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Immobilization of bacterial association in alginate beads for bioremediation of oil-contaminated lands

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Abstract

Entrapment in alginate gel is a widely used approach for immobilization of microorganisms to improve their viability. A natural bacterial association consisting of seven strains isolated from acid tar-contaminated soil was used for immobilization in alginate gel. The aim of this study was to identify conditions for entrapment of a bacterial association and to determine conditions for its prolonged storage. The association was successfully immobilized in 1.5% alginate beads that were 2 mm in diameter. All tested treatments, with or without 5% clay, with or without 10% glycerol, and with or without incubation of prepared beads in nutrient medium or 0.2 M solution of $CaCl_2$, showed good results in terms of survival of bacteria immediately after entrapment in gel and during at least six months storage of beads at temperature of 4 and 20 °C. The number of bacterial colony-forming units increased by 1.1 to 1.3 log g^{-1} in clay-containing beads during the experiment, regardless of storage temperature but it remained at baseline levels in other treatments. Further studies are necessary to evaluate bioremediation activities of the immobilized association in laboratory and field conditions.

Key words: alginate, bacteria, bacterial association, gel entrapment, immobilization.
Abbreviations: CFU, colony-forming units; MHB, Mueller Hinton Broth.

Introduction

Environmental bioremediation is a profitable and promising technology, which can lead to complete mineralization of organic pollution. Bioaugmentation (introduction of selected microorganisms to supplement indigenous populations) is one of the bioremediation approaches (Hassanshahian et al. 2014). Successful bioaugmentation can be expected by enrichment cultures obtained from previously contaminated sites. However, problems arising from competition between introduced and indigenous microorganisms and between mixed microbial strains in the cultivated consortium cannot be precluded (reviewed in McCarty, Criddle 2012). Thus, Hassanshahian et al. (2014) showed reduced biodegradation capability of the consortium (*Alcanivorax borkumensis* and *Thalassolituus oleivorans*), suggesting unfavourable interaction. Success of bioaugmentation also largely depends on the ability of inoculants to survive in the usually harsh conditions (toxic or inhibitory substances, unsuitable pH, osmotic stress etc.) of polluted environments. One of the biggest problems is survival of inocula after administration (Bento et al. 2005). Promotion of biodegradation may be achieved by use of immobilized cells (reviewed in Mrozik, Piotrowska-Seget 2010). The carrier provides a protective niche to microorganisms and ensures viability over a prolonged period of release. Several organic carriers have been tested for soil bioremediation, including chitin, chitosan, gellan gum, polyvinyl alcohol etc. (reviewed in Lebeau 2011) as well as an organomineral complex prepared from humic acids bound on zeolite (Dercova et al. 2007).

Many biodegrading microorganisms belong to the genera *Acinetobacter* (Luckarift et al. 2011), *Alcanivorax* (Hassanshahian et al. 2014), *Bacillus* (Gupta et al. 2000), *Burkholderia* (Mohanty, Mukherji 2008), *Marinobacter*, *Pseudomonas* (Abed et al. 2014), *Rhodococcus* (Kundu et al. 2016), *Sphingomonas* (Matsumura et al. 2015) and *Thalassolituus* (Crisafi et al. 2016). Szulc et al. (2014) selected a consortium consisting of *Aeromonas hydrophila, Alcaligenes xylosoxidans, Gordonia* sp., *Pseudomonas fluorescens, Pseudomonas putida, Rhodococcus equi, Stenotrophomonas maltophilia* and *Xanthomonas* sp. for diesel-oil contaminated soil bioremediation. Crisafi et al. (2016) investigated a hydrocarbonoclastic consortium consisting of *Alcanivorax borkumensis, Alcanivorax dieselolei, Marinobacter hydrocarbonoclasticus, Cycloclasticus* sp. 78-ME and *Thalassolituus oleivorans*. A consortium of *Acinetobacter, Pseudomonas, Microbacterium* and *Ralstonia* species was found to have polycyclic aromatic hydrocarbons-degradation capability (Simarro et al. 2013).

