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EXPERIMENTAL ARTICLES

Utilization of H_2O_2 as the Oxygen Source by Bacteria of the Genera *Pseudomonas* and *Rhodococcus*

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Abstract—The growth of bacteria of the genera *Pseudomonas* and *Rhodococcus* in the presence of hydrogen peroxide as the sole source of oxygen was studied. The toxic effect of H_2O_2 in the concentration range of 100–200 μg/ml was shown to extend the lag phase by two to three days. Apart from the peroxide toxicity, the bacterial growth was inhibited by the toxic effect of dissolved oxygen in concentrations over 100 μg O_2/ml; in the presence of a liquid hydrocarbon phase, this effect was alleviated. Under decreased partial pressure of oxygen in the presence of hydrocarbons (12–15 vol %), culture growth was initiated at high initial concentrations of H_2O_2 (300 μg/ml). When hydrogen peroxide concentrations exceeded 320 μg/ml, no growth occurred, regardless of how much hydrocarbon was added.

Key words: oil-oxidizing microorganisms, hydrogen peroxide, microbial biotechnology for the enhancement of oil recovery.

In natural environments, the growth of aerobic microorganisms often occurs under oxygen deficiency; optimal oxygen concentrations possibly occur rather seldom. Aerobic bacterial growth under the conditions of oxygen excess, i.e., its high partial pressure, occurs, as a rule, only under artificial conditions or in biotechnological installations, e.g., those designed for the oxidation of alkanes [1], aromatic hydrocarbons [2], or tetrachloroethylene [3].

A variety of biotechnological methods for oil recovery enhancement exists in which oxygen supply is achieved by pumping a water–air mixture into the oil layer by means of high-pressure compressors [4–6]. This is a difficult technical task since the pressure inside the oil layer is often higher than the pressure created by the compressor. Moreover, the expense of pumping the inactive nitrogen of the air is not always economically acceptable. Supplying oxygen in the form of hydrogen peroxide solution could be a viable alternative. Oxygen, produced via hydrogen peroxide decomposition, is necessary during the initial stage of the microbial processes leading to increased oil extraction.

Hydrogen peroxide solution is not an exotic reagent for oil recovery. The possible application of hydrogen peroxide for such technical tasks as thermal treatment of the oil stratum, effective oil displacement by vapor, hydrate melting in underwater equipment, decontamination of the near-bottom zones of wells, etc., is considered in the professional literature [7]. Hydrogen peroxide decomposition with the subsequent oxidation of hydrocarbons in high-temperature oil strata can increase oil recovery from deposits of heavy oil [8, 9].

The goal of the present work was to investigate the possibility of aerobic growth of hydrocarbon- and organic acid–utilizing microorganisms with H_2O_2 as the sole oxygen source.

MATERIALS AND METHODS

The investigation was performed using cultures of *Pseudomonas aeruginosa*, strain 202, and *Rhodococcus erythropolis*, strain 367-6. *R. erythropolis* 367-6 was isolated from the Bondyuzhskoe oil field [10], and *P. aeruginosa* 202 was isolated from the White Tiger marine oil reservoir (Vietnam). Both strains are used in biotechnologies for the enhancement of oil recovery.

For bacterial cultivation, modified Raymond medium was used, which had the following composition (g/l): NaCl, 5.0; NH_4Cl, 1.0; $MgCl_2 \cdot 6H_2O$, 0.2; $CaCl_2 \cdot 2H_2$, 0.01; $MnSO_4 \cdot 5H_2O$, 0.02; $FeSO_4 \cdot 7H_2O$, 0.01; K_2HPO_4, 0.04; Tris, 2.0; CH_3COONa, 1.4–2.0; yeast extract (Difco), 1.0; pH 6.9–7.1.

Fourteen- or 20-ml bottles were filled with the medium completely and sealed with rubber stoppers secured with aluminum caps. The initial optical density of the culture was 0.04–0.06 (λ = 540 nm). The inoculum, H_2O_2, and other additives were introduced with syringes; air bubbles were avoided. Growth on acetate was measured by the radioisotopic method as incorporation of labeled carbon into cell biomass. Methyl carbon–labeled acetate (specific activity, 49 GBq/g) intro-

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duced in an amount of 0.1 μCi per milliliter medium was used as the label. During the incubation period, the bottles were kept at 30°C. Samples of 0.5 ml were taken from the bottles and filtered through 0.22-μm nitrocellulose membrane filters. The biomass retained on the filters was washed twice with isotonic salt solution, dried, and placed into vials with ZhS-106 scintillation liquid. The activity of the biomass was measured using a Rackbeta scintillation counter (LKB, Sweden).

