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Influence of Support Materials on Phosphate Removal by the Pure Culture of *Acinetobacter calcoaceticus*

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Summary

The aim of this study was to investigate the influence and performance of the addition of support material on the phosphate uptake in a pure culture of phosphate-accumulating bacteria *Acinetobacter calcoaceticus* (DSM, 1532). Materials (natural zeolite and clay from Hrvatsko zagorje, Croatia) of different physical, chemical and mineral characteristics as well as different particle size were tested. In anaerobic/aerobic sequencing batch reactors with the pure culture of *A. calcoaceticus*, the addition of natural zeolite or clay in the aerobic phase resulted in a significantly higher final efficiency of phosphate removal. The amount of phosphate removed depended on particle size and type of material used. The number of *A. calcoaceticus* cells was significantly higher in reactors with support materials than in control reactors. After 24 h of incubation with support materials, the cells were present in colonies on the outer layer, strongly adsorbed and adhering to one another by extracellular substances. The main contribution of the support material to the phosphate removal was the increase of biomass, and in a lesser extent the adsorption of phosphate on the material particles.

Key words: *Acinetobacter calcoaceticus*, clay, natural zeolite, phosphate removal, wastewater

Introduction

Phosphate-accumulating bacteria play an important role in an enhanced biological phosphorus removal (EBPR) from wastewaters. Bacteria from the genus *Acinetobacter* have become the model organism for EBPR since it was isolated from a P-removing activated sludge plant (*1*). Although *Acinetobacter* spp. were present in extremely low number in the activated sludge plant, their capacity to remove orthophosphate (o-P) was the highest among all the P-accumulating isolates (*2*).

During the anaerobic stage of the wastewater treatment, these bacteria convert volatile fatty acids into intracellularly stored poly-hydroxy-alkanoates (PHA), using the energy liberated by the hydrolysis of polyphosphate (poly-P) to o-P, which is released from the cell to the liquid. During the aerobic stage, the stored PHA is used to generate cell growth, glycogen formation, maintenance, and o-P is removed from the wastewater in a quantity greater than the amount originally released (*3*,*4*).

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The addition of natural zeolite (NZ) in wastewater treatment evidently reduces the final concentration of P (*5–8*), but little is known about this mechanism. Among the support materials, NZ has been shown as a promising material for the immobilization of microorganisms (*9–11*).

The aim of this study was to investigate the influence and performance of the addition of support material on the o-P uptake in a pure culture of *A. calcoaceticus*. Materials of different physical, chemical and mineral characteristics as well as different particle size were tested.

Material and Methods

Microorganism

A P-accumulating bacterium *A. calcoaceticus* (DSM, 1532) was taken from the Deutsche Sammlung von Microorganismen und Zellkulturen GmbH (*12*).

Support materials

Natural zeolite

The zeolitized tuff from Hrvatsko zagorje, Croatia contained more than 50 % of zeolite of the heulandite group, some quartz and plagioclase, and accessory minerals from mica group (illite-celadonite and biotite), as estimated by X-ray powder diffraction method. The chemical composition (estimated by X-ray fluorescent spectroscopy) is shown in Table 1. Among the exchangeable cations, potassium was the dominant one in the sample. The NZ tuff was washed three times with demineralised water and then dried at 105 °C for 16 h before use in the experiments.

Table 1. Chemical composition (in mass fraction/%) of natural zeolite and clay from Hrvatsko zagorje, Croatia

w / %	Natural zeolite	Clay
SiO_2	65.91	59.12
TiO_2	0.27	0.61
Al_2O_3	14.78	12.21
Fe_2O_3	0.14	3.35
MnO	0.01	0.03
MgO	0.29	2.23
CaO	2.53	2.40
Na_2O	3.30	1.79
K_2O	3.41	0.63
P_2O_5	0.05	0.12
H_2O^-	2.76	10.75
H_2O^+	4.24	4.86
Sum	98.62	98.87

Clay

The clay sample from Hrvatsko zagorje, Croatia consisted mainly of montmorillonite, minor plagioclase, opal-CT and zeolite of the heulandite group, and accessory quartz. Dominant cations in the sample were calcium and sodium. The chemical composition of the sample is shown in Table 1. Clay was dried at 70 °C for 24 h before use in the experiments.

Synthetic wastewater

The composition of the synthetic medium used to simulate the sewage was (in mg/L of distilled water): Na-propionate 500; peptone 100; $MgSO_4$ 10; $CaCl_2$ 6; KCl 30; yeast extract 20. The concentration of KH_2PO_4 varied from 4 up to 440 mg/L to obtain a concentration of total P in the wastewater in the range of 1, 10, 50 and 100 mg/L. The pH of the synthetic wastewater was adjusted to 7±0.1 with 1 M NaOH or 1 M HCl before autoclaving (121 °C, 15 min).

