Technical paper

Aluminosilicates as Carriers of Phosphate-accumulating Bacteria

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Abstract

Five different aluminosilicate materials: natural altered tuff, synthetic mordenite, ammonium-exchanged mordenite, synthetic perlite and zeolite A were used for the immobilisation of metabolically active P-accumulating bacteria *A. junii*. After 24 h of bacterial cultivation in reactors containing different carriers, one part of the total cell population was immobilised onto the carriers by means of adsorptive growth while the other part remained as free cells in the supernatant. Taking in the consideration the number of immobilised viable cells and reactor performance, perlite of the particle size 0.1 to 1.0 mm was classified as the most suitable carrier of P-accumulating bacteria *A. junii* among the tested aluminosilicate materials.

Key words: Aluminosilicate, bacteria, immobilisation, phosphate, wastewater.

1. Introduction

The process of the enhanced biological phosphorus removal (EBPR) from wastewater is based on the activity of a physiological group of P-accumulating bacteria. Although the bacteria from the genus *Acinetobacter* does not have to be the predominant in the wastewater system, it have been reported to be the most efficient P-accumulating species and model organisms for EBPR process.[1–4]

The EBPR process is characterised by alternating anaerobic/aerobic conditions in the reactors where the wastewater treatment takes place. The P-accumulating bacteria in the absence of oxygen transport volatile fatty acids (*e.g.* propionate) into the cell, and subsequently convert and store these as poly-hydroxy-alkanoates (PHA). The energy for this transport and storage is supplied by hydrolysis of intracellular stored poly-P to o-P, which is released from the cell to the liquid. Under aerobic conditions, stored PHA would be catabolised, using oxygen as electron acceptor to generate energy for cell growth, maintenance, glycogen formation and poly-P synthesis, resulting in the o-P uptake in a quantity greater than the amount previously released.[1–4]

In order to achieve better efficiency of the wastewater treatment, currently attention is being drawn to the immobilisation of desired bacteria on different carrier materials. Immobilisation of microorganisms has been investigated using the synthetic carriers such as alginate[1] and ceramic[5] or natural zeolites and sand.[6–9] The material suitability is usually considered in terms of reactor performance or microbial biomass concentration on the carrier. Both factors should be taken in account when choosing the material as the carrier of microorganisms in order to improve the particular process.

The aim of this study was to determine the capacity of different aluminosilicate materials: natural clay, synthetic mordenite, ammonium-exchanged mordenite, synthetic perlite and zeolite A, for the immobilisation of metabolically active P-accumulating bacteria *A. junii*. The attempt was made to find the cheap commercially available material, which as a supplement can improve the EBPR process.

2. Materials and Methods

2. 1. Micro-organism

A culture of a P-accumulating bacterium *A. junii* 1532 was obtained from the Deutsche Sammlung von Microorganismen und Zellkulturen GmbH.[3]

2. 2. Carriers

Altered tuff (AT): The AT sample from Cerje Jesenjsko, Hrvatsko zagorje, Croatia consisted mainly of montmorillonite, mordenite and quartz, and accessory K-feldspar. Dominant cations in the sample were potassium and calcium. The chemical composition of AT (estimated by X-ray fluorescence, apart from FeO content that was determined by Wilson method) is given in Table 1. The particles of the sample were smaller than 0.122 mm.

Mordenite (M): The synthetic mordenite was a commercial product of JM Hubber Corp, USA. The M sample, with particles smaller than 15 μm was in sodium form.

Ammonium-exchanged mordenite (M–NH_4): The M–NH_4 was prepared by treating 10 g of hydratised original M (dried at 105 °C for 3 h) in the 150 ml of 0.5 M NH_4Cl solution by constant mixing on magnetic stirrer at 70 °C for 2 h. The supernatant was decanted and material was washed out with 100 ml of 0.5 M NH_4Cl solution. The washed material was treated again in the 150 ml of 0.5 M NH_4Cl solution and washed out with redistillate water until a negative chloride ion test with 1% $AgNO_3$ solution was obtained. After preparing, material was dried at 50 °C for 24 h.

