average phosphate uptake exhibited by the environmental strains (27.65 $mg.l^{-1}$) if compared to the medical strains (21.14 $mg.l^{-1}$) would indicate a degree of adaption to their natural habitat.

In conclusion, the heterogeneity of the protein profiles, even within a cluster, resulted in no correlation being found between the ability to accumulate phosphate and the genetic variation between the strains. Phosphate uptake is thus strain specific and not specie or even sub-specie related. Our work, together with the similar results obtained by Nishimura *et al.* [31], using different isolates, strongly questions the placement of *A. baumannii* and *A. haemolyticus* as separate species rather than sub-species of *A. calcoaceticus*. However, more work needs to be done (*e.g.* DNA homology studies) before any specific suggestions can be made.

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Table 1. Bacterial strains identified using the API 20 NE system.

Bacterial strains	API 20 NE identification	% Probability	Origin
B1	*A. baumannii*	91.4	Dam
ϕ5	*A. baumannii*	97.6	Dam
Ac	*A. baumannii*	99.9	Anaerobic digester
Ao8	*A. baumannii*	99.9	Unknown
M3	*A. baumannii*	99.9	Clinical
M4	*A. baumannii*	99.9	Clinical
M9	*A. baumannii*	99.9	Clinical
M16	*A. baumannii*	99.9	Clinical
M21	*A. baumannii*	99.9	Clinical
M26	*A. baumannii*	99.9	Clinical
M27	*A. baumannii*	99.9	Clinical
M29	*A. baumannii*	99.9	Clinical
AS33	*A. junii*	92.0	Activated Sludge
AS60	*A. junii*	92.0	Activated Sludge
AS64	*A. junii*	92.0	Activated Sludge
AS78	*A. junii*	75.5	Activated Sludge
AS79	*A. junii*	70.5	Activated Sludge
AS81	*A. junii*	34.5	Activated Sludge
AS87	*A. junii*	70.5	Activated Sludge
AS89	*A. junii*	70.5	Activated Sludge
AS92	*A. lwoffii*	88.3	Activated Sludge
AS93	*A. lwoffii*	88.3	Activated Sludge
AS96	*A. junii*	70.5	Activated Sludge

Table 1. Continued.

Bacterial strains	API 20 NE identification	% Probability	Origin
AS97	*A. lwoffii*	94.9	Activated sludge
AS98	*A. baumannii*	99.9	Activated Sludge
AS99	*A. baumannii*	99.9	Activated Sludge
AS100	*A. baumannii*	99.9	Activated Sludge
AS101	*A. baumannii*	99.9	Activated Sludge
AS104	*A. baumannii*	99.9	Activated Sludge
AS104	*A. baumannii*	99.9	Activated Sludge
AS105	*A. baumannii*	99.9	Activated Sludge
DSM 30006^T	*A. baumannii*	99.9	Quinate enrichment
DSM 1139	*A. baumannii*	99.9	Hexadecane enrichment

Table 2. ATCC reference strains.

Bacterial strains	ATCC identification	Origin
ATCC 23055^T	*A. calcoaceticus*	Quinate enrichment
ATCC 17908^T	*A. junii*	Urine
ATCC 19606^T	*A. baumannii*	Urine
ATCC 17906^T	*A. haemolyticus*	Unknown
ATCC 17909^T	*A. johnsonii*	Duodenum
ATCC 17912	*A. calcoaceticus*	Unknown
ATCC 21130	*A. lwoffii*	Soil

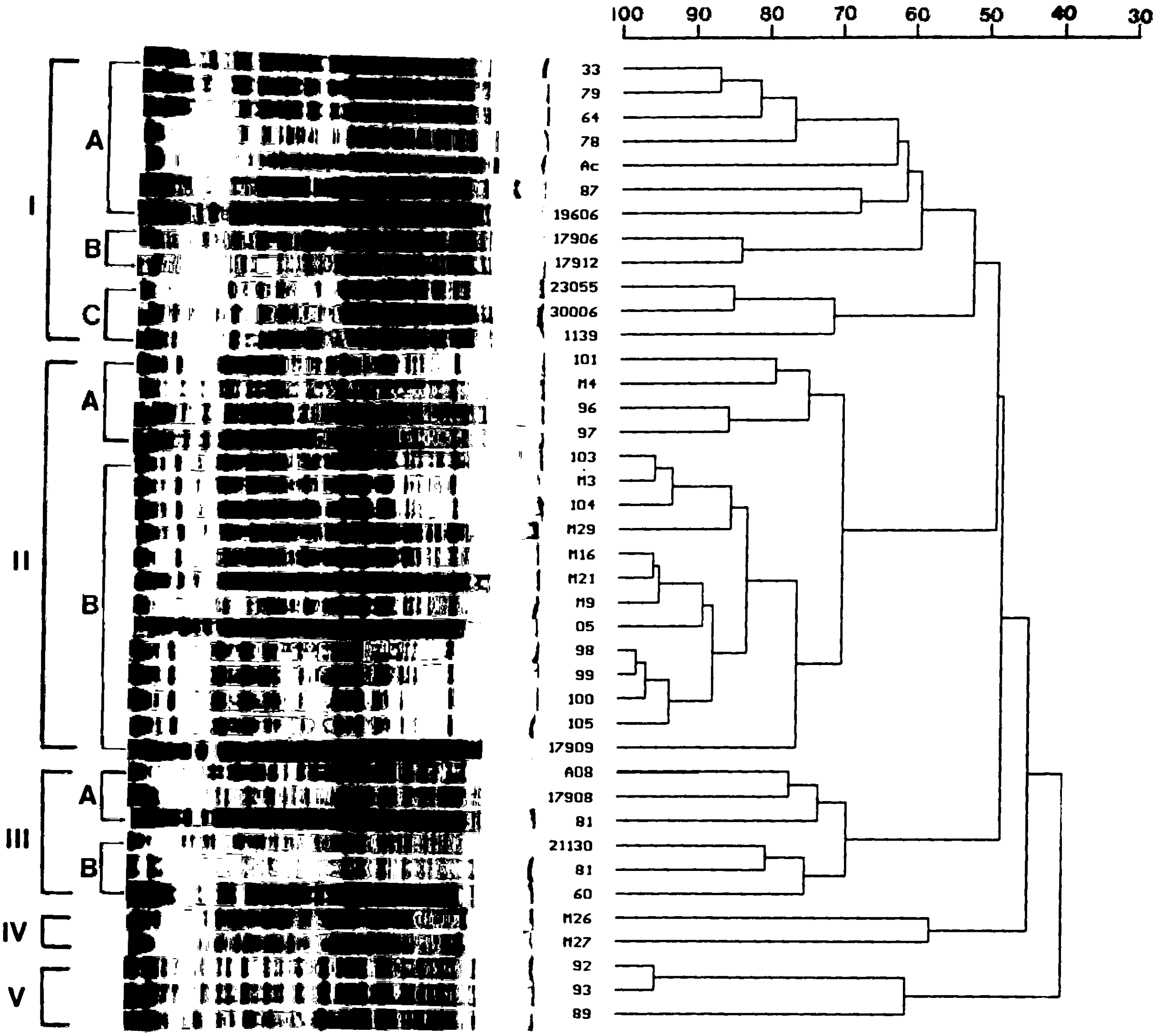

Fig. 1. Dendogram of the correlation coefficient (r), determined using the unweighted average linkage cluster analysis for PAGE of whole cell proteins, by the Gel Compar (Version 1.3) program (Helix C.V., Gent, Belgium), showing the taxonomic relationships between the various *Acinetobacter* strains.