Phosphorus adsorption capacity of support materials

The P-adsorption capacity of support materials was determined by equilibrating a quantity of substrate within a range of o-P solution made from KH_2PO_4 (*7*). Erlenmeyer flasks containing 4 g of dry material and a set of flasks with no material (blanks) were set up in triplicate, each with 100 mL of o-P solution (0, 5, 50, 500 and 5000 mg/L). Two drops of chloroform were added in each flask to inhibit microbial growth. Flasks were shaken on a mechanical shaker at 200 rpm for 72 h at 25 °C. The amount of 10 mL of the sample was taken from each flask at 24, 48 and 72 h. The samples were centrifuged (4000 rpm, 10 min), and the supernatant was analysed for o-P. The o-P that disappeared from the solution was considered to have been adsorbed by NZ or clay. The P retained by the support material was taken to calculate the P adsorption in mg/kg.

Phosphate release and uptake kinetics

In order to follow the growth kinetics and o-P release, the bacteria were pregrown in a nutrient broth for 24 h at (30±0.1) °C. The biomass was centrifuged (7000 rpm, 15 min), washed with sterile water, centrifuged, and resuspended in Erlenmeyer flask with 250 mL of synthetic wastewater. The flasks were sealed with sterile gum caps (*2*) and anaerobically incubated as triplicates in a water bath controlled with thermostat ((30±0.1) °C) and shaker (70 rpm). In order to follow the growth kinetics and o-P uptake, the cell biomass was resuspended in Erlenmeyer flask with 250 mL of synthetic wastewater without the carbon source. Cultures (set up as triplicates) were shaken at 70 rpm and aerated (around 4 L/min) with sterile air at (30±0.1) °C. Growth kinetics, o-P release and uptake were monitored until the constant o-P concentration in the supernatant was reached.

Experimental methods

The experiments were carried out as triplicate sequencing batch tests in alternating 24 h anaerobic/24 h aerobic stages. The bacteria were pregrown in a nutrient broth for 24 h at (30±0.1) °C. The biomass was centrifuged (7000 rpm, 15 min), washed with sterile distilled water, centrifuged, and resuspended in the synthetic wastewater, following the anaerobic incubation (70 rpm, (30±0.1) °C) (*12*). After the anaerobic stage, the volume of each reactor was aseptically divided into two reactors (*12*). In one reactor 15 g/L of NZ or clay was added, and the other reactor was left without NZ or clay addition, serving as a control reactor. In the aerobic phase that followed, reactors were shaken at 70 rpm, aerated

(around 4 L/min) with sterile air and incubated at (30±0.1) °C.

Analytical methods

All measurements were done according to the Standard Methods for the Examination of Water and Wastewater (*13*). pH value, temperature and dissolved oxygen in water were measured with WTW 330 SET supplied with pH electrode, temperature sensor and dissolved oxygen electrode. The samples were filtered before o-P measurements through Sartorius nitrocellulose filters, pore diameter 0.2 μm. The pH of samples having pH above 7.8 was adjusted between 6.8 and 7.5 before o-P measurement (*12*). The o-P ($P\text{-}PO_4^{3-}$) concentrations in water were measured colorimetrically in a DR/890 Hach colorimeter by the molybdovanadate method.

Bacterial numbers of *A. calcoaceticus* were determined as colony forming units (CFU) (*12*). At the end of the experiment, the particles of support material were washed three times with sterile distilled water, and viable cell counts were performed in order to determine the number of immobilised cells. Immobilisation of *A. calcoaceticus* cells was also determined microscopically (*12*). Cell shape and size were determined after a Gram stain. Neisser stain was performed to confirm poly-P granules in cells.

Data analysis

The data analyses were performed as described by Hrenović *et al.* (*12*).

Results

During the determination of the P-adsorption capacity of the materials the equilibrium in the flasks containing support materials was reached after 48 h. At a lower initial P concentration (up to 5 mg ($P\text{-}PO_4$)/L) more than 20 % for NZ and 50 % for clay of the applied P was adsorbed. At higher P concentrations, both materials adsorbed less than 25 % of the applied P. According to the P removal efficiency the equilibrium adsorption capacity of 25.0 mg/kg for the NZ and 50.0 mg/kg for the clay were estimated.

