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EXPERIMENTAL ARTICLES

Role of Alkylhydroxybenzenes in Bacterial Adaptation to Unfavorable Growth Conditions

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Abstract—The adaptogenic effect of the chemical analogues of alkylhydroxybenzenes (AHBs), bacterial extracellular autoregulators (the individual compound C7-AHB and its technical preparation Sidovit), was demonstrated for two pseudomonad species, *Pseudomonas aeruginosa* and *P. fluorescens*. The protective effect of AHBs resulted in increased growth rate and biomass accumulation in bacteria grown under suboptimal conditions within the species tolerance range. The adaptogenic effect of AHBs (10–50 μmg/l) was more pronounced under more unfavorable growth conditions. In the case of *P. fluorescens*, the individual compound C7-AHB increased the biomass yield by 30% under alkaline conditions (pH 9.5), when the growth rate decreased by 80–90% compared to the optimum (pH 5.5–7.5). The Sidovit preparation, containing a mixture of natural AHBs with C7-AHB as the main component, increased the growth rate of *P. aeruginosa* by 40–60% at nonoptimal temperatures (45 and 10°C) or under enhanced salinity (1% NaCl). The action of AHBs as regulators of the *rpoS* and SOS response stress regulons was demonstrated to be among the mechanisms of their adaptogenic effect, as was demonstrated with the relevant reporter genes in the model strains *E. coli* C600 *thi, thr, leuΔ(pro-lac)* with the *osmE-lacZ* and *umuD-lacZ* hybrid operons, respectively. AHBs are technologically and economically acceptable as adaptogenic supplements for bacterial preparations used in soil bioremediation and oil spillage removal under conditions unfavorable for microbial growth, including increased salinity, extreme pH, and fluctuating sub- or supraoptimal temperatures.

Keywords: bacteria, stress, adaptation, alkylhydroxybenzenes, protectors, efficiency of bacterial preparations, soil bioremediation.

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The effect of microbial alkylhydroxybenzenes (AHBs) on the resistance of various pro- and eukaryotic organisms under sublethal and lethal levels of oxidation or temperature stress and shock factors has been demonstrated previously [1, 2]. Little is known, however, concerning the effect of adaptogens, including AHBs, on microbial growth under conditions when some physicochemical parameter (temperature, pH, salinity, etc.) is constantly nonoptimal. A positive effect of AHBs on the indices of microbial growth in the extreme zone was demonstrated only for the case of yeasts growing under hyperosmotic conditions [3].

The protective effect of AHBs, resulting in decreased cell death, depends partially on their antioxidant functions, which cause a decrease in the level of reactive oxygen species (ROS) [4, 5]. Moreover, since AHBs may modify the structure of biopolymers, this possibly results in increased activity and functional and operational stability of enzyme proteins in a wide range of conditions nonoptimal for catalysis [6–8]. The stress-protective effect of AHBs results also from their ability to interact with the membrane phospholipids, causing structural reorganization of the lipid stroma of the membrane and affecting its rigidity. This has an effect on the functional activity of the membrane [8, 9]. AHBs are also capable of interaction with DNA, enhancing its resistance to denaturing factors [9, 10].

The protective effect of AHBs was found to depend on their structure and concentration [1, 3]. Since the mentioned mechanisms of AHB action increasing cell resistance to damaging factors are probably universal, the possibility of an adaptogenic effect of AHBs on bacterial cultures under nonoptimal, growth-inhibiting conditions (similar to those occurring in natural microbial habitats) seemed interesting. The problem of microbial adaptation to stress, apart from its fundamental importance, is related to numerous practical issues when bacterial preparations of various composition and purpose are used under conditions nonoptimal for microbial growth. Such conditions occur, for example, in the course of ensilage, application of plant protection techniques, soil bioremediation, or decontamination of environmental objects polluted by xenobiotics or petroleum products.

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The goal of the present work was to investigate the effect of alkylhydroxybenzenes (both as individual compounds, the chemical analogues of microbial autoregulators, and as a technical AHB preparation) on stress resistance and the range of active growth of pseudomonads developing under the influence of the factors most commonly occurring in natural environments (elevated NaCl concentration and supra- and suboptimal values of pH and temperature).

