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1. Introduction

The practice of the wastewater treatment started at the beginning of the twentieth century in order to protect the waterborne diseases and the eutrophication of natural recipients. Today, numerous wastewater facilities have been constructed to treat domestic and industrial wastewaters. The wastewater treatment process comprises four steps: preliminary, primary, secondary or tertiary advanced treatment of wastewater. In some sensitive water bodies, very low phosphorus discharge limits (below 0.1 g of total P per m$^3$) have been promulgated. The process of enhanced biological phosphate (P) removal from wastewater using microorganisms is carried out by controlling the soluble P present in the wastewater as a nonsoluble intracellular polyP. The P removal from wastewater as polyP has been found to be related to the population of P-accumulating bacteria, which are able to accumulate polyP among all the cultivable isolates (Sidat et al., 1999) and could accumulate up to 100 mg of P per gram of cell protein, which represents more than 10% of dry biomass (Auling et al., 1999). None of the Acinetobacter isolates possesses the typical metabolic characteristics of the PAB since they failed to accumulate the polyhydroxy-alkanoates from extracellular volatile fatty acids in anaerobic conditions of growth (Kortstee et al., 2000). These isolates are however able to accumulate polyP in the absence of extracellular carbon sources in aerobic conditions of growth. The Acinetobacter spp. had the highest capacity to accumulate polyP among all the cultivable isolates (Sidat et al., 1999) and could accumulate up to 100 mg of P per gram of cell protein, which represents more than 10% of dry biomass (Auling et al., 1999).

Within the serpentine body near Gornje Oresje, Croatia, sepiolite was found as alteration product of hydrothermal activity inside peridotitic rocks, developed as monomineral veins (10 cm wide and up to few meters long). Sepiolite was tested for the immobilization of phosphate (P)-accumulating bacteria Acinetobacter junii under sterile (synthetic wastewater) and non-sterile conditions (effluent from the secondary stage of wastewater treatment). In sterile conditions the number of immobilized A. junii was 5.60 × 10$^9$ CFU g$^{-1}$. The A. junii were successfully immobilized onto non-sterile sepiolite in the original effluent water and the prepared bio-particles contained 2.43 × 10$^8$ CFU g$^{-1}$ of A. junii and 1.19 × 10$^6$ CFU g$^{-1}$ of heterotrophs. After 24 h of incubation of bio-particles in effluent water with P concentration adjusted to 20 mg L$^{-1}$, the number of immobilized A. junii increased to 6.64 × 10$^9$ CFU g$^{-1}$. The P removal from effluent water was more efficient in a reactor with bio-particles (94.1%) than in a reactor with planktonic A. junii (73.5%).

By the use of immobilized Acinetobacter spp. onto carriers a higher number of bacteria in bioreactor and thus improved efficiency of the P removal can be achieved. The recommended procedure for the immobilization of bacteria onto carriers implied the mixing of pure bacterial culture with the pre-sterilized carrier in the filtered culture medium (Pedersen and Weiergang, 2007). The use of sterile conditions and culture medium increases the price of the immobilization process thus making them less cost-effective. The certain species of bacteria may have a preferable carrier and one species of bacteria can be selectively adsorbed from mixed bacterial culture onto one carrier (Kubota et al., 2008). Sepiolite is a needle structured clay mineral, hydrated magnesium silicate and has a variety of applications derived from its adsorptive properties (Alkan et al., 2005). However, the studies using the sepiolite as a carrier of bacteria are relatively scarce (Albareda et al., 2008; Hrenovic et al., 2010a; Pedersen and Weiergang, 2007).

A variety of minerals were detected within the serpentine body near Gornje Oresje, Croatia (Palinkas et al., 2006). This association was a result of hydrothermal alteration of former peridotitic rock. Later weathering process produced layer of lateritic crust which contains goethite and it is rich in Upper Cretaceous fossils. Millerite was common inside green clay pockets. Magnezite was an alteration product of peridotite. Siderite was preserved as a subparallel group of crystals on the contact of Cretaceous limestone and montmorillonite clay. Nontronite was found between serpentine and laterite. Calcite was widespread in Cretaceous limestone as part of preserved fossils, as sediment but also as
alteration product of serpentine. Pyrite was found within cracks and vugs of Cretaceous limestones as well as in fossils. Quartz was found as final alteration product inside serpentine (Palinkas et al., 2006). Sepiolite veins are up to 10 cm wide and up to few meters long. Sepiolite was of light grayish brown in color. It was phyllosilicate with layers mostly parallel to the parent rock.

