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EXPERIMENTAL ARTICLES

The Strategy of Strain Selection for a Mixed Culture Performing Rapid Conversion of a Mixture of Polyaromatic Compounds

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Abstract—The strain *Sphingomonas* sp. VKM V-2434 converts the mixture of seven polyaromatic compounds (PACs): fluorene, dibenzothiophene, carbazole, phenanthrene, anthracene, fluoranthene, and pyrene. The effect of each of the above PACs on the rate of mixture conversion was determined. The following two strains, which utilize the substances inhibiting the studied process, were added to the culture: strain FON-11 utilizing 9-fluorenone (fluorene metabolite) and strain CBZ-21 utilizing carbazole. In the case of the mixed culture of three strains, conversion rates were 1.5 and 1.2–3.8 times higher for the PAC mixture and its individual components, respectively, than the rates for *Sphingomonas* sp. VKM V-2434 monoculture. The degree of degradation of PAC conversion products increased from 32 to 44%. The rate of PAC conversion by the mixed culture exceeded the sum of conversion rates for the individual component strains; this cooperative effect was particularly marked for anthracene and pyrene.

Key words: mixture of polycyclic aromatic compounds, mixed culture.

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Polycyclic aromatic hydrocarbons and their heterocyclic analogues constitute a large class of polyaromatic compounds (PAC). Environmental contamination with these substances is dangerous for the health of humans and animals. Microbial activity is the main factor of PAC degradation in various ecosystems [1]; hence, PAC-degrading microorganisms are intensely studied as a prospective object of biotechnology [2]. Microbial habitats usually contain a complex PAC mixture rather than a single compound; PAC in soil originate from pollution with crude oil or coal tar. Biodegradation of PAC mixtures is exposed to positive and negative impacts of substrates and their conversion products. PAC conversion becomes slower at substrate inhibition [3–5] and inhibition by products [6, 7], while cross induction [8], cometabolism [9, 10], and increase of biomass concentration during growth [4] accelerate it. The above effects, in their turn, depend on the composition of the microbial community present in the ecosystem, inasmuch as microorganisms utilize PACs and produce or utilize PAC conversion products. This process can be significantly accelerated by microorganisms degrading the inhibitors of PAC conversion [6]. Natural microbial communities from polluted soils are usually more efficient against PAC mixtures than individual strains isolated from these communities [10]; however, the composition of such communities is unknown and variable. Mixed cultures artificially composed of separate strains seem more promising objects of research and practical application. This work pursues the search of a technique for formation of a mixed culture for quick conversion of a PAC mixture. The strain *Sphingomonas* sp. VKM V-2434, which is able to oxidize a wide range of polycyclic hydrocarbons [11], was used as the major component of the community, which was supplemented with the strains utilizing the inhibitors of PAC conversion by the strain VKM V-2434.

The goal of the present work was to propose a procedure for selecting strains for the formation of a mixed culture of known composition for rapid conversion of PAC mixtures.

The following challenges were issued to attain this goal:

—study of PAC mixture conversion by the strain *Sphingomonas* sp. VKM V-2434, in particular, characterization of the effect of each substrate on conversion of other substrates comprising the mixture;

—isolation from polluted soils of the strains degrading PAC mixture components, which slow down PAC conversion by strain VKM V-2434; and

—assessment of the efficiency of PAC mixture conversion by the mixed culture including VKM V-2434 and strains decomposing the inhibitors of PAC conversion.

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MATERIALS AND METHODS

Strains. The strain *Sphingomonas* sp. VKM V-2434 has been isolated and characterized previously [11]. This strain converts all tested polycyclic hydrocarbons: naphthalene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz[*a*]anthracene, chrysene, and benzo[*a*]pyrene; the extent of molecular degradation was different for different substrates: from aromatic ring hydroxylation (fluorene and pyrene) to supposedly complete mineralization (acenaphthene and fluoranthene). Strains FON-11 and CBZ-21 were isolated from the mixture of soils and bottom sediments, which had been contaminated with coal tar for a long time, by the method of enrichment cultures maintained in a mineral medium with 9-fluorenone and carbazole, respectively. The strain FON-11 (99% 16S rRNA nucleotide sequence similarity to the type strain of *Pseudomonas stutzeri*) utilizes 9-fluorenone, a product of fluorene oxidation by many microorganisms including strain VKM V-2434, as a sole carbon and energy source. The still unidentified strain CBZ-21 utilizes carbazole as a sole carbon and energy source.

Reagents. The inocula of strains VKM V-2434, FON-11, and CBZ-21 were grown on acenaphthene, 9-fluorenone, and carbazole, respectively. The substrate was a mixture of seven PACs of medium molecular mass: fluorene, dibenzothiophene, phenanthrene, anthracene, carbazole, fluoranthene, and pyrene. All PAC reagents used in this work were highly pure (>98%; Sigma-Aldrich). Other reagents were graded no less than "analytically pure." Acetone and ethyl acetate were distilled before usage.

