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Effect of the various carbon sources and growth conditions on phosphate release and uptake by *Acinetobacter calcoaceticus*

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The aim of this study was to investigate the influence of various carbon sources applied at different initial concentrations of phosphorus and growth conditions, on the phosphate release and uptake in a pure culture of *Acinetobacter calcoaceticus DSM1532*. P-release and uptake studies with various carbon sources applied at different initial concentrations of phosphorus were carried out in alternating anaerobic/aerobic batch experiments. The best P-release and P-uptake rates were obtained when sodium propionate was the carbon source. Glucose addition in the synthetic wastewater caused lower P-release and uptake ratios, but did not have any significant influence on the final percentage of P-removal. The highest P-release and P-uptake rates were obtained with the highest phosphorus loadings. The highest P-release resulted in the highest P-uptake rates. The observed aerobic P-uptakes in a batch test, without preliminary resort to anaerobic conditions indicate the probable aerobic mechanism of P-uptake by *A. calcoaceticus DSM1532*. The results of the semi-continuous test indicate the possibility of the adaptation of *A. calcoaceticus DSM1532* to high phosphorus loads (up to 100 mg P L^{-1}) in wastewater.

Key words: *Acinetobacter calcoaceticus*, phosphorus removal, release, uptake.

Introduction

Phosphorus is considered the main limiting factor for algal growth in internal and coastal waters. Phosphorus removal from municipal and industrial wastewater is required to protect receiving waters from nutrient enrichment – eutrophication, which is one of the most serious environmental problems involving water sources in all industrialised countries.

Conventional activated sludge wastewater treatment processes can remove 30–40% of the P content of municipal wastewater. Enhanced biological phosphorus removal (EBPR) from wastewater is based on the enrichment of activated sludge with phosphate accumulating organisms. These microorganisms (also called P-removing or poly-P bacteria) are able to store intracellular phosphorus as polyphosphate. The requirement to achieve a phospho-

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rus removing bacterial population in an activated sludge system is the exposure of activated sludge to anaerobic and aerobic (or anoxic) conditions (JONES and STEPHENSON 1996).

Under anaerobic conditions, P-removing bacteria transport volatile fatty acids (VFA, e. g. acetate) into the cell and subsequently convert and store these as poly-hydroxy--alkanoates (PHA, e.g. poly-beta-hydroxy-butyrate; PHB). The energy for this transport and storage is supplied by the hydrolysis of intracellularly stored polyphosphate (poly-P) to ortho-phosphate, which is released from the cell to the liquid. Anaerobic P-release is strongly influenced by pH. Reducing equivalents required for the conversion of acetate to PHB are supplied by the conversion of intracellular stored glycogen through the glycolytic pathway to PHB and CO_2. Glycogen plays an essential role in maintaining the redox balance in the cell. In the absence of glycogen, anaerobic uptake of the organic substrate by poly-P bacteria may not occur (BRDJANOVIC et al. 1998).

Under aerobic conditions, anaerobically formed PHB or available external carbon substances are used to generate energy for cell growth, poly-P synthesis, glycogen formation and maintenance, resulting in the uptake of phosphate. Alternatively, bio-P bacteria capable of using oxidised nitrogen (nitrate or nitrite) could produce energy under anoxic conditions (absence of dissolved oxygen but presence of oxidised nitrogen). The P-accumulating bacteria can be divided into two groups: one group capable of utilising only oxygen as oxidant (electron acceptor) and another group capable of utilising both oxygen and nitrate as oxidant. The P uptake is more rapid under aerobic conditions than under anoxic conditions. The explanation of this is that all P-accumulating bacteria take up phosphate under aerobic conditions, whereas only part of the P-accumulating bacteria take up phosphate under anoxic conditions (COMEAU et al. 1987, KERRN-JESPERSEN and HENZE 1993).

Anaerobic release and aerobic uptake of P has been observed in bacteria from the *Acinetobacter* genus such as *A. calcoaceticus*, in other bacteria, yeasts and fungi.