Perlite 1 (P1): The synthetic perlite sample was an expanded volcanic rock, commercially available and provided by KGM-Ozalj, Croatia. The P1 was the fraction of the particle size 0.1 to 1.0 mm.

Perlite 2 (P2): This was the same commercial perlite sample (KGM-Ozalj) only with grain size within the 0.1 to 2.0 mm range.

Zeolite (Z): The commercial synthetic zeolite A, type ZP-4A of Silkem d.o.o., Kidricevo, Slovenia was used. The material was sodium aluminosilicate. The average particle size of sample was 3–5 μm. Since the main application of the material was the production of detergents, material was neutralised with H_2SO_4 at the end of the process of synthesis, which results in the presence of 1–2% of Na_2SO_4 in the final product.

Table 1. Chemical composition (mass %) of altered tuff (AT).

	Mass %
SiO_2	70.44
TiO_2	0.19
Al_2O_3	11.34
Fe_2O_3	1.04
FeO	0.13
MnO	0.02
MgO	0.81
CaO	1.75
Na_2O	0.94
K_2O	1.97
P_2O_5	0.03
H_2O^-	5.38
H_2O^+	5.72
Sum	99.76

All carriers were washed three times with 300 ml of demineralised water and then dried at 105 °C in oven for 16 h (only M–NH_4 was dried at 60 °C/24 h) before the experiments were to commence.

2.3. Synthetic Wastewater

The composition of medium for the anaerobic precultivation of *A. junii* (in mg per 1 l of distilled water) was: Na-propionate 1000; peptone 100; $MgSO_4$ 10; $CaCl_2$ 6; KCl 30; yeast extract 20; KH_2PO_4 44. The composition of the synthetic wastewater (in mg per 1 l of distilled water) was: Na-propionate 300; peptone 100; $MgSO_4$ 10; $CaCl_2$ 6; KCl 30; yeast extract 20; KH_2PO_4 88. The pH of the synthetic wastewater was adjusted to 7.00 ± 0.02 with 1 M NaOH or 1 M HCl before autoclaving (121 °C/15 min).

2. 4. Phosphorus Adsorption Capacity of Carriers

The P-adsorption capacity of each carrier was determined by equilibrating a quantity of material within a range of P solutions (0–5000 mg/l) made from KH_2PO_4.[10, 4]

2. 5. Experimental Methods

The bacteria were pre-grown in nutrient broth (Biolife, Italy) for 24 h at 30.0 ± 0.1 °C and then cultivated anaerobically for next 24 h at 30.0 ± 0.1 °C before experiments. The experiments were carried out as triplicate batch tests in 300-ml Erlenmeyer flasks. The biomass was centrifuged at 7000 r/min for 15 min, washed once with 10 ml of sterile distilled water, centrifuged (7000 r/min for 15 min), and resuspended in 200 ml of synthetic wastewater. In each flask 2 g of carrier was added. The control reactor was left without the addition of carrier. The flasks were sealed with a sterile gum cap and thereafter aerobically agitated (70 r/min) in a water bath controlled with a thermostat (30.0 ± 0.1 °C). The aeration rate of 1 l/min with sterile air was provided during the 24 h of experiment.

2.6. Analytical Methods

The pH-value in the synthetic wastewater was measured with a WTW 330 SET. The samples were filtered before P measurements using Sartorius nitrocellulose filters, with a pore diameter of 0.2 μm. The P (P–PO_4^{3-}) concentration in the synthetic wastewater was measured spectrophotometrically in a DR/2500 Hach spectrophotometer using the molybdovanadate method (Hach method 8114). After 24 h, the particles of each carrier were washed three times with 300 ml of sterile 0.3% NaCl, and viable cell counts were performed in order to determine the number of immobilised cells. Each carrier was aseptically placed

in a tube containing 9 ml of sterile 0.3% NaCl, crushed with a sterile glass rod and dispersed by mixing (2700 r/min for 10 min using the test tube shaker Kartell TK3S) prior to performing serial dilutions of each sample (10^{-1} to 10^{-9}). Volumes of 0.1 ml were plated (spread plate method) onto nutrient agar (Biolife, Italy) and plates were incubated at 30 ± 0.1 °C for 72 h. After incubation, the bacterial colonies were counted and reported as colony-forming units (CFUs) per one gram of dry carrier. Simultaneously, the viable cell counts were performed on the supernatant in order to determine the number of free cells. Neisser stain was performed to confirm poly-P granules in cells of *A. junii.*