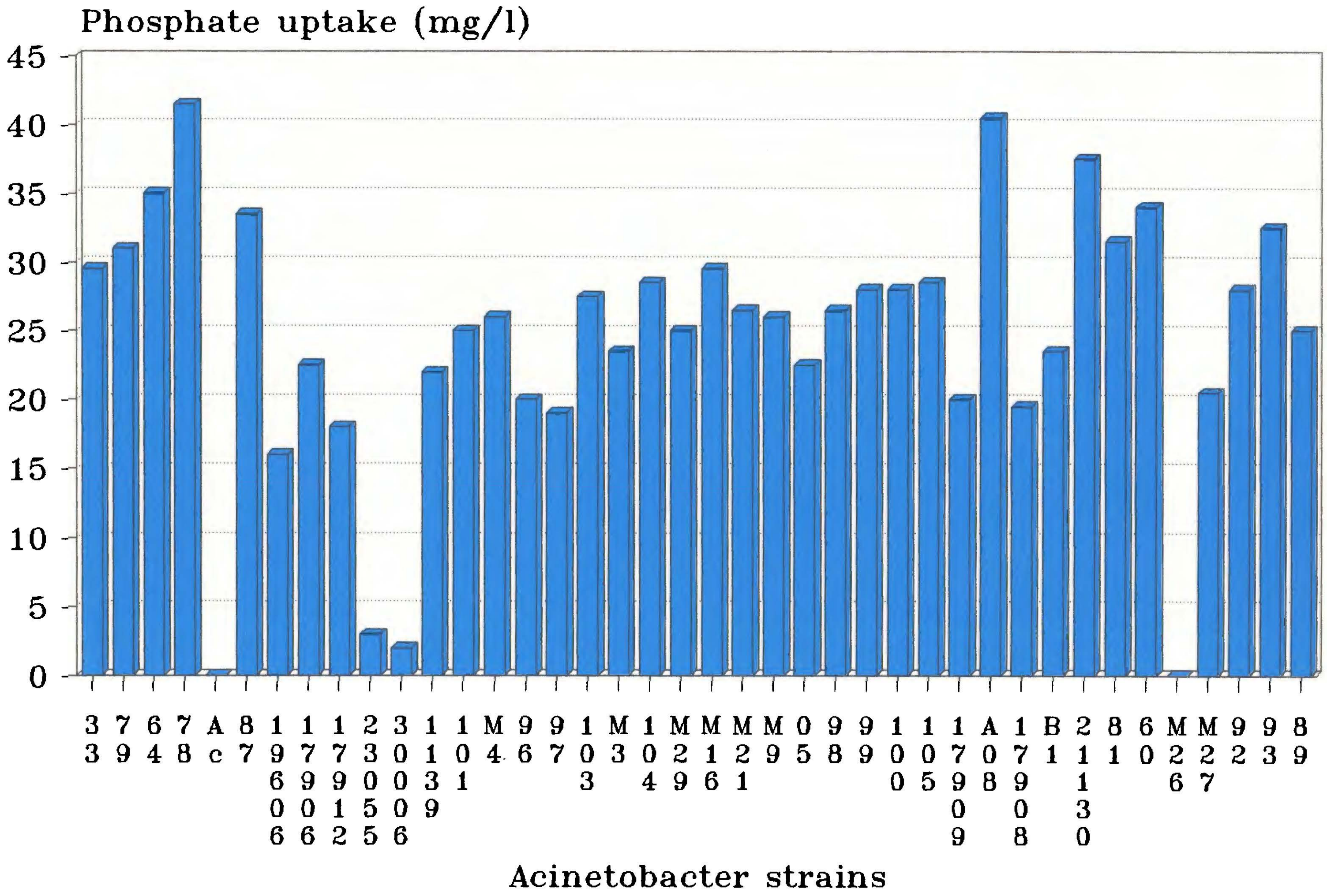

Fig. 2 Phosphate uptake by the *Acinetobacter* strains.

CHAPTER 4

(Submitted for publication in The Journal of General and Applied Microbiology[*]*)*

GROWTH AND PHOSPHORUS UPTAKE OF FREE AND IMMOBILIZED *ACINETOBACTER* STRAINS IN MIXED LIQUOR

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[*] *The editorial style used in this chapter are in accordance with the requirements of The Journal of General and Applied Microbiology.*

SUMMARY

Biological phosphorus removal from activated sludge does not always reduce phosphorus concentrations to the legislated levels. Immobilization was therefore investigated as a possible technique for conducting *in situ* studies of biological phosphorus removal. Five *Acinetobacter* strains were tested for their ability to grow and accumulate phosphorus in mixed liquor. All the strains were able to grow in the mixed liquor and phosphorus was accumulated, mostly in the lag phase and not during exponential growth. Two of the strains were then immobilized in 2% sodium alginate and their survival and phosphorus accumulation ability was monitored. The strains were able to grow and accumulate phosphorus in the immobilized state. The alginate concentration was however not high enough and cells leached out of the alginate balls. Research is therefore needed to optimize the immobilization technique.

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 [17]. 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.l^{-1} [15]. 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 [1], 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 $mg.l^{-1}$ by additional chemical precipitation with $FeCl_3$. 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 [7]. *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 [3, 5, 13, 16]. Cloete and Steyn [4] 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 μm^3. Du Preez [6] 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 [4].

Immobilization is a technique which allows the study of pure cultures in their natural habitat without disruption of the ecosystem [18]. Immobilization would therefore make *in situ* studies of polyphosphate accumulating bacteria in the activated sludge system possible, without costly manipulation of the process design or operation. In this way valuable insight into the behaviour of the cells in the activated sludge system and the mean cell retention times required for maximal phosphorus removal could be determined. *In situ* studies would provide direct insight to the problems and possible solutions regarding the functioning of activated sludge plants without the use of pilot plants. The latter being difficult to operate and simulation of the exact conditions prevailing in a full scale plant is impossible. The immobilization of phosphorus removing organisms could therefore assist in determining the optimal operating conditions for activated sludge treatment systems and possibly even be used to increase the efficiency of biological phosphorus removal systems. The aim of this study was therefore firstly to determine the relationship between phosphorus uptake and growth and secondly to study the effect of immobilization on the survival, growth and phosphorus uptake of *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 [2]. 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 [2]. The phosphorus uptake ability of the strains in mixed liquor was determined [2] 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 4^0C and subcultured monthly.