Investigation of growth kinetics and o-P release in the pure culture of *A. calcoaceticus* subjected to the anaerobic conditions (Fig. 1a) resulted in a short lag phase (approx. 1 h), after which cell multiplication occurred, which stopped after 5 h of incubation. o-P was released during the lag and stationary phases. During the aerobic cultivation (Fig. 1b), *A. calcoaceticus* displayed a short lag phase (approx. 1 h), after which cell multiplication began that turned to the logarithmic growth after 7 to 9 h of incubation. o-P was removed from the media during the lag and stationary phases. The concentrations of o-P, anaerobically released and aerobically removed during the 24 h of monitoring, stayed almost constant during the next 12 h. Therefore, in the next experiment a 24-hour anaerobic phase was followed by a 24-hour aerobic phase, where the primary goal was to ascertain whether the addition of support material (NZ or clay) to the culture medium increased the o-P removal using pure cultures of *A. calcoaceticus*.

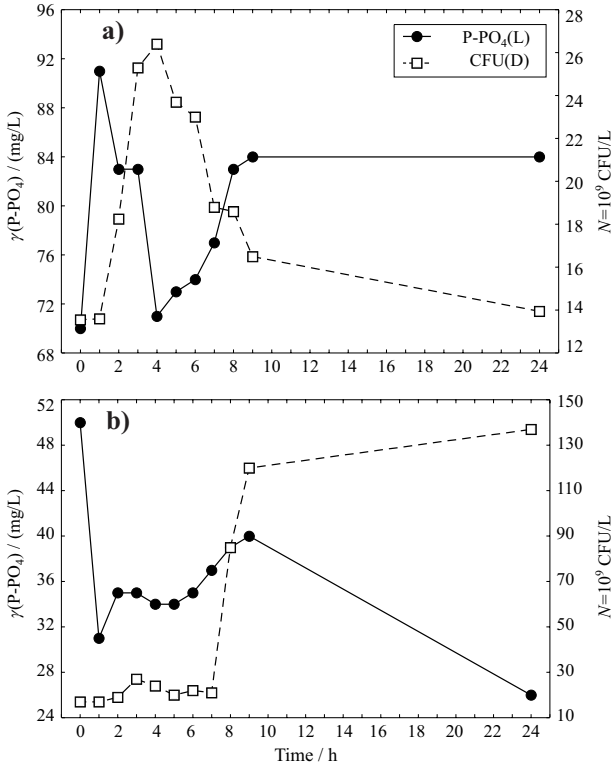
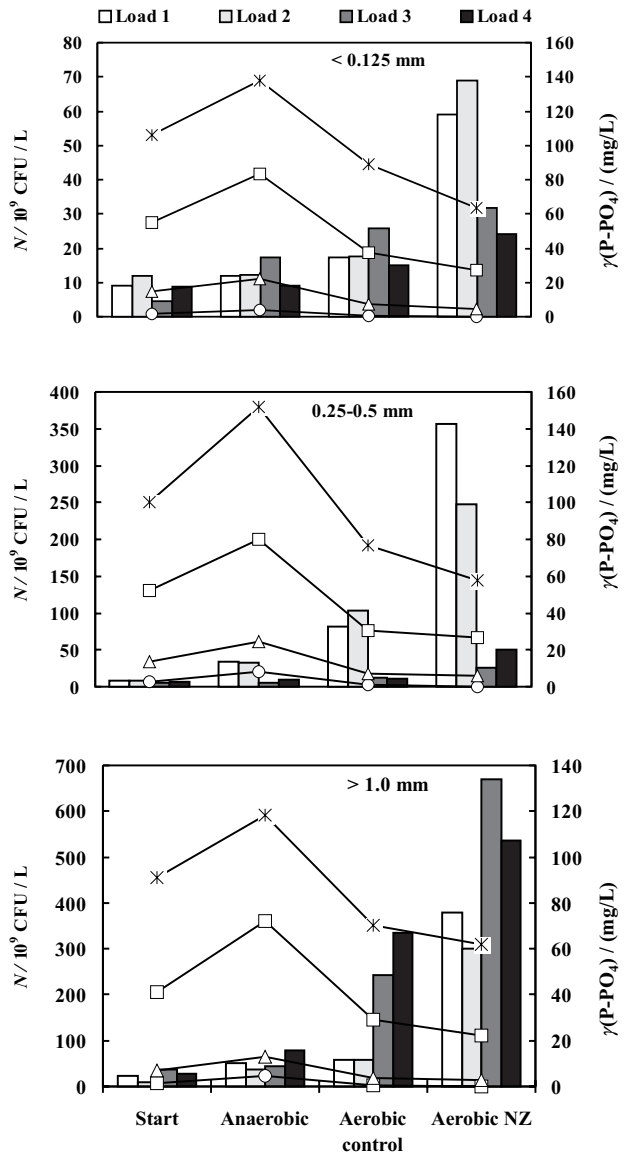


Fig. 1. Kinetics of anaerobic phosphate release (a) and aerobic phosphate uptake (b) depending on the growth phase of phosphate-accumulating bacteria *Acinetobacter calcoaceticus*

In anaerobic/aerobic sequencing batch reactors with the pure culture of *A. calcoaceticus* with the addition of support materials in the aerobic phase, anaerobic o-P release followed by the aerobic o-P uptake in control reactors as well in reactors with NZ or clay addition can be seen (Figs. 2 and 3). A significantly ($p<0.05$) higher o-P removal was achieved in reactors with NZ or clay addition, compared to the control reactors, regarding each starting o-P load and particle size of the materials used. The efficiency of o-P removal in reactors with the addition of support materials depended on the efficiency of biological component in the corresponding control reactors, which was indicated by a significant positive correlation ($r=0.99$, $p<0.05$).