2. 7. Data Analysis

Results were statistically analysed using the Statistica program.[11] The results obtained for the six carriers and control were compared. Since the data was independent ordinary Student's t-tests were performed. The null hypothesis tested by the analysis was that reactors with different types of material and control reactor showed no difference in performance. Results were considered significant at the 5% level ($p = 0.05$). The correlation between variables was estimated using the Pearson linear correlation.

3. Results and Discussion

According to the results the carriers obtained equilibrium for P-adsorption after 48 h. At the lowest initial P concentration (5 mg P–PO_4/l) 18% of applied P was adsorbed by AT, 4–7% P by M, M–NH_4, P1 and P2, while Z did not show any P adsorption. At higher P concentrations (50–5000 mg/l) the carriers adsorbed smaller percent of the applied P. It was estimated that the P removal efficiency of the average equilibrium adsorption capacity (Table 2) was the highest for AT, followed by M, P1, M–NH_4 and P2, and zero for Z.

The estimated P adsorption capacity of AT is something higher than reported 50 mg/kg for AT consisted mainly of montmorillonite.[4] The P adsorption capacity of M and M–NH_4 was notably lower than for its natural mixture AT (mordenite-rich tuff), which is explained by impurities (especially Fe- oxides/hydroxides) and more different exchangeable cations and sorption sites on AT. The particle size of P1 which range between 0.1 to 1.0 mm showed a higher P-adsorption capacity than the particle size of P2 (0.1–2.0 mm), which is in agreement with the reported negative correlation of the P-adsorption capacity and particle size of materials.[12, 9] The P adsorption capacity of P1 and P2 fits in the range between 2 to 15 mg/kg,[13] 25 mg/kg[4] and 48.5 mg/kg[3] reported for the natural zeolite samples. The absence of sorption sites on the synthetic Z, which has no natural analogue, for electrical negative species $H_2PO_4^-$ resulted in a zero P sorption capacity. Synthetic and natural hydrated crystalline aluminosilicates have little affinity for inorganic anions. Permanent negative surface charges result in repulsion and negative sorption of anionic species.[14]

After 24 h of bacterial cultivation in reactors containing different carriers, one part of the total cell population was immobilised onto the carriers by means of adsorptive growth while the other part remained as free cells in the supernatant. The number of immobilised viable cells (CFU) depended on the type of carrier (Fig. 1). Regarding the type of carrier; the highest number of immobilised cells was achieved with AT (42.30 ± 6.38 x 10^8 CFU/g), followed by P1 (31.64 ± 4.26 × 10^8 CFU/g), P2 (17.66 ± 3.07 × 10^8 CFU/g), M–NH_4 (13.51 ± 1.38 × 10^8 CFU/g), M (12.47 ± 1.48 × 10^8 CFU/g) and Z (0.07 ± 0.01 × 10^8 CFU/g). The number of immobilised cells onto P1 was higher than onto P2, which is in agreement with a previously observation that the loading rate of immobilized cells decrease with the increase of particle size of material.[9] The number of immobilised cells on the AT and P1 did not differ significantly ($p < 0.05$) but they were significantly ($p < 0.05$) higher than for other materials. The

Table 2. Phosphorus adsorption capacity for natural altered tuff (AT), synthetic mordenite (M), ammonium-exchanged mordenite (M–NH_4), synthetic perlites (P1 and P2) and zeolite A (Z).