MATERIALS AND METHODS

Bacteria *Pseudomonas fluorescens* (strain NCIMB 9046) and *P. aeruginosa* (strain 202 obtained from the collection of I.A. Borzenkov) were used in the work. These species are widespread in nature. Bacteria were grown in liquid M9 medium [11] with glucose (2 g/l) or acetate (1 g/l) in 60-ml vials (20 ml of the medium) under shaking (180 rpm). Stationary cultures were used as inocula (2–5% vol/vol). The values of pH, temperature, and salinity were adjusted according to specific experimental tasks.

The adaptogens used were preparations of individual alkylhydroxybenzenes, amphiphilic C7-AHB and hydrophobic C12-AHB (99%, synthesized in the Moscow Academy of High Chemical Technology), and the Sidovit technical preparation (New Technologies Ltd., Russia), which contains 65% of C7-AHB and AHBs with longer chains.

The respiration rate as CO_2 emission determined on an Infralit infrared analyzer (Germany) and the number of colony-forming units (CFU) developing on nutrient agar were used as criteria of the effect of AHBs on bacterial growth. For respiration rate measurement, the vials were purged with CO_2-free air (bubbled through 20% KOH), sealed with rubber stoppers, and incubated for 1–3 h, depending on the growth phase. CO_2 content was then determined. The respiration rate for 20 ml of the culture was expressed in μg C-CO_2/h. Since preliminary experiments demonstrated that CO_2 accumulation was linear within a 1- to 3-h period, the respiration rate was considered proportional to the biomass (yield). The growth rate was expressed as a daily increment of respiration rate [μg C-CO_2/h 20 ml culture day].

The regulatory properties of AHBs were investigated using the reporter gene method. Based on *Escherichia coli* C600 *thi, thr, leu*Δ*(pro-lac)*, the tester (reporter) strains were constructed [12], with the *osmE-lacZ* (*osmE* encodes the stress-inducible lipoprotein of the outer membrane and is a part of the *rpoS* regulon of the stationary phase) and *umuD-lacZ* hybrid operons (*umuD* encodes the low-precision polY polymerase and is a part of the SOS response operon). The hybrid operons were inserted into a pJEL250 low-copy transcription vector containing an ampicillin resistance determinant and transferred into *E. coli* cells via transformation. The constructed strains um250 (*E. coli* C600 *thi, thr, leu*Δ*(pro-lac)*/pJEL250Amp^R*umuD-lacZ*) and os250 (*E. coli* C600 *thi, thr, leu*Δ*(pro-lac)*/pJEL250Amp^R*osmE-lacZ*) were used to assess the effect of adaptogens on expression of the SOS response genes and *rpoS* regulon genes, respectively. Expression of the hybrid operons *osmE-lacZ* and *umuD-lacZ* resulted in β-galactosidase accumulation. The specific activity of this enzyme (per unit OD) was determined by the standard procedure as cleavage of *o*-nitrophenyl-β-galactopyranoside with formation of a spectrophotometrically determined colored product. The amount of the enzyme resulting in 1 U OD_{420} increase per 1 min was used as the unit of β-galactosidase activity.

E. coli cells were grown in LB medium [11] with 50 mg/l ampicillin in shaken (160 rpm) 250-ml flasks with 50 ml of the medium. The inoculum (stationary-phase cultures) was added to the initial optical density of 0.2 (Specord, $\lambda = 650$ nm, $l = 10$ mm).

For determination of the effect of AHBs on bacterial stress reactions, an aliquot of the exponential (strain um250) or stationary-phase (strain os250) *E. coli* culture was supplemented with a required concentration of AHBs (as an aqueous solution) and incubated at 28°C for 80 min under static conditions. The cells were then separated by centrifugation (4000 *g*, 15 min) and washed twice with Z buffer and β-galactosidase activity was determined [13]. In the experiments on protective effect of AHB, the samples of strain um250 were preincubated with AHB for 1 h under static conditions and UV-irradiated for 10 min (DB 30-1W lamp, 0.8 m from the light source). After irradiation, the samples were incubated for 10 min prior to determination of β-galactosidase activity. In experiments with strain os250, after 1-h incubation with AHB, 1.5% NaCl was added to the sample and β-galactosidase activity was determined after 20-min incubation. In the control variants, the samples were incubated with an equal volume of the solvent (water).

