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Immobilisation of Selected Bacteria for Remediation on Various Media

Immobilizacja wybranych bakterii o znaczeniu remediacyjnym na różnych nośnikach

Soil degradation generates the need for reclamation measures. Bioaugmentation represents a promising method to treat degraded soils. The introduction of microorganisms into the soil often requires immobilisation. Porous media, such as biochar, have been used for the purpose. The introduction of individual bacteria into the soil requires the choice of a suitable medium to ensure high bacterial survival rate and effective bioaugmentation. The possibility of immobilization of *Bacillus subtilis*, *Pseudomonas fluorescens*, *Azospirillum brasilense* and a bacterial consortium by means of encapsulation in alginate with the addition of biochar, nanosilica, perlite or vermiculite was analysed in the study. The best effects of *B. subtilis* immobilization were obtained with alginate with 5 ml of biochar, *P. fluorescens* - with 25% (v/v) perlite and vermiculite, while *A. brasilense* - with 25% (v/v) alginate and 50% (v/v) perlite. The most effective immobilization of the bacterial consortium was achieved using alginate with addition of 50% (v/v) of biochar.

Keywords: **immobilization, bioaugmentation, degraded soil, soil contamination, bioremediation**

Introduction

Soil quality represents a determinant of its suitability for agricultural use. Soil contamination, resulting mainly from the progress of industry and urbanization, excludes such a possibility [1]. The process of self-treatment of contaminated soil is time-consuming. Therefore, human intervention and the use of soil treatment techniques are necessary [2]. Biological methods are of particular importance. Bioremediation is a process using the ability of select organisms (microorganisms, plants) to grow in toxic conditions and to decompose toxic substances. Enzymes produced by microorganisms can also be used [3, 4]. Bioremediation is a promising technique due to a number of its advantages, which include in particular the opportunities for *in situ* biological reclamation, cost-effectiveness, and no need for specialized equipment. Another great advantage is processing of the toxic substance,

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which, unlike other methods, involves transport of the toxic agent to other locations. The use of bioremediation allows for neutralization of substances that are difficult to decompose, such as petroleum derivatives and heavy metals [5-7]. Despite many advantages, this process is time-consuming and difficult to control [7, 8].

Bioaugmentation, i.e. introduction of microorganisms (inoculum) into the soil, leads to biological decomposition of contaminants present in the soil [9]. This technique uses plant growth-promoting bacteria (PGPR) and can be used in agriculture to improve yields. The microorganisms used must show both high rate of decomposition, high mobility and the ability to adapt quickly to new environmental conditions [8, 9]. Microorganisms resistant to heavy metals (e.g. *Pseudomonas aureginosa*, *Bacillus sp.*, *Aspergillus sp.*, *Pseudomonas sp.*, *Fusarium flocciferum*, *Ascophyllum nodosum*, *Proteus mirabilis*) are most commonly used in biological reclamation of areas contaminated with these elements. Furthermore, microorganisms such as *Micrococcus sp.*, *Chyseomonas luteola*, *Bacillus sp.*, *P. aureginosa*, *Flavobacterium*, *Phanaerochaete chrysosporium*, *Mycobacterium flavescens* are used in bioaugmentation of soils contaminated with petroleum derivatives [10-13].

An important aspect of bioaugmentation is immobilisation of microorganisms, which allows for preservation of their potential (catalytic properties). The choice of a suitable medium is a factor that limits the effectiveness of bioaugmentation. Different materials with high porosity such as biochar or silica have been used for the preparation of media [14]. This paper discusses the opportunities for immobilization of microorganisms on various media for the bioaugmentation process.

The aim of the study was to find the most effective method of immobilization of *Bacillus subtilis, Pseudomonas fluorescens, Azospirillum brasilense* and a bacterial consortium consisting of the above mentioned bacteria to be used in the process of bioaugmentation of degraded soils.

1. Materials and methods

1.1. Materials

Biological material. Three strains of bacteria that promote plant growth were used in the study: *B. subtilis*, *P. fluorescens*, *A. brasilense*. These bacteria were obtained from the collection of the Institute of Environmental Engineering. Properties of individual bacteria strains are shown in Table 1. Growth media used to prepare biological material were LB, LB agar, NFB and Agar King B.