Material	Capacity (mg P–PO_4/kg)
AT	60
M	40
M–NH_4	20
P1	30
P2	20
Z	0

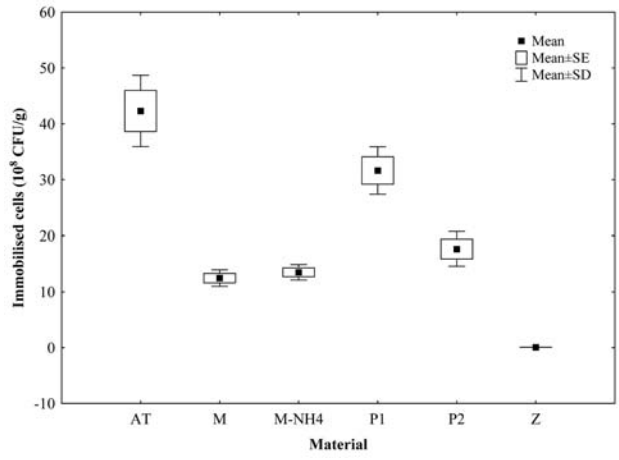

Figure 1: Number of immobilised viable cells (CFU) per dry mass of natural altered tuff (AT), synthetic mordenite (M), ammonium-exchanged mordenite (M–NH_4), synthetic perlites (P1 and P2) and zeolite A (Z). [t_0 CFU (10^9 CFU/l)] = 23.16 ± 3.85.

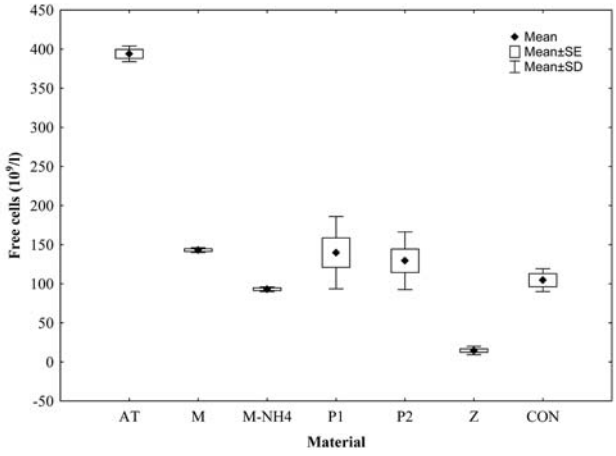

Figure 2: Number of free viable cells (CFU) in reactors with natural altered tuff (AT), synthetic mordenite (M), ammonium-exchanged mordenite (M–NH_4), synthetic perlites (P1 and P2), zeolite A (Z) and in control reactor (CON). [t_0 CFU (10^9 CFU/l)] = 23.14 ± 3.63.

numbers of immobilised cells grown per volume of AT, M–NH_4, M, P1, and P2 were higher than the numbers of free cells (Fig. 2), showing the average ratio of 13.73, 6.86, 4.81, 1.91 and 1.02, respectively. However, the number of immobilised cells grown per volume of Z was lower than the number of free cells (ratio 0.20).

It can be seen that in the presented well aerated and mixed system the cells of *A. junii* were immobilized by means of adsorptive growth within 24 h onto all examined carriers, but in different extent. The highest rates of immobilised cells achieved for AT, M, M–NH_4, P1 and P2 are comparable to the reported: 2.5×10^8 CFU/g of *A. johnsonii* cells entrapped inside alginate beads;[1] 9×10^8 CFU/g of *Pseudomonas aeruginosa* immobilised onto a type-Z carrier consisting of silica, alumina and zeolite molecular sieves;[6] and 2.9×10^9 CFU/g of *Acinetobacter* spp. immobilised onto ceramics by the vacuum method.[5] However, the best result obtained in this study is lower than the reported 6.86×10^9 CFU/g of *A. calcoaceticus* immobilised onto magnesium-exchanged natural zeolite.[9] The number of cells immobilised onto Z is very low and not comparable to the literature data.

The observed ratios of immobilised cells could be explained by the predominantly smooth surface of Z, and rough surface of other used materials, as well as great cavities of P1,2 particles, which are therefore better microenvironment for the adsorption of bacteria. The microscopic examination after Neisser stain confirmed the bacterial colonization of the surface of carriers, as well as cavities of P1,2. This is consistent with Chang et al.[15] and Hrenovic et al.[9] who reported that the bacteria grown on natural zeolite are numerous to the sand particles.