Immobilized cell systems are classified into four categories based on the physical mechanism of the localisation and nature of the support mechanisms: adsorption, aggregation, confinement and entrapment (reviewed in Karel et al. 1985; Cassidy et al. 1996). Gel entrapment with natural polymers, such as agar, alginate,

carrageenan and cellulose and its derivatives, collagen and gelatin, is a mild technique and takes place under ambient conditions when damage to living cells can be minimized. Disadvantages are the poor mechanical strength and the limited longevity of the gel structure. Algal polysaccharides: alginate, carrageenan and agar are the most widely used (reviewed in Willaert, Baron 1996). Alginate is a binary linear heteropolymer containing 1,4-linked β-D-mannuronic and α-L-guluronic acid residues in varying proportion and sequence and are characterized by a selective binding of calcium ions in solution (Smidsrød 1974). The droplets form a gel instantaneously and entrap cells in a three-dimensional porous matrix of cross-linked alginate. The most common forms are small beads about 1 to 5 mm in diameter. Entrapment of microbial cells within beads of calcium alginate has become one of the most widely used immobilization methods. The major disadvantage of alginate beads is its sensitivity to chelating agents such as EDTA, citrate, lactate, phosphate, or anti-gelling cations such as sodium and magnesium. They can be used to dissolve gel (Mørch et al. 2006). Alginate, being a natural polymer, is biodegradable and therefore environmentally friendly. Several genera of bacteria are reported to contain alginate-lyases producing and alginate degrading strains, for example, *Gracilibacillus* (Tang et al. 2009), marine *Streptomyces* sp. (Kim et al. 2009), and the production of an extracellular alginate-degrading enzyme is one of the characteristics of genus *Vibrio* (Kitamikado et al. 1990).

Alginate is known as a biomaterial with numerous applications in biomedicine due to its biocompatibility and low toxicity (reviewed in Lee, Mooney 2012). Alginate-clay composites are suitable for environmental remediation as sorbents of heavy metals (Shawky 2011) and persistent organic pollutants (Barreca et al. 2014). Alginate is also used as an inoculant carrier for plant growth promoting bacteria (Bashan 1986; Trivedi et al. 2005), for biocontrol agents (Fravel et al. 1985; Pradeep, Subbaiah 2016) and for bacteria with biodegradation ability (Li et al. 2005). Alginate-immobilized bacteria have shown an higher tolerance to toxic compounds (Kim et al. 2006) and better xenobiotic degradation rates (Li et al. 2005; Pradeep, Subbaiah 2016) than non-immobilized cells. Formulations containing inorganic and organic materials such as kaolin clay or wheat bran (Lewis, Papavizas 1985), montmorillonite clay (Weir et al. 1995), glycerol (Zohar-Perez et al. 2002) or skim milk (Trivedi et al. 2005) have demonstrated increased viability during storage.

The aim of this study was to identify conditions for entrapment of a new bacterial association suitable for bioremediation of oil-contaminated lands in alginate beads and to determine conditions for its prolonged storage. Effect of clay and glycerol in bead formulations and effect of incubation medium were evaluated. Survival of bacteria was determined during six months of storage of beads at temperature of 4 and 20 °C.

Materials and methods

Microorganisms

A natural microbial association containing seven bacterial strains was used. Components of the association were *Acinetobacter* sp., *Bacillus circulans, Bacillus licheniformis, Brevibacillus brevis, Burkholderia cepacia, Leifsonia aquatica* and *Sphingomonas paucimobilis*. This association was isolated by Dr. biol. Līvija Vulfa about 20 years ago from soil contaminated with acid-tar in the neighbourhood of Inčukalns (Latvia) and the association is maintained in the Microbial Strain Collection of Latvia, University of Latvia, accession number MSCL 311.