Incubation medium. The mineral medium contained the following (g/l): NH_4NO_3, 1.0; KH_2PO_4, 1.0; K_2HPO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.02; $FeCl_2$, two drops of the saturated solution; pH ~7.5. The mineral medium was filtered, dispensed into flasks by 100 ml, and sterilized. After sterilization, vitamin B_{12} (1 μg/l) and 10 mg/l of each PAC were added into the flasks. All PACs, except for anthracene, were introduced jointly as 0.2 ml of acetone solution containing 5 g/l of each PAC. Anthracene was added separately: also as a 0.2-ml solution in acetone with a concentration of 5 g/l (0.2 ml of acetone was added in the experiments without anthracene).

Experimental procedure. The appropriate culture (1 ml) was inoculated into a 750-ml flask containing 100 ml of the medium. The VKM V-2434, FON-11 and CBZ-21 cultures used as inocula were grown for 3 days in the medium with acenaphthene (0.5 g/l), 9-fluorenone (0.1 g/l), and carbazole (0.1 g/l), respectively. The flasks were cultivated on a shaker (29°C, 120 rpm) for 72 h, being removed from the shaker every 12 h or more often. The contents of a flask was twice extracted by ethyl acetate (30 + 10 ml). The extract volumes were brought to 30 ml with ethyl acetate and PAC concentration was assayed by gas chromatography.

Gas chromatography of the extracts. PAC concentration in the extracts was assayed by gas chromatography in a Crystal 2000M chromatograph (Chromatek, Yoshkar-Ola) with a flame ionization detector and an HP-5 column (30 m × 0.32 mm × 0.25 μm). The temperatures of evaporator and detector were 250 and 290°C, respectively. The column temperature in the course of analysis increased from 160 to 260°C at a rate of 10°C/min. Samples (1 μl) were introduced with a DAZh-2M autosampler.

Measurement of COD of the culture liquid extract. The technique is based on the methods of chemical oxygen demand (COD) measurement in wastewater [12]. One-third (10 ml) of culture liquid extract was transferred to a round-bottom flask (50 ml). The solvent was removed in a rotor evaporator until dry; 5 ml of acetone was then added to the dry residue and evaporated as well. The flask was kept open overnight for volatilization of residual acetone; then, 10 ml of 0.25 N $K_2Cr_2O_7$ solution and 10 ml of sulfuric acid were added into it and boiled for 2 h with a backflow condenser. Then, the solution was quantitatively transferred into a glass, supplemented with two drops of 0.4% diphenylamine solution in 80% sulfuric acid and 3 ml of phosphoric acid, and titrated with 0.25 N Mohr's salt solution until the disappearance of blue color. The chemical oxygen demand (COD) of culture liquid extract, i.e. the amount of oxygen (mg) needed for complete oxidation of organic substances extracted from 1 l of the culture liquid, was calculated by the formula:

$$h = (V_X - V) \times \frac{10}{V_X} \times 0.25 \times 8 \times 3 \times 10 = \left(1 - \frac{V}{V_X}\right) \times 600, \quad (1)$$

where V_X and V are Mohr's salt volumes used for titration of an idle and tested sample, respectively; $\frac{10}{V_X}$ is the correction factor for bringing Mohr's salt concentration to 0.25 N; 0.25 is Mohr's salt concentration; 8 is the oxygen equivalent; and 3 × 10 is the coefficient for recalculation per 1 l of the culture liquid. In the COD measurement experiments, the culture liquid was acidified with 0.5 ml of 25% H_2SO_4 before the extraction; this procedure provided the extraction of the PAC metabolites containing a carboxyl group.

Assessment of the effect of a given substrate on mixture conversion. The effect of a substrate on PAC mixture conversion by the strain VKM V-2434 was assessed as follows: all substrates, except for the tested one, were introduced into the incubation medium; the control medium contained all seven PACs. The effect of one substrate (S_k) on conversion of another substrate (S_m) was characterized by the influence index,

i.e., the relative change of S_m conversion rate at a transition from incubation in the absence of S_k to incubation in the presence of S_k:

$$\alpha_m^k = \frac{v_m - v_m^k}{v_m}, \quad (2)$$

where v_m is the S_m conversion rate in the presence of S_k and v_m^k is the S_m conversion rate in the absence of S_k. The index α_m^k is positive or negative when S_k activates or inhibits S_m conversion, respectively.