Media. Soil additives, such as biochar, liquid biochar, perlite, vermiculite and hydrophilic nanosilica, were used as bacterial media.

Soil. Anthropogenic soil was sampled from an open-pit coal mine in central Poland (51°15′54″N 19°4′41″E). Detailed soil characterization was contained in the previous publication [16].

Table 1. **Characteristics of bacteria [15]**

Bacteria	*Bacillus subtilis*	*Pseudomonas fluorescens*	*Azospirillum brasilense*
Respiration	Aerobic, relatively aerobic	Aerobic	Aerobic
Gram staining	G (–)	G (–)	G (–)
Fluorescence under UV light	+	+	–
Nitrogen fixation	+	+	+
Production of siderophores	+	+	+
Production of IAA[1]	+	+	+
ACC[2] activity	+	+	+

[1] IAA - indole-3-acetic acid
[2] ACC deaminase activity; conversion of 1-aminocyclopropane-1-carboxylic acid, a precursor of ethylene, to α-ketobutyric acid and ammonia

1.2. Methods

1.2.1. Biomass preparation

The microorganisms were first identified by culture on microbiological media: LB agar (*B. subtilis*), Agar King B (selective medium for *P. fluorescens*) and NFB (selective medium for *A. brasilense*). Microorganisms were incubated for 7 days at 32°C. Characterization of the colonies grown on individual media was used as a basis for strain identification. Next, pure bacterial cultures were grown in the same liquid media in 32 sterile bottles protected with parafilm to allow access to oxygen. At this stage, a bacterial consortium consisting of the same proportions of individual microorganisms was also prepared. Incubation performed on liquid agar with shaking was performed for 24 hours at 28°C. After the incubation period, the number of grown microorganisms was determined by measuring the suspended solids density using the HACH DR/4000V (USA) spectrophotometer at a wavelength of $\lambda = 600$ nm and compared to the reference curve.

1.2.2. Media preparation

The media were processed mechanically and then sterilized. Biochar and perlite were ground in the frozen state in liquid nitrogen using the cryogenic mill Frezer/Mill 6875 (Spex® SamplePrep, UK). The media were then sieved through a sieve with a mesh diameter of 2 mm.

1.2.3. Preparation of alginate capsules

The 1.5% alginate solidified in 0.5% calcium carbonate was used to prepare the capsules of media. Appropriate weighed amounts of soil additives with 1 ml of LB growth medium and 5 ml of PBS buffer (phosphate-buffered saline) were introduced (Table 2) to individual bottles containing grown bacterial strains or bacterial

consortium. The media volumes were chosen based on the optimization in order to allow for easy and efficient preparation of the capsules in the medium-alginate system. The volume of alginate was such that it supplemented the medium to the volume of 50 ml.

Table 2. **Volumes of individual soil additives used to immobilise bacteria**

Medium	Volume
Biochar	5 ml
	25 ml
Perlite	12.5 ml
	25 ml
Vermiculite	12.5 ml
	25 ml
Nanosilica	12.5 ml
	25 ml

1.2.4. Bioaugmentation

The amounts of 10 g media and media without microorganisms were weighed and introduced into sterilized soil (300 g). In the case of media without alginate, 5 ml of PBS and 1 ml of LB medium were added. The samples were mixed and placed in pots. Control pots containing individual pure bacterial cultures or a consortium without media with 5 ml of PBS buffer and 1 ml of LB medium were also prepared. Incubation was continued for 21 days, with the pots watered every second day with an equal amount of sterile water. The process diagram is shown in Figure 1.