Immobilization and storage of bacteria

Immobilization was carried out using a modification of the method described by Bashan (1986). The bacterial association was cultivated on Plate Count Agar (Bio-Rad, France) for 7 days at 20 °C. Encapsulation was performed using a sterile 3% solution of sodium alginate (Duchefa Biochemie, Netherlands) and 0.2 M $CaCl_2$ (Ing. Pert Švec – PENTA s.r.o., Czech Republic). Treatments with addition of clay powder (Ceplis Ltd., Latvia) 5 g per 100 mL of 3% alginate and analytical glycerol (P.P.H. „Stanlab", Poland) 10 g per 100 mL of 3% alginate also were prepared. All chemicals were sterilized separately by autoclaving 15 min at 121 °C.

A bacterial suspension of 10^9 colony-forming units (CFU) mL^{-1} was prepared in sterile water and mixed with 3% sodium alginate in volume ratios 1:1, thereby producing 1.5% alginate containing bacteria in the amount 5×10^8 CFU mL^{-1}. The obtained mixture was stirred for 30 min, then filled into a sterile syringe and dispensed dropwise with a needle 21G into a stirred solution of 0.2 M $CaCl_2$, where formation of beads took place. The beads were allowed to solidify at room temperature for 60 min.

Several types of alginate beads were prepared: with and without clay and/or glycerol, and with and without incubation of clay-containing beads in incubation medium with additives (Table 1). One portion of the beads was washed twice with sterile water, and the remainder was left in $CaCl_2$ solution without washing. Wet beads were distributed in sterile glass tubes with screw caps, two grams in each and maintained at temperatures of 4 or 20 °C for

Table 1. Treatments of incubation medium MHB with additives [MHB : additive = 1:1 (v/v)] and storage conditions of clay-containing beads

No.	Additive	Storage conditions
1	Distilled water	2 g beads, 0.2 mL 0.2 M $CaCl_2$, 20 °C
2	Distilled water	2 g beads, 0.2 mL 0.2 M $CaCl_2$, 4 °C
3	Distilled water	2 g beads, 0.2 mL distilled water, 20 °C
4	Distilled water	2 g beads, 0.2 mL distilled water, 4 °C
5	0.2 M $CaCl_2$	2 g beads, 0.2 mL distilled water, 20 °C
6	0.2 M $CaCl_2$	2 g beads, 0.2 mL distilled water, 4 °C

up to 74 days, but duration of one experiment was seven months. In part of the experiment, beads were incubated for an additional 24 h at room temperature in sterile Mueller Hinton Broth (MHB, Laboratorios Conda, Spain) containing acid casein peptone 17.5 g L^{-1}, beef infusion 2.0 g L^{-1} and corn starch 1.5 g L^{-1} and supplemented with yeast extract (Bio-Rad, France) 2.5 g L^{-1} with or without addition of 0.2 M $CaCl_2$ (Table 1). After 24 h, the liquid was decanted, and the beads were washed with sterile water, distributed in glass tubes as above and covered with distilled water or 0.2 M $CaCl_2$ for storage at 4 or 20 °C.

Microbiological analyses

Samples (2 g) of beads were ground in a sterile mortar with a pestle in a certain volume of sterile water to recover the bacteria. The number of bacterial CFU was determined by plating tenfold serial dilutions on Plate Count Agar (Bio-Rad, France) plates using the spread plate method. Plates were incubated at 20 °C for 7 days before counting colonies. Results were calculated and expressed as CFU g^{-1} of moist beads. Experiments were conducted in duplicate.

Viability of bacteria was analysed in slices of alginate beads using a LIVE/DEAD® BacLight™ Bacterial Viability Kit (Molecular Probes, USA) and fluorescence microscope (Leica DM 2000, Germany). Images were taken with camera Leica DFC 420 and processed by Image-Pro Express 6.0 (Media Cybernetics, USA) software.