In this paper we report about the occurrence of sepiolite near Gornje Oresje, Croatia. The immobilization of P-accumulating bacteria Acinetobacter junii onto non-sterilized sepiolite in the effluent water from the secondary stage of the biological wastewater treatment was tested.

2. Materials and methods

2.1. Sepiolite

The sepiolite was obtained from Gornje Oresje, Croatia. The rock was crushed and by wet sieving trough the 0.100 and 0.212 mm mineral sieve, the aggregate size of 0.100–0.212 mm was prepared. In the experiments the sample sterilized by classical microbiological autoclaving (121 °C/15 min) and the original sample were tested.

2.2. Bacteria

The culture of P-accumulating bacteria A. junii strain DSM 1532 was obtained from the Deutsche Sammlung von Microorganismen und Zellkulturen GmbH (Hrenovic et al., 2003).

2.3. Wastewater

The composition of synthetic wastewater was as follows: Na-propionate 300 mg; peptone 100 mg; MgSO4 10 mg; CaCl2 6 mg; KCl 30 mg; yeast extract 10 mg; KH2PO4 88 mg; distilled water 1000 mL. The pH value was adjusted to 7.00±0.02 with 1 M NaOH or 1 M HCl (Kemika, Croatia) before autoclaving (121 °C/15 min).

The effluent from the secondary stage of the biological wastewater treatment in the wastewater treatment plant of Zagreb, Croatia was used. The fresh sample of effluent was used in the experiments within 2 h after the transportation to the laboratory. The chemical composition of effluent was: pH = 7.92, chemical oxygen demand = 24.3 mg O2 L−1, total nitrogen = 20.8 mg L−1, total phosphorus = 2.12 mg L−1. The effluent was filtered through a Buchner funnel and filter paper (blue band) and the pH value was adjusted to 7.00±0.02. In order to accurately follow the performance of P-accumulating bacteria A. junii, the P concentration in effluent was adjusted to 20 mg L−1 with KH2PO4.

2.4. Experimental design

In order to establish the extent of immobilization of bacteria onto sepiolite, A. junii were pre-grown on a nutrient agar (Bioloft, Italy) for 16 h at 30±0.1 °C. The biomass was then suspended in sterile 0.05 M NaCl solution. One milliliter of suspended biomass was inoculated into Schott bottles which contained 100 mL of synthetic wastewater, giving the starting number of colony forming units (CFU) of A. junii 109 mL−1. Into one bottle a 1.0 g of autoclaved sepiolite was added, while other bottle was left without addition of sepiolite. The third bottle contained only synthetic wastewater and 1.0 g of sepiolite (without bacteria). The bottles were sealed with a sterile gum cap with a central hole, through which the aeration with filtered air (1 L min−1) was provided in order to avoid the contamination with microorganisms present in air. The bottles were incubated during 24 h of experiment in a water bath (Memmert WNB22) with stirring of 70 rpm to assure the complete mixing at 30.0±0.5 °C.

In the following experiment the behavior of bioparticles, consisting of A. junii immobilized onto non-sterilized sepiolite, in the real effluent water was tested. The bioparticles were prepared by incubating a suspension of A. junii (concentration of 106 CFU mL−1) and 1.0 g of original sepiolite in Schott bottles which contained 100 mL of original effluent water. The bottles were incubated during 24 h as described earlier. After the incubation the bioparticles consisting of A. junii immobilized onto sepiolite were gently washed with 0.05 M NaCl and used in experiment. The experiment was set up in Schott bottles which contained the 100 mL of effluent water with the P concentration adjusted to 20 mg L−1. In one bottle a 1.0 g of bioparticles was added, in the second bottle the suspension of A. junii was added (concentration of 106 CFU mL−1), while the third bottle was left blank and served as control. The bottles were incubated during 24 h as described earlier.