Acinetobacter has become the model organism for biological phosphorus removal since it was isolated from a phosphorus-removing activated sludge plant (FUHS and CHEN 1975). There are opposite hypotheses that *Acinetobacter* spp. is the predominant micro-organism involved in EBPR. Some researchers who conducted studies on full-scale plants have reported that *Acinetobacter* spp. formed 50–70% of the total population isolated from the mixed liquor (BUCHAN 1983, LOTTER and MURPHY 1985, KORTSTEE et al. 2000). Other studies with activated sludge showing EBPR indicated that *Acinetobacter* spp. accounted for 1–10% of the bacterial communities (HIRAISHI et al. 1989, KORTSTEE et al. 2000). SIDAT et al. (1999) found that although *Acinetobacter* spp. were present in extremely low numbers, their capacity to accumulate polyphosphate intracellulary was the highest amongst all the isolates from the activated sludge plant.

It can be deduced that only some *Acinetobacter* spp. contained in the sludge are able to accumulate great amounts of phosphorus under aerobiosis. These strictly aerobic bacteria, belonging to the gamma-subclass proteobacteria, when exposed to alternating anaerobic/aerobic conditions, are subjected to a stress analogous to that preceding the overplus accumulation, with the difference that oxygen is lacking instead substrate. *Acinetobacter*, in the absence of oxygen, would hydrolyse polyphosphate to get sufficient energy for synthesising PBH or other polysaccharides from products of the intermediate metabolism of heterotrophic populations or, alternatively, from glucose. Under aerobic conditions, on the other hand, PBH would be catabolized using oxygen as electron acceptor. Thus *Acineto-*

bacter organisms would not be forced to compete with the other species for any external substrate and could grow and re-establish the original content of P (CONVERTI et al. 1993).

Present knowledge indicates that many strains of *Acinetobacter* are able to accumulate P while utilizing VFA (acetic, propionic, butyric, isobutyric, valeric, isovaleric, formic acid). *Acinetobacter* organisms prefer VFA, especially acetate, as a growth substrate present in or able to be produced from wastewater in an activated sludge system. VFA consumption rate and P assimilation rate are strain-dependent (RUSTRIAN et al. 1996, RUSTRIAN et al. 1997).

The aim of this study was to investigate the influence of various carbon sources applied at different initial concentrations of phosphorus and growth conditions on phosphate release and uptake in pure cultures of *A. calcoaceticus DSM1532*.

Material and methods

Microorganism. Lyophilised cultures of *A. calcoaceticus DSM1532*, which has been described as a phosphate-accumulating bacteria, was taken from DSM-Deutsche Sammlung von Microorganismen und Zellkulturen GmbH. A strain of *A. calcoaceticus DSM1532* was maintained on nutrient agar medium, subcultured monthly and stored at 4 °C.

Experimental operation. The bacteria were pregrown in a nutrient broth for 24 h at 30 °C. The biomass was centrifuged (15 min, 7000 g), washed with sterile distilled water, centrifuged, and resuspended in an Erlenmeyer flask with 500 mL of phosphate uptake medium. The pH of the experimental reactors was adjusted to 7 ± 0.1 pH units with 1 M NaOH or 1 M HCl at the start of each run. Temperature was maintained at 30 °C. The aeration (about 4 L min^{-1}) with sterile air in the aerobic stage was provided by aquarium pumps. Phosphate release and uptake studies with various carbon sources were carried out as batch experiments in alternating 24 h anaerobic / 24 h aerobic stages. Aerobic phosphate uptake without preliminary resort to anaerobic conditions was studied in a batch test for 5 days. Semi-continuous testing of phosphate removal without preliminary resort to anaerobic conditions with a high phosphorus concentration was carried out for 9 days; every 24 h 25 mL was taken from the reactor and 25 mL of synthetic wastewater was added into the reactor.

Synthetic wastewater. The composition of the synthetic medium used to simulate sewage is reported in Tab. 1. The concentration of KH_2PO_4, the only sole source of P in tests, varied from 4 up to 440 mg L^{-1} to obtain a concentration of phosphorus in the wastewater ranging from 1 to 100 mg L^{-1}. The sterilisation of the synthetic medium was done at 121 °C / 15 min.

Analytical methods. All measurements were done according to the Standard Methods for the Examination of Water and Wastewater (APHA 1992). The samples were filtered before measurements through nitrocellulose filters (Sartorius) of a pore diameter of 0.2 μm. pH-values were measured with WTW pH 323 for temperature and pH measurement. Orthophosphate ($P\text{-}PO_4^{3-}$) concentrations in the water were measured colorimetricaly in a DR/890 Hach colorimeter by the ascorbic acid method.