Beside the electrostatic interactions, the cell immobilization on inorganic material depends mainly on the ratio of external and internal surface area of porous carrier. As higher the external surface area, the higher is the immobilization capacity. The P-accumulating bacteria are preferentially fixed on the surface, cavities and macropores of the material. The materials with high porosity and adequate pore size distribution (larger than 2.5 μm) are suitable for the immobilization of bacterial cells. Considering this, the prelite is the most attractive carrier for the immobilization of *A. junni,* due to its amorphous macroporous system and huge surface area.

The increase of the total population of cells correlated positively with the number of immobilised cells ($r^2 = 0.89$, $p < 0.05$). The increase of the total number of cells was most intensive in reactors containing AT (16.90 times), which was significantly ($p < 0.05$) higher than in reactors containing M, M–NH_4, P1 and P2 or in control reactor (7.73–5.58 times). A significantly better multiplication of *A. junii* in reactors containing the AT can be explained by the positive influence of different exchangeable cations on the yield of biomass of P-accumulating bacteria.[9] In reactors containing Z decay of total population of cells was evident with the average ratio of final and starting cell numbers of 0.66. This indicated the antibacterial properties of examined Z and eliminates it as a possible carrier of the P-accumulating bacteria *A. junii* or material which stimulates its growth. The observed antibacterial property of Z is consistent with the material safety data sheet where aquatic toxicity against alga, *Daphnia* and fish is reported. The presence of Na_2SO_4 can also contribute to the material toxicity against investigated P-accumulating bacteria.

The P-uptake rates per total CFU of *A. junii* (Fig. 3) were on average the highest in reactors containing M–NH_4 ($1.41 \pm 0.03 \times 10^{-10}$ mg P–PO_4/CFU), which was insignificantly ($p > 0.05$) higher than control ($1.34 \pm 0.14 \times 10^{-10}$ mg P–PO_4/CFU) and significantly ($p < 0.05$) higher than in reactors containing other materials. Neisser stain at the end

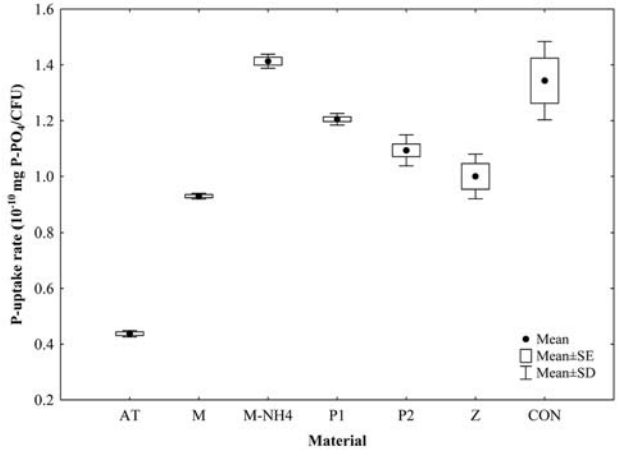

Figure 3: Phosphate uptake rate per colony forming units (CFU) of *Acinetobacter junii* in reactors with natural altered tuff (AT), synthetic mordenite (M), ammonium-exchanged mordenite (M–NH_4), synthetic perlites (P1 and P2), zeolite A (Z) and in control reactor (CON). [t_0 P–PO_4 (mg/l)] = 22.10 ± 1.94.

of experiments confirmed the presence of poly-P granules in cells of *A. junii.* The uptake of P and poly-P accumulation by *Acinetobacter* occurs when cells are not actively multiplying i.e. during the stationary phase of growth.[16, 17, 1] The highest P-uptake rates in reactors containing M–NH_4 can be explained by the positive influence of NH_4^+ on the rate of multiplication of *A. junii* and therefore quicker achievement of the stationary phase of growth. On the other way, the positive influence of AT on the yield of biomass of *A. junii* and the greater part of cells in the log phase of growth, can explain the lowest P-uptake rates.

However, more P-accumulating cells can remove more P from wastewater. The highest percent of the applied P was removed in reactors containing AT (88.89%), followed by P1 (82.46%), M–NH_4 (69.31%), M (68.53%), P2 (67.70%), control reactor (59.89%) and reactors containing Z (7.39%). In reactors containing all carriers except Z the significantly higher percent of P was removed than in control reactors.