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} $MgSO_4.7H_2O$ (Merck) and 0.18 g.l^{-1} KNO_3 (Merck) were added and the pH adjusted to pH7 with 2N HCL, before autoclaving (121^{0}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^{0}C with shaking (80 rpm) and placed in a shaking waterbath (80rpm) at 28^{0}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^{0}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 [11]. 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 65^0C for at least 48h to allow the resin to polymerize. Silver-gold sections were obtained using a Reichert-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 [14] 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 [4].

Immobilization studies. Cells were cultured for 48h at 28^0C in Nutrient broth (Biolab), by shaking in a shaking waterbath at 80 rpm. To a 20ml sterile 2% sodium alginate (Merck) solution, 4ml of the 48h culture was added. The solution was thoroughly mixed before being induced to gel by dropping it into a 1.1% sterile calcium chloride solution (Merck) with the use of a syringe and 26G needle. The balls obtained were *ca.* 2 mm in diameter and were left in the $CaCl_2$ solution for one hour to allow the balls to harden sufficiently.

Determination of survival and growth of immobilized cells. The alginate balls, containing the immobilized cells, were washed with sterile distilled water before being placed in 100 ml sterile mixed liquor medium and incubated at 28^0C in a shaking waterbath (80 rpm). Uninoculated alginate balls were used as control and treated in the same manner as the inoculated alginate balls. Before starting the experiment it was determined that 0.1g of alginate balls (*ca.* 8 balls) contained the same number of cells as 1ml of medium containing unimmobilized cells. Growth in the balls was therefore monitored hourly by dissolving 0.1g balls in 1 ml 1M phosphate buffer (pH 7) by mixing on a vortex (Heidolph, REAX 2000) for *ca.* 60s, making a dilution series of the dissolved balls and doing viable counts thereof on Nutrient agar (Biolab) plates which were incubated at 28^0C for 48h. A serial dilution and viable counts were also made of the mixed liquor medium in which the balls were suspended to determine if any of the cells leached out of the alginate balls into the mixed liquor medium. The phosphorus uptake by the immobilized cells was determined as described above.

RESULTS

Growth and phosphorus accumulation of *Acinetobacter* strains in activated sludge mixed liquor. Although strains ϕ5 and AS93 were capable of limited growth in the mixed liquor medium, containing 200 mg.l^{-1} sodium acetate, very small quantities of phosphorus was removed from the medium (Fig. 1-2). *Acinetobacter* strains AS 60, AS 78, AS 93, ATCC 17908 and ϕ5 all had the ability to grow and remove phosphorus in the mixed liquor medium containing 5g.l^{-1} sodium acetate (Fig. 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 ϕ5 after 4 and 7 h respectively (Fig. 8).

Growth and phosphorus accumulation of immobilized *Acinetobacter* strains. Strains $\phi 5$ and AS93 were immobilized and in both cases the cells did multiply within the alginate balls and phosphorus was also removed from the mixed liquor medium (Fig 9-10). Cells did however leach out of the balls and were already present in the medium after 1 h (Fig. 9-10). The uninoculated alginate balls removed *ca.* 2 $mg.l^{-1}$ phosphorus from the medium, indicating that only phosphorus removal above 2 $mg.l^{-1}$ could be ascribed to the cells entrapped within the alginate balls (Fig. 11).

DISCUSSION

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 [19]. 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 [4, 6]. 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 [8]. The author postulated that once nucleic acid synthesis began (*i.e* growth) competition for the available phosphorus would prevent the accumulation of polyphosphate [8]. 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 [7]. 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). Since similar quantities of phosphorus were removed per cell, *i.e.* 3.7×10^{-11} and 1.51×10^{-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 availabilty. 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 ϕ5, Table 2).

Soil, water and sewage, being the natural habitat of these organisms, is an environment 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 [9]. In the biochemical model for polyphosphate accumulation as proposed by Wentzel *et al.* [20], 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 ϕ5 accumulated the most per cell. The larger quantity of phosphorus removed was therefore a function of the larger cell numbers and not due a greater affinity of the cells for polyphosphate accumulation. This suggests that biomass is critically important to phosphorus removal. *Acinetobacter* strain ϕ5 therefore effectively accumulated the most phosphorus per cell (Table 2). Nutrient availability would therefore appear 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). The phosphorus concentration of the mixed liquor medium decreased dramatically in the first 5h, *i.e.* the lag phase (Fig. 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 [10] 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 [4] 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 μm^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 [6, 12]. Du Preez [6] 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 [6]. Du Preez [6] also found that oxygen limitation alone could not induce pleomorphism and that the growth rate did not play a role in this phenomenon. Growth rate did however influence the cell size. Slow growing cells were smaller than fast growing cells [6]. 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.

Once it had been determined that all the strains tested could both grow and accumulate phosphorus in the unimmobilized state, strains $\phi 5$ and AS93 were immobilized in sodium alginate to determine whether the immobilization process would affect the viability and phosphorus uptake ability of the cells.

Phosphorus removal by immobilized cells occurred within the first 5h, similarly to the pattern observed for the unimmobilized cells. Phosphorus was however actively being accumulated while the cells were also leaching out of the balls and growing in the medium. The cell leaching was however only monitored to 5h. After 5h, 3.79 x 10^5 and 4.16 x 10^5 cells had leached from the alginate balls for strains ϕ5 and AS93 respectively, while the alginate balls contained 3.7 x 10^7 and 1.44 x 10^7 cells respectively. The leached cells could therefore only account for 1% of the cell biomass present for strain ϕ5 and 3% for strain AS93. Only 1 and 3% of the phosphorus removed could therefore be ascribed to the leached cells of strains ϕ5 and AS93 respectively. Since a large percentage of the phosphorus removed, had already been removed after 5h, *i.e.* 16 $mg.l^{-1}$ and 30 $mg.l^{-1}$ for strains ϕ5 and AS93 respectively, further leaching and growth after 5h would not have had any significant effect on the total phosphorus removed. The phosphorus removed from the medium was 9.3 $mg.l^{-1}$ and 14.5 $mg.l^{-1}$ more than the unimmobilized cells for strains ϕ5 and AS93 respectively. The phosphorus uptake per cell indicated that the immobilized cells accumulated *ca.* 10 times more phosphorus than the unimmobilized cells (Table 2). The alginate balls themselves were responsible for the removal of *ca.* 2 $mg.l^{-1}$ phosphorus, indicating that the increased phosphorus removal was not an artifact of the immobilization process, but removal due to accumulation by the immobilized cells (Fig. 11).