The *A. calcoaceticus DSM1532* count was determined as colony forming units (CFU) on nutrient agar using the spread plate method. Plates were incubated at 30 °C for 72 h.

Dissolved oxygen and temperature were controlled with Jenway 9071 dissolved oxygen meter.

Tab. 1. Composition of the synthetic sewage.

Component / mg L^{-1}	a)	b)	c)
Sodium acetate	–	1000	500
Sodium propionate	1000	–	40
Glucose	–	–	40
Peptone	100	100	100
$MgSO_4$	10	10	10
$CaCl_2$	6	6	6
KCl	30	30	30
Yeast extract	20	20	20
KH_2PO_4	Variable	Variable	Variable

Methylene blue stains were performed to confirm phosphate accumulation and cell growth stage.

Calculations

The statistical analysis was performed using the program Statistica, Version 6.0.

A = phosphorus load at time zero (P-PO_4 mg L^{-1})
B = phosphorus load at the end of anaerobic stage (P-PO_4 mg L^{-1})
C = phosphorus load at the end of aerobic stage (P-PO_4 mg L^{-1})
a = CFU L^{-1} after incubation at the end of anaerobic stage
b = CFU L^{-1} after incubation at the end of aerobic stage

Phosphorus release ratio:
P-release ratio (mg $cell^{-1}$) = (B – A) / a

Phosphorus uptake ratio:
P-uptake ratio (mg $cell^{-1}$) = (A – C) / b

Percentage of released phosphorus:
P-released (%) = (B – A) / A × 100

Percentage of phosphorus removal:
P-removal (%) = (A – C) / A × 100

Results and discussion

Batch experiments (anaerobic/aerobic). *A. calcoaceticus DSM1532* tested in alternating anaerobic/aerobic batch experiments showed a phosphate removal potential that varied with type of carbon source and initial concentrations of phosphorus.

With each type of carbon source and initial concentrations of P, P-release under anaerobic conditions and P-uptake under aerobic conditions can be seen. The P-uptake ratio was lower than the P-release ratio (Tabs. 2, 3, 4).

It was observed that the highest P-release resulted in the highest P-uptake rates. The highest P-release and P-uptake rates were obtained by the highest phosphorus loadings. The highest P-release and P-uptake rates were obtained when sodium propionate was the

Tab. 2. Phosphate, bacteria viable count, pH, phosphate release and uptake ratios and percent of phosphate release and removal obtained with *Acinetobacter calcoaceticus DSM1532* cultured in sodium propionate.

Carbon source Period	Experiment 1	*Experiment 2*	Experiment 3	Experiment 4
a) Na-propionate				
Influent				
$P\text{-}PO_4$ (mg L^{-1})	2.00	10.60	67.00	130.00
CFU L^{-1}	4.50×10^7	7.90×10^7	5.20×10^7	3.10×10^7
pH	7.00	7.00	7.10	6.80
Anaerobic stage (end)				
$P\text{-}PO_4$ (mg L^{-1})	7.25	30.50	114.00	198.00
CFU L^{-1}	3.60×10^7	6.80×10^7	5.20×10^7	2.10×10^7
pH	6.41	6.41	6.42	6.40
P-release ratio	1.46×10^{-7}	2.93×10^{-7}	9.04×10^{-7}	3.24×10^{-6}
P-released (%)	262.50	187.74	70.15	51.31
Aerobic stage (end)				
$P\text{-}PO_4$ (mg L^{-1})	0.65	4.50	37.50	80.00
CFU L^{-1}	6.20×10^7	1.20×10^8	9.30×10^7	2.20×10^7
pH	7.30	7.20	6.80	6.70
P-uptake ratio	2.18×10^{-8}	5.08×10^{-8}	3.17×10^{-7}	2.27×10^{-6}
P-removal (%)	67.50	57.55	44.03	38.46

Tab. 3. Phosphate, bacteria viable count, pH, phosphate release and uptake ratios and percent of phosphate release and removal obtained with *Acinetobacter calcoaceticus DSM1532* cultured in sodium acetate.