The effect of the substrate S_k on conversion of all other substrates of the mixture was assessed by the arithmetic average of indices of influence of this substrate on other six substrates,

$$\alpha_A^k = \frac{1}{6}\sum_{\substack{i=1 \\ i\neq k}}^{7} \alpha_i^k, \quad (3)$$

and by the general influence index calculated from consideration of all other substrates as a single substance,

$$\alpha_O^k = \frac{\sum_{i=1}^{7} v_i - \sum_{i=1}^{7} v_i^k}{\sum_{i=1}^{7} v_i}, \quad i \neq k. \quad (4)$$

Selectivity of the influence of the given substrate S_k on conversion of other substrates was characterized by the sum of squares of deviations of influence indices from their arithmetical average:

$$D_k = \sum_{\substack{i=1 \\ i\neq k}}^{7} (\alpha_i^k - \alpha_A^k)^2. \quad (5)$$

Assessment of the cross-effect of strains as components of a mixed culture. Acceleration of substrate conversion by a binary culture including strains *L* and *M*, as compared with the conversion of this substrate by individual strains *L* and *M*, was characterized by the cooperation index:

$$c_k^{L,M} = \frac{v_k^{L,M}}{v_k^L + v_k^M}, \quad (6)$$

where $v_k^{L,M}$ is the rate of conversion of a substrate S_k by mixed culture and v_k^L and v_k^M are the rates of conversion of a substrate S_k by individual strains. The cooperation index is equal to 1 if the rate of substrate conversion by a mixed culture is merely a sum of the rates of substrate conversion by single strains; at reciprocal intensification and reciprocal weakening of substrate conversion rate, $c > 1$ and $c < 1$, respectively. The formula (6) can be easily generalized for a mixed culture containing more than two strains. The indices similar to the cooperation index used here, designated as symbiotic indexes, have been used previously in ecological studies [13, 14].

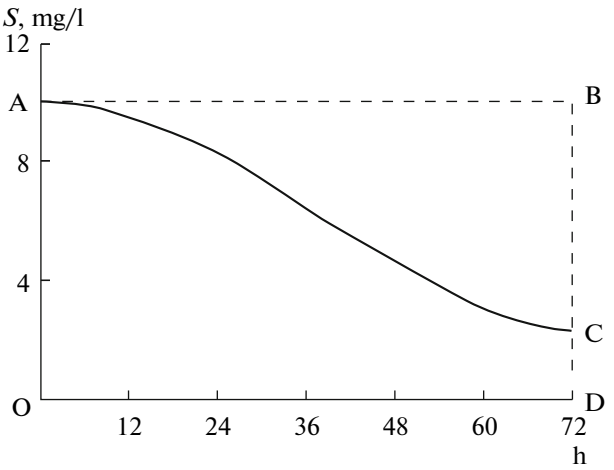

Fig. 1. Geometric interpretation of the average degree of substrate conversion. The average degree of conversion during 72 h of incubation is equal to the ratio of the area of the curvilinear triangle ABC to the area of the rectangle ABDO: $v = \frac{S_{ABC}}{S_{ABDO}}$.

The measure of the substrate conversion rate. Calculation of the indices (2–6) requires the data on the rates of PAC conversion by microbial cultures. Since the rate of conversion varied during the incubation in a very intricate manner, we had to use a certain mean value. The average degree of substrate conversion during the period of observation (72 h) was taken as a measure of substrate conversion rate. It was calculated on the basis of the definitions of conversion degree and the mean of function on an interval:

$$v = \frac{1}{t}\int_0^t \frac{S_0 - S}{S_0} dt = 1 - \frac{1}{S_0 t}\int_0^t S dt, \quad (7)$$

where t is time interval, S_0 is the initial substrate concentration, and S is the current value of substrate concentration. The integral (7) was calculated from experimental data using the trapezium rule:

$$v \approx 1 - \frac{1}{2S_0 t}\sum_{i=1}^{n} (S_{i-1} + S_i)(t_i - t_{i-1}), \quad (8)$$

where n is the number of points on the kinetic curve, i is the point number, and S_i is the substrate concentration value corresponding to the time value t_i. A geometrical interpretation of the average degree of conversion is presented in Figure 1.

Estimation of the degree of degradation of PAC conversion products. The degree of degradation of the products was characterized by the ratio of COD decrease expressed in substrate concentration equiva-

lents $\left(\frac{S_0}{h_0}dh\right)$, to the corresponding decrease of total substrate concentration (dS):

$$p = \frac{S_0}{h_0}\frac{dh}{dS}. \quad (9)$$

This value shows the portion of the formed PAC conversion products, which is transformed into compounds not extracted by ethyl acetate. The p value was assessed by the angular coefficient of the diagram in coordinates $\left(\frac{S}{S_0};\ \frac{h}{h_0}\right)$. The formula (9) suggests that the initial COD value of the culture liquid extract (h_0) is entirely determined by PAC concentration; this assumption was confirmed experimentally.