The difference in o-P removed at the end of the aerobic phase between units with NZ or clay addition and control units appeared to depend on particle size and type of material used. With regard to the particle size (Table 2), the biggest difference in o-P removed between units was achieved with the smallest particle size (< 0.125 mm) of NZ, and 0.25–0.5 mm particle size of clay. With regard to the type of material (Table 2), bigger difference in o-P removed was achieved with the addition of NZ ((6.84±7.87) mg/L) than with the addition of clay ((6.03±6.46) mg/L).

Viable cell counts of *A. calcoaceticus* increased during the experiment, especially in the aerobic phase (Figs. 2 and 3). The final viable cell counts were higher ($p<0.05$) in reactors with the addition of support materials than in control reactors. P-uptake rates per cell of *A. calcoaceticus* were similar ($p>0.05$) in control reactors ($m(P\text{-}PO_4)=(2.42\pm4.34)\cdot10^{-10}$ mg/cell) and in reactors



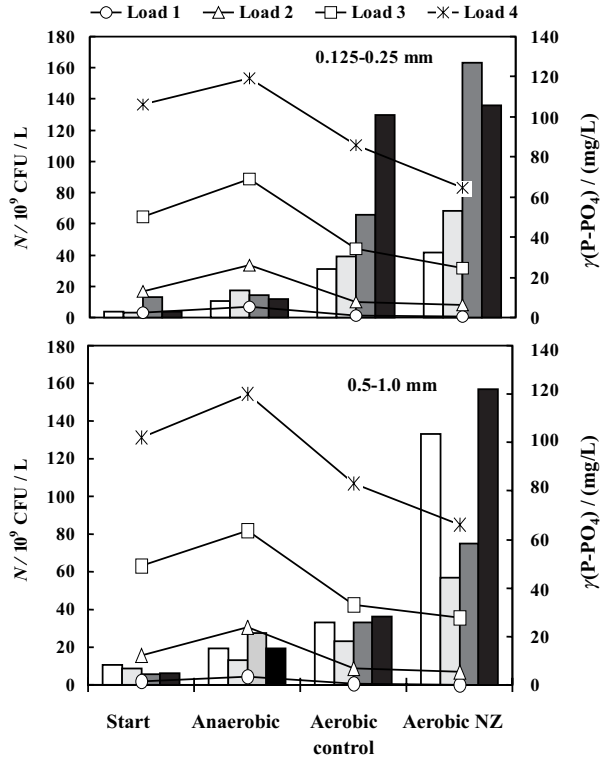


Fig. 2. Phosphate concentration and viable cell count (CFU) in the pure culture of *Acinetobacter calcoaceticus* with the addition of different particle size of natural zeolite (NZ) in the aerobic phase. Phosphate concentration by different starting phosphate loads is shown in lines and viable cell count in boxes. [t_0 ($P\text{-}PO_4^{3-}$)/(mg/L)] = Load 1 2.02±0.62, Load 2 12.08±2.97, Load 3 49.45±5.12, Load 4 101.05±6.06; [t_0 (CFU)/(10^9 CFU/L)] = Load 1 10.82±6.85, Load 2 8.36±3.32, Load 3 12.92±13.37, Load 4 10.56±9.47

with the addition of support materials (m(P-PO_4) = (2.49 ±5.68)·10^{-10} mg/cell).

After washing the support material particles with sterile distilled water, bacteriological examination showed that after 24 h of incubation the cells of *A. calcoaceticus* were present in colonies on the outer layer of NZ (Fig. 4) and clay particles. The viable cell counts from the supernatant samples were similar to the bacterial counts obtained in control units.