Alginate was a suitable matrix since it could be induced to gel under relatively mild conditions. It allowed a certain amount of operational ease and dissolved easily in phosphate buffer, which facilitated enumeration. Higher concentrations however need to be examined to limit cells leaching out of the alginate. Immobilization of polyphosphate bacteria therefore has potential for ***in situ*** studies whereby the effects of the various conditions prevailing in an activated sludge plant, on the bacteria, can be determined. Immobilization therefore has potential as a mechanism whereby conditions in the activated sludge system can be manipulated without disrupting the operation of the system. Research is however needed to find the most suitable matrix and the most suitable polyphosphate bacteria for this purpose.

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Table 1. *Acinetobacter* strains used in growth, phosphorus removal and immobilization studies.

Strain	**SDS-PAGE identification**[a]	**Origin**
ϕ5	*Acinetobacter johnsonnii*	Dam
AS60	*Acinetobacter lwoffii*	Activated sludge
AS78	*Acinetobacter calcoaceticus* subsp. *baumannii*	Activated sludge
AS93	*Acinetobacter lwoffii*	Activated sludge
ATCC[b] 17908^T	*Acinetobacter junii*	Urine

[a] Identified by Bosch and Cloete [2].

[b] American type culture collection (ATCC, Rockville, Maryland 20852, U.S.A.); Type strain of *A. junii*.

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)*:				
φ5	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)*:				
φ5	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}
Immobilized cells (5 g.l^{-1} sodium acetate)*:				
φ5	1.07 x 10^{7}	1.40 x 10^{8}	29.00	2.08 x 10^{-10}
AS93	9.67 x 10^{6}	9.85 x 10^{7}	31.00	3.11 x 10^{-10}

[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 φ5: 24h
[b] Strains AS60, AS78 and 17908: 13h;
Strain φ5 and AS93: 12h;
Immobilized cells: Strain φ5 and AS93: 14h.

Table 3. Cell volume of *Acinetobacter* cells containing polyphosphate granules.

Electron micrograph*	Strain	Cell volume μm^3
a	AS93	0.65
b (1)	AS93	0.22
b (2)	AS93	0.17
b (3)	AS93	0.12
b (4)	AS93	0.18
c	AS93	3.29
d	$\phi 5$	2.53
e (1)	$\phi 5$	0.53
e (2)	$\phi 5$	0.10
f (1)	$\phi 5$	0.14
f (2)	$\phi 5$	0.48
f (3)	$\phi 5$	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

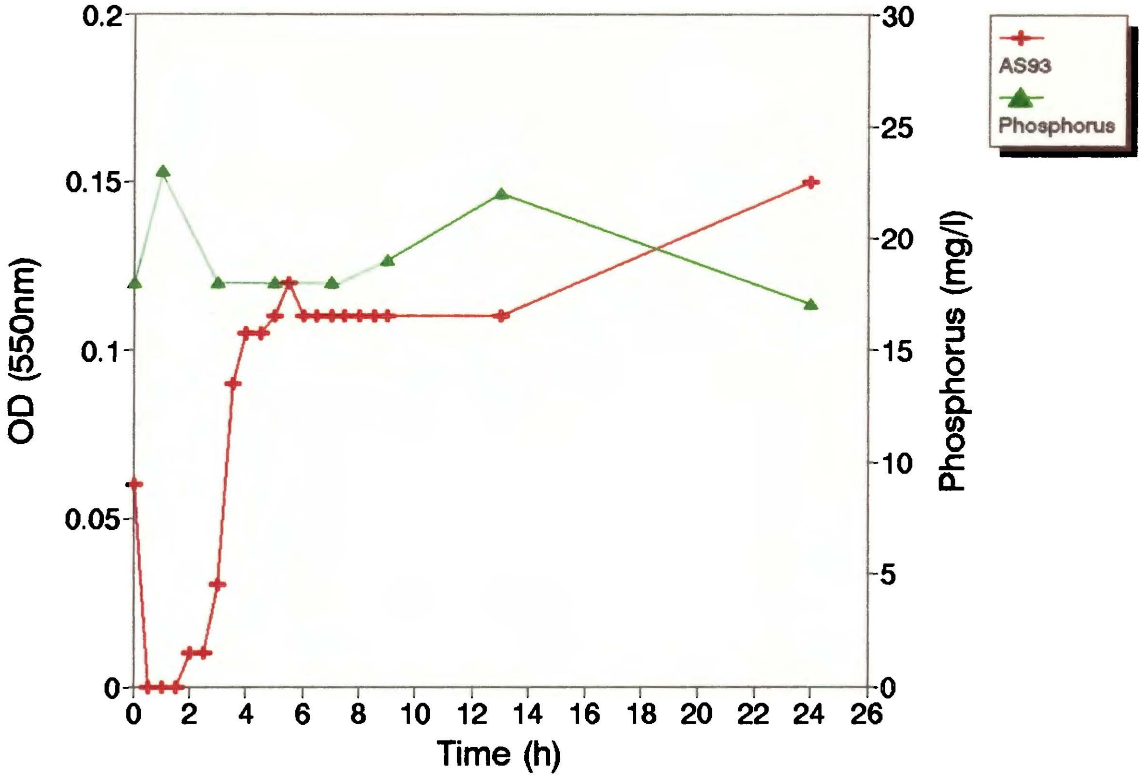


Fig. 1 Growth and phosphorus uptake of A. lwoffii strain AS93 in mixed liquor medium (200 mg/l sodium acetate).

