Immobilisation of *Acinetobacter calcoaceticus* using natural carriers

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Abstract

There is a growing interest to immobilize desired bacteria using inexpensive materials in order to improve the wastewater treatment process. Three different types of carriers namely natural zeolite, magnesium-exchanged natural zeolite and quartz sand of different particle size were used to immobilize the phosphate-accumulating bacteria *Acinetobacter calcoaceticus* and to determine which one was the most effective. Bacteria were cultured for 24 h in various reactors containing different particle sizes of each of the carriers. The majority of the cultured bacterial population was immobilised onto the different carriers by means of adsorptive growth while a minority of free cells was observed in the supernatant. The number of immobilised viable cells (CFU) depended on the type of carrier and the particle size. The highest loading rate of immobilised cells (68.61±1.11 x 10^8 CFU/g) was observed with the smallest particle size (<0.125 mm) of magnesium-exchanged natural zeolite.

Keywords: *Acinetobacter calcoaceticus*, immobilisation, phosphate, quartz sand, wastewater, zeolite

Introduction

Enhanced biological phosphorus removal (EBPR) from wastewater, a biological alternative to chemical phosphate (P) precipitation, is based on the activity of P-accumulating bacteria. Bacteria from the genus *Acinetobacter* have been reported to be the most efficient P-accumulating species (Muyima and Cloete, 1995; Sidat et al., 1999; Hrenović et al., 2003a; Hrenović et al., 2003b).

Currently attention is being drawn to the immobilisation of bacteria in order to achieve a higher cell density in bioreactors; based on this, smaller reactors, shorter residence/retention time or higher flow rates can be employed. Immobilisation of *Acinetobacter* spp. has been investigated using alginate (Muyima and Cloete, 1995) or ceramic (Kariminiaae-Hamedaani et al., 2003) carriers. Besides the synthetic carriers, natural zeolite (NZ) has been shown as a promising material for the immobilisation of micro-organisms (Shindo et al., 2001) due to its high porosity and large surface area. The extent of bacterial colonization depends on the chemical properties and particle size of NZ.

It has been suggested that available magnesium ions (Mg^{2+}) are important for the stable EBPR and efficient P-removal (Seviour et al., 2003). The negative charge of the NZ, which is attributed to the tetrahedrally co-ordinated aluminium, is balanced by exchangeable cations. Mg^{2+} is one of the most common cations in some NZ. Based on the presence of Mg^{2+}, the use of NZ as carrier of immobilised P-accumulating bacteria may improve the P-uptake capacity of the system.

The aim of this study was to determine the capacity of NZ and quartz sand for the immobilisation of P-accumulating bacteria *A. calcoaceticus*. In addition, the influence of Mg^{2+} present in the NZ on the P-uptake ability of immobilised cells was also determined.

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Material and methods

Micro-organism

A culture of a P-accumulating bacterium *A. calcoaceticus* (DSM, 1532) was obtained from the *Deutsche Sammlung von Microorganismen und Zellkulturen GmbH* (Hrenović et al., 2003a).

Carriers

Natural zeolite (NZ): The zeolitised tuff from Donje Jesenje, Croatia contained more than 50% of zeolite of the heulandite group (clinoptilolite), some quartz and plagioclase and accessory minerals from the mica group (illite-celadonite and biotite). Among the exchangeable cations, potassium was the dominant one in the sample. The NZ (10 g of each particle size) was washed three times with demineralised water (300 mℓ) and then dried at 105°C in oven for 16 h before the experiments were to commence.

Magnesium-exchanged natural zeolite (NZMg): The NZMg was prepared by treating 10 g of the particular fraction of original NZ with 250 mℓ of neutral 1 M $MgCl_2$ (Kemika, Croatia) solution. Erlenmeyer flasks were agitated on a mechanical shaker (Inko SP17) at 200 r/min for 48 h at room temperature (24±2°C). The supernatant was decanted and particles were washed with demineralised water (1 ℓ) until a negative chloride ion test with 1% silver nitrate solution was obtained. The NZMg was air-dried for 7 d, followed by drying at 105°C in an oven for 16 h before the experiments were to commence.