Biomass growth per milliliter medium at a given time was estimated using the amount of incorporated carbon from the acetate methyl group according to the equation

$$P = (r \times C_{ac} \times W \times M_c \times 1000)/(R \times V \times M_{ac}),\ \mu g\ C/ml,$$

where P is acetate carbon activity in the medium; r, carbon activity on the filter; C_{ac}, acetate concentration in the medium; W, the amount of medium in the bottle; V, the aliquot volume; M_c, the molecular mass of the acetate carbon; M_{ac}, the molecular mass of acetate; and 1000, a coefficient to render milligrams into micrograms. A preliminary comparison was performed of the data on culture growth obtained by optical density measurement and by the radioisotopic method. The correlation coefficient for various growth stages of *P. aeruginosa* was 0.98, thus enabling the use of the radioisotopic method to obtain objective data on growth dynamics.

To exclude the possible effect of changing the gas pressure in the bottle, for studies of bacterial growth dynamics samples were taken once from each bottle. Oxygen concentration in the aqueous phase was calculated using the equation $2H_2O_2 \longrightarrow O_2 + 2H_2O$.

The sterile mixture of liquid paraffins Parex was introduced into the bottles as a hydrocarbon phase. In calculating oxygen concentration in the aqueous phase, its solubility in hydrocarbons was taken into account. To estimate the solubility of oxygen in paraffins and in oil, the volume of oxygen dissolved in a volume of paraffin (or oil) under normal conditions was determined. In order to degas the paraffin aliquot, it was vacuumized for 15 min. The volume of oxygen absorbed by the paraffin was determined using a calibrated syringe.

Fatty acid analysis was performed by gas chromatography after extraction and methanolysis. Fatty acids (FA) were extracted from the hydrocarbon phase with an alkaline solution (0.05 N NaOH), which was subsequently acidified to pH 5; FA were then extracted with hexane. Methyl ethers of fatty acids were obtained by acidic methanolysis; acetyl chloride solution in methanol was applied for 1.5 h at 80°C [11], followed by hexane extraction. The analysis was performed on a model 3700 gas–liquid chromatograph (Russia) with a flame-ionization detector. Diethyleneglycol succinate (15%) on Chromosorb W (40–60 mesh) was used as the solid phase; the mode was isothermic at 180°C; the carrier gas was argon (40 ml/min). Methyl ethers of fatty acids with 8 to 18 carbon atoms were used as standards.

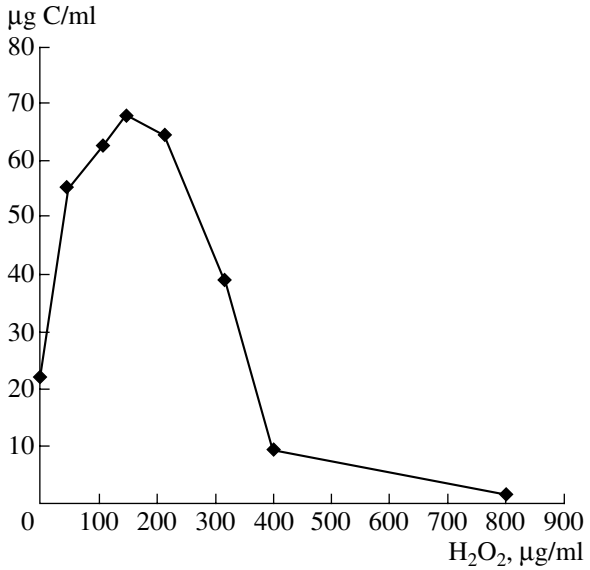

Fig. 1. Effect of hydrogen peroxide concentration on the growth of *R. erythropolis* 367-6 in the presence of a gas phase (1-day culture).