Carbon source Period	Experiment 1	Experiment 2	Experiment 3	Experiment 4
b) Na-acetate				
Influent				
$P\text{-}PO_4$ (mg L^{-1})	1.90	11.00	61.00	130.00
CFU L^{-1}	3.65×10^7	2.40×10^7	2.20×10^7	2.95×10^7
pH	6.90	7.20	7.20	6.80
Anaerobic stage (end)				
$P\text{-}PO_4$ (mg L^{-1})	7.00	37.50	104.00	192.50
CFU L^{-1}	4.60×10^7	3.25×10^7	2.85×10^7	3.90×10^7
pH	6.30	6.60	6.80	6.55
P-release ratio	1.11×10^{-7}	8.15×10^{-7}	1.51×10^{-6}	1.60×10^{-6}
P-released (%)	268.42	240.91	70.49	48.08
Aerobic stage (end)				
$P\text{-}PO_4$ (mg L^{-1})	0.43	3.48	31.92	69.00
CFU L^{-1}	1.04×10^8	8.00×10^8	6.70×10^8	1.03×10^8
pH	7.70	8.40	8.60	6.80
P-uptake ratio	1.41×10^{-8}	9.40×10^{-9}	4.34×10^{-8}	5.92×10^{-7}
P-removal (%)	77.37	68.36	47.67	46.92

Tab. 4. Phosphate, bacteria viable count, pH, phosphate release and uptake ratios and percent of phosphate release and removal obtained with *Acinetobacter calcoaceticus DSM1532* cultured in sodium acetate, sodium propionate and glucose.

Carbon source Period	Experiment 1	Experiment 2	Experiment 3	Experiment 4
c) Na-acetate, Na-propionate, glucose				
Influent				
$P\text{-}PO_4$ (mg L^{-1})	2.70	10.50	42.00	108.00
CFU L^{-1}	5.00×10^7	1.00×10^7	5.00×10^7	1.00×10^7
pH	7.00	7.10	7.30	6.90
Anaerobic stage (end)				
$P\text{-}PO_4$ (mg L^{-1})	7.00	26.00	126.00	210.00
CFU L^{-1}	1.00×10^8	1.00×10^8	1.00×10^8	1.60×10^9
pH	5.40	5.70	6.20	6.25
P-release ratio	4.30×10^{-8}	1.55×10^{-7}	8.40×10^{-7}	6.38×10^{-8}
P-released (%)	159.26	147.62	200.00	94.44
Aerobic stage (end)				
$P\text{-}PO_4$ (mg L^{-1})	0.70	4.20	21.50	44.50
CFU L^{-1}	2.50×10^8	6.00×10^8	7.45×10^8	2.44×10^9
pH	7.60	7.40	8.00	7.70
P-uptake ratio	8.00×10^{-9}	1.05×10^{-8}	2.75×10^{-8}	2.60×10^{-8}
P-removal (%)	74.07	60.00	48.81	58.80

carbon source (Fig. 1). Similar results were obtained by RUSTRIAN et al. (1997) with pure cultures of *A. calcoaceticus NRRL8058* cultured on propionic acid. But, these authors did not find phosphorus uptake in the aerobic stage to be dependent on the phosphorus release rate in the pure culture of other *A. calcoacetius* strains cultured on acetic, butyric and propionic acid.

In general, anaerobic conditions alone are not able to induce P-release. The P-release phenomenon is primarily dependent on the nature of the feed rather than the anaerobic condition as such. Phosphate-accumulating bacteria release phosphorus under anaerobic, anoxic, and aerobic conditions when VFA are present (RUSTRIAN et al. 1997). Various biochemical models (COMEAU et al. 1987, BRDJANOVIC et al. 1998, KORTSTEE et al. 2000) have been proposed to explain the EBPR mechanisms, and these models agree that VFA (especially acetate) play a key role as a substrate in EBPR mechanism.

It is generally accepted that poly-P organisms are unable directly to utilize glucose under anaerobic conditions in the EBPR system, and, moreover, glucose is even detrimental to EBPR unless it is first converted to VFA by non-poly-P microorganisms (acidogenic bacteria) (JEON and PARK 2000). Glucose cannot serve as a substrate for the growth and multiplication of *A. calcoaceticus* in aerobic conditions. This organism can only oxidize glucose to gluconic acid, which accumulates as a dead-end product in the culture media (HARTIG et al. 1999). In our experiment, with glucose addition in the synthetic wastewater (Tabs. 3, 4), the P-release and uptake ratios obtained were lower. Nevertheless, this does not have any significant influence on the multiplication of *A. calcoaceticus* and the final percentage of P-removal (no significant differences at the level of $p<0.05$, Student t-test).