In order to verify whether the addition of carriers induced changes in the pH profiles, the final pH values of the wastewater was measured. The final pH-values were on average the highest in the reactors containing Z (8.25 ± 0.11), followed by control (7.42 ± 0.06), reactors containing AT (7.26 ± 0.07), P1 (7.19 ± 0.20), P2 (7.15 ± 0.28), M (7.03 ± 0.01) and M–NH_4 (6.91 ± 0.04). The increase of pH in the reactors containing AT, P1 and P2 was not higher than the increase of pH due to the P uptake in control reactors containing the pure culture of *A. junii* without the addition of carrier. This suggests that the AT, as well as P1 and P2, did not act as a pH regulator, which is in agreement with the observed property of natural zeolites.[7, 8] The final pH values were significantly ($p < 0.05$) higher in reactors containing Z than in control reactors. As reported in material safety data sheet, 5% slurry of Z has a typical pH 11–12. Zeolites with high Al content like zeolite A, which have Si:Al ratio of 1, have a high density of extraframework cations and high density of lattice anions. The high number of Na^+ cations present in material can be exchanged with H_3O^+ cations present in the water solution via the hydrolysis of zeolite A, which results in the increase of the pH value of system.[18] The final pH values in reactors containing M and M–NH_4 were significantly ($p < 0.05$) lower than in control reactors; moreover the final pH in reactors containing M–NH_4 were lower than initial pH. The probable explanation is that the Na^+ contained in M was completely ionised, while NH_4^+ contained in M–NH_4 was partially ionised, because in the case of M–NH_4 low dissociated NH_4OH is generated and therefore the concentration of H^+ in solution increased. Therefore the M–NH_4 reacted more acidic than M.

When considering suitability of different carrier materials the procedure of their removal from EBPR reactors have to be considered as well. In this respect the synthetic perlite is the most suitable material due to the fact that it has low specific weight and remains at the surface of the wastewater system and therefore can easily be removed from the system. Based on the practical experiences, the low specific weight of the material is further decreasing in the process of the immobilization with the biomass. In the aerated wastewater system this material is often incorporated in the foam in the surface of the system and thus not functional for EBPR in the main stream off the reactor. Therefore, the further investigation will be focused on the impregnation of the surface and macroporous system of the perlite with the oxides of the nontoxic transition metals. We expect that this impregnation will increase the specific gravity of perlite close to the water specific gravity.

4. Conclusions

It is obvious from the results presented that P removal was significantly improved by using the various carriers, except Z, as opposed to the absence of a carrier. Taking in the consideration all mentioned influences of different aluminosilicate materials, P1 can be classified as the most suitable carrier of P-accumulating bacteria *A. junii.* The number of cells immobilised on the P1 ($31.64 \pm 4.26 \times 10^8$ CFU/g) was significantly higher than for other materials excepting the AT. At the same time, the P-uptake rate ($1.21 \pm 0.02 \times 10^{-10}$ mg P-PO_4/CFU) was slightly lower (14%) than the best obtained in reactors containing M–NH_4. In the reactors containing P1 the average percent of P removal was very high (82.46%) and only 6% lower than the best obtained in reactors with AT. The P1 did not influence the final pH of the medium. Moreover, P1 material due to its low specific weight remains at the surface of the wastewater system and can easily be removed from the system. The P-accumulating bacteria immobilised onto P1 could be easily implemented in the wastewater treatment, giving a cheap alternative for the improving EBPR process.

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Povzetek

Za imobilizacijo aktivnih fosfat-akumulirajočih bakterij *A.junii* je bilo uporabljenih pet različnih alumosilikatnih nosilcev: naravni lehnjak, sintetični mordenit, amonij-mordenit, sintetični perlit in zeolit A. Po 24-urnem gojenju omenjene bakterije v reaktorjih z naštetimi nosilci so se poskusi nadaljevali z adsorbiranimi in prostimi celicami v supernatantu. Na osnovi analize živih imobiliziranih in adsorbiranih celic ter obratovalnih karakteristik reaktorja je bilo ugotovljeno, da je med testiranimi materiali najprimernejši nosilec perlit z velikostjo delcev 0.1 do 1.0 mm.