Quartz sand (QS): The QS from Vrtlinska, Croatia contained dominantly quartz as well as minor quantities of feldspars and micas. The QS (10 g of each particle size) was washed three

TABLE 1
Chemical composition (mass %) of the natural zeolite (NZ), magnesium-exchanged natural zeolite (NZMg) and quartz sand (QS) determined by XRF (*) or ICP-ES (**) method

Mass %	NZ*	NZMg**	QS**
SiO_2	65.91	66.45	77.70
TiO_2	0.27	0.25	0.75
Al_2O_3	14.78	15.42	11.29
Fe_2O_3	0.14	1.16	1.02
MnO	0.01	0.01	0.03
MgO	0.29	0.46	0.12
CaO	2.53	2.99	0.64
Na_2O	3.30	2.81	1.54
K_2O	3.41	4.00	3.91
P_2O_5	0.05	0.02	0.10
H_2O^-	2.76		
H_2O^+	4.24	6.3	2.7
Sum	98.62	99.87	99.80

times with demineralised water (300 mℓ) and then dried at 105°C in the oven for 16 h before the experiments were to commence.

Different size fractions of NZ, NZMg and QS were used for the experiments. The chemical composition of each carrier (estimated by X-ray fluorescence or ICP- emission spectroscopy) is given in Table 1.

Synthetic wastewater

The composition of the synthetic wastewater (prepared in 1 ℓ of distilled water) was as follows: Na-propionate 300 mg/ℓ; peptone 100 mg/ℓ; $MgSO_4$ 10 mg/ℓ; $CaCl_2$ 6 mg/ℓ; KCl 30 mg/ℓ; yeast extract 20 mg/ℓ; KH_2PO_4 88 mg/ℓ. The pH of the synthetic wastewater was adjusted to 7.03±0.04 with 1 M NaOH (Kemika, Croatia) or 1 M HCl (Kemika, Croatia) before autoclaving (121°C/15 min).

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 to 5 000 mg/ℓ) made from KH_2PO_4 (Kemika, Croatia) (Sakadevan and Bavor, 1998; Hrenović et al., 2003b).

Experimental methods

The experiments were carried out as triplicate batch tests in 300 mℓ Erlenmeyer flasks. The bacteria were pre-grown in nutrient broth (Biolife, Italy) for 24 h at 30.0±0.1°C. The biomass was centrifuged (Rotanta 46R) at 7 000 r/min for 15 min, washed once with 10 mℓ of sterile distilled water, centrifuged (7 000 r/min for 15 min), and resuspended in 200 mℓ of synthetic wastewater. In each flask 2 g of carrier was added. The flasks were sealed with a sterile gum cap and thereafter aerobically agitated (70 r/min) in a water bath (Julabo SW23) controlled with a thermostat (30.0±0.1°C). An aeration rate of 1 ℓ/min with sterile air was provided. According to the previously estimated growth and P uptake kinetics of *A. calcoaceticus* (Hrenović et al., 2003b), experiments were carried out over 24 h.

Analytical methods

The pH-value, temperature and dissolved oxygen in the synthetic wastewater were measured with a WTW 330 SET supplied with a pH-electrode, temperature sensor and dissolved oxygen electrode. The samples were filtered before P measurements using Sartorius nitrocellulose filters, with a pore diameter of 0.2 µm. The P ($P\text{-}PO_4^{3-}$) concentration in the synthetic wastewater was measured colorimetrically in a DR/890 Hach colorimeter using the molybdovanadate method (Hach method 8114).

After 24 h, the particles of each carrier were washed three times with sterile distilled water (300 mℓ), 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 distilled water, crushed with a sterile glass rod and dispersed by mixing (2 700 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 1g of carrier. Simultaneously, the viable cell counts were performed on the supernatant in order to determine the number of free cells. Immobilisation of the bacterial cells was also confirmed by direct microscopy. Unstained samples before washing the carriers were examined using an inverted microscope (Axiovert 200 MAT; Carl Zeiss MicroImaging, Inc.) under the phase contrast at magnification of 1 000x. Microphotographs were taken using a digital camera (AxioCam MRc; Carl Zeiss MicroImaging, Inc.) connected to a PC equipped with Axiovision (Carl Zeiss MicroImaging, Inc.).