In order to establish whether the addition of support material influenced the cell shape and size of *A. calcoaceticus*, cells grown in the presence and absence of material were microscopically observed (Fig. 5). Spherical cells of *A. calcoaceticus* grown without NZ (Fig. 5a) were on average 0.75–1.05 μm, and rod cells were 0.65–0.80 μm wide and 1.44–1.65 μm long. When cultured in the media containing NZ (Fig. 5b), the spherical cells had an average size of 0.75–1.19 μm, and rod cells were 0.69–0.81 μm wide and 1.67–2.14 μm long. The difference in cell size was not detected with the addition of

Table 2. Difference in the amount of phosphate removed (in mg/L) between units with the addition of natural zeolite or clay and control units, by different initial phosphate loads. [t_0 ($P\text{-}PO_4^{3-}$)/(mg/L)] = Load 1 2.09±0.48, Load 2 12.69±2.39, Load 3 49.73±5.33, Load 4 96.05±9.59

Support material, fraction	Load 1	Load 2	Load 3	Load 4
Natural zeolite				
< 0.125 mm	0.16	2.61	10.20	25.30
0.125–0.25 mm	0.40	1.52	9.60	21.00
0.25–0.5 mm	0.86	1.17	3.77	19.35
0.5–1.0 mm	0.50	1.44	5.30	16.90
> 1.0 mm	0.21	1.18	7.00	8.40
Clay				
< 0.125 mm	0.49	2.11	11.00	16.20
0.125–0.25 mm	0.35	0.70	5.50	7.87
0.25–0.5 mm	0.60	2.16	12.00	23.70
0.5–1.0 mm	0.10	1.00	9.00	10.83
> 1.0 mm	0.20	0.70	7.50	8.50