RESULTS AND DISCUSSION

Preliminary experiments on the effect of H_2O_2 were performed in the presence of the gas phase in sealed 20-ml bottles with 5 ml of medium inoculated with *R. erythropolis* 367-6. During the first day, the culture development was observed under H_2O_2 concentrations up to 300 μg/ml (Fig. 1). Under higher H_2O_2 concentrations, growth commenced later. The presence of the gas phase can increase the threshold toxic concentration of H_2O_2 in the medium due to the exchange of free oxygen between the phases. Therefore, the subsequent experiments were carried out in bottles completely filled with liquid. Under these conditions, growth of *P. aeruginosa* 202 and *R. erythropolis* 367-6 was limited to an H_2O_2 concentration of about 220 μg/ml (Fig. 2).

Investigation of the growth dynamics of *R. erythropolis* 367-6 demonstrated that the lag period increased by two to three days with the increase of the H_2O_2 concentration from 100 to 200 μg/ml (Fig. 3). This is an indication of increased toxic effect of peroxide on the cells. The final cell yield in the experiments with peroxide concentrations from 100 to 200 μg/ml corresponds to the amount of peroxide, and thus the final biomass is possibly limited by the amount of oxygen in the bottle. No growth was observed when peroxide concentrations exceeded 300 μg/ml. The elongation of the lag phase concomitant with the increase in the cell yield gives grounds to the suggestion that within the range of peroxide concentrations from 100 to 200 μg/ml a part of the population died out during the short (2–3 h) period of peroxide decomposition. The hydrogen peroxide concentrations optimal for the initiation of cell growth are evidently considerably below 100 μg/ml, i.e., about 50 μg O_2/ml. According to published data, such oxygen

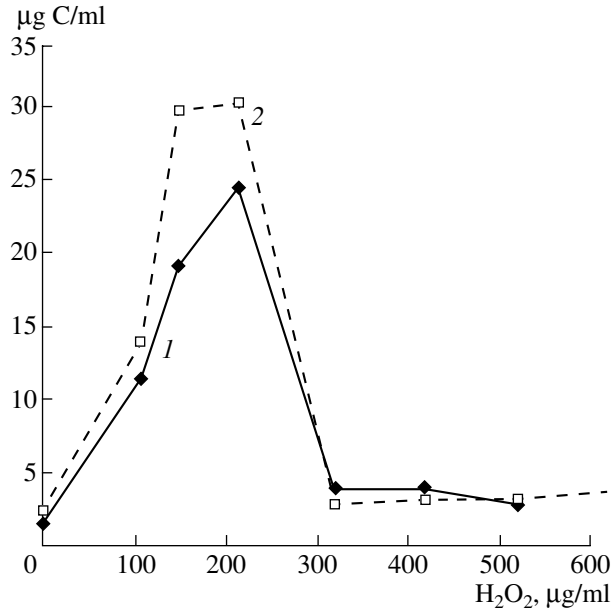

Fig. 2. Effect of hydrogen peroxide concentration on the growth of (*1*) *R. erythropolis* 367-6 and (*2*) *P. aeruginosa* 202 in the absence of the gas phase (1-day culture).

concentrations, corresponding to a partial pressure of more than 1 atm, will decrease the growth rate and increase the respiration rate considerably [12]. During the lag phase, the cells are possibly adapting to these conditions.

Hydrogen peroxide toxicity was tested by incubating the cells for 4 h with different concentrations of H_2O_2. An aliquot of bacterial suspension was afterwards plated on petri dishes with agarized PYG medium [10]. Colony growth occurred under the normal atmospheric pressure of ambient air. The incubation time duration was chosen since calculations show that is peroxide practically completely decomposed by cellular catalases within 2–3 h. The data shown in Fig. 4 demonstrate that the concentrations below 100 μg/ml did not affect the number of viable cells. At H_2O_2 concentrations of about 150 μg/ml, the viable cell numbers decreased noticeably, while above the 200 μg/ml concentration limit, only single colonies were formed.

This estimate of peroxide toxicity coincides with the data provided by the culture dynamics experiments with H_2O_2 as the sole oxygen source. Cell growth at the peroxide concentrations of 150–200 μg/ml occurred possibly because some cells survived the period of H_2O_2 decomposition by catalase. Increased resistance of these cells was possibly the result of their physiological condition, similar to stationary-phase culture cells [13, 14].