Data analysis

Results were statistically analysed using the Statistica (StatSoft, 2004) program. The results obtained for the three carriers were compared. Since the data were independent, ordinary Student's *t*-tests were performed. The null hypothesis tested by the analysis was that reactors with different types of material 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.

Results and discussion

According to the results, the carriers obtained equilibrium for P-adsorption after 48 h. At a lower initial P concentration (up to 5 mg $P\text{-}PO_4$/ℓ) more than 30% P was adsorbed by NZMg and NZ while only 9% P was absorbed by QS. At higher P concentrations (50 to 5 000 mg/ℓ), all the carriers adsorbed smaller amounts of the applied P. It was estimated that the P removal efficiency of the average equilibrium adsorption capacity of each carrier was as follows: 30.0±6.5 mg/kg for NZMg, 25.0±4.6 mg/kg for NZ and 6.7±5.2 mg/kg for QS. The P adsorption capacity (Table 2) depended on the particle size and type of material used. The highest P adsorption capacity was observed for all three carriers when the smallest particle size was tested, and decreased with increasing particle sizes ($r=-0.53$, $p>0.05$). The average P adsorption capacity of NZ was not significantly ($p>0.05$) improved by the presence of Mg^{2+}. The average P adsorption capacity for NZMg and NZ was significantly ($p<0.05$) higher than for QS.

The estimated P adsorption capacity of NZ fits in the range between 2 to 15 mg/kg reported by Lopez-Ruiz et al. (1997) and

TABLE 2
Phosphorus adsorption capacity for different fractions of natural zeolite (NZ), magnesium-exchanged natural zeolite (NZMg) and quartz sand (QS)

Material, fraction	Capacity (mg/kg)
NZ	
1) < 0.125 mm	30.0
2) 0.125-0.25 mm	27.5
3) 0.25-0.5 mm	22.5
4) 0.5-1.0 mm	20.0
NZMg	
1) < 0.125 mm	37.5
2) 0.125-0.25 mm	32.5
3) 0.25-0.5 mm	27.5
4) 0.5-1.0 mm	22.5
QS	
2) 0.125-0.25 mm	12.5
3) 0.25-0.5 mm	5.0
4) 0.5-1.0 mm	2.5

48.5 mg/kg according to Hrenović et al. (2003a). The notably higher potential of P sorption for NZMg is consistent with results reported by Lopez-Ruiz and colleagues (1997). The absence of exchangeable cations and sorption sites on QS resulted in a poor P sorption capacity, which was lower than the estimated 20 mg/kg for Danish QS (Arias et al., 2001). The particle size that ranged between 0 to 1.0 mm of NZMg, NZ and QS was negatively correlated with the P-adsorption capacity, which is in agreement with the reported relation for the different sands (Arias et al., 2001).

After 24 h of bacterial cultivation in reactors containing different particle sizes of carriers, most of the total cell population was immobilised onto carriers by adsorptive growth while the rest of the bacteria remained as free cells. The number of immobilised viable cells (CFU) depended on the type of carrier as well as on its particle size (Fig. 1). The highest loading rate of immobilised cells (68.61±1.11 x 10^8 CFU/g) was achieved with the smallest particle size (<0.125 mm) of NZMg. Regarding the type of carrier; the highest number of immobilised cells was obtained with NZMg (35.13±26.21 x 10^8 CFU/g), followed by NZ (23.27±19.74 x 10^8 CFU/g) and QS (14.17±13.49 x 10^8 CFU/g). The number of immobilised cells for all carriers was the highest with the smallest particle size, and decreased with the increase of particle size. However, the number of immobilised cells grown on NZMg, NZ and QS was higher than the number of free cells. The number of free cells (Fig. 2) correlated significantly negatively (r=-0.94 to -0.98, $p<0.05$) with the number of immobilised cells on each type of carrier. The ratio between the immobilised and free cells was the highest for the NZMg (309), followed by NZ (90) and QS (54).