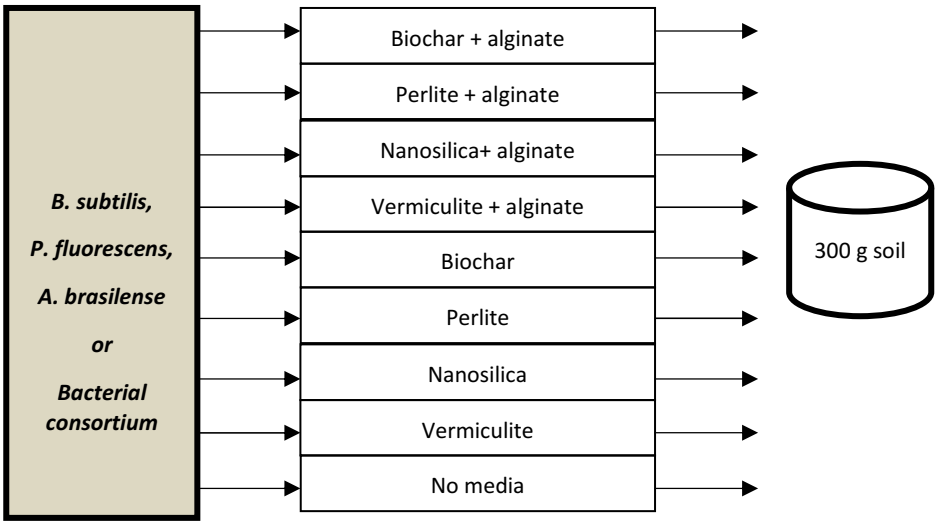

Fig. 1. Bioaugmentation variants used in the study

1.2.5. Measurement of bacterial count on media

The amounts of 1 g of prepared capsules and the respective amounts of media without alginate were subjected to tests determining the amount of immobilized microorganisms by maceration in 9 ml of PBS buffer for 10 min at 300 rpm and 28°C, and spectrophotometric measurement with $\lambda = 600$ nm. Cultivation on appropriate agar media was also carried out. Then the microorganisms were incubated for 24 hours at 32°C, with growing colonies counted.

1.2.6. Batch-test for microorganism leachability

During the test, 75 ml of sterile distilled water was added to an equal amount of 2.5 g of alginate capsules and an adequate amount of media without alginate. The prepared mixtures were shaken intensively for 2 hours. Next, the samples were centrifuged for 5 minutes at a rate of 11200 x g (Centrifuge 5804R) and the procedure described in 1.2.3 was used.

1.2.7. Measurement of bacterial population in soil after 21-day incubation

Equal amounts of 1 g of soil from each sample were macerated in 9 ml of sterile PBS buffer for 10 minutes by shaking them at 300 rpm. Next, the procedure was the same as in 1.2.3.

2. Results and analysis

2.1. Leachability of bacteria from media

The leachability of bacteria from media was an important indicator of the ability of media to immobilise microorganisms and may indicate the potential for their transfer. The results of the BATCH-TEST are illustrated in Figure 2.

A high percentage of leaching of *B. subtilis* from the media was obtained in samples of P1+A, P2+A, S1+A, W2+A and S1. Furthermore, insignificant leaching of bacteria *B. subtilis* was recorded for media without alginate except for the S1 sample. In the case of *P. fluorescens* bacteria, large leaching of bacteria, oscillating between 85÷95%, was observed in samples of B2+A, W2+A and S2+A. Low leaching of *P. fluorescens* was observed for samples of media without alginate. In the case of *A. brasilense* bacteria, a high degree of microbial leaching was observed for samples of media with alginate and perlite without alginate. A low level of leaching of *A. brasilense* microorganisms was found for samples without alginate (except for perlite), especially in samples B1 and W1. Large leaching of the bacterial consortium was observed for media P1+A, S2+A, W2+A, P2, while low leaching was found in samples: B1+A, B1, S2, W1 and W2.

Microorganism transfer to degraded soil is important from the standpoint of biological soil treatment using microorganisms. The number of microorganisms present in contaminated soil is a limiting factor in the effectiveness of the bioremediation process. The effectiveness of the process of anthropogenic soil bioaugmentation with individual bacteria and bacterial consortium with the use of the tested media is presented in Figures 3-6.