Statistical analysis

Means, standard deviations and correlation coefficients were calculated. Analysis of variance (ANOVA) and the Student *t*-test was used to test differences among groups. Values of $p < 0.05$ was considered statistically significant.

Results

Characteristic of beads

During the experiment bacteria containing alginate beads with and without clay additive were prepared. The mean diameter of beads was 2 mm, but clay-containing beads had 16% greater weight and density than beads prepared without clay (Table 2). Beads with clay contained 53% more bacterial CFU than the same size beads without clay. Visually, beads without clay were bright and before hardening almost transparent (Fig. 1A) while clay-containing beads were pale yellow (Fig. 1B). It was observed that clay additive reduced the edge smoothness (Fig. 1C-D). The LIVE/DEAD test demonstrated viability of all bacteria in both kinds of beads as well as even distribution of cells inside the bead (Fig. 2). Morphological changes were not observed during storage of beads.

Survival of bacteria in alginate beads

Bacteria remained alive in beads with and without clay additive during the experiment (74 days) at temperatures of 4 and 20 °C (Fig. 3). After 74 days of storage, the number of CFU g^{-1} increased by 1.1 to 1.3 ($p < 0.05$) and 0.1 to 0.2 log ($p > 0.05$) in the case of alginate beads with and without clay, respectively, in comparison with the initial concentration regardless of the storage temperature.

Table 2. Characteristics of alginate beads containing bacteria

Parameter	Beads without clay	Beads with clay
Number of beads in 2 g	41	35
Mean bead diameter, mm	2	2
Calculated weight of one bead, mg	49	57
Calculated volume of one bead, mm^3	4.187	4.187
Calculated density, g cm^{-3}	11.67	13.57
Calculated number of bacteria in one bead after four days of storage, CFU	1.7×10^5	2.6×10^5
Calculated number of bacteria in one g of beads after four days of storage, CFU	3.5×10^6	4.6×10^6

The immobilized bacterial association consisted of seven species and it was possible to distinguish colonies of one of these species, i.e. *Burkholderia cepacia*. This species formed smooth round colonies with a fluorescent greenish-yellow colour in the Plate Count Agar. Decrease of the proportion of *B. cepacia* was observed during storage, especially in clay-containing beads, but data were not obtained in this experiment.

Fluctuations in the number of CFU in the range of 1.8 log CFU g^{-1} at the beginning (day 0) of the experiment, exceeding the limits of possible errors, were observed in beads with and without clay and glycerol (Fig. 4). The number of CFU tended to increase during the first two weeks ($p > 0.05$), but significantly decreased on day 27. Then, the number of colonies increased and at the end of the experiment (203 days) did not significantly differ from the number at the beginning, thereby indicating good survival of bacteria.

The number of CFU increased by 0.47 to 0.57 log after day-long incubation in nutrient medium of clay-containing beads, which was followed by a reduction. Fluctuations in the range of 3 log CFU g^{-1} were observed in tests carried out in the first 22-day period in all three treatments especially when beads were stored at temperature 4 °C (Fig. 5). The lowest number of colonies was after 8 days of storage in treatments 1 to 4 (incubation in MHB with water) and after 11 days of storage in treatments 5 and 6 (incubation in MHB with $CaCl_2$ solution), regardless of storage medium. Treatments 4 and 6 had the largest number of colonies at the end of the experiment after 183 days, i.e. 7.7 log CFU g^{-1}. Number of colonies was 2.3 (treatment 4) and