The toxicity of oxygen proper can be another factor limiting the culture growth; at 100–150 μg/ml H_2O_2, the oxygen concentration exceeds the air equilibrium 6- to 9-fold. The decrease of partial pressure caused by removing some portion of the liquid results in formation of a gas bubble and in the gas redistribution between the gas and liquid phases. It must be noted, that, in the experiments with high peroxide concentrations (310–414 μg/ml), the culture growth resumed when 0.5–1.0 ml of the liquid was substituted by an air

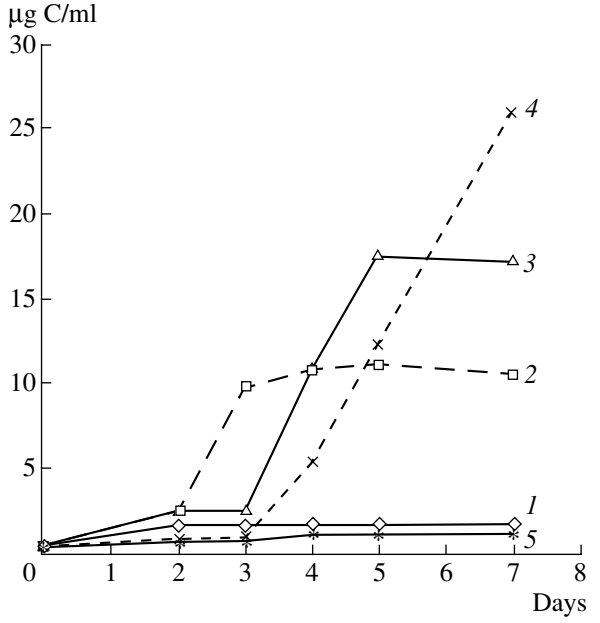

Fig. 3. Growth dynamics of *R. erythropolis* 367-6 at different concentrations of hydrogen peroxide: (*1*) 0, (*2*) 103, (*3*) 145, (*4*) 207, and (*5*) 310 μg/ml H_2O_2.

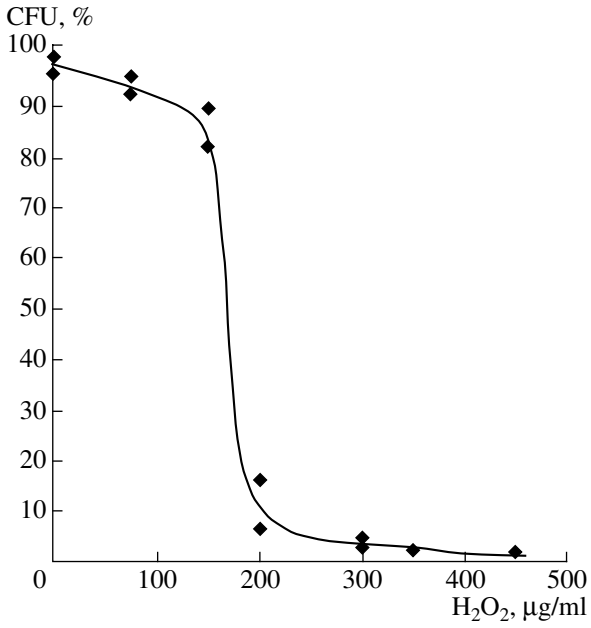

Fig. 4. Effect of hydrogen peroxide concentration on the viability of *R. erythropolis* 367-6 cells.

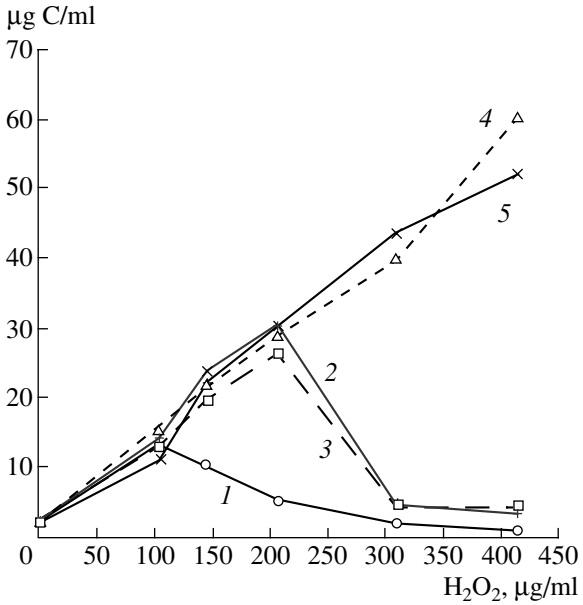

Fig. 5. Growth of *R. erythropolis* 367-6 at different concentrations of hydrogen peroxide: (*1*) 4th day; (*2*) 5th day; (*3*) 6th day; (*4*) 6th day at atmospheric pressure; (*5*) 9th day at atmospheric pressure.