Direct microscopy (Figs. 3 a, b, c) before washing the carriers revealed the extensive colonization of the three carriers with *A. calcoaceticus*. The cells were present in colonies, mainly on the outer layer of carriers, strongly adsorbed and adhering to one another by extracellular substances, such as polysaccharides (Abd El-Haleem, 2003). Immobilised cells were numerous in comparison with free cells.

Muyima and Cloete (1995) reported that 2.5 x 10^8 CFU/g of *A. johnsonii* cells were entrapped inside alginate beads. Viable *Pseudomonas aeruginosa* population of 9 x 10^8 CFU/g was attainable by the immobilisation onto a type-Z carrier consisting of silica, alumina and zeolite molecular sieves (Durham et al., 1994). A high *Acinetobacter* spp. loading rate (2.9 x 10^9 CFU/g) was immobi-

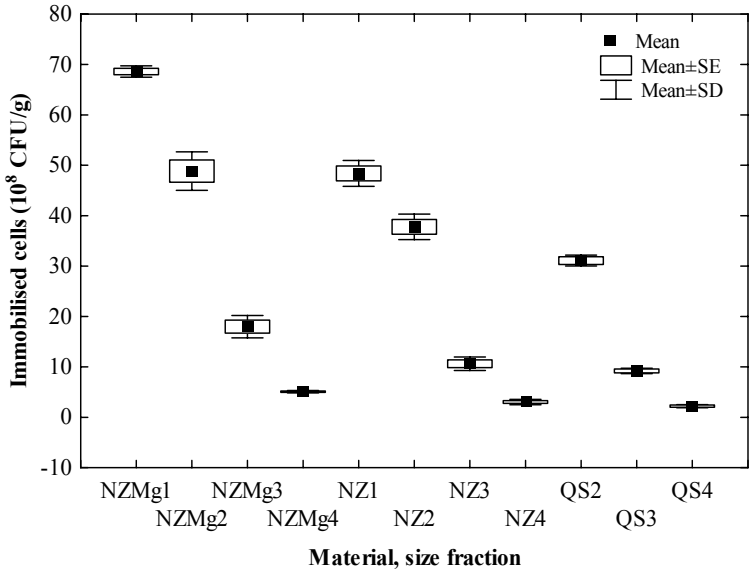


Figure 1
Number of immobilised viable cells (CFU) per dry mass of magnesium-exchanged natural zeolite (NZMg), natural zeolite (NZ) and quartz sand (QS). [t_0 CFU (10^6 CFU/mℓ)] = 18.94±4.10. Numbers 1 to 4 designate the size fraction (see Table 2).

