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SHORT COMMUNICATIONS

Effect of Reconstituted Biofilm Composition on Bacterial Hydrocarbon-Oxidizing Activity

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Two major approaches are used in *bioremediation* of natural objects and ecosystems, i.e., in elimination of pollutants, including oil components: *biostimulation* of native microbial flora (either by aeration or by introduction of additional nutrients, such as sources of nitrogen and phosphorus) and *bioaugmentation* (introduction of highly potent natural or genetically modified bacteria into the ecosystem). A mixture of microorganisms capable of pollutant biodegradation is generally used in bioaugmentation to enhance its efficiency. The biological polyculture product "Devoroil," containing five species of hydrocarbon-oxidizing bacteria and yeasts [1], and a number of preparations comprising microorganisms of a single genus, e.g., "Roder" [2], exemplify preparations aimed at oil pollution control. The efficiency of polycomponent preparations may be based on cometabolism, on activity towards different oil fractions, or on utilization of an additional nitrogen source by one of the components, as in the case with an *Azotobacter*-containing community [3]. However, in natural substrates, hydrocarbon-oxidizing microorganisms interact with many other, aboriginal, microbial populations (by mechanisms for the most part unknown), and these interactions may have both positive and negative effects on oil degradation efficiency [4].

In trophic chains of oil-fields, oil-oxidizing microorganisms may be viewed as primary producers, since they provide products of oil degradation to companion consumers unable to oxidize oil. The metabolic dependence of consumers on producers is evident. However, an inverse action of consumers upon producers should also exist in such communities, promoting the activity of producers and thus providing additional nutrient substrates for consumers. Indeed, earlier we managed to show that microbial satellites, isolated via an intermediate reconstitution of the biofilms of oil reservoir waters, significantly influence the oil oxidizer activity by excreting activating compounds into the media [5] or by exerting a protective effect in extreme environments [6].

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The aim of the present work was a comparative study of the types of interaction between microorganisms isolated from oil reservoir water under different conditions: in liquid suspensions, on the surface of solid media, and in reconstituted biofilms.

We used bacterial cultures isolated from Dagan (China) oil reservoir water. More than 40 strains were obtained. The following cultures were elected for a detailed study: oil-oxidizing strains 44a and 14-3 and satellite strains 44b, 45, 14-1, and 14-2 (the first numeral indicates the number of the association from which the culture was isolated).

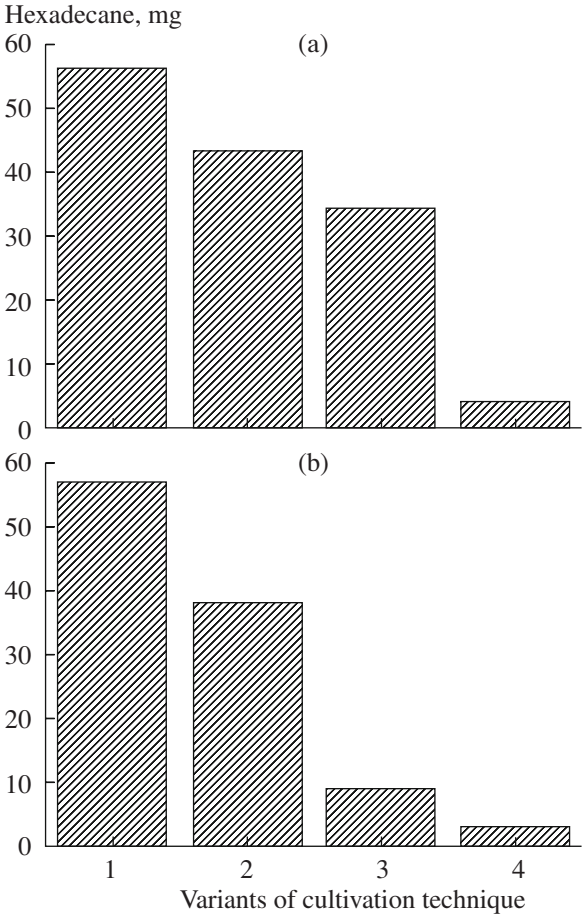
Biofilm reconstitution and the isolation, cultivation, identification, and storage of microorganisms were performed as described in our previous work [6]. To identify the isolates, polyphasic taxonomy approaches were used. Physiological and biochemical tests (determination of cultural and physiological features and of the activity of certain enzymes) were supplemented with determination of 16S rRNA gene sequences and their comparison with the database sequences). The oil-oxidizing strain 44a was found to be a representative of the genus *Dietzia* of the suborder *Corynebacterineae*; another oil-oxidizing strain, 14-3, was shown to represent the genus *Rhodococcus* genus of the same suborder; and the satellite 44b strain was assigned to the genus *Brevundimonas* of the family *Caulobacteraceae.* Identification of other cultures is currently in progress.