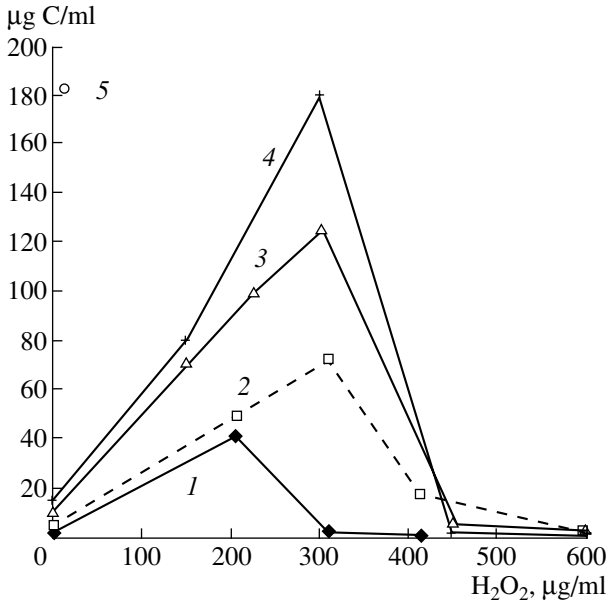

Fig. 6. Growth of *P. aeruginosa* 202 as dependent on hydrogen peroxide concentration and paraffin addition: (*1*) no paraffins; (*2*) 1 ml paraffins; (*3*) 2.5 ml paraffins; (*4*) 3 ml paraffins; (*5*) no paraffins in the presence of a gas phase.

bubble of normal atmospheric pressure (Fig. 5). This is possibly the result of the decrease in oxygen partial pressure caused by its removal into the gas phase. The culture growth in such cases was characterized by a high biomass yield. In a 14-ml bottle with an initial H_2O_2 concentration of 414 μg/ml, creating a 1-ml gas bubble at atmospheric pressure resulted in the drop of the dissolved oxygen concentration from 195 to 93 μg/ml.

In an oil layer, oxygen toxicity may be reduced by the presence of two liquid phases (water and hydrocarbons) since oxygen is more soluble in hydrocarbons than in water, e.g., 5-fold in toluene [15] and 6.7-fold in hexadecane [16]. Thus, the introduction of a hydrocarbon phase into the experiment was bound to decrease the toxicity of high oxygen concentrations.

According to our experiments, oxygen solubility in paraffins was close to that in hexadecane, while oxygen solubility in oil was 1.4 times less than in paraffins. For the calculations of the equilibrium oxygen concentration in the water–paraffins two-phase system, oxygen solubility values for hexadecane [16] were used.

Since the rhodococcal cells are lipophilic and therefore concentrate in the hydrocarbon phase when grown on hydrocarbons, the culture of *P. aeruginosa* 202, with hydrophilic cell surfaces, was used for this set of experiments.

Preliminary tests indicated that the presence of hydrocarbons (as the paraffin mixture) as a potential substrate did not affect the rate of acetate incorporation into the biomass.

The possibility of production of fatty acids as potential bacterial growth substrates in the course of interaction between paraffins and H_2O_2 was tested in the following experiment. Liquid paraffins and hydrogen peroxide in a high concentration (6000 μg/ml) were added to the medium. After three days of incubation at 30°C, no fatty acids were found.

In the experiments with the hydrocarbon phase, H_2O_2 was introduced in a manner similar to the previous experiments; 20-ml bottles contained microorganisms suspended in the growth medium and up to 3 ml of liquid hydrocarbons. The results of acetate carbon incorporation at the stationary growth phase of *P. aeruginosa* 202 incubated with different concentrations of hydrogen peroxide in the presence of paraffins are shown in Fig. 6 and Table 1. It was found that an amount of paraffins comprising 5% of the medium volume was sufficient to ensure cell growth at initial peroxide concentrations of about 300 μg/ml medium. Increasing the paraffin volume to 3 ml (15% of the medium volume) caused an increase in biomass to a level comparable to the cell yield under normal conditions of aeration. The calculated oxygen concentration in the medium was 64 μg/ml. No cell growth was observed at initial peroxide concentrations above 450 μg/ml. Under such conditions, the amount of surviving cells is possibly not sufficient to initiate growth (Table 1, Fig. 6). Thus, the presence of hydrocarbons enables initial peroxide concentrations in the medium to be increased from 200 to 300 μg/ml.

To test the ability of microorganisms to grow in medium with a high oxygen concentration, the bottles with medium and paraffins were inoculated twice: together with hydrogen peroxide addition and 3 h later.

Table 1. Incorporation of carbon into *P. aeruginosa* 202 cells in the presence of paraffin

Initial H_2O_2 concentration, μg/ml	Paraffin, vol %	Calculated O_2 concentration, μg/ml	Carbon incorporation into cells, μg C/ml
0	0	0	2
207	0	97	41
310	0	146	2
414	0	195	1
0	7	0	5
207	7	65	50
310	7	97	72
414	7	130	18
621	7	195	1
828	7	259	5
1034	7	324	2
0	12.5	0	11
150	12.5	36	71
225	12.5	54	100
300	12.5	72	124
450	12.5	108	5
600	12.5	144	2
750	12.5	180	1
0	15	0	14
150	15	32	80
300	15	64	181
450	15	97	2
600	15	129	1
Air	–	7.8	182

Table 2. Incorporation of carbon into *P. aeruginosa* 202 cells in the presence of hydrogen sulfide

H_2O_2 concentration, μg/ml	$\Sigma H_2S + HS^-$ initial concentration, μg/ml	Calculated O_2 concentration, μg/ml	Carbon incorporation into cells, μg C/ml
414	0	195	1
414	110	85	52
414	166	29	23
414	248	0	1

The density of the culture was 0.04 (cuvette, 0.5 cm, $\lambda = 540$ nm) during peroxide decomposition and 0.08–0.1 after the second inoculation. In this case, growth at 300 μg/ml, which corresponds to initial oxygen concentrations of about 141 μg/ml or its equilibrium in the aqueous phase at 64 μg/ml, commenced without a prolonged lag phase. Bacterial growth was observed at H_2O_2 concentrations of 375–450 μg/ml; in these cases, the lag phase duration was 1–2 days. The density of the inoculum affected the inhibiting concentration, possibly because it implied different catalase concentrations in the medium. An exposure duration increase to 18 h did not result in any change in the threshold inhibitory concentration. The maximal oxygen concentration enabling growth was somewhat higher than in the experiments without preliminary inoculation. Thus, the threshold oxygen concentration enabling growth of the culture was 59–97 μg O_2/ml.

The water in the oil layers usually contains H_2S and its derivatives or iron ions (mostly Fe^{2+}). When hydrogen peroxide is pumped into the layer, the oxygen produced can both be dissolved in oil and be consumed for the oxidation of these compounds if they are available. It was demonstrated in a series of experiments that introduction into the bottles of appropriate amounts of H_2S can lead to a decrease in high oxygen concentrations to below the toxic level (Table 2). Bacterial growth was consistent with the amount of oxygen remaining after its inactivation by hydrogen sulfide: $2H_2S + O_2 = 2S + 2H_2O$.

Hydrogen peroxide in concentrations of about 100 μg/ml can therefore provide oxygen for the growth of aerobic microbial cells in the absence of a gas phase. At H_2O_2 concentrations of 100 to 300 μg/ml, part of the population dies and subsequent growth is postponed. Higher H_2O_2 concentrations inhibit cell growth. In 2–3 h, H_2O_2 is completely decomposed in the medium by catalases and inorganic catalysts, while the oxygen produced acts in its turn as another inhibiting factor with a threshold concentration of around 100 μg/ml. The presence of a hydrocarbon phase ameliorates the toxic effect of dissolved oxygen.

Hydroperite, a complex of H_2O_2 with urea, was tested as a potential H_2O_2 analogue. Hydroperite is not only a source of oxygen but also a potential source of nitrogen, which is also necessary for the activation of microbial processes [5]. The possibility of cultivating hydrocarbon-oxidizing bacteria on hydroperite was tested on *P. aeruginosa* 202. The maximal growth was shown to occur at hydroperite concentrations of from 300 to 450 μg/ml, which is consistent with the data obtained for H_2O_2 (Fig. 7).

These data allow us to suggest that pumping hydrogen peroxide into the layer, its subsequent decomposition, and preferential dissolution of the