Statistical treatment by the standard Student test was carried out for the results of three experimental series with three repeats in each using the Microsoft Excel software package. The parameters were calculated for $P < 0.05$. The figures represent the typical experimental results. In the tables, average values for three series are given.

RESULTS AND DISCUSSION

The adaptogenic effect of individual alkylhydroxybenzenes (C7- and C12-AHB) on the growth characteristics of *P. fluorescens* was studied under extreme pH (optimal pH for growth is 5.5–7.5). At pH 4.5 and 8.5 and pH 3.5 and 9.4–10, the growth rate was two and ten times lower, respectively. The effect of C7- and C12-AHB of *P. fluorescens* growth was studied at pH values of 3.5 and 9.4. C7- and C12-AHB were added in the concentrations of 30 and 10 mg/l, respectively. Preliminary experiments revealed no effect of AHB homologues in such concentrations on the growth

parameters under standard conditions (pH 6.5). At pH 3.3–3.6, addition of C7- and C12-AHB had no stimulatory effect. At pH 9.4, growth stimulation was reliably observed; in a 48-h culture with C7-AHB (30 mg/l) and C12-AHB (10 mg/l), the yield increased by 30 and 10%, respectively.

The protonophore properties of AHBs may be responsible for their protective effect under alkaline conditions and the absence of such an effect under acidic conditions [7]. Under alkaline conditions, protonophore action of AHBs enhances the processes of substrate oxidation and, consequently, CO_2 production, which results in a pH shift to neutral values. Under acidic conditions, the protonophore effect of AHB is more pronounced, since the protons in its hydroxyl groups are not dissociated. High AHB concentrations may uncouple the respiration and the energy-yielding processes, with no pronounced protective effect.

Unlike C12-AHB, C7-AHB was previously shown to exhibit protective features under conditions of oxidative stress [1] and heat shock [2]. The subsequent experiments were therefore carried out with C7-AHB and Sidovit, a C7-AHB-based technical preparation.

The adaptogenic effect of Sidovit was studied on a culture of the petroleum-oxidizing *P. aeruginosa* grown in M9 medium with acetate under conditions of elevated salinity, simulating natural stress. The *P. aeruginosa* strain used in this work was highly sensitive to salt concentration in the medium. At 1% NaCl without the preparation (control), the growth rate was 23% lower than under optimal conditions. No growth occurred at NaCl concentrations above 5%. In the experimental variants, Sidovit (10 mg/l) was used as an adaptogen. In the variant with Sidovit and 1% NaCl, the growth rate and the biomass were 39 and 43%, respectively, higher than in the control. In the presence of AHBs without NaCl, these parameters also increased, albeit less markedly, by 23 and 10%, respectively (Fig. 1).

These results demonstrate that Sidovit stimulates the growth of *P. aeruginosa* under both normal and stress conditions (1% NaCl), although its effect is more pronounced under stress conditions. However, at 3% NaCl, the adaptogenic effect of Sidovit did not equalize the inhibitory action of NaCl. Both in the presence and absence of AHB, the biomass increase on the third day of cultivation with 3% NaCl was only 33% of the control value (Fig. 1b). Thus, the adaptogenic action of AHB resulted in a considerable increase in the growth rate under moderately nonoptimal cultivation conditions.

The cultivation temperature is another environmental factor affecting bacterial growth. The optimal growth temperature for *P. aeruginosa* is 28°C, with the growth rate at 10 and 40°C being 25 and 50% of the maximum, respectively. Under these nonoptimal conditions, Sidovit stimulated bacterial growth (Fig. 2). Its effect was lowest (17%) at the optimal temperature