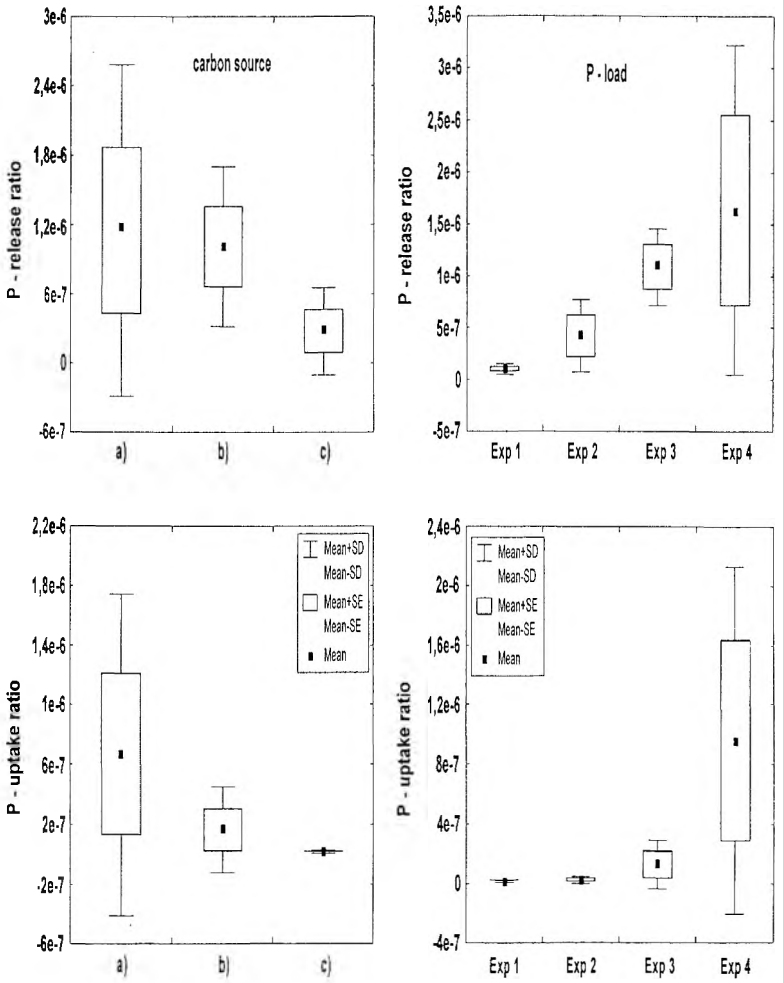

Fig. 1. Box-whisker plots for P-release and uptake ratios in the pure culture of *Acinetobacter calcoaceticus DSM1532* cultured in: a) sodium propionate, b) sodium acetate, c) sodium propionate, sodium acetate, glucose and different initial P-loads [$\gamma_0(P\text{-}PO_4^{3-})$ / mg L^{-1}]: Exp1 2.20 ± 0.44, Exp2 10.70 ± 0.26, Exp3 56.67 ± 13.05, Exp4 122.67 ± 12.70.

It has been found that the percentage of phosphate removal is much more dependent on the phosphorus load than on the type of feed. Cluster analysis groups exp1 and exp2 (low P-load) with a high percentage of P-removal and exp3 and exp4 (high P-load) with a lower percentage of P-removal. For this statement, surely the biomass also plays a role; more organisms can remove more P and if organisms remain constant at term out experimentation, it is obvious that more P (based on %) will be removed by low P loading.

A. calcoaceticus DSM1532 cells presented rapid adaptation ability to both anaerobic an aerobic conditions while shifting from one environment to the other. Bacterial numbers of *A. calcoaceticus* increased during experimentation, especially in the aerobic phase. Multiplication of cells and increase in numbers of *A. calcoaceticus* were not influenced by the

source of carbon in the synthetic wastewater (Student t-test, $p < 0.05$). Release of P increased during active growth, and uptake occurred when cells reached the stationary growth phase (microscopic examinations confirmed the spherical cells with volutin granules).