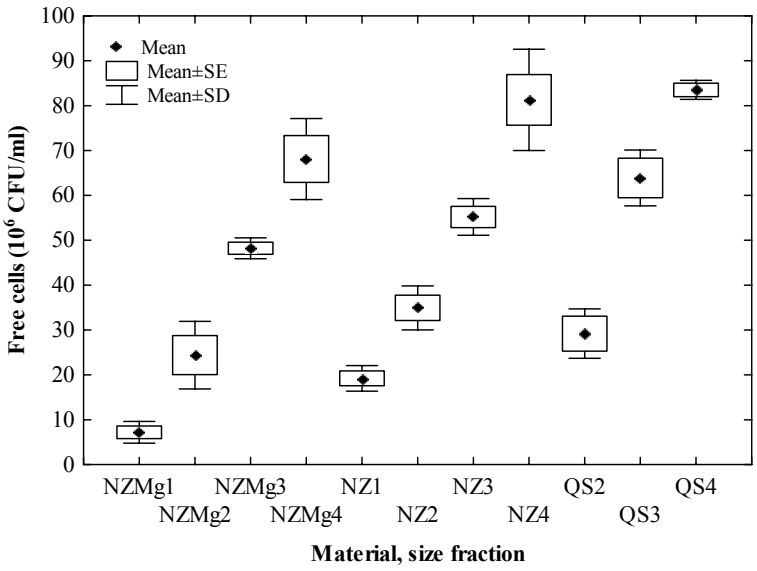
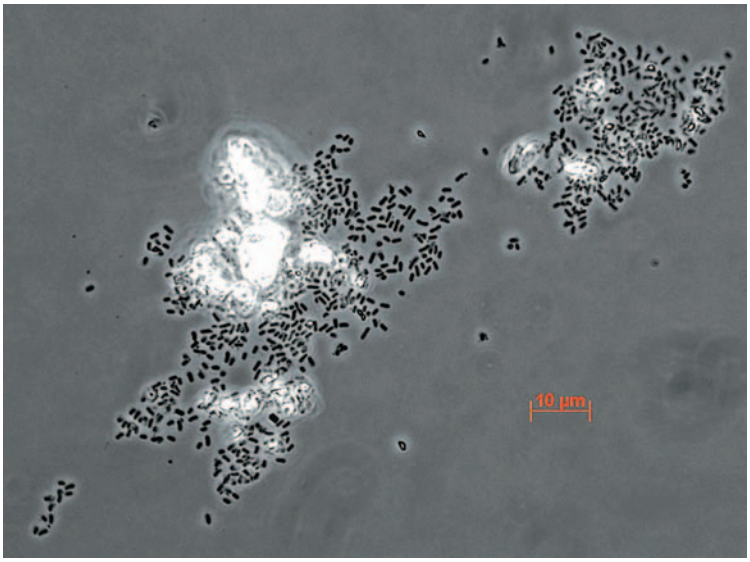
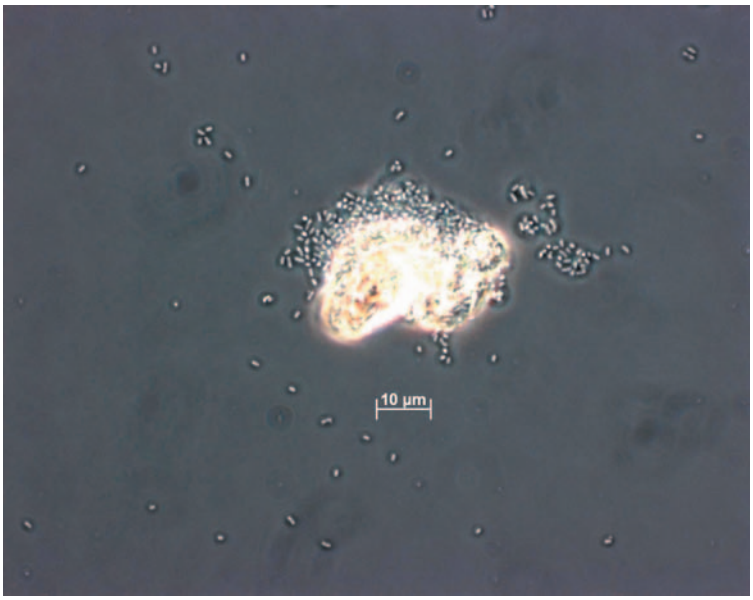
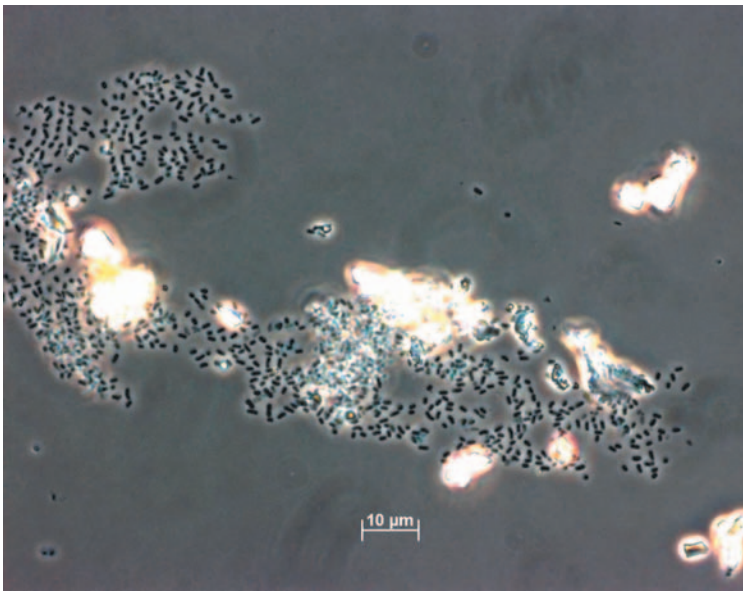