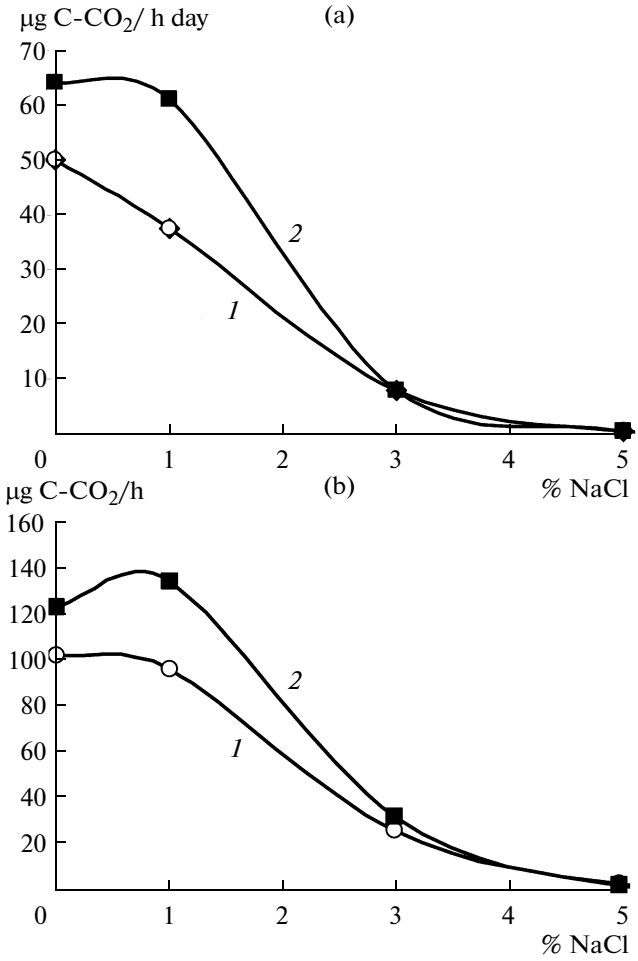

Fig. 1. Effect of Sidovit (10 mg/l) on the growth rate (a) and biomass yield (b) of *P. aeruginosa* at different NaCl concentrations. Without Sidovit (*1*) and with Sidovit (*2*).

(28°C) and increased at the borders of the temperature range. In the control, the growth rate at 10 and 40°C decreased by 75 and 50%, respectively. In the presence of an AHB preparation, the decrease in growth rates was less pronounced (65 and 22%, respectively). Thus, the growth rate increased by 40–55%, respectively (Fig. 2).

The adaptogenic effect of AHBs results partially from their antioxidant activity, resulting in decreased levels of reactive oxygen species, which are abundantly formed under stress conditions [1, 5]. Moreover, AHBs are capable of direct interaction with enzyme proteins, with the changed conformation and dynamics of their molecules resulting in their increased catalytic activity, as well as higher functional and operational stability [6]. The latter is probably especially important in the case of bacteria developing under nonoptimal temperatures close to the borderline for the species.

Participation in the regulation of expression of the stress regulon genes may be another component of the

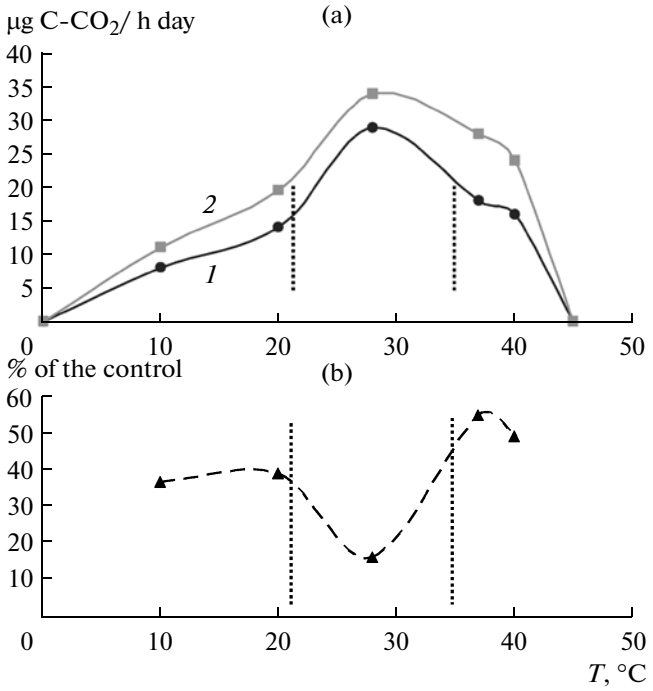

Fig. 2. Effect of Sidovit (10 mg/l) on the growth rate of *P. aeruginosa* at different temperatures: control (without AHBs) (*1*) and with AHBs (*2*). Efficiency of the increase in the growth rate in the presence of the preparation, % of the control (b).

adaptogenic effect of AHBs under nonoptimal growth conditions. This statement was confirmed by the experiments involving *E. coli* tester strains (um250 and os250) carrying the *umuD-lacZ* and *osmE-lacZ* hybrid operons on the transcriptional plasmid transformed into the cell. The levels of UV irradiation or increased salinity used in our experiments resulted in decreased growth rates, though not in growth inhibition, which occurs at higher intensities of the stress factors.