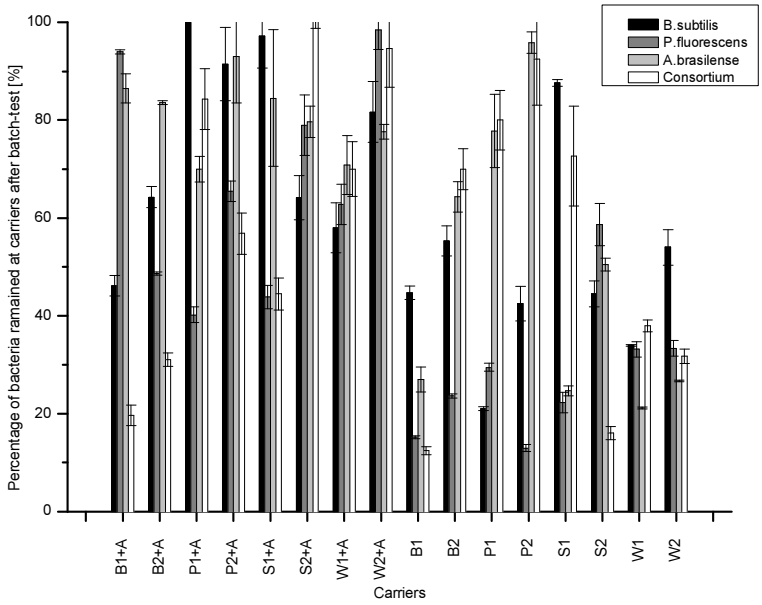

Fig. 2. Percentage of bacteria remaining on media after leaching (B1+A - alginate medium with addition of 10% (v/v) of biochar, B2+A - alginate medium with addition of 50% (v/v) of biochar, P1+A - alginate medium with addition of 25% (v/v) of perlite, P2+A - alginate medium with addition of 50% (v/v) of perlite, S1+A - medium of alginate with addition of 25% (v/v) of nanosilica, S2+A - medium of alginate with addition of 50% (v/v) of nanosilica, W1+A - medium of alginate with addition of 25% (v/v) of vermiculite, W2+A - medium of alginate with addition of 50% (v/v) vermiculite; B1, B2, P1, P2, S1, S2, W1, W2 - respective media in analogous volumes without alginate)

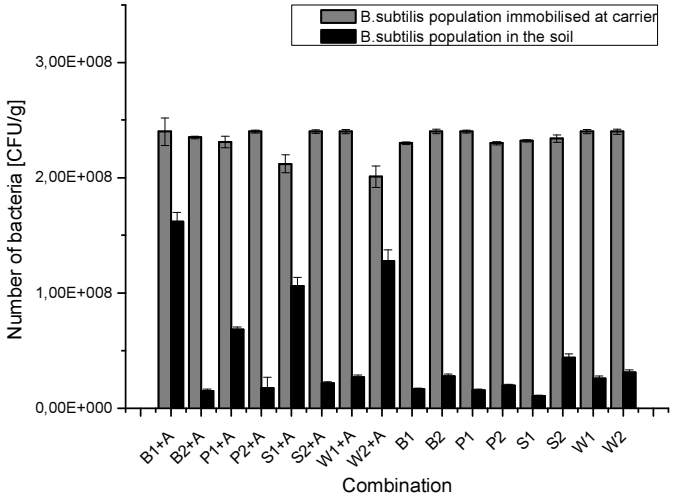

Fig. 3. Count of *B. subtilis* bacteria immobilized on media and in soil after 21 days of incubation (B1+A - alginate medium with addition of 10% (v/v) of biochar, B2+A - alginate medium with addition of 50% (v/v) of biochar, P1+A - alginate medium with addition of 25% (v/v) of perlite, P2+A - alginate medium with addition of 50% (v/v) of perlite, S1+A - medium of alginate with addition of 25% (v/v) of nanosilica, S2+A - medium of alginate with addition of 50% (v/v) of nanosilica, W1+A - medium of alginate with addition of 25% (v/v) of vermiculite, W2+A - medium of alginate with addition of 50% (v/v) vermiculite, B1, B2, P1, P2, S1, S2, W1, W2 - respective media in analogous volumes without alginate)