Figure 2
Number of free viable cells (CFU) in reactors with magnesium-exchanged natural zeolite (NZMg), natural zeolite (NZ) and quartz sand (QS). [t_0 CFU (10^6 CFU/mℓ)] = 18.94±4.10. Numbers 1 to 4 designate the size fraction (see Table 2).







lised onto ceramics by the vacuum method (Kariminiaae-Hamedaani et al., 2003). In our well-aerated and mixed system, the cells of *A. calcoacetcus* adsorbed within 24 h onto all examined carriers. The higher loading capacity (58.73±11.11, 43.09±6.24 and 31.10±1.08 x 10^8 CFU/g) of these immobilised cells was achieved with NZMg, NZ and QS with particle sizes smaller than 0.25 mm (Fig. 1). However, the number of immobilised cells was lower than 17.99±2.22 x 10^8 CFU/g when cultivated using particle sizes ranging between 0.25-1.0 mm of NZMg, NZ and QS (Fig. 1) which have a smaller surface area. The number of cells that were immobilised onto NZMg and NZ were higher than those immobilised onto QS, regardless of which particle size was used. This is consistent with Chang et al. (2002) who reported that the numbers of heterotrophic bacteria and *Nitrobacter* spp. were greater in biofilm grown on NZ than sand. This could be explained by the predominantly smooth surface of QS and rough surface of NZ particles, which is therefore a better microenvironment for the adsorption of bacteria.

The increase of the total number of cells was most intensive in reactors containing NZMg (4.58±0.95 times), which was significantly ($p<0.05$) higher than in reactors containing NZ (3.62±0.55 times) and QS (3.39±0.33 times). The increase of the total population/numbers of cells correlated positively with the number of immobilised cells onto NZMg ($r=0.71$, $p<0.05$), NZ ($r=0.70$, $p<0.05$) and QS ($r=0.64$, $p>0.05$). The P-uptake rates per total CFU of *A. calcoaceticus* (Fig. 4) were on average the highest in reactors containing NZMg (1.45±0.25 x 10^{-10} mg $P\text{-}PO_4$/CFU), which was insignificantly ($p>0.05$) higher than NZ (1.28±0.25 x 10^{-10} mg $P\text{-}PO_4$/CFU) and significantly ($p<0.05$) higher than QS (1.11±0.21 x 10^{-10} mg $P\text{-}PO_4$/CFU). P-uptake rates in the reactors with NZ and QS did not differ significantly ($p>0.05$).

Several studies (Imai et al., 1988; Boting et al., 1993; Schonborn et al., 2001; Seviour et al., 2003) pointed out that the availability of Mg^{2+} is particularly important for successful EBPR. This effect is ascribed to the fact that Mg^{2+} besides K^+ are bound as counter ions of P anions in the poly-P chains and are released and taken up simultaneously with P (Boting et al., 1993; Schonborn et al., 2001). In this study, a significantly better multiplication of *A. calcoaceticus* was observed in reactors containing the NZMg than in reactors containing NZ and QS, which suggests the positive influence of Mg^{2+} on the yield of biomass of P-accumulating bacteria. With the addition of NZMg the mean P-uptake rates per CFU of *A. calcoaceticus* were 23% higher than with the addition of QS and 12% higher than with the addition of NZ. In addition, the P-uptake rates were 13% higher with the addition of NZ than with QS.