In our experiments, β-galactosidase activity was determined in the exponential-phase cells of strain um250 after preincubation with Sidovit for 1 h and UV irradiation for 10 min (Table 1).

In the control variants (without AHBs), UV-irradiation resulting in decreased growth rates caused a 30% increase in the expression of the reference enzyme. This was an indication of weak activation of the SOS response genes. Incubation with AHBs had a concentration-dependent effect on the cell responses. The dependence was not linear. Introduction of the preparation (250 and 500 mg/l) had the same effect on the activation of the reference gene (30%) as irradiation (X_n/Y_0). The similarity of the cell responses to a natural stressor and to enhanced AHB concentrations suggests the role of AHB as a population stress signal.

In the variants with irradiation of the cells preincubated with AHB (50 and 100 mg/l), expression of the *umuD* gene decreased by ~30%, indicating the protective function of C7-AHB (Y_n/Y_0), probably resulting from its previously demonstrated antioxidant effect [4, 5]. Preincubation of the cells with higher AHB concentrations (250 and 500 mg/l), which resulted in enhanced expression of the stress genes in the control variants, leveled out the activation caused by UV-irradiation. This phenomenon may be interpreted as a protective effect (Table 1), which has been previously described for other stress reactions. Analysis of the levels of regulation of the SOS response gene expression by AHBs makes it possible to differentiate the types of protective effect. The levels of regulation were calculated as the ratio of β-galactosidase activity in cells preincubated with AHBs and irradiated with UV to the enzymatic activity in the cells: (i) preincubated with AHBs and not irradiated (Y_n/X_n) as the preadaptation index and (ii) irradiated and not preincubated (Y_n/Y_0) as an index of protection due to neutralization of the stressor effect. The ratio for the cells preincubated with AHBs and not irradiated to those irradiated and not preincubated (X_n/Y_0) was calculated as an index of expression stimulation.

The calculated values confirmed the concentration dependencies for C7-AHB in protection from irradiation (50–100 mg/l) and preadaptation (250–500 mg/l) of *E. coli* cells.

In the experiments with strain os250, 1.5% NaCl was used as a stressor. This concentration resulted in an ~50% inhibited growth of *P. aeruginosa* (Fig. 1). The stationary culture of strain os250 was preincubated with Sidovit for 1 h and incubated for 20 min with 1.5% NaCl. β-galactosidase activity was then determined (Table 2).

In the control variants (without AHBs), addition of NaCl resulted in 10% increase of *osmE* expression, which correlates with the literature data on the 15–20% activation of osmotic stress genes, e.g., *osmB*, in the presence of 1.5% NaCl [15]. Incubation with AHBs (50–500 mg/l) without addition of NaCl resulted in a 13–77% increase of *osmE* expression, depending on AHB concentration. This was an indication of the signal function of AHB. No increase in β-galactosidase activity occurred when NaCl was added to the cultures preincubated with AHBs, probably indicating the preadaptation reaction (Y_n/X_n), while the protective effect (Y_n/Y_0) was not pronounced. The calculated level of the regulation of expression of the *osmE* gene in the *rpoS* regulon by AHB revealed the action of alkylhydroxybenzenes as the activators of gene expression for this regulon. At 100–250 mg/l of the preparation, activity of *osmE* increased by 70%. Thus, the role of AHBs in preadaptation of the cells to the subsequent osmotic stress was demonstrated, although no protective effect of AHB was observed for this type of stressor.