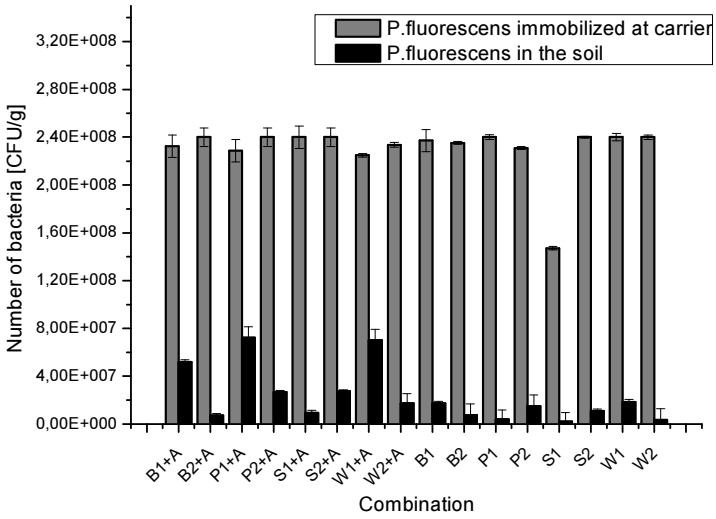

Fig. 4. Number of *P. fluorescens* bacteria immobilized on media and in soil after 21 days of incubation (B1+A - alginate medium with addition of 10% (v/v) of biochar, B2+A - alginate medium with addition of 50% (v/v) of biochar, P1+A - alginate medium with addition of 25% (v/v) of perlite, P2+A - alginate medium with addition of 50% (v/v) of perlite, S1+A - medium of alginate with addition of 25% (v/v) of nanosilica, S2+A - medium of alginate with addition of 50% (v/v) of nanosilica, W1+A - medium of alginate with addition of 25% (v/v) of vermiculite, W2+A - medium of alginate with addition of 50% (v/v) vermiculite, B1, B2, P1, P2, S1, S2, W1, W2 - respective media in analogous volumes without alginate)

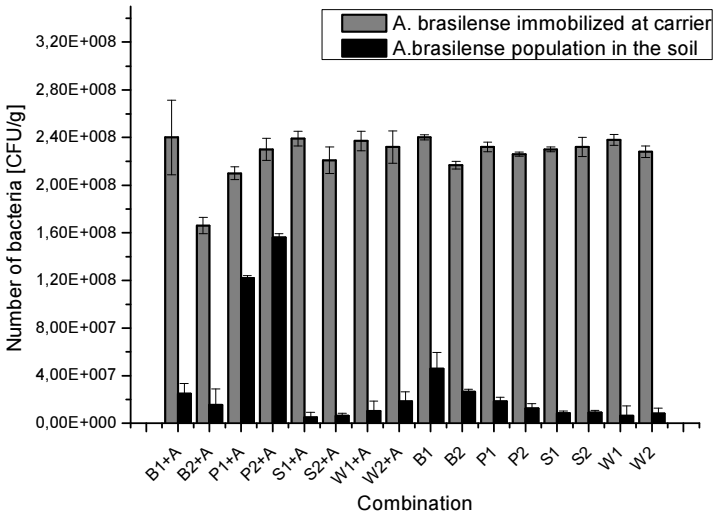

Fig. 5. Number of *A. brasilense* bacteria immobilized on media and in soil after 21 days of incubation (B1+A - alginate medium with addition of 10% (v/v) of biochar, B2+A - alginate medium with addition of 50% (v/v) of biochar, P1+A - alginate medium with addition of 25% (v/v) of perlite, P2+A - alginate medium with addition of 50% (v/v) of perlite, S1+A - medium of alginate with addition of 25% (v/v) of nanosilica, S2+A - medium of alginate with addition of 50% (v/v) of nanosilica, W1+A - medium of alginate with addition of 25% (v/v) of vermiculite, W2+A - medium of alginate with addition of 50% (v/v) vermiculite, B1, B2, P1, P2, S1, S2, W1, W2 - respective media in analogous volumes without alginate)