Figure 3 (left)
Cells of Acinetobacter calcoaceticus immobilised on (a) magnesium-exchanged natural zeolite (NZMg), (b) natural zeolite (NZ) and (c) quartz sand (QS). Direct microscopy, magnification 1 000x

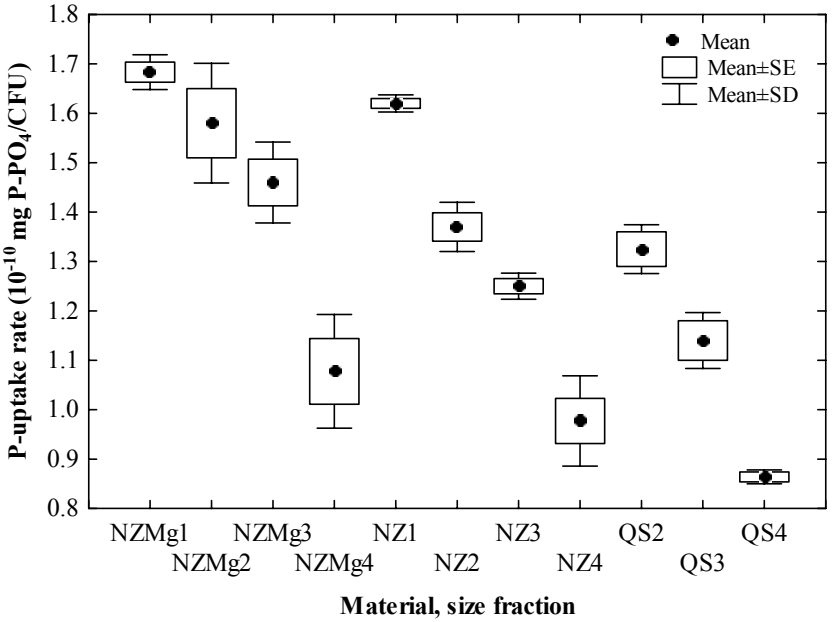


Figure 4
Phosphate uptake rate per colony-forming units (CFU) of Acinetobacter calcoaceticus in reactors with magnesium-exchanged natural zeolite (NZMg), natural zeolite (NZ) and quartz sand (QS). [t_0 P-PO_4 (mg/ℓ)] = 20.68±2.02. Numbers 1 to 4 designate the size fraction (see Table 2).

It can be concluded that by increasing the Mg^{2+} concentration in the carrier, in addition to the biomass yield, the P-removal capacity of the system was enhanced. A beneficial influence of NZ when compared to the QS can be ascribed to the higher level of exchangeable Mg^{2+} occurring naturally in the original NZ.

In order to verify whether the addition of carriers induced changes in the pH profiles, the pH of the material-supplemented cultures was measured. The final pH-values were on average the highest in the NZMg-supplemented reactors (7.63±0.13), followed by QS-supplemented reactors (7.56±0.05) and NZ-supplemented reactors (7.53±0.09). According to the low difference in final pH (up to 0.1 unit) in reactors containing different carriers, the examined carriers did not act as a pH regulator, which is in agreement with the observed property of other NZ (Bauman et al., 2001; Shindo et al., 2001). The increase of pH in the reactors containing carriers was not higher than the increase of pH due to the P uptake in reactors containing the pure culture of *A. calcoaceticus* without the addition of carrier (Hrenović et al., 2003b). Therefore, it is quite unlikely that the chemical precipitation with Mg^{2+} or other exchangeable cations present in the carriers was the predominant mechanism of P removal. The contribution of the immobilisation of *A. calcoaceticus* on natural carriers to the EBPR was mainly a function of the higher density of metabolically active cells. The increased Mg^{2+} concentration in the carriers of immobilised cells improved the P-removal capacity of the EBPR system.

Conclusions

It is shown that naturally occurring materials such as NZ and QS and adsorptive growth can be successfully used for the immobilisation of P-accumulating bacteria. They can replace the commercial synthetic carriers and special methods of cell immobilisation. The available data suggest that the Mg^{2+} contained in the NZ (naturally or artificially modified) can help to achieve high cell density in bioreactor resulting in an increased P-removal capacity of the system. It therefore seems promising for improving EBPR. A particle size of NZMg <0.125 mm is recommended for successful immobilisation of metabolically active P-accumulating bacteria. In practice, NZMg, NZ or QS of the particle size <0.25 mm can be used. The P-accumulating bacteria immobilised onto naturally occurring materials could be implemented in the wastewater treatment plants, giving a cheap alternative for improving the EBPR process.