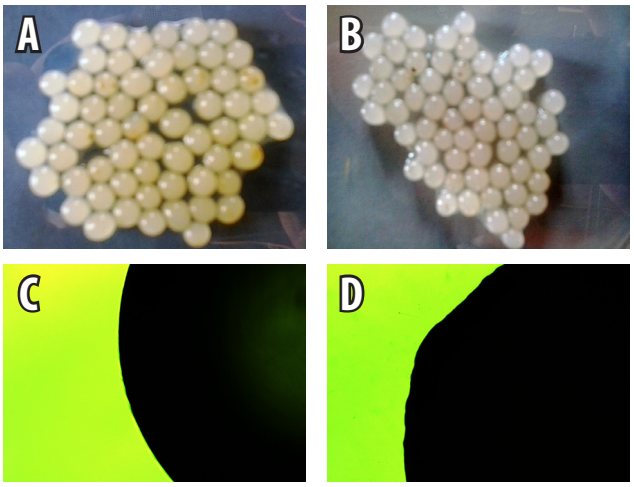

Fig. 1. Alginate beads 2 mm in diameter without clay (B, C) and with clay (A, D). C and D, shape of bead edges under magnification of 100 ×.

1.9 (treatment 6) times more than in the corresponding treatments that were stored at 20 °C. Beads of treatments 1 and 2 had the smallest amount of CFU. The number of bacterial CFU g^{-1} did not significantly differ from the number at the beginning in treatments 1, 2, 3 and 5, but were 4.0 to 4.4 times higher ($p < 0.05$) than at the beginning in treatments 4 and 6.

Discussion

In this study, a bacterial association was successfully immobilized in alginate beads that were 2 mm in diameter. All tested treatments, with or without clay or glycerol, with or without incubation in nutrient medium or $CaCl_2$ solution, showed good results in terms of survival of bacteria immediately after entrapment in gel and during at least six months storage of beads at temperature 4 °C and 20 °C. The number of bacterial CFU increased by 1.1 to 1.3 log g^{-1} in clay-containing beads during the experiment regardless of storage temperature (Fig. 3) but it remained at baseline levels in other treatments. Power et al. (2011) also showed 100% recovery of viable *Pseudomonas fluorescens* F113 after 250 days of storage in alginate beads under ambient temperature and concluded that alginate beads are an effective storage system for polychlorinated biphenyls degrading inocula. Like us, they also observed a reduction of the number of CFU, which occured 40 days after preparation of beads, and later the population began to increase.

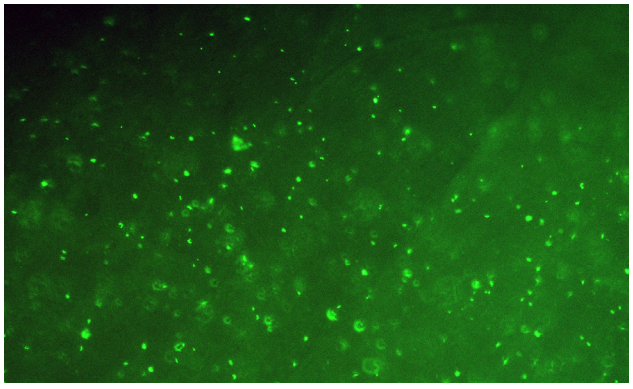

Fig. 2. Distribution of live (green) bacteria inside the alginate bead (LIVE/DEAD test).

Five percent clay addition resulted in reduced smoothness of bead edges, probably because a portion of the clay particles protruded outward. It has been demonstrated that also the addition of attapulgite (magnesium aluminium phyllosilicate) in the amount of 0.5 to 1% and calcium carbonate in the amount of 3 to 7% changed the roughness and resulted in unevenly shaped alginate granules (Wang et al. 2014).

Trevors et al. (1993) amended alginate with TY medium (ingredients: tryptone 10 g L^{-1}, yeast extract 5 g L^{-1} and NaCl 10 g L^{-1}) or soil extract and concluded that there was a negligible effect on long-term survival of *P. fluorescens* Rsf cells. However, amendment with skim milk or with skim milk plus bentonite clay significantly promoted survival of the cells. In our study, addition of 10% glycerol did not have significant impact on the survival of bacteria. Nassif et al. (2002) added glycerol to a suspension of *E. coli* to 10% final concentration before entrapment in silica gel. After one month, the percentage of culturable bacteria remained 30% higher than in silica gels without glycerol. Some authors have described additional incubation of bacteria-containing prepared alginate beads in nutrient broth for 24 to 48 h (Bashan 1986), so that bacteria could multiply inside the beads. In our study, this approach did not give any additional benefit in any of the six treatments of incubation and storage media, and storage temperature and the number of bacterial CFU remained at the initial level (Fig. 5).