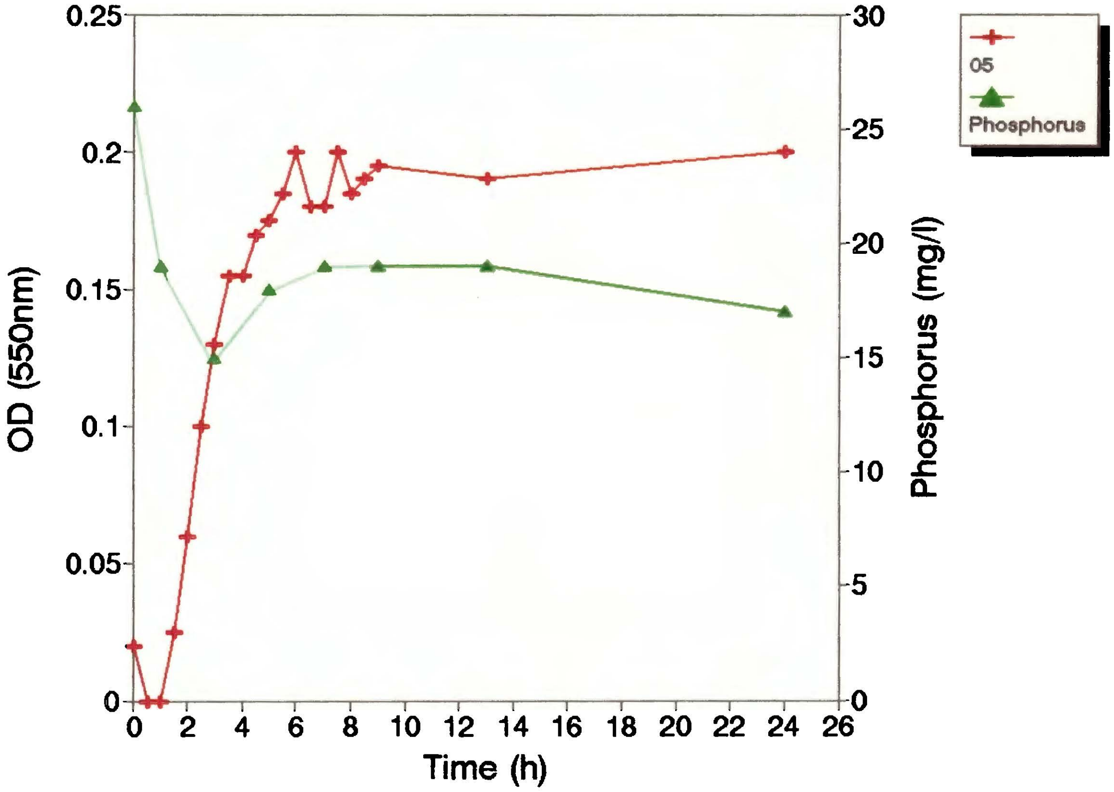


Fig. 2 Growth and phosphorus uptake of A. johnsonnii strain 05 in mixed liquor medium (200 mg/l sodium acetate).

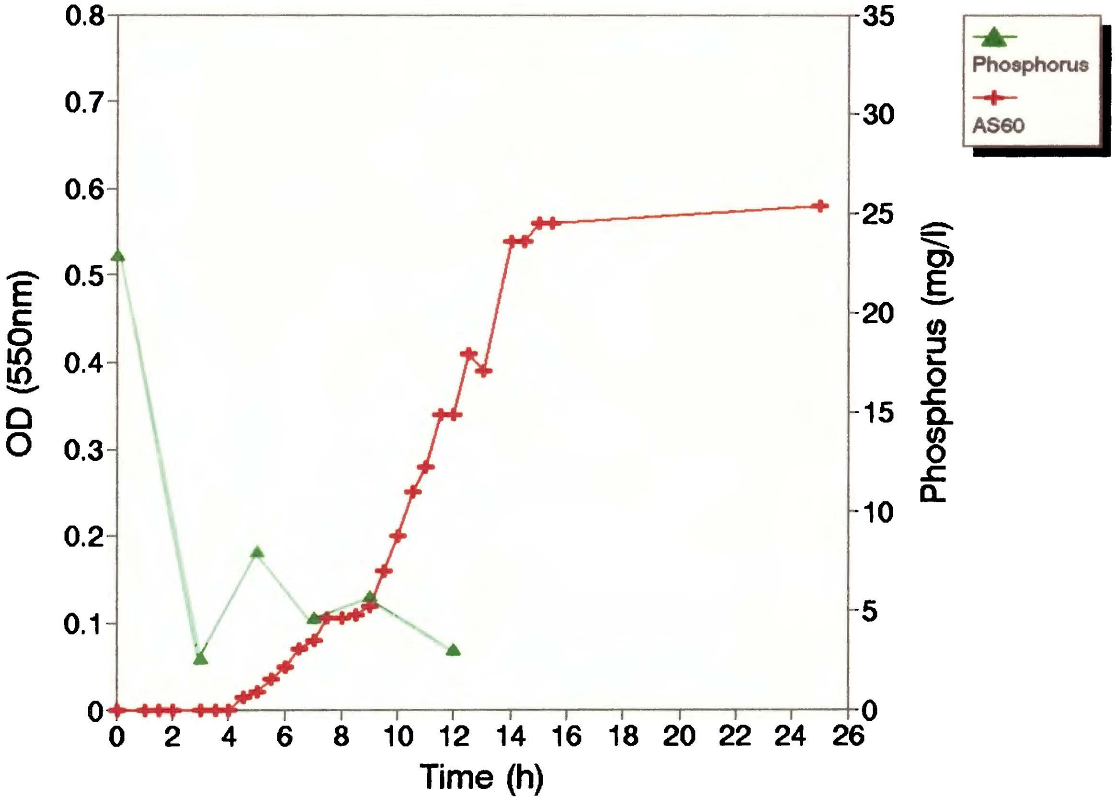


Fig. 3 Growth and phosphorus uptake o f A. lwoffii strain AS60 in mixed liquor medium (5 g/l sodium acetate).

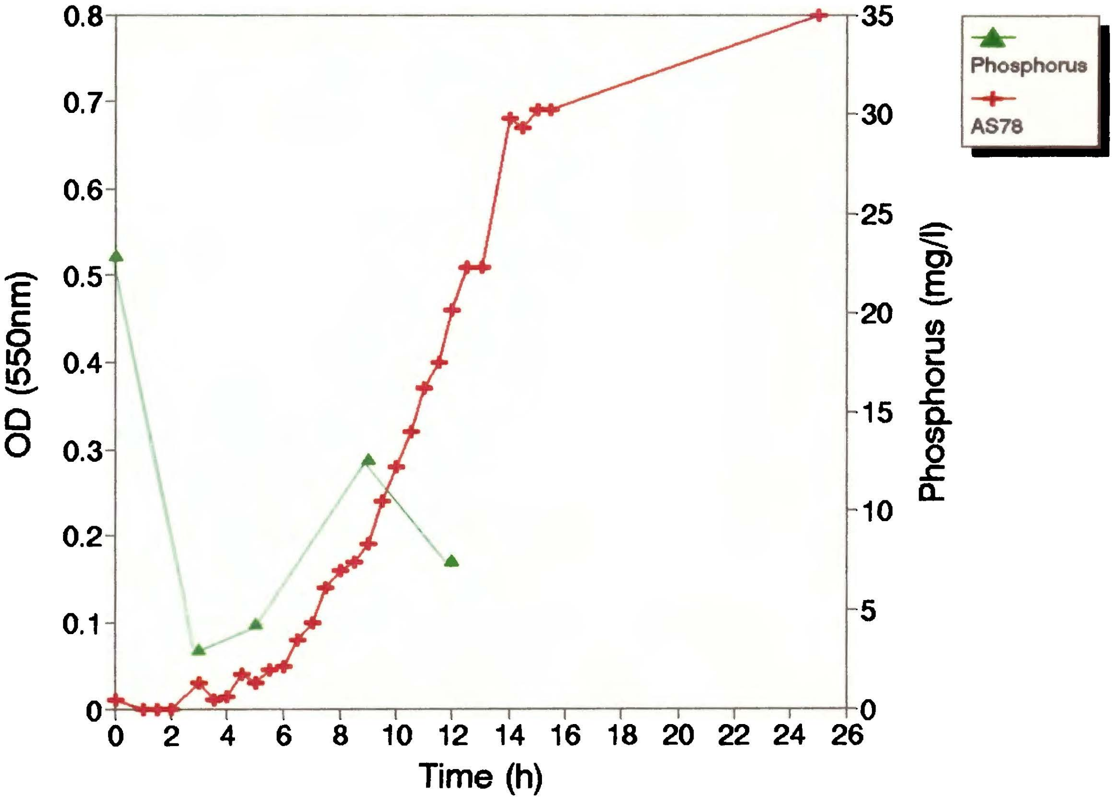


Fig. 4 Growth and phosphorus uptake of A. calcoaceticus subsp. baumannii strain AS 78 in mixed liquor medium (5g/l sodium acetate).