2.5. Analytical methods

The X-ray powder diffraction data were collected using X-ray powder diffractometer Philips PW3050/60 XPert PRO with Cu tube, accelerated by 40 kV and current of 40 mA. Step size was 0.02° and counting time 1 s per step. X-ray pattern analyses were performed by assignment of peaks using HighScore Plus software. The unit-cell calculations were performed using Unit Cell software. The scanning electron microscopy (Field Emission Scanning Electron Microscope, jeol JSM 7000 F, Japan) and Gram stain followed by light microscopy were performed to confirm the morphology of sepiolite, the shape of bacteria and immobilization of bacterial cells onto the sepiolite fibers. The Neisser stain was performed to confirm polyP granules in cells of A. junii. The pH value was measured with the WTW 330 pH-meter. Before the measurement of P the pH of samples was adjusted between 6.8 and 7.5 and the samples were filtered using Sartorius nitrocellulose filters with pore diameter of 0.2 μm (Hrenovic et al., 2003). The P concentration in the water was measured spectrophotometrically in a DR/2500 Hach spectrophotometer using the molybdovanadate method (Hach method 8114).

For the determination of planktonic bacteria, 1 mL of supernatant was serially diluted (10−1 to 10−9) and volumes of 0.1 mL were aseptically inoculated onto the nutrient agar (spread plate method). After the incubation (30 ± 0.1 °C/24 h), the bacterial colonies were counted and the number of viable cells was reported as CFU mL−1.

To determine the number of immobilized bacteria, the sepiolite was taken from the bottle, washed three times with sterile 0.05 M NaCl solution, and aseptically placed into a tube which contained 9 mL of 0.05 M NaCl. The samples were crushed with a sterile glass rod and vigorously shaken on a mechanical shaker (40 Hz/3 min, Kartell TK3S). This procedure (Durham et al., 1994) detaches immobilized cells from the carrier. Serial dilutions were made from these suspensions and nutrient agar plates were inoculated and incubated as already described. After the incubation the colonies were counted and the remaining samples were dried (105 °C/2 h) and weight. The number of cells was reported as immobilized CFUs per 1 g of the dry carrier. All measurements were done in triplicate.

Statistical analyses were carried out using Statistica Software 9.1 (StatSoft, Tulsa, USA). The numbers of bacterial CFU were logarithmically transformed beforehand to normalize distribution and to equalize variances of the measured parameters. The comparisons between samples were done using the one-way analysis of variance (ANOVA) and subsequently the post-hoc Duncan test was performed for the calculations concerning pair-wise comparisons. The correlation between variables was estimated using the Pearson linear correlation. Statistical decisions were made at a significance level of p<0.05.

3. Results and discussion

3.1. Occurrence and characterization of sepiolite

Main deposits of sepiolite are located in Anatolia in Turkey, Ceeerbuer in Somalia, South-central China and Spain with 70% of world reserves (Alvarez-Ayuso and Garcia-Sanchez, 2003).
3.2. Immobilization of A. junii onto sepiolite

After 24 h of incubation of sepiolite in the synthetic wastewater (Table 1), 11.1% of P was removed from wastewater. The P adsorbed on sepiolite (2.0 mg P g$^{-1}$) gave no signal when examined by X-ray powder diffraction. This relatively low P adsorption capacity was higher than 1 and 1.5% of P removal obtained for natural mineral which contained 40–50% and 50–55% of sepiolite, respectively (Hrenovic et al., 2010a). The experimental approach of P removal by sepiolite used in this study is preliminary and construction of a complete adsorption isotherm is required. In a complex medium the competitive adsorption of P and other anions is expected. Therefore, the P adsorption onto sepiolite in wastewater will vary with type of wastewater and can be different from the P adsorption in solution of P salt.

In contact with sepiolite the majority of bacteria A. junii (5.60 × 10$^9$ CFU g$^{-1}$) were immobilized on the surface of the sepiolite fibers by extracellular substances (Fig. 2), while the minority of A. junii was present as planktonic bacteria (Table 1). The number of immobilized A. junii onto sepiolite was higher than 1.1 × 10$^9$ CFU g$^{-1}$ of Pseudomonas fluorescens (Pedersen and Weiergang, 2007) and comparable to 10$^8$ CFU g$^{-1}$ of Bradyrhizobium japonicum and 10$^9$ CFU g$^{-1}$ of Sinorhizobium fredii (Albareda et al., 2008) immobilized onto sepiolite. This number was almost the same as 5.57 × 10$^9$ CFU g$^{-1}$ of A. junii immobilized onto sepiolite rock which contained 40–50% of sepiolite, but lower than 8.12 × 10$^9$ CFU g$^{-1}$ of A. junii immobilized onto acid treated sepiolite with 50–55% of active compound (Hrenovic et al., 2010a). This indicated that the sepiolite content in the natural samples was not the prevailing factor for the efficiency of the immobilization of A. junii. The enhanced immobilization of bacteria onto acid treated sepiolite could be due to the changes in surface reactivity (Corma et al., 1987) and increased specific surface area of acid treated sepiolite (Myriam et al., 1998). The particle size of the material can have a great influence on the extent of immobilization of bacteria, where the increase of particle size resulted in decrease of immobilized bacteria (Hrenovic et al., 2003). The size of sepiolite aggregates used in this work (0.100–0.212 mm) was overlapping with the range of sepiolite aggregates used in previous work (0.044–0.149 mm, Hrenovic et al., 2010a). Therefore, a different extent of the immobilization of A. junii in these two works cannot be ascribed to the difference in aggregate size of sepiolite.