To the best of our knowledge, this study is one of a few conducted on immobilization of a natural bacterial association instead of immobilization of individual species or an artificial microbial consortium. For example, El-Komy (2005) co-immobilized *Azospirillum lipoferum* and *Bacillus megaterium* in alginate beads to improve phosphorus solubilization. Huang and Yang (1998) co-immobilized homolactic and homoacetic bacteria in a fibrous-bed bioreactor for acetate production from whey lactose. A mixed culture of bacterium *Zymomonas mobilis* and yeast *Saccharomyces cerevisiae* was immobilized to improve fermentation performance for ethanol production (De Almeida, de Angelis 2016). Bergero et al. (2017) used immobilization of a consortium of *Aeromonas hydrophila* and *Pseudomonas putida* in Ca-alginate beads to achieve degradation of cationic surfactants. Problems with multi-strain associations or consortia arise due to different requirements and response of individual strains. For example, all fungi [*Gliocladium virens* (currently

Trichoderma virens), *Penicillium oxalicum, Talaromyces flavus* and *Trichoderma viride*], but not *Pseudomonas cepacia* (currently *Burkholderia cepacia*), were viable after pellet formation in $CaCl_2$ (Fravel et al. 1985). In our study, decrease of the proportion of *B. cepacia* was observed during the storage of alginate beads. At the same time, the total number of bacteria increased (Fig. 3). This means that populations of one or several bacterial strains grew which

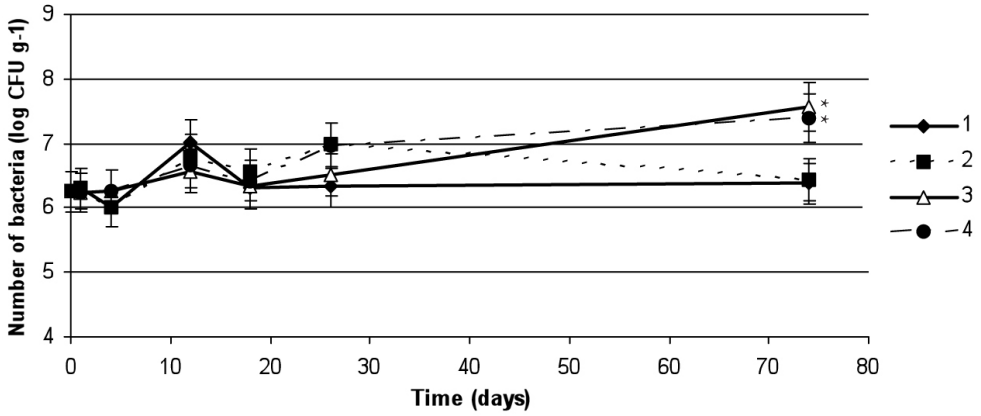

Fig. 3. Survival of bacteria in alginate beads with or without clay during storage at temperature of 4 and 20 °C. 1, without clay, 4 °C; 2, without clay, 20 °C; 3, with clay, 4 °C; 4, with clay, 20 °C. Values are mean ± SD of two replicates. *, significant difference from the day 0.