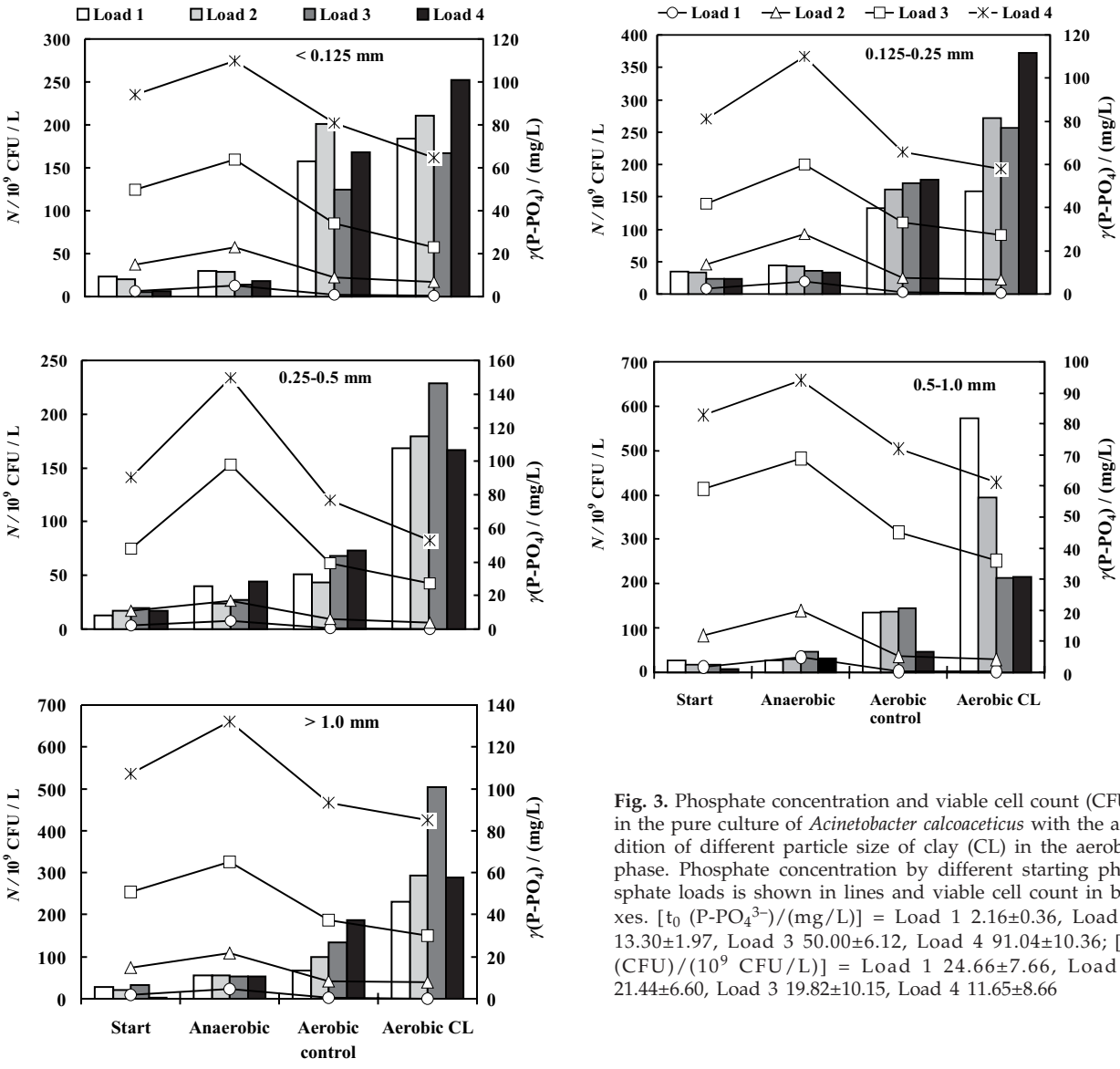


Fig. 3. Phosphate concentration and viable cell count (CFU) in the pure culture of *Acinetobacter calcoaceticus* with the addition of different particle size of clay (CL) in the aerobic phase. Phosphate concentration by different starting phosphate loads is shown in lines and viable cell count in boxes. [t_0 ($P\text{-}PO_4^{3-}$)/(mg/L)] = Load 1 2.16±0.36, Load 2 13.30±1.97, Load 3 50.00±6.12, Load 4 91.04±10.36; [t_0 (CFU)/(10^9 CFU/L)] = Load 1 24.66±7.66, Load 2 21.44±6.60, Load 3 19.82±10.15, Load 4 11.65±8.66

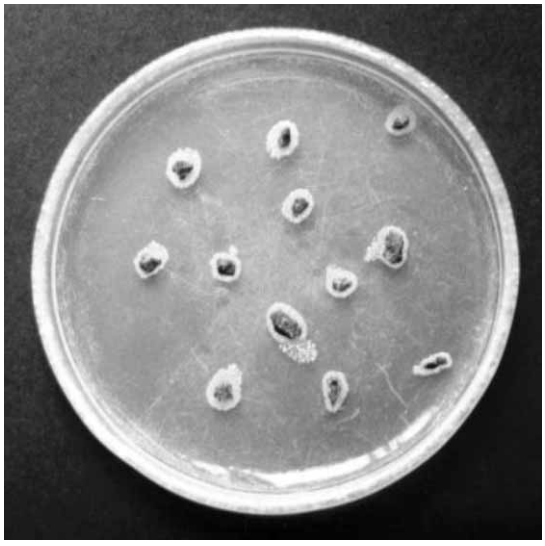


Fig. 4. Colonies of *Acinetobacter calcoaceticus* grown on the natural zeolite particles

clay. The cells of *A. calcoaceticus* were present in colonies on the outer layer of NZ and clay particles, strongly adsorbed and adhering to one another by extracellular substances. Neisser staining showed that the greater part (more than 80 %) of the spherical cells cultivated either with or without support materials contained different granules of intracellular poly-P at the end of aerobic phase.

In order to verify whether the addition of support material induced changes in the pH profiles, the pH of the control and support material-supplemented cultures was measured. The pH values were on average higher in the NZ-supplemented reactors (7.63±0.18) and in the clay-supplemented reactors (8.01±0.22) in comparison with corresponding control reactors (7.46±0.13 and 7.73±0.33). pH values in reactors with the addition of support materials showed a significant positive correlation ($r=0.83$, $p<0.05$) with those in control reactors.