Culture growth was estimated by optical density and biomass dry weight. *n*-Hexadecane quantitative determination in M9 medium was carried out according to a technique developed earlier. After separation of cells by centrifugation, the culture liquid was filtered using water jet air pump through 1.5–2 g of hydrophobic silica gel (Octadecyl=Si300Polyol, Serva) on a fiber glass filter (Whatman, GF/D). Unconsumed hexadecane present in the culture fluid binds to silica gel, while cells and other components of the medium stay in permeate. The filter was then washed with distilled water, and hexadecane was eluted with 5–10 ml of *n*-hexane (>98%, Fluka). The solution was evaporated to dryness in completely desiccated porcelain cups, and *n*-hexadecane was weighed using analytical balance.

Optical density of the oil-oxidizing culture 44a after cultivation using the turn-over technique (% of the control without satellites)

Cultures	Optical density, %
44a	100
44a + 44b	140
44a + 45	139

A modified "reversed agar plate" method was used to evaluate the influence of satellites on oil oxidizers in mixed cultures. An oil oxidizer and a corresponding satellite were plated (in an amount such as to produce a continuous lawn and in approximately equal proportions) onto a 4–5 mm thick plate of solid M9 medium containing *n*-hexadecane. In control plates, a sole oil oxidizer was plated. After 4 days of growth, the biomass was removed with a sterile spreader, the plate was turned over, and its clear side was inoculated with a suspension of an oil oxidizer. In 3 days, cells were washed off the plates with two 3 ml portions of 1% NaCl solution and optical density of the obtained suspension was measured. In some of the experiments, dry biomass weight was also determined. Typical experimental data are presented in the table; they demonstrate considerable (by about 40%) stimulation of the oil oxidizer growth.



n-Hexadecane remainder in 20 ml of culture fluid. (a) Preformed biofilms under stationary conditions: 1, control (without inoculation); 2, oil-oxidizing strain 44a; 3, strain 44a with satellite 44b; 4, strain 44a with satellite 45. (b) Biofilms grown in the presence of hydrophobic silica gel: 1, control (without inoculation); 2, oil-oxidizing strain 14-3; 3, strain 14-3 with satellites 14-1 and 14-2; 4, strain 14-3 with satellite 14-1.

The results of our experiments suggest two possible mechanisms of the influence of satellites on oil oxidizers: (1) excretion, in the course of cocultivation, by satellite microorganisms of substances that stimulate growth of oil oxidizers and (2) consumption by satellites of metabolic products of oil oxidizers that adversely affect the growth of the latter organisms.

The first explanation seems to be more probable, since the effect was observed even after turning over the agar plate, in the absence of the satellite.

The stimulatory effect of the satellite may be evaluated by measuring *n*-hexadecane remainder in the media after growth of a mixed culture in liquid medium. However, no significant difference in *n*-hexadecane content was registered for suspension cocultures of oil oxidizers and satellites, although the biomass yield was greater than in the pure culture of the oil oxidizer. Apparently, biomass augmentation was due to utilization by the satellite of paraffin degradation products unavailable for the oil oxidizer.

We assumed that the absence of stimulatory effect on oil oxidation in suspension cultures could be due to the fact that the cell density of the mixed population in suspension is considerably lower than in biofilms, where quorum sensing comes into action and genes silent in suspension cultures are expressed [7].

Therefore, we attempted to imitate conditions close to those in natural biofilms but allowing measurement of remainders of *n*-hexadecane. Two techniques were used. The first one involved preformation of biofilms on filters on solid M9 medium containing *n*-hexadecane [6] and further aseptic transfer of these filters to liquid medium of the same composition. After the biofilms had been incubated in liquid medium for two weeks under stationary conditions, the remainder of *n*-hexadecane was determined. In the second case, biofilms were formed directly in liquid medium in the presence of hydrophobic silica gel, which adsorbed *n*-hexadecane present in the medium. Microorganism growth occurred mainly within silica gel volume. The results are presented in the figure. In all variants, cultivation was performed for the same time period.

One can see that recombining oil oxidizers and satellites that comprise the same natural association increases the *n*-hexadecane uptake manifold. Thus, the efficiency of *n*-hexadecane utilization by oil oxidizers under conditions imitating natural structured associa-

tions increases significantly. The chemical nature of the stimulatory substances is currently under study.

From the obtained results, we conclude that, in the models studied, the interactions between oil oxidizers and their satellites are close to protocooperative: the producers form hydrocarbons degradation products, thus providing nutrients for the growth of consumers, which, in turn, secrete substances that stimulate producer growth. We are the first to report microbial interaction of this kind within the oil-field microflora. These interactions are not only of undoubted theoretical interest but also are to be taken into consideration in development of procedures aimed at improvement of oil waste removal from natural environments.

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