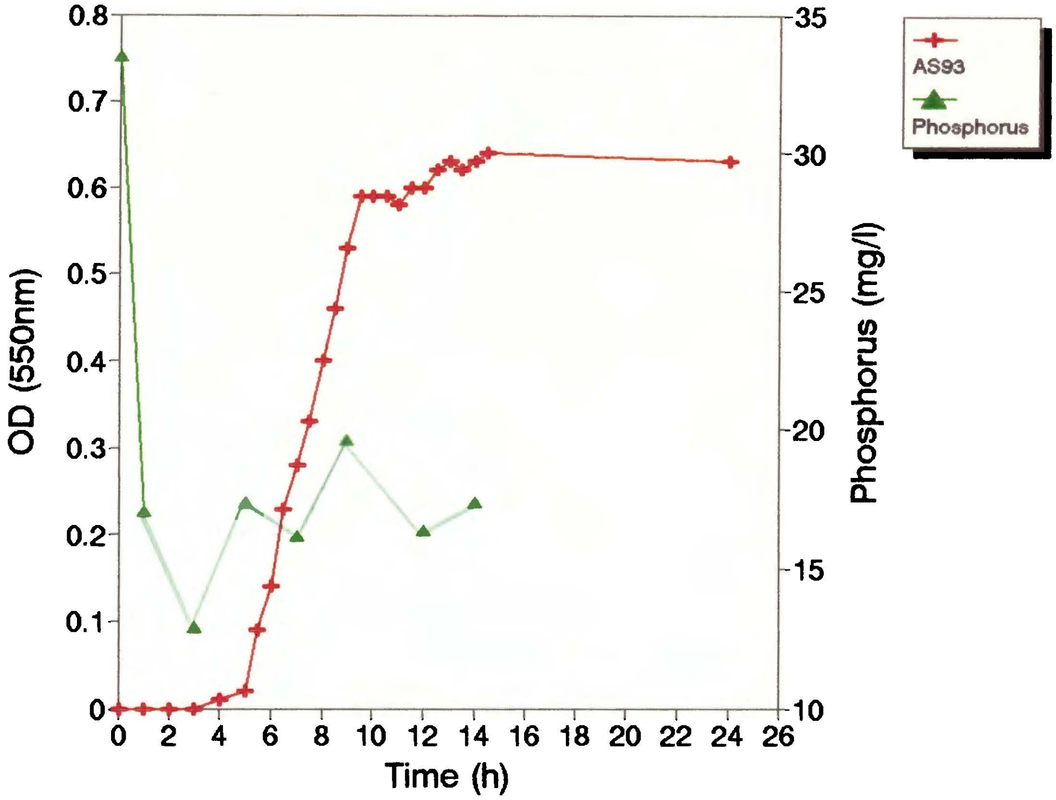


Fig. 5 Growth and phosphorus uptake of <u>A. lwoffii</u> strain AS93 in mixed liquor medium (5g/l sodium acetate).

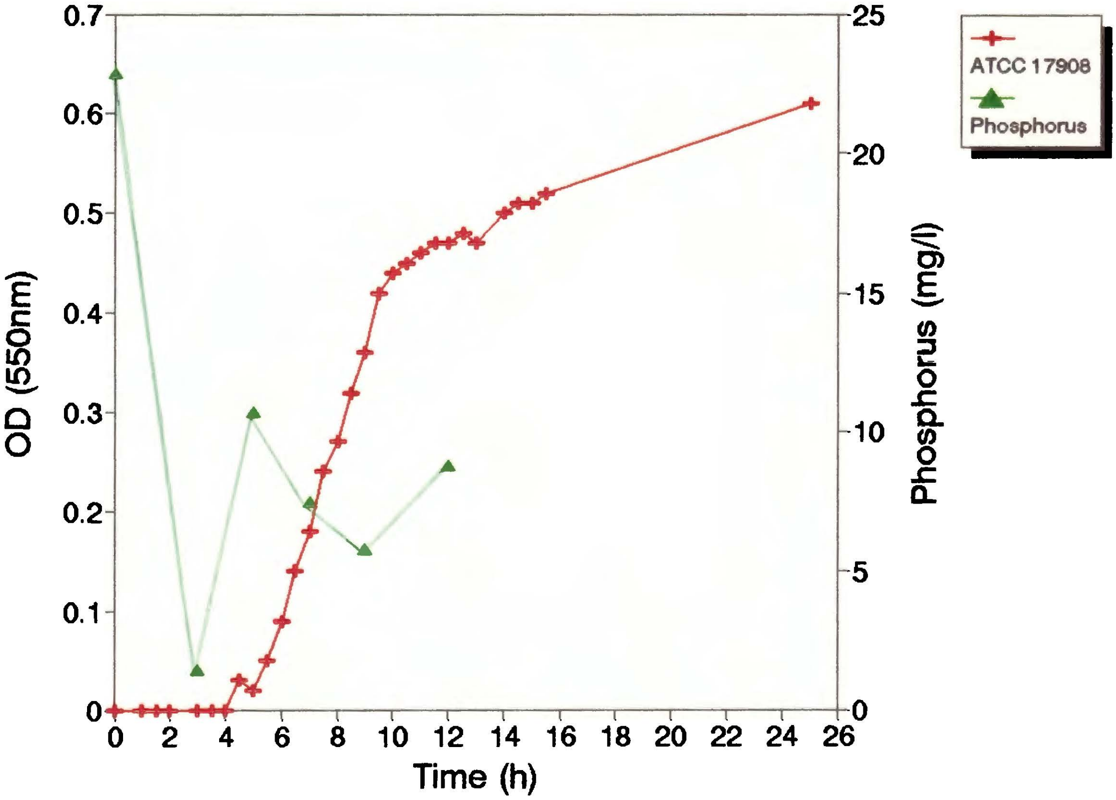


Fig. 6 Growth and phosphorus uptake of <u>A. junii</u> strain ATCC 17908 in mixed liquor medium (5g/l sodium acetate).