RESULTS

PAC mixture conversion by strain VKM V-2434: the effect of each substrate on mixture conversion. The curves of conversion of individual substrates component in the mixture of six PACs (with one substrate excluded) by strain VKM V-2434 are shown in Figure 2. These data were used to calculate the influence index α of each substrate for conversion of each of the remaining substrates (Fig. 3a–3g) and of the mixture as a whole (Fig. 3h). All of the tested PACs, except for dibenzothiophene, had a significant effect on the rates of conversion of other PAC from the mixture. They can be legibly divided into two groups by the sign of effect: (1) substrates stimulating the conversion of other substrates (fluoranthene, phenanthrene) and (2) substrates inhibiting conversion of other substrates (carbazole, fluorene, anthracene, and pyrene); the compounds are listed in the order of decrease of their average influence indices (α_A). The values of the sum of squares of deviation of influence indices from their arithmetic average (D) were 0.67 for fluorene, 0.01 for dibenzothiophene, 0.05 for phenanthrene, 0.10 for anthracene, 0.08 for carbazole, 0.24 for fluoranthene, and 0.26 for pyrene. This fact evidences that the effect of fluorene on PAC conversion was more selective; indeed, fluorene selectively inhibited conversion of pyrene, anthracene, and carbazole (Fig. 3a). As a result of preferential influence of fluorene on conversion of the substrates against which the tested strain showed low activity (anthracene, pyrene), the total index of influence of fluorene on the PAC mixture conversion (α_O^{FL}) was half the arithmetic average (α_A^{FL}) (see Fig. 3h). A rather selective effect on PAC conversion was also demonstrate by pyrene (inhibits the conversion of fluoranthene) and fluoranthene (activates the conversion of all substrates, but of pyrene to a greater extent and of phenanthrene to a lesser extent than the rest).

Conversion of PAC mixture by the binary culture comprising strains VKM V-2434 and FON-11. The rate of PAC mixture conversion by the 9-fluorenone-degrading strain FON-11 is insignificant. Nevertheless, the addition of FON-11 to the culture of VKM V-2434 considerably accelerated conversion of three out of the seven PACs: anthracene, carbazole, and pyrene (Fig. 4a). While the stationary level of 9-fluorenone (1.2–1.5 mg/l) was established in the monoculture of VKM V-2434, 9-fluorenone was not revealed in the binary culture with FON-11 after the first 20 h of incubation.

PAC mixture conversion by the binary culture comprising strains VKM V-2434 and CBZ-21. The rate of PAC conversion by the carbazole-degrading strain CBZ-21 is insignificant (with the exception of carbazole). However, the addition of CBZ-21 to the culture of VKM V-2434 accelerated conversion of all the components of the mixture except for carbazole; the greatest effect was observed for anthracene and pyrene (Fig. 4b).

PAC mixture conversion by the mixed culture comprising three strains: VKM V-2434, FON-11, and CBZ-21. The addition of strains FON-11 and CBZ-21 to the culture of VKM V-2434 accelerated conversion of individual PAC mixture components by 1.2–3.8 times (Fig. 5) and the conversion of mixture as a whole by 1.5 times (Fig. 6a). The rate of PAC conversion by the mixed culture exceeded the sum of the rates of PAC conversion by component strains (dotted line in Fig. 6a), i.e. the cooperative effect was observed. Cooperation index for conversion of the mixture as a whole was 1.1, but the special cooperation indices for anthracene and pyrene conversion reached the values of 2.9 and 3.8, respectively. The COD of the culture liquid extract of the triple culture decreased twice compared to the COD of the monoculture of VKM V-2434 (Fig. 6a). The extent of degradation of PAC conversion products (p) in the triple culture and in the monoculture of VKM V-2434 was 0.32 and 0.44, respectively (Fig. 6b).

DISCUSSION

Strains with definite properties were selected to make a mixed culture for rapid conversion of PAC mixture. The strain (VKM V-2434) that converted (with different rates and depths of degradation) all components of the PAC mixture was selected first. The components inhibiting the process were determined: carbazole, fluorene, anthracene, and pyrene. It was assumed that the introduction into the system of a strain decomposing one of these compounds would result in accelerated conversion of not only this particular substance, but of other components of the mixture as well. The carbazole-degrading strain (CBZ-21) actually showed an activating effect on PAC mixture