The difference in protective effects of AHB (Sidovit) in the case of UV-irradiation and hyperosmotic stress probably depends on the nature of the stress factors. Excessive ROS produced by UV irradia-

Table 1. Activity of β-galactosidase in *E. coli* cells containing the *umuD::lacZ* transcriptional fuse (SOS system reporter gene) in the presence of AHB (Sidovit) under UV irradiation, U (preincubation with AHB for 1 h, UV irradiation for 10 min, postincubation for 10 min, and activity measurement)

Concentration of the preparation, mg/l (*n*)	Enzyme activity, U/min (changes in expression level compared to the variant without AHB, times)		Regulation level, times*		
			1	2	3
	Without UV-irradiation (X)	With UV-irradiation (Y)	Y_n/X_n preadaptation	Y_n/Y_0 protection	X_n/Y_0 induction
0	148.5 (1.0)	192.0 (1.0)	1.30	1.0	0.77
25	138.0 (0.92)	192.9 (1.0)	1.40	1.0	0.71
50	125.7 (0.84)	126.0 (0.65)	1.00	0.65	0.65
100	114.9 (0.77)	141.9 (0.73)	1.23	0.73	0.59
250	184.8 (1.24)	178.5 (0.92)	0.96	0.92	0.96
500	195.6 (1.31)	184.2 (0.95)	0.94	0.95	1.00

* The regulation level was calculated as the ratio of β-galactosidase activity in the cells preincubated with AHBs and UV irradiated (Y_n) to the enzymatic activity in the cells: preincubated and not irradiated (Y_n/X_n), a measure of preadaptation (1); irradiated and not preincubated (Y_n/Y_0), a measure of protection (2); and the ratio of preincubated, but not irradiated cells to not preincubated irradiated ones (X_n/Y_0) as a measure of stimulation of the *umuD* gene expression (3). The index is the concentration of the preparation in the experiment for which the regulation level was calculated.

Table 2. Activity of β-galactosidase in *E. coli* cells containing the *osmE::lacZ* transcriptional fuse (*rpoS* regulon reporter gene) in the presence of AHBs (Sidovit) under enhanced salinity (1.5% NaCl), U (preincubation with AHBs for 1 h, exposure to NaCl for 20 min, and activity measurement)

Concentration of the preparation, mg/l (*n*)	Enzyme activity, U/min (changes in expression level compared to the variant without AHBs, times)		Regulation level, times*		
	Without NaCl (X)	With NaCl (Y)	Y_n/X_n preadaptation index	Y_n/Y_0 protection index	X_n/Y_0 induction index
0	5920 (1.0)	6556 (1.0)	1.10	1.0	0.90
25	6166 (1.04)	6565 (1.00)	1.06	1.0	0.94
50	6730 (1.13)	6586 (1.00)	0.97	1.0	1.02
100	9846 (1.66)	7673 (1.17)	0.77	1.17	1.5
250	10439 (1.76)	7036 (1.07)	0.67	1.07	1.6
500	7981 (1.34)	5490 (0.83)	0.68	0.83	1.21

* The regulation level was calculated as described in Table 1.

tion are neutralized by AHBs, which act as traps for these particles. Thus, in the case of irradiation, AHBs exhibited a protective effect. On the other hand, in the case of introduction of 1.5% NaCl, preincubation with AHBs resulted in enhanced adaptive functions of the organism (preadaptation), while the protective effect of AHBs was not pronounced.

It may, therefore, be concluded that alkylhydroxybenzenes have a pronounced effect, enhancing the physiological activity of bacteria under nonoptimal growth conditions, adapting microbial cultures to these conditions. Their adaptogenic effect manifests itself in the broadened range of active proliferation, rather than tolerance. Thus, the growth rate at suboptimal temperature or osmotic pressure becomes equal to the maximal growth rate under optimal conditions.

Investigation of the mechanisms of adaptogenic effect of C7-AHB (Sidovit) demonstrated its stress-protective efficiency both for actively growing, exponential-phase bacterial cells and for the stationary-phase cells. This finding broadens significantly the range of practical applications for AHB preparations. These results are important for the understanding of development of the adaptive reaction in a microbial population as a whole organism. We demonstrated that alkylhydroxybenzenes are adaptogens against stressors of various nature, including osmotic stress. Previously, their role has been demonstrated only for heat shock [2] and oxidative stress [1, 5].

Our results make it possible to consider the AHB-based preparations as efficient adaptogenic supplements for bacterial preparations, which are often used under conditions nonoptimal or unfavorable for

microbial growth. Such preparations include microbial compositions used for bioremediation of petroleum-contaminated soils. The preparations are usually applied under conditions of unstable temperature and pH, as well as elevated salinity. Application of AHB-based preparations will enhance the economic and environmental efficiency of preparations used for bioremediation of natural ecosystems.

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