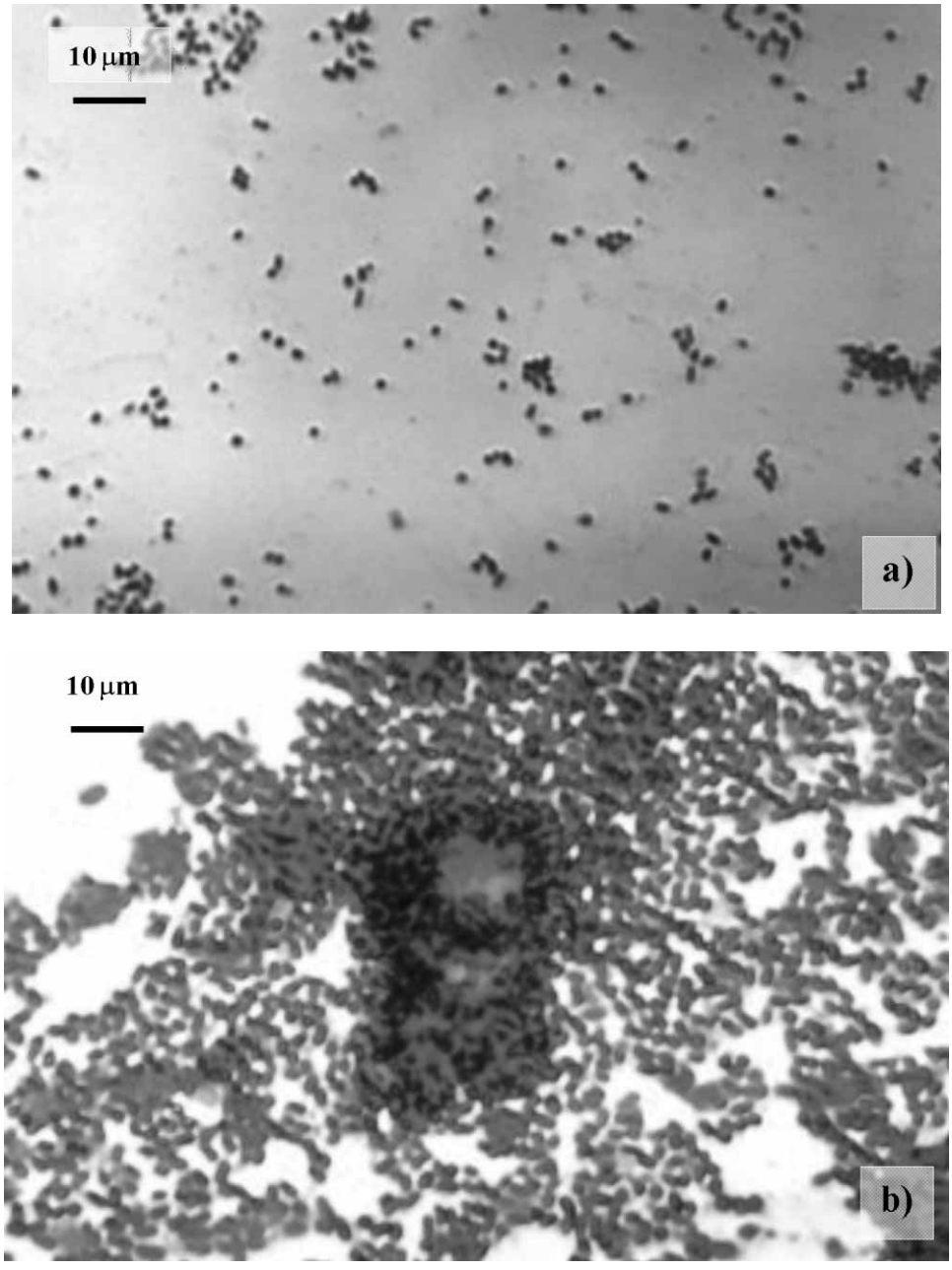


Fig. 5. Cells of *Acinetobacter calcoaceticus* at the end of the experiment cultivated without the addition of natural zeolite (a) and with the addition of natural zeolite (b). Gram stain, magnification 2000x

Discussion

Predominantly negative surface charge and the lack of sorption sites in zeolite suggest the absence of P sorption (*14*). P can be incorporated into zeolites as surface phosphite or phosphate groups (*15*). Literature data on the o-P ion adsorption of NZ reported little or no adsorption (especially in the pH range of 7–10) (*16*), or 2–15 μg/g (*6*) and even 48.5 mg/kg (*12*), to the high adsorption capacity of 2.15 g/kg (*7*). For the clay samples, higher o-P adsorption capacity (4.24–5.21 g/kg) was reported (*7*). Although the clay sample used in this work showed a higher o-P adsorption capacity than NZ, the clay material showed a tendency to swelling, particle coagulation, difficulties in sedimentation and filtration, and resulting system blockage.

The results of o-P release and uptake kinetics (Fig. 1) are in agreement with the findings of other authors (*17–19*) that the release of o-P and poly-P accumulation by *Acinetobacter* occurs when cells are not actively multiplying. The cells showed a limit of o-P, which could be accumulated per cell, in spite of o-P accessibility. It was concluded that the cell biomass and growth phase were the key factors affecting the amount of o-P removed by P-accumulating bacteria such as *Acinetobacter*. For the purpose of better o-P removal from wastewater, it is recommended to achieve the maximum cell concentration in the system and to avoid logarithmic growth in the aerobic stage.

The addition of support materials positively influenced the o-P removal in pure cultures of *A. calcoaceticus*

(Figs. 2 and 3, Table 2). The dimensions of bacteria are comparable to the sizes of zeolite crystallites and also to the corresponding intercrystalline pores. The adsorption of the microorganisms on the NZ depends on the texture and morphology of the particles, and adsorption selectivity of the particle surface for different groups of microorganisms (*9*). Therefore, the P-accumulating bacteria can be biosorbed, either by active or passive adhesion, on the outer accessible grooves and cavities on the rough surface of NZ particles. The NZ and clay particles were good carriers of the P-accumulating bacteria *A. calcoaceticus*, which adsorbed themselves on the particle surface, resulting in the increased biological activity of the system.