In each system, a decrease of the pH values during the anaerobic phase and an increase during aerobic P uptake can be seen.

The successful reduction of P levels in synthetic wastewater strongly contaminated with P, up to 100 mg L^{-1}, in the pure culture of *Ac. calcoaceticus*, prove the ability of this strain of poly-P accumulating bacteria to face P overloads.

Batch experiment (aerobic).

An aerobic batch experiment without a previous anaerobic stage was carried out to examine the ability of *A. calcoaceticus DSM1532* to remove phosphorus from wastewater without preliminary anaerobic stress.

The highest P-uptake was observed after 72 h of aeration, when the bacterial numbers of *A. calcoaceticus* reached the maximum and the pH values were also the highest (Fig. 2). At this time the calculated aerobic P-uptake ratios were low (between 1.23×10^{-10} and 9.43×10^{-11}) in comparison with P-removal in anaerobic/aerobic batch experiments fed with all the carbon sources tested (Tabs. 2, 3, 4). The percentages of aerobic P-removal were also much lower with each phosphorus load (except for exp1), although the numbers of *A. calcoaceticus* were much higher. The percentages of aerobic P-removal achieved were, in decreasing order: exp1 90.91%, exp2 33.33%, exp3 25.86%, exp4 9.09%. After 96 h of experiment, P-release and the decrease of pH-values were observed. This was probably connected with decay of bacteria in the flask.

The observed aerobic P-uptakes, without preliminary resort to anaerobic conditions indicate the probable aerobic mechanism of P-uptake by *A. calcoaceticus DSM1532*. GHIGLIAZZA et al. (1998) verified in aerobic batch tests the ability of *Acinetobacter lwoffi* to remove phosphorus by »luxury uptake« and »overplus accumulation« without anaerobic stress. CONVERTI et al. (1999) observed in experiments with aerobic sludge enriched with *A. lwoffi*, that *A. lwoffi* behaved as a poly-P bacterium without the necessity of resorting to intermediate anaerobiosis.

Semi-continuous system. The semi-continuous test was carried out to examine the possibility of *A. calcoaceticus DSM1532* adaptation to high phosphorus loads in the wastewater.

GERBER et al. (1987) reported that phosphorus-accumulating bacteria release phosphorus under anaerobic, anoxic and aerobic conditions when acetate or propionate is present. We observed P-uptake under aerobic conditions 2 h after the start of the experiment (data not shown). The maximal percentage of P-removal (28.07%) and maximal calculated P-uptake ratio (6.25×10^{-6}) were achieved after 48 h of experiment (Fig. 3). At this time a strong increase in the pH-value was observed. After 96 h of experiment, P-removal, the number of *A. calcoaceticus* and pH-value became almost stabile. The percentages of P-removal during that time varied between 15.79 and 21.05%, and P-uptake ratios between 1.25×10^{-10} and 8.28×10^{-11}.

Although the system proved capable of tolerating and reducing high levels of P (more than 100 mg L^{-1}) in the synthetic wastewater, the observed efficiency was poor in compari-