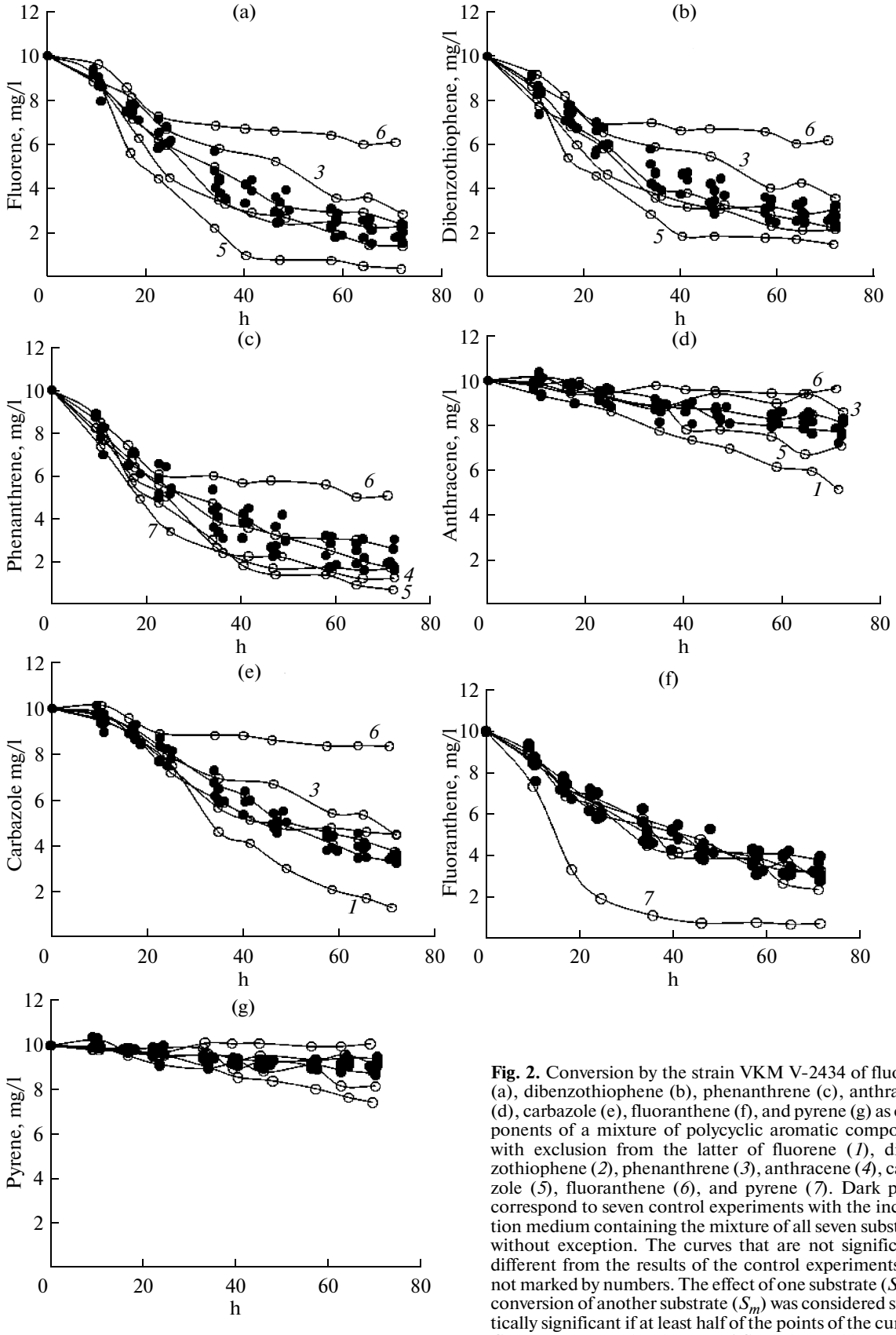

Fig. 2. Conversion by the strain VKM V-2434 of fluorene (a), dibenzothiophene (b), phenanthrene (c), anthracene (d), carbazole (e), fluoranthene (f), and pyrene (g) as components of a mixture of polycyclic aromatic compounds with exclusion from the latter of fluorene (*1*), dibenzothiophene (*2*), phenanthrene (*3*), anthracene (*4*), carbazole (*5*), fluoranthene (*6*), and pyrene (*7*). Dark points correspond to seven control experiments with the incubation medium containing the mixture of all seven substrates without exception. The curves that are not significantly different from the results of the control experiments, are not marked by numbers. The effect of one substrate (S_k) on conversion of another substrate (S_m) was considered statistically significant if at least half of the points of the curve of S_m conversion in the absence of S_k are located beyond the region filled with the points of the control experiments by S_m conversion in the presence of all substrates.