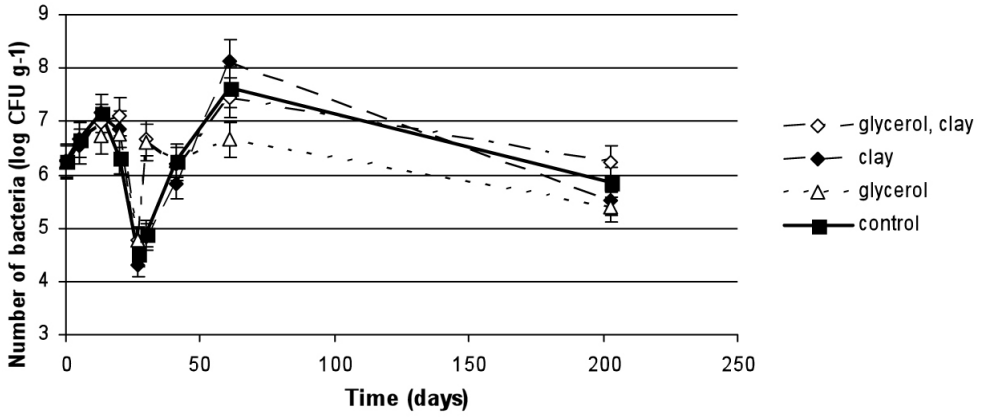

Fig. 4. Effect of clay and glycerol addition in beads on viability of bacteria during storage at 4 °C. Values are mean ± SD of two replicates.

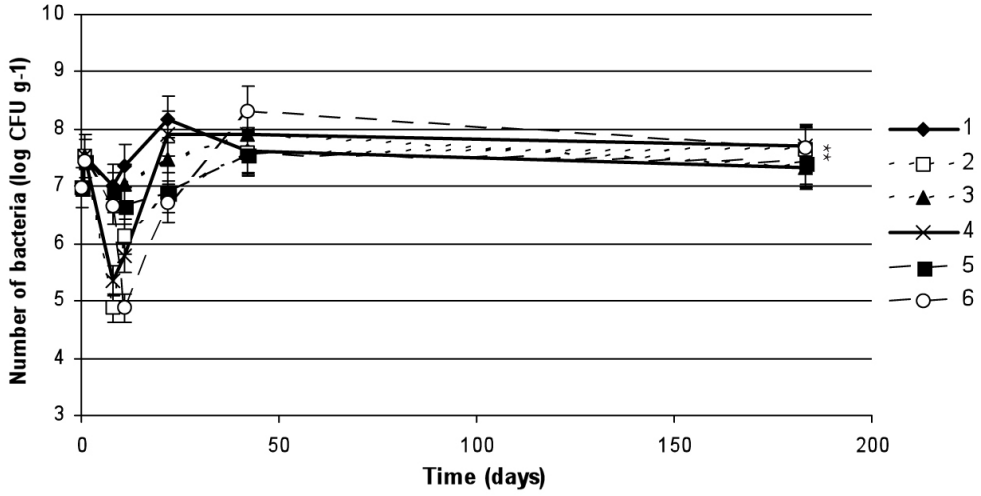

Fig. 5. Effect of incubation conditions of clay-containing beads on viability of bacteria. Treatments (Table 1) 1 to 4 were incubated in MHB with added water but treatments 5 and 6 were incubated in MHB with $CaCl_2$ solution. After incubation, the beads were stored in $CaCl_2$ solution in treatments 1 and 2 but in water in the other treatments. Treatments 1, 3 and 5 were stored at 20 °C. Treatments 2, 4 and 6 were stored at 4 °C. Values are mean ± SD of two replicates. *, significant difference with the day 0.

in turn led to the changes in strain proportions. This may affect not only composition but also metabolic activity of the multi-strain association.

The investigated association consisted of seven bacterial strains naturally adapted to acid tar-contaminated soil and, therefore, suitable for bioremediation applications (unpublished data) in the presence of toxic substances. Attempts were made to improve the survival of the association through immobilization in alginate gel to make it more resistant to different kinds of pollution. Further studies are necessary to evaluate bioremediation activities of immobilized associations in laboratory and field conditions and molecular approaches are needed to investigate fate of individual components.

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