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References

ABD EL-HALEEM D (2003) *Acinetobacter*: Environmental and biotechnological applications. *Afr. J. Biotechnol.* **2** (4) 71-74.

ARIAS CA, DEL BUBBA M and BRIX H (2001) Phosphorus removal by sands for use as media in subsurface flow constructed reed beds. *Water Res.* **35** (5) 1159-1168.

BAUMAN M, MESARIĆ M, RIBAR S, MARIĆ V and TUDJA M (2001) Natural zeolite clinoptilolite increases the concentrations of sphingoid bases in the yeast *Yarrowia lipolytica*. *J. Basic Microbiol.* **41** (1) 7-16.

BOTING CFC, KORTSTEE GJJ, BOEKESTEIN A and ZEHNDER AJB (1993) The elemental composition dynamics of large polyphosphate granules in *Acinetobacter* Strain 210A. *Arch. Microbiol.* **159** (5) 428-434.

CHANG WS, HONG SW and PARK J (2002) Effect of zeolite media for the treatment of textile wastewater in a biological aerated filter. *Process Biochem.* **37** (7) 693-698.

DURHAM DR, MARSHALL LC, MILLER JG and CHMURNY AB (1994) Characterization of inorganic biocarriers that moderate system upsets during fixed-film biotreatment process. *Appl. Environ. Microbiol.* **60** (9) 3329-3335.

HRENOVIĆ J, BÜYÜKGÜNGÖR H and ORHAN Y (2003a) Use of natural zeolite to upgrade activated sludge process. *Food Technol. Biotechnol.* **41** (2) 157-165.

HRENOVIĆ J, TIBLJAŠ D, BÜYÜKGÜNGÖR H and ORHAN Y (2003b) Influence of support materials on phosphate removal by the pure culture of *Acinetobacter calcoaceticus*. *Food Technol. Biotechnol.* **41** (4) 331-338.

IMAI H, ENDOH K and KOZUKA T (1988) Magnesium requirement for biological removal of phosphate by activated sludge. *J. Ferment. Technol.* **66** (6) 657-666.

KARIMINIAAE-HAMEDAANI HR, KANDA K and KATO F (2003) Wastewater treatment with bacteria immobilized onto a ceramic carrier in an aerated system. *J. Biosci. Bioeng.* **95** (2) 128-132.

LOPEZ-RUIZ JL, LOPEZ-ALCALA JM, TORRES-FERNANDEZ JC and RODRIGUEZ-FUENTES G (1997) Elimination of phosphates by natural zeolites. *5th Int. Conf. on the Occurrence, Properties, and Utilization of Natural Zeolites – Ischia*. De Frede, Napoli. 209-211.

MUYIMA NYO and CLOETE TE (1995) Immobilisation of *Acinetobacter johnsonii* cells within alginate beads. *Water SA* **21** (3) 239-244.

SAKADEVAN K and BAVOR HJ (1998) Phosphate adsorption characteristics of soil, slags and zeolite to be used as substrates in constructed wetland systems. *Water Res.* **32** (2) 393-399.

SCHONBORN C, BAUER HD and ROSKE I (2001) Stability of enhanced biological phosphorus removal and composition of polyphosphate granules. *Water Res.* **35** (13) 3190-3196.

SEVIOUR RJ, MINO T and ONUKI M (2003) The microbiology of biological phosphorus removal in activated sludge systems. *FEMS Microbiol. Rev.* **27** (1) 99-127.

SHINDO S, TAKATA S, TAGUCHI H and YOSHIMURA N (2001) Development of novel carrier using natural zeolite and continuous ethanol fermentation with immobilized *Saccharomyces cerevisiae* in a bioreactor. *Biotechnol. Lett.* **23** (24) 2001-2004.

SIDAT M, BUX F and KASAN HC (1999) Phosphate accumulation by bacteria isolated from activated sludge. *Water SA* **25** (2) 175-179**.**

STATSOFT, INC. (2004) *Statistica* (data analysis software system) *Version 6*. Tulsa.