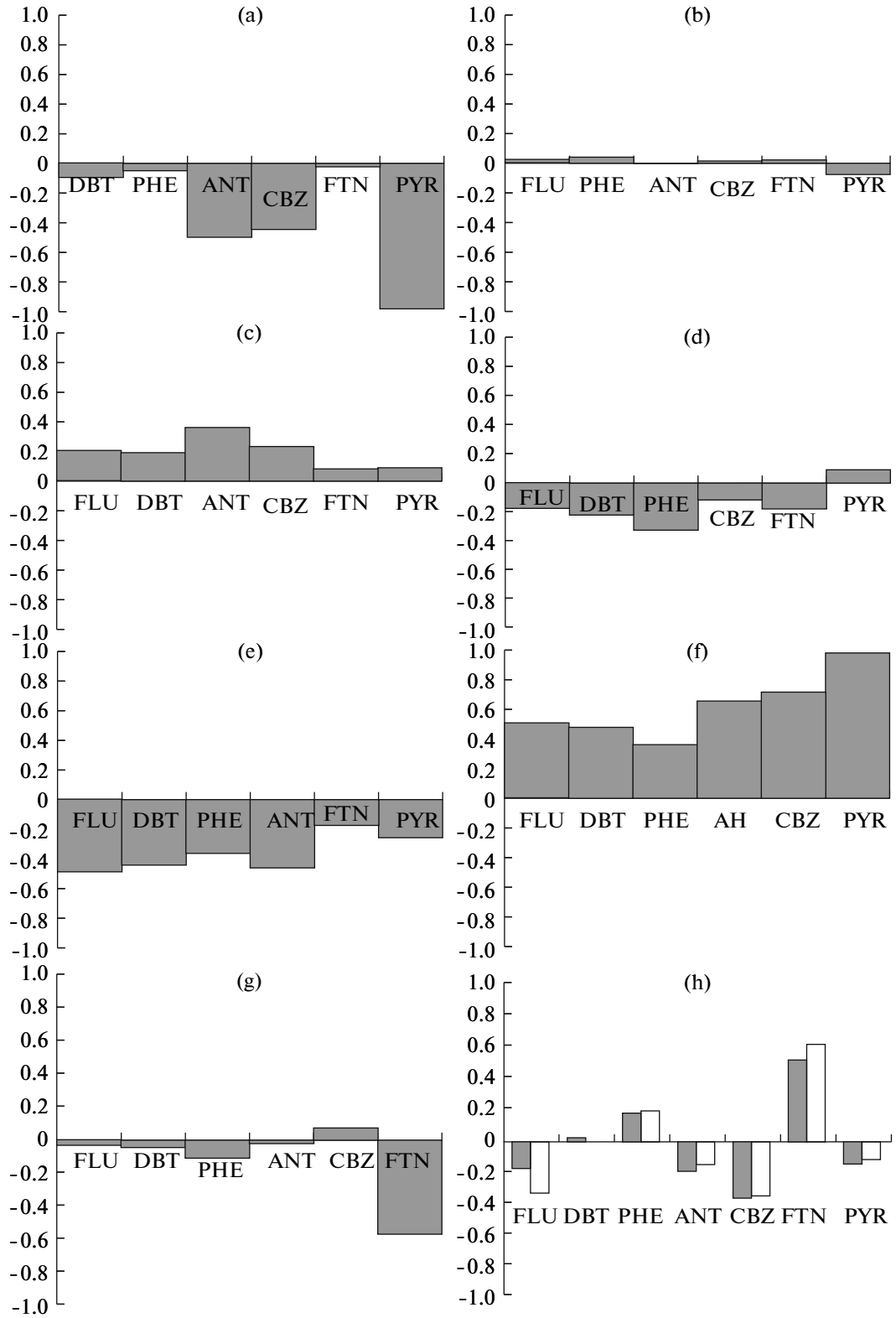

Fig. 3. The indices of effects of fluorene (a), dibenzothiophene (b), phenanthrene (c), anthracene (d), carbazole (e), fluoranthene (f), and pyrene (g) on conversion of fluorene (FLU), dibenzothiophene (DBT), phenanthrene (PHE), anthracene (ANT), carbazole (CBZ), fluoranthene (FTN), and pyrene (PYR) by the strain VKM V-2434 as components of the mixture of polyaromatic compounds. The influence of individual substrates on conversion of the mixture as a whole (h): light columns are the arithmetic average index of influence (α_A); dark columns are the total index of influence (α_O).

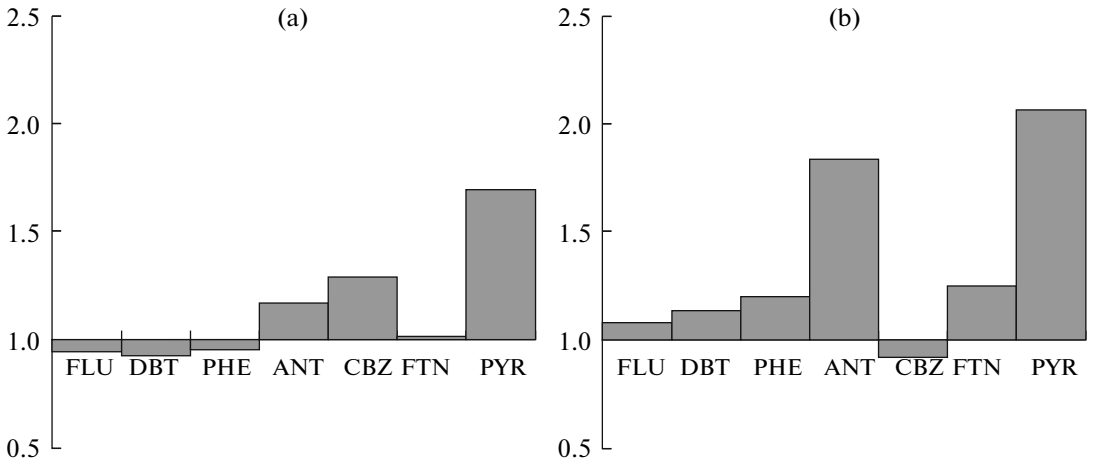

Fig. 4. Cooperation indices relative to PAC mixture conversion: for the pair of strains VKM V-2434 and FON-11 (a) and for the pair of strains VKV V-2324 and CBZ-21 (b).