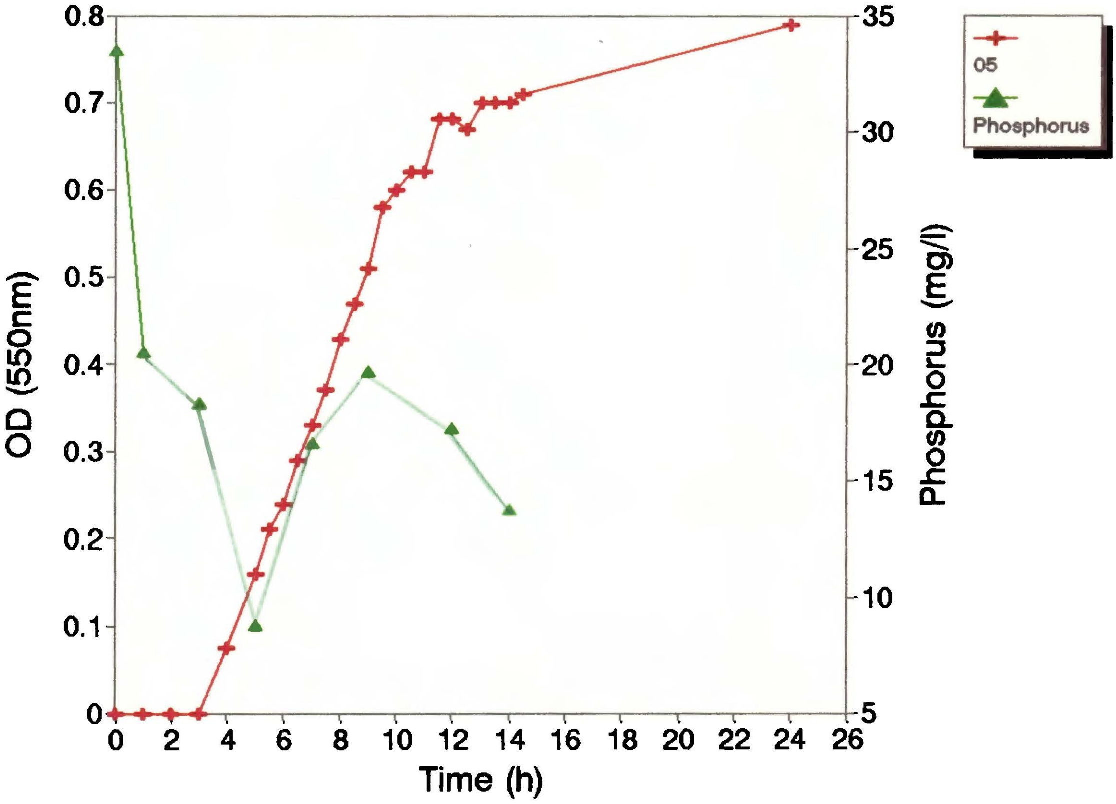
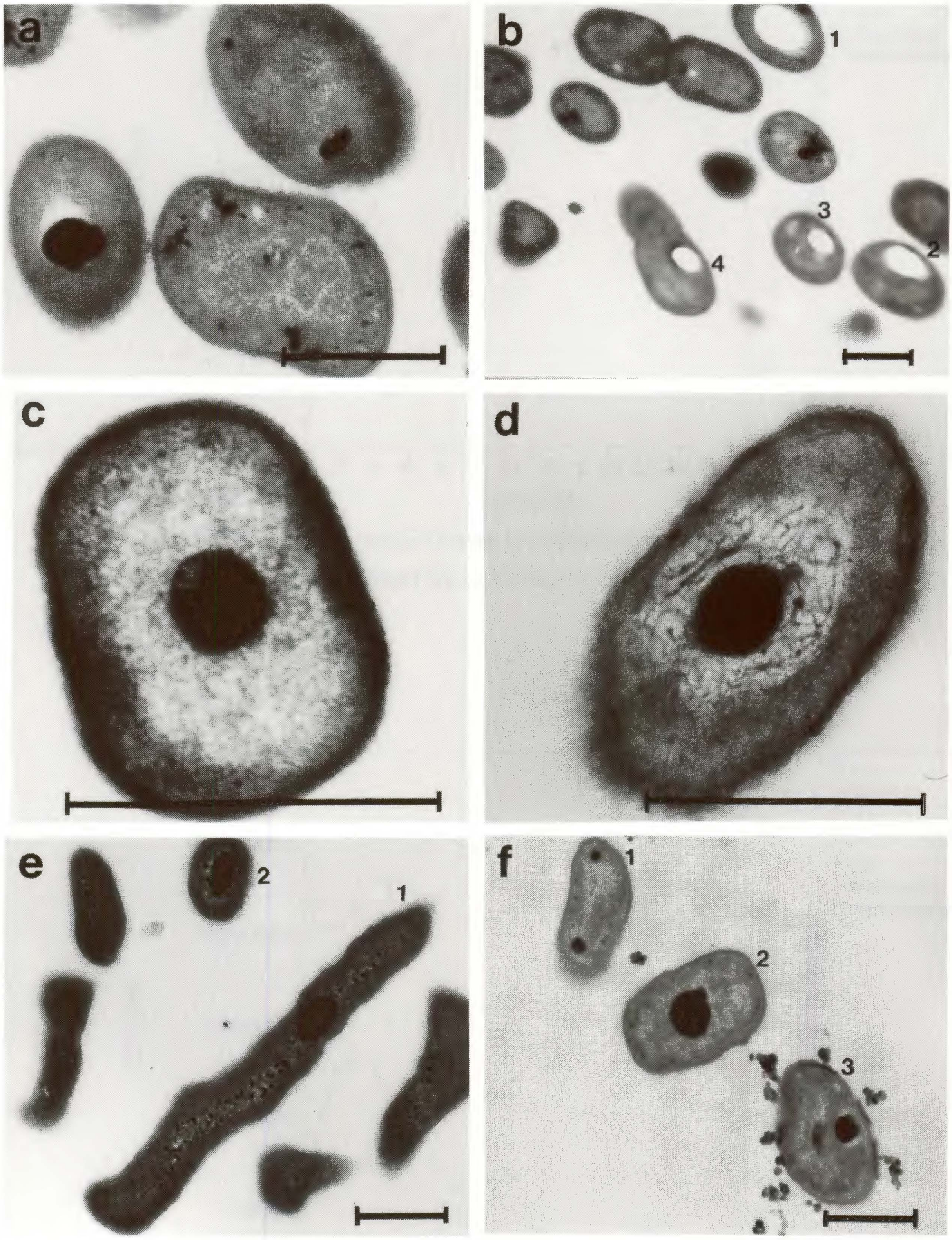


Fig. 7 Growth and phosphorus uptake of *A. johnsonnii* strain 05 in mixed liquor medium (5g/l sodium acetate).