The number of total bacteria and the ratio of final and starting number of A. junii in the reactor with sepiolite were significantly higher than in the reactor without sepiolite (Table 1). Probably the high content of Mg$^{2+}$ ions in sepiolite was available for bacterial metabolism by ion exchange and thus enhanced the yield of biomass of A. junii (Hrenovic et al., 2010b). The P-uptake rate per CFU of A. junii was lower in the reactor with sepiolite than in the reactor without sepiolite. Better multiplication of A. junii in the reactor with sepiolite resulted in a higher proportion of bacteria in log phase (rod shaped cells) than in stationary phase (spherical cells) of growth, as confirmed by Gram stain and SEM. Since the P-accumulation in A. junii occurs only in the stationary phase of growth (Hrenovic et al., 2003), lower P-uptake rates were observed in reactor with sepiolite. Significantly higher P removal (Table 1) was achieved in reactor with sepiolite (55.4%) than in reactor without sepiolite (25.4%). The percent of P removal showed a significantly positive correlation with the total number of A. junii (p = 0.992). Better P removal in the reactor with sepiolite was due to the increased bacterial biomass and not due to the increased bacterial P-uptake rate. Although statistically significant, the difference in final pH values among reactors (Table 1) was only 0.10 pH units. The possible negative influence of pH on the growth and metabolism of A. junii can be excluded since these bacteria grow in the pH range 6–8 (Garry et al., 2005). The quick (within 10 min) alkaline reaction of 1% of sepiolite in neutral synthetic wastewater (Table 1) was congruent

Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sepiolite</th>
<th>A. junii</th>
<th>A. junii + sepiolite</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immobilized cells (10$^9$ CFU g$^{-1}$)</td>
<td>-</td>
<td>-</td>
<td>5.60 ± 0.83</td>
</tr>
<tr>
<td>Planktonic cells (10$^9$ CFU mL$^{-1}$)</td>
<td>-</td>
<td>-</td>
<td>3.56 ± 0.23$^B$</td>
</tr>
<tr>
<td>Total cells (10$^9$ CFU mL$^{-1}$)</td>
<td>1.24 ± 0.05</td>
<td>4.12 ± 0.16$^B$</td>
<td>39 ± 3</td>
</tr>
<tr>
<td>CFU immobilized/planktonic</td>
<td>-</td>
<td>-</td>
<td>21.92 ± 2.04</td>
</tr>
<tr>
<td>CFU final/CFU start</td>
<td>-</td>
<td>-</td>
<td>70.41 ± 3.51$^B$</td>
</tr>
<tr>
<td>P-uptake rate</td>
<td>-</td>
<td>-</td>
<td>5.60 ± 0.83</td>
</tr>
<tr>
<td>(10$^{-11}$ mg P CFU$^{-1}$)</td>
<td>1.24 ± 0.05</td>
<td>4.12 ± 0.16$^B$</td>
<td>39 ± 3</td>
</tr>
<tr>
<td>P removed (%)</td>
<td>11.1 ± 1.3</td>
<td>25.4 ± 1.8$^A$</td>
<td>55.4 ± 1.8$^A$</td>
</tr>
<tr>
<td>Final pH</td>
<td>7.83 ± 0.02</td>
<td>7.82 ± 0.02</td>
<td>7.73 ± 0.02$^AB$</td>
</tr>
</tbody>
</table>
to values obtained for 3% of sepiolite suspension in distilled water (Cinar et al., 2009).