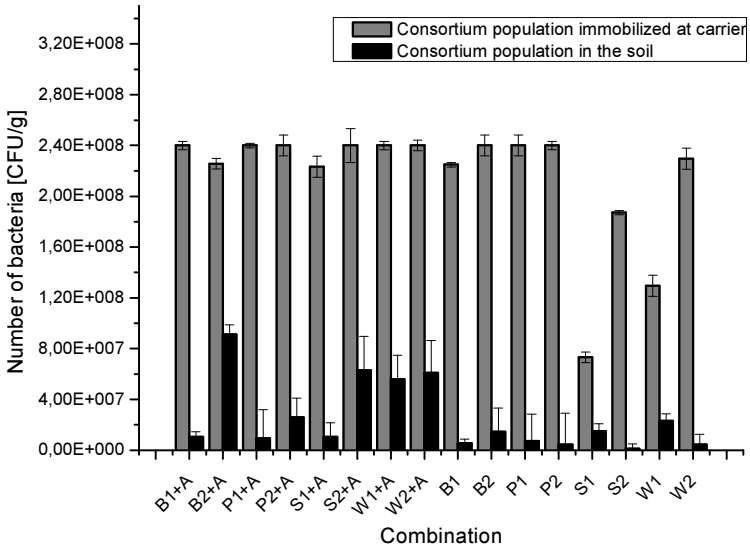

Fig. 6. Number of bacterial consortium immobilized on media and in soil after 21 days of incubation (B1+A - alginate medium with addition of 10% (v/v) of biochar, B2+A - alginate medium with addition of 50% (v/v) of biochar, P1+A - alginate medium with addition of 25% (v/v) of perlite, P2+A - alginate medium with addition of 50% (v/v) of perlite, S1+A - medium of alginate with addition of 25% (v/v) of nanosilica, S2+A - medium of alginate with addition of 50% (v/v) of nanosilica, W1+A - medium of alginate with addition of 25% (v/v) of vermiculite, W2+A - medium of alginate with addition of 50% (v/v) vermiculite, B1, B2, P1, P2, S1, S2, W1, W2 - respective media in analogous volumes without alginate)

A high bacterial count of *B. subtilis* in soil was obtained for samples of B1+A, S1+A and W2+A. In media without alginate, a low *B. subtilis* count was observed in soil after 21 days of incubation. The smallest number of bacteria *B. subtilis* in soil was recorded for sample S1.

In the case of *P. fluorescens* bacteria, their count in soil was lower compared to *B. subtilis* bacteria. Among the media tested, the highest bacterial transfer to soil was observed for samples of B1+A, P1+A and W1+A. However, the increase in the volume of these alginate capsules significantly decreased the efficiency of the transfer to the soil. The lowest bacteria count in the soil after 21 days of the process was observed in S1 and W1 samples. In the *P. fluorescens* immobilization samples using media without alginate, bacterial transfer to soil was lower compared to media with alginate.

In the case of *A. brasilense* bacteria, the highest bacterial transfer to soil after 21 days of incubation was observed for samples of P1+A and P2+A. In the remaining samples of media, the number of bacteria in soil was significantly lower compared to the dominant samples.

As in the case of *A. brasilense* bacteria, much lower bacterial transfer into the soil was observed in the samples of media without alginate compared to those with alginate. The highest bacterial count in soil after 21 days of process was found for B2+A, S2+A, W1+A and W2+A samples.

3. Discussion

Many studies in the literature have analysed immobilization of microorganisms for industrial processes, but few of them concerned application in the soil. High efficiency of immobilization has been demonstrated for immobilization of *Pseudomonas sp.* on alginate, acrylamide [17], 3-chloroaniline [18] and 2-chloroethanol [19]. Frequently used media also include polyurethane [20], agar [21-23] and kappa-carrageenan [24]. The highest number of microorganisms in the soil for the applied bacterial consortium immobilized on alginate with 50% (v/v) of biocarbonate was noted in the conducted studies. Similar results were obtained in Eklahout et al., where positive effects of immobilization of *Rhodobacter capsulatus* using agar on pH stability during the process were documented [23]. Immobilized bacteria also impact on the control of phytopathogenic fungal populations [24]. Immobilization of microorganisms on media is an effective method of reducing the effect of stress factors on the populations of microorganisms introduced into the soil environment. This phenomenon was also confirmed by Chen et al., who studied the effect of chitosan used as a medium for *P. putid* bacteria and found an increase in bacterial resistance to higher TCE concentrations [25]. The best effects of *B. subtilis* immobilization were obtained with the use of alginate with addition of 5 ml of biochar. A similar tendency for the applied carbon addition, i.e. an increase in the bacterial population (CFU) on activated carbon reaching 10^{10} g^{-1} compared to zeolite (10^{6} g^{-1}) was obtained in the study by Liang et al. [26]. Furthermore, immobilisation of microorganisms on activated carbon resulted in a 12-fold increase in the activity of microorganisms compared to immobilisation based on the use of zeolite. A larger population of immobilised organisms introduced into a specific environment intensifies the target effect. The study also demonstrated a species preference for specific media and the associated higher survival of microorganisms in the soil. It can therefore be concluded that there is no universal medium. Consequently, the type of medium, in addition to the species of microorganisms used, has an effect on the intensity of microbiological processes such as bioremediation. Liang et al. found increased petroleum biodegradation in soil (by almost 50%) with the use of immobilization of microorganisms on activated carbon [26]. Furthermore, in a study by Balfranz and Rehm, the results of immobilization of *Alcaligenes* sp. A 7-2 on granulated clay in order to degrade 4-chlorophenol allowed to investigate and intensify degradation of 4-chlorophenol by adding immobilized bacteria [27]. In a study by Ajao et al., the researchers attempted to immobilize *B. subtilis* and *P. aureginosa* bacteria on an agar-agar medium in order to intensify treatment of wastewater from the textile industry [28] and achieved a significant process improvement. High microbiological activity was also strongly correlated with intensification of PAH degradation in industrial soils [16]. The basic material used for immobilisation should have a large active surface, accessible to microorganisms, and a matrix that physically binds the microorganisms to the medium. In a study by Liffourren and Lucchesi [29], an attempt was made to immobilize *P. fluorescens* in alginate-perlite capsules. Similar tendencies were