conversion by strain VKM V-2434. This effect may be due to several factors: decreased carbazole concentration, utilization of its conversion products, slowdown of product formation resulting from the competitive reaction of carbazole utilization for the growth of CBZ-21, and conversion of the metabolites of other PAC present in the system by strain CBZ-21. The inhibiting effect of fluorene was eliminated by the introduction of strain FON-11 utilizing 9-fluorenone; this compound is the first intermediate of fluorene conversion by the strain VKM V-2434 [11]. In the mixed culture, 9-fluorenon was not found (in contrast to the monoculture of VKM V-2434); hence, all subsequent products of fluorene metabolism were absent. The effect of addition of FON-11 to the culture of VKM V-2434 on PAC conversion was equivalent to exclusion of fluorene from the substrate mixture: selective acceleration of pyrene, carbazole, and anthracene conversion was observed. Thus, the inhibiting effect of fluorene on PAC mixture conversion by the culture of VKM V-2434 is mediated by the products of fluorene metabolism. It is known that 9-fluorenone is a toxic product of nonspecific fluorene oxidation by bacteria and fungi. Elimination of the toxic effect of 9-fluorenone on bacterial degradation of fluorene by addition of the 9-fluorenone-utilizing strain *Pseudomonas mendocina* MC2 was previously demonstrated [6]. Our results confirm the information concerning the inhibiting effect of fluorene metabolites and natural abundance of 9-fluorene-degrading bacteria.

The tested anthracene- and pyrene-degrading strains had no significant influence on conversion of the PAC mixture (the data not presented). It probably results from the high resistance of anthracene and pyrene to biodegradation [15] and, therefore, the low rate of their conversion by additional strains, which is insufficient for elimination of the inhibiting effect. The observed selective inhibition of fluoranthene conversion by pyrene is of interest. It may be a result of formation of pyrene 4–5-bond oxidation products by strain VKM V-2434 [11]; the toxicity of these compounds was demonstrated in work [7].

Thus, our efforts resulted in creation of a mixed culture consisting of three strains (VKM V-2434, FON-11, and CBZ-21). This culture converts the PAC mixture much more quickly than each of the strains separately and much more quickly than in the case of mere summation of the rates (cooperative effect). The strongest cooperative effect was shown for conversion of the most stable components: pyrene and anthracene. At the qualitative level, it means that a mixed culture (community) possesses the properties missing in the component strains (populations); in ecology, such properties of communities are called emergent ones [16].

The side effect of adding new strains was the higher degree of degradation of PAC conversion products in the triple mixed culture than in the monoculture of VKM V-2324. However, the degree of product degradation by the mixed culture (44%) was still insufficient for complete purification. Therefore, we think that degradation of PAC conversion products is an individual task. The scheme of purification of a solid material (e.g., soil) may include quick conversion of PAC into water-soluble compounds followed by purification of the resultant wastewater.

Addition of the strains degrading the components that inhibit conversion of the mixture as a whole resulted in a mixed culture performing rapid conversion of the PAC mixture. Such a strategy of strain selection seems to be prospective for creation of mixed cultures.