3.3. Performance of bioparticles in effluent water

The bacteria A. junii were successfully immobilized onto non-sterile sepiolite in the original effluent water which contained $1.30 \pm 0.15 \times 10^4$ CFU mL$^{-1}$ of heterotrophic bacteria grown at 30 $^\circ$C/24 h. The prepared bioparticles contained $2.43 \pm 0.43 \times 10^9$ CFU g$^{-1}$ of A. junii and $1.19 \pm 0.08 \times 10^8$ CFU g$^{-1}$ of heterotrophs.

After 24 h of incubation of bioparticles in effluent water with P concentration adjusted to 20 mg L$^{-1}$, the number of immobilized A. junii increased for 27 times ($6.64 \times 10^9$ CFU g$^{-1}$) while the number of immobilized heterotrophs increased for 13 times ($1.56 \times 10^9$ CFU g$^{-1}$).

The number of A. junii immobilized in non-sterile conditions (Table 2) was not significantly different than the number immobilized in sterile conditions of experiment (Table 1). This indicated that the immobilization experiments performed with pure culture of bacteria were applicable to the ecological conditions of immobilization in real scale and that the aggregates of sepiolite had their maximum capacity of immobilization of bacteria. When the capacity of immobilization of bacteria was reached, the A. junii were detached from the bioparticles and continued to grow as planktonic population (Table 2). The similar behavior was observed for A. johnsonii immobilized into alginate beads where the minor population was leaking out of the beds and was grown as planktonic bacteria (Muyima and Cloete, 1995). After the use in experiment, the aggregates of sepiolite were solid without changes in X-ray powder diffraction pattern, which indicated that the sepiolite was a good inert material for the immobilization of bacteria.

In reactor containing bioparticles the highest total number of A. junii and the lowest total number of heterotrophs was detected when compared to reactor which contained planktonic A. junii or control reactor (Table 2). Probable the development of population of A. junii inhibited the growth of heterotrophs present in the effluent water. The highest percent of P removal from water (Table 2) was observed in the reactor with bioparticles (94.1%), followed by the reactor with planktonic A. junii (73.5%) and control reactor (36.2%).

The percent of P removal from water showed a significantly positive correlation with the total number of A. junii ($p = 0.937$) and significantly negative correlation with the total number of heterotrophs ($p = -0.953$). This indicated that the P removal from water was the function of increased biomass of P-accumulating bacteria A. junii. Moreover, the population of A. junii in the form of bioparticles was 24 h older than in reactor with planktonic A. junii, which resulted in a higher proportion of cells in the stationary phase of growth that were more efficient on P removal from water. The extracellular substances produced by the immobilized bacteria onto solid surfaces could also in lower extent contribute to P removal, then acting as a P reservoir (Cloete and Oosthuizen, 2001).

The efficiency of P removal in the experiment with effluent water by lower total number of A. junii (Table 2) was higher than in the experiment with synthetic wastewater by higher total number of A. junii (Table 1). These results could be explained by the acclimatization of A. junii to the effluent during the preparation of biosolids for experiments and are in agreement with the observation (Hollender et al., 2002) that real wastewater contains the heat sensitive compounds that are important for metabolism of P-accumulating bacteria.

Low P removal in reactors with sepiolite only (Table 1) suggested that the P adsorption on sepiolite was not the dominant mechanism of P removal. Medium pH of above 7.8 would have shown that P had precipitated as either calcium or magnesium salts, which could erroneously account for the decrease in P concentration in the sample (Sidat et al., 1999). The influence of the high final pH (Table 2) on the P concentration in water can be excluded since the pH of samples was adjusted between 6.8 and 7.5 before P measurement and the difference in final pH values among reactors was only 0.13 pH units.

4. Conclusions

From the practical aspect, it was shown that the P-accumulating bacteria A. junii were successfully immobilized at 30 °C onto non-sterile sepiolite in the effluent water which contained $10^4$ CFU mL$^{-1}$ of heterotrophic bacteria. The efficiency of selective immobilization of A. junii in the effluent water at temperatures below 30 °C should be further examined in order to simplify the method of immobilization. The bioparticles, consisting of A. junii immobilized onto sepiolite, prepared under non-sterile conditions were very efficient and removed 94.1% of the starting P from effluent water. The presence of other heterotrophic bacteria did not disturb the growth and metabolic activity of A. junii.

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References