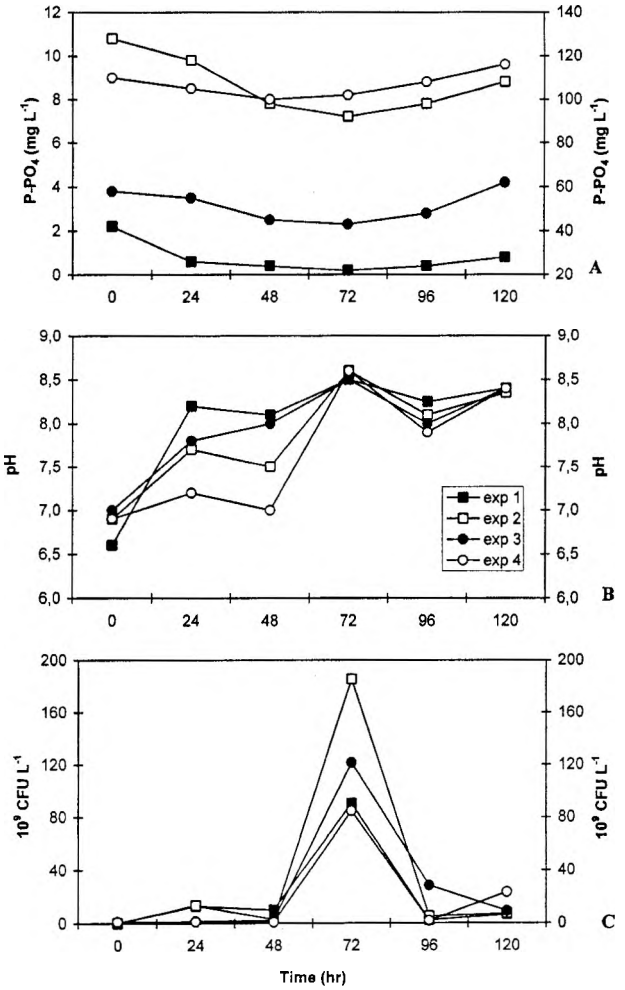

Fig. 2. Kinetics of aerobic phosphate uptake (A), variations of the pH-values (B) and viable cell count (C), for the pure culture of *Acinetobacter calcoaceticus DSM1532* cultured in sodium acetate, in batch experiment without preliminary resort to the anaerobic conditions. Exp1 $\gamma_0(P\text{-}PO_4^{3-}) = 2.20$ mg L^{-1}; exp2 $\gamma_0(P\text{-}PO_4^{3-}) = 10.80$ mg L^{-1}; exp3 $\gamma_0(P\text{-}PO_4^{3-}) = 58.00$ mg L^{-1}; exp4 $\gamma_0(P\text{-}PO_4^{3-}) = 110.00$ mg L^{-1}.

son with the anaerobic/aerobic system (Figs. 2, 3, 4). Nevertheless, P-removals observed were much higher than those observed in the aerobic batch experiment (Fig. 2), thus indicating the possibility of *A. calcoaceticus DSM1532* adaptation to high phosphorus loads in the wastewater.

The experimental results indicate the ability of phosphate-accumulating bacteria *A. calcoaceticus DSM1532* to consume orthophosphate and accumulate it as polyphosphate in alternating anaerobic/aerobic conditions as well as in aerobic conditions without the necessity of a preliminary resort to the anaerobic stage.

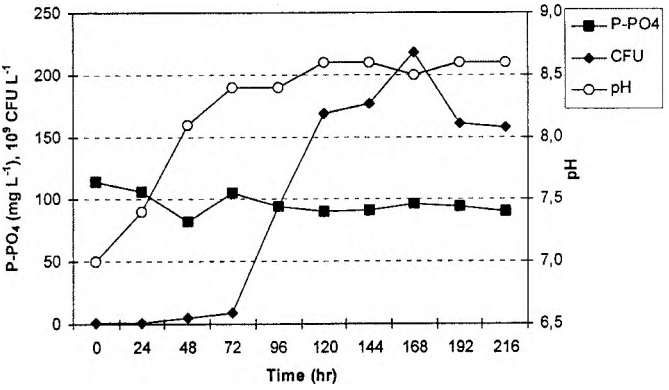

Fig. 3. Variations of phosphate, bacteria viable count and pH-values, in the semi-continuous system without preliminary resort to the anaerobic conditions, for the pure culture of *Acinetobacter calcoaceticus DSM1532* cultured in sodium acetate; $\gamma_0(P\text{-}PO_4^{3-}) = 114.00$ mg L^{-1}.

From the practical aspect, this is potentially interesting for the bioaugmentation of activated sludge in either anaerobic/aerobic or aerobic wastewater treatment systems to improve the efficiency of P-removal.

Conclusions

The highest P-release and P-uptake rates by a pure culture of *A. calcoaceticus DSM1532* were obtained when sodium propionate was the carbon source. Glucose addition in synthetic wastewater caused lower P-release and uptake rates, but it did not have any significant influence on the final percentage of P-removal. The highest P-release and P-uptake rates were obtained with the highest phosphorus loadings. The highest P-release resulted in the highest P-uptake rates.

The observed aerobic P-uptakes in a batch test, without a preliminary resort to anaerobic conditions indicate the probable aerobic mechanism of P-uptake by *A. calcoaceticus DSM1532*. The results of the semi-continous test indicate the possibility of *A. calcoaceticus DSM1532* adaptation to high phosphorus loads (more than 100 mg L^{-1}) in the wastewater.

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