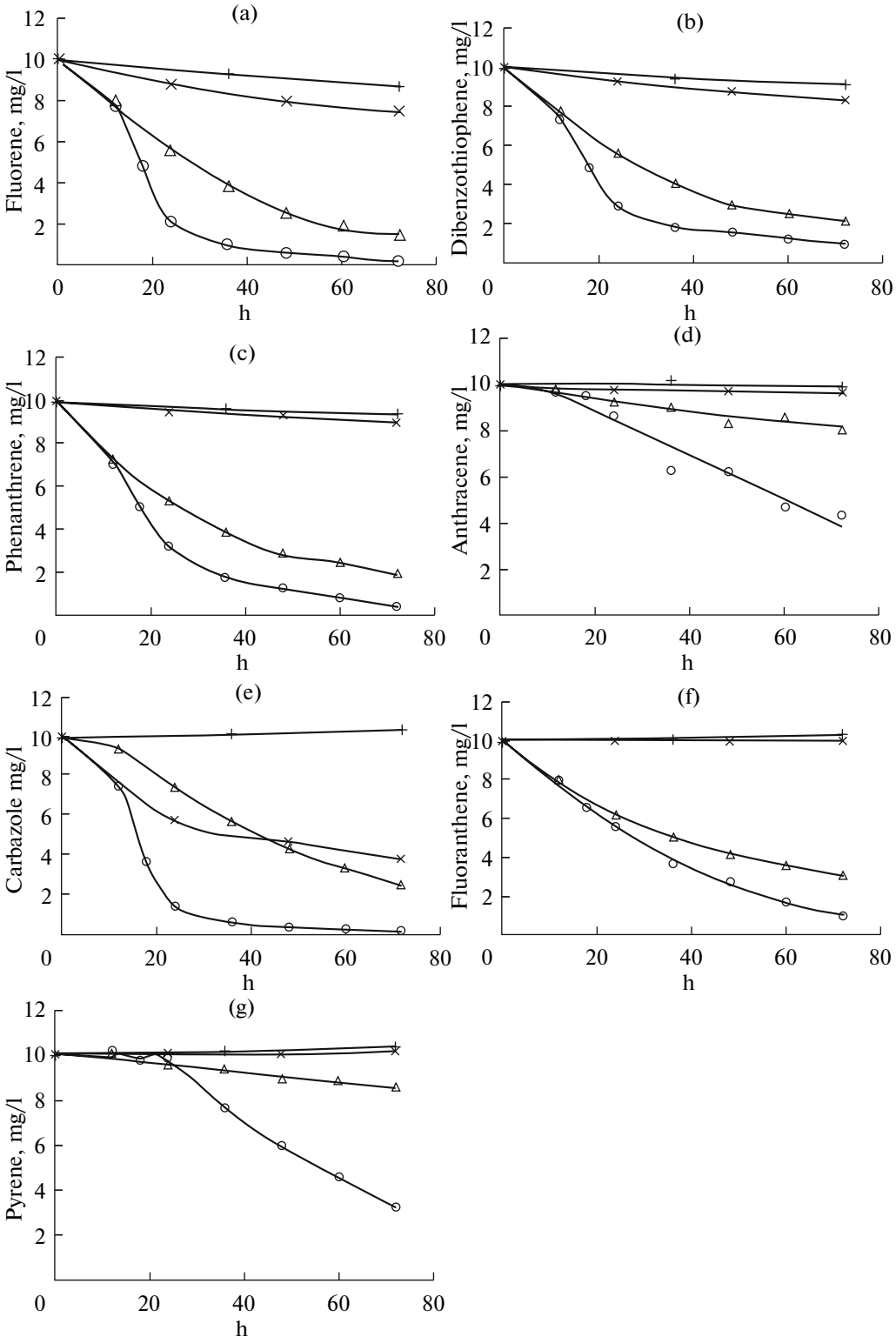

Fig. 5. The dynamics of concentration of fluorene (a), dibenzothiophene (b), phenanthrene (c), anthracene (d), carbazole (e), fluoranthene (f), and pyrene (g) at incubation of the strains VKM V-2434 (Δ), FON-11 (+), CBZ-21 (×), and mixed culture including all three strains (○).

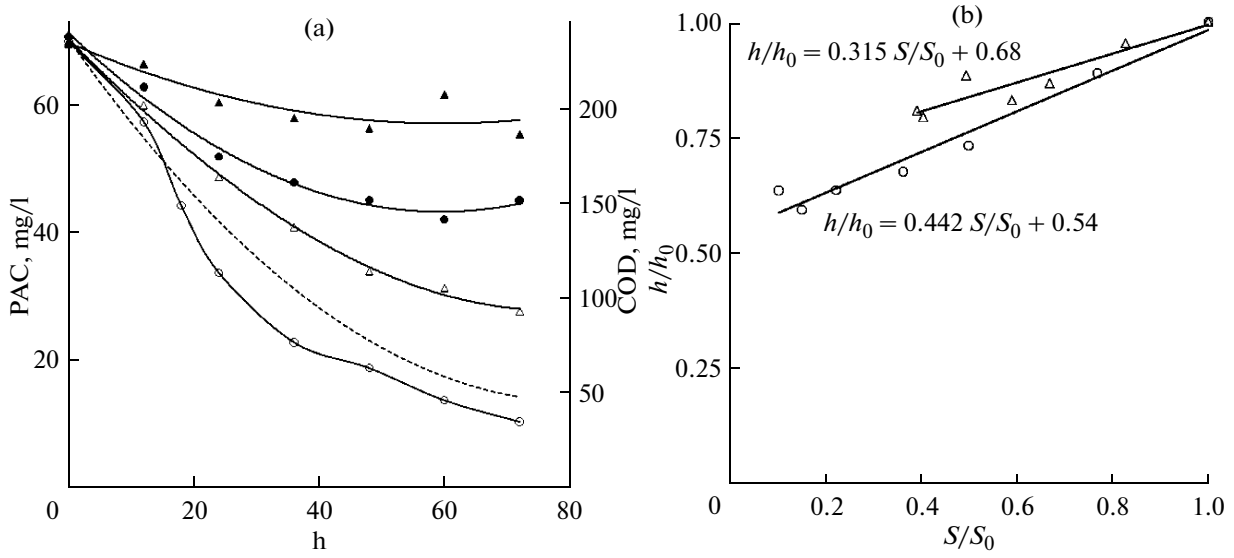

Fig. 6. a: The dynamics of total PAC concentration at incubation of strain VKM V-2434 (Δ) and the triple mixed culture (○); dotted line shows the curve of PAC mixture conversion in the case when the rate of PAC conversion by the mixed culture would be equal to the sum of the rates of PAC conversion by individual strains. The COD dynamics of the culture liquid extract at incubation of the strain VKM V-2324 (▲) and triple mixed culture (●). b: Graphs in the coordinates $\left(\frac{S}{S_0}; \frac{h}{h_0}\right)$ for incubation of strain VKM V-2324 (Δ) and the triple mixed culture (○).

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