The best improvements of o-P removal in the pure culture experiments with *A. calcoaceticus* with NZ addition were achieved with the smallest particle size (< 0.125 mm) (Table 2). The smallest particle size of NZ provided a larger surface area available for colonisation with P-accumulating bacteria, resulting in the highest biological activity. When the NZ tuff was used for filtration (*5*), better efficiency of o-P removal was achieved using 0.5–1.0 mm than 0.25–0.5 mm particle size. The best improvements of o-P removal achieved with the 0.25–0.5 mm particle size of clay (Table 2) are explained by coagulation and adhesion of the very fine clay particles in water media, so that the smallest particles were not the particles of the largest surface area available for the bacterial colonisation. Better o-P removals in the pure culture experiments with *A. calcoaceticus* achieved with the addition of NZ than with clay (Table 2) were most probably the result of the chemical and morphological characteristics of the material, which resulted in adsorption selectivity for these P-accumulating bacteria.

The number of *A. calcoaceticus* cells was significantly higher in reactors with support materials than in control reactors, which suggest the positive influence of natural materials on the cell multiplication and biomass yield. Without support material addition, the cells of *A. calcoaceticus* in the media were distributed as single cells or in small aggregates (Fig. 5a). After 24 h of incubation with support material, the cells were present in colonies on the outer layer of NZ and clay particles (Fig. 4), strongly adsorbed and adhering to one another by extracellular substances (Fig. 5b). The presence of the extracellular substances may help the adsorption of *A. calcoaceticus* cells on the particle surface. Since no cell colony was observed before the addition of support materials, colonies were the result of growth on the material particles. Growth was referred to as the change in the total population, rather than an increase in size or mass of an individual cell. *A. calcoaceticus* showed the ability of o-P accumulation when cultivated with support materials, since the number of cells with poly-P granules and P-uptake rates per cell did not differ significantly, in comparison with the control reactors. Similarly, the immobilised cells of *A. johnsonii* within alginate beads (*19*) were present in the colonies, bound to one another by extracellular substances to form a biofilm and were metabolically active as they removed o-P from the medium.

Significantly longer cells of *A. calcoaceticus* were observed for cells adhered to the NZ particles as well for suspended cells in the media containing NZ, in relation to the cells cultivated without NZ addition. This was probably caused by the presence of oligoelements in the NZ, which were accessible for the cells. No difference in the cell size of *A. calcoaceticus* cultivated with clay could be caused by unsuitable physical and chemical characteristics of the mineral.

Synthetic zeolite could act as a pH regulator (*20*) due to its ion-exchange capacity, but this property was not observed when using the NZ (*10,12*). It is more likely that the observed low difference in pH between NZ-supplemented and control reactors in this study is the result of higher intensity of aerobic o-P uptake than the ion-exchange capacity of NZ.

It has been reported that zeolites improve the yeast metabolism by the catalytic activity on enzyme levels (*10,20*). However, the contribution of the NZ and clay to the o-P removal by the pure culture of *A. calcoaceticus* was mainly a function of the increased biomass, which therefore took up more o-P for the biomass and poly-P synthesis, and less o-P adsorption on the material particles. Finally, these results may provide a better basis for optimising the growth conditions of P-accumulating bacteria, such as *Acinetobacter*, in wastewater treatment plants and therefore help in improving EBPR process. In this study, NZ and clay were used as support materials instead of commercial plastic and other materials. The use of such naturally occurring materials gives a cheaper alternative, since they could be taken near many wastewater treatment plants.

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Učinkovitost nosača za uklanjanje fosfata u čistoj kulturi bakterije *Acinetobacter calcoaceticus*

Sažetak

Svrha je ovog istraživanja bila ispitati učinkovitost dodanog nosača za vezanje fosfata u čistoj kulturi fosfat-akumulirajuće bakterije *Acinetobacter calcoaceticus* (DSM, 1532). Ispitan je materijal (prirodni zeolit i glina iz Hrvatskoga zagorja) različitih fizikalnih, kemijskih i mineralnih svojstava, a i različite veličine čestica. U anaerobno/aerobnim šaržnim reaktorima sa čistom kulturom *A. calcoaceticus*, dodatkom prirodnog zeolita ili gline u aerobnoj fazi, na kraju je procesa postignuta puno veća učinkovitost uklanjanja fosfata. Količina uklonjenih fosfata ovisila je o veličini čestica i vrsti upotrijebljenog materijala. Broj stanica *A. calcoaceticus* bio je kudikamo veći u reaktorima s nosačima nego u kontrolnim reaktorima. Nakon 24 h inkubacije na nosačima, stanice *A. calcoaceticus* nalazile su se u kolonijama na vanjskom sloju nosača, jako adsorbirane i međusobno povezane ekstracelularnim supstancijama. Glavni doprinos nosača pri uklanjanju fosfata očitovao se u povećanju biomase, a manjim dijelom u adsorpciji fosfata na čestice materijala.