found in the present study. In this study, the count of bacteria immobilized on alginate perlite media was much higher compared to alginate medium. Thirumal et al. found higher efficiency of microorganism cell immobilization with alginate and a higher cell transfer to the environment [30]. A study on immobilization of *Micro* sp. and *Bacillus* sp. on vermiculite showed immobilization of 10^6 CFU *Bacillus* sp./g and 10^{12} CFU *Micro* sp./g [31]. Similar high immobilization of cells was achieved in the present study. Scientists remain to be interested in selection of media for microorganisms in order to achieve effective bioaugmentation.

Conclusions

Bacterial immobilization on media is an effective method for soil application. The experiment presented in the study allows for determination of the most effective method of immobilization of individual bacteria and bacterial consortium. The most effective method of immobilization of *B. subitilis* was encapsulation in alginate with the addition of 5 ml of biochar. An alternative method may be encapsulation in alginate with 25% (v/v) addition of nanosilica or 50% (v/v) addition of vermiculite. In the case of *P. fluorescens,* the most effective method was encapsulation with addition of 25% (v/v) perlite or 25% (v/v) vermiculite. The most effective method in bioaugmentation of *A. brasilense* bacteria was encapsulation with addition of 25% (v/v) or 50% (v/v) perlite. The addition of 50% (v/v) of perlite improved the effectiveness of bioaugmentation by 33.33% compared to encapsulation with addition of 25% (v/v) of perlite. In the case of a bacterial consortium, the most effective method of bioaugmentation was the use of bacteria immobilized on alginate with 50% (v/v) of biochar.

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Streszczenie

Degradacja gleb generuje konieczność podjęcia działań rekultywacyjnych. Bioaugmentacja stanowi obiecującą metodę oczyszczania gleb zdegradowanych. Wprowadzenie mikroorganizmów do gleby bardzo często wymaga zastosowania immobilizacji. Do tego celu wykorzystuje się porowate nośniki, takie jak biowęgiel. Wprowadzenie poszczególnych bakterii do gleby wymaga doboru odpowiedniego nośnika zapewniającego wysoką przeżywalność bakterii oraz umożliwiającego efektywną bioaugmentację. W niniejszej pracy określono możliwość immobilizacji *Bacillus subtilis*, *Pseudomonas fluorescens*, *Azospirillum brasilense* oraz konsorcjum bakteryjnego poprzez kapsułkowanie w alginianie z dodatkiem biowęgla, nanokrzemionki, perlitu lub wermikulitu. Najlepsze efekty immobilizacji *B. subtilis* uzyskano z zastosowaniem alginianu z dodatkiem 5 ml biowęgla, *P. fluorescens* z wykorzystaniem 25% (v/v) perlitu i wermikulitu, natomiast *A. brasilense* 25% (v/v) alginianu i 50% (v/v) perlitu. Najefektywniejszą immobilizację konsorcjum bakteryjnego uzyskano z wykorzystaniem alginianu z dodatkiem 50% (v/v) biowęgla.

Słowa kluczowe: **immobilizacja, bioaugmentacja, gleba zdegradowana, bioremediacja**