Fig.8 TEM photographs of *Acinetobacter* strains ϕ5 and AS93 showing intracellular polyphosphate granules: **a)** Strain AS93 after 4h (35 000 x); **b)** Strain AS93 after 4h, the translucent holes are where the polyphosphate granules were ripped out during sectioning (15 000 x); **c)** Strain AS93 after 7h (80 000 x); **d)** Strain ϕ5 after 7h (60 000 x); **e)** Strain ϕ5 after 7h, showing pleomorphism (20 000 x); **f)** Strain ϕ5 after 9h still contained polyphosphate granules (20 000 x). In each electron micrograph the bar represents 1μm.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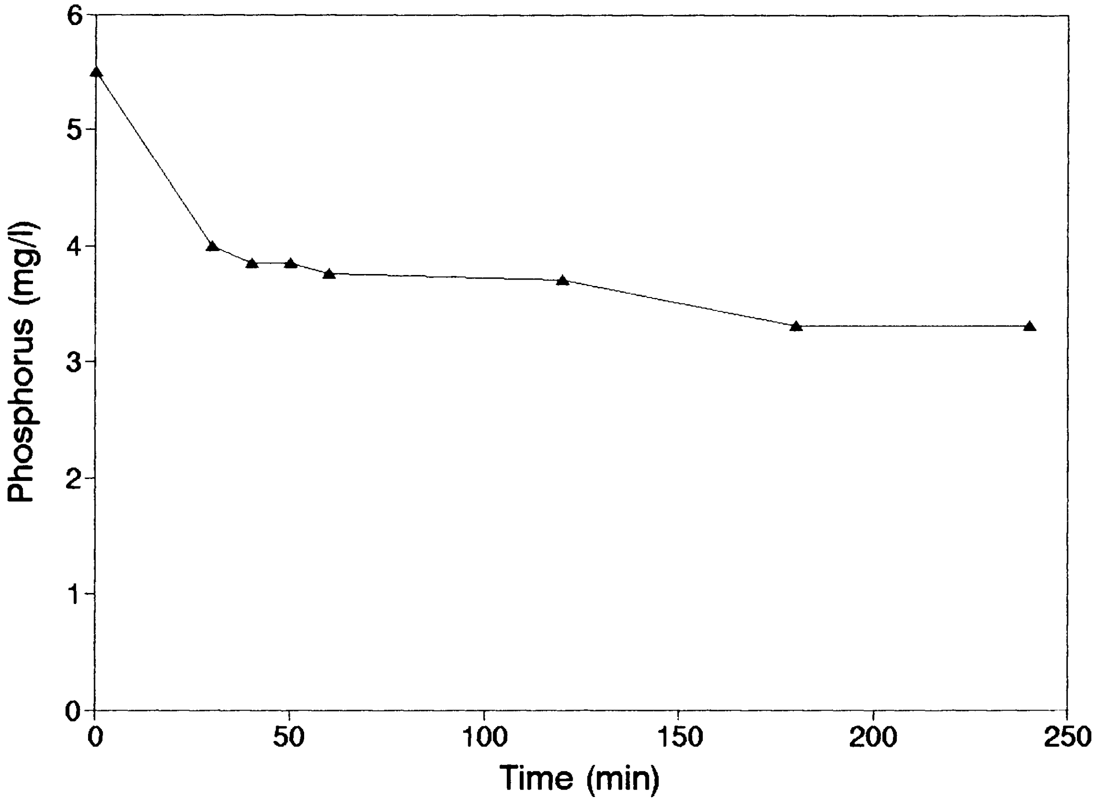

Fig. 11 Phosphorus uptake by uninnoculated alginate balls in mixed liquor medium (5g/l sodium acetate).

CHAPTER 5

CONCLUSIONS

The editorial style of the Journal of Bacteriology was followed in this chapter.

The numerical analysis of the protein profiles obtained by SDS-PAGE indicated that *Acinetobacter* was a heterogeneous genus, especially within clusters 1,3 and 4. *A. baumannii* (ATCC 19606) and *A. haemolyticus* (ATCC 17906) fall into cluster 1 together with *A. calcoaceticus* and are proposed to be subspecies of *A. calcoaceticus*, instead of separate species as proposed by Bouvet and Grimont (1986). This proposal supports the earlier findings of Nishimura *et al.* (1988). The reclassification of *A. baumannii* (ATCC 19606) and *A. haemolyticus* (ATCC 17906) as *A. calcoaceticus* subsp. *baumannii* and *A. calcoaceticus* subsp *haemolyticus* respectively, is proposed.

Acinetobacter strains were isolated from activated sludge and a number of strains were obtained from culture collections. Two strains (Ac and M26), obtained from culture collections, were unable to accumulate phosphorus when cultivated in mixed liquor medium. All the other strains tested were able to accumulate phosphorus to varying degrees.

The heterogeneity of the genus is reflected in the large variation in the phosphorus uptake ability of the strains within a cluster. This indicated that phosphate uptake ability was strain specific rather than specie specific. These findings have recently been supported by Beacham *et al.* (1992) who investigated 156 *Acinetobacter* strains isolated from a nutrient removal plant and also found variations in phosphorus accumulation amongst strains.

Concurrent growth and phosphorus uptake studies indicated that phosphorus was accumulated in the lag phase of the normal growth cycle. Little or no phosphorus was accumulated in the logarithmic growth phase, instead phosphorus was released at the beginning of logarithmic growth. Further phosphorus accumulation took place in the stationary phase, once active growth had ceased. Harold (1963) found a reciprocal relationship between phosphate uptake and growth. Stress conditions were not a prerequisite for 'luxury uptake' as cells accumulated polyphosphates as part of their natural growth cycle (*i.e.* lag phase).

Since cells grown in lower nutrient environment accumulated similar quantities of phosphorus per cell it would appear that cells have a limit to the amount of phosphorus that can be accumulated irrespective of nutrient availability. Under conditions of increased nutrient availability, phosphorus removal will increase due to the resultant increase in biomass and not due to an enhanced polyphosphate accumulating ability of the cell.

The cell volume measurements and frequency distribution of polyphosphate containing cells indicated that mostly small cells contained polyphosphate granules. Since phosphorus was mostly accumulated in the lag phase of growth it was concluded that these relatively small cells were also very slow growing cells.

Acinetobacter cells, immobilized in 2% sodium alginate, remained viable, multiplied and accumulated phosphate within the alginate balls. The immobilized cells removed 10 times more phosphorus from the medium than the free cells. The concentration of the alginate matrix was however not optimal since cells leached out of the matrix into the medium. Further research is needed to optimize the alginate concentration and also to investigate other suitable matrixes. The cells were easily recovered from the matrix for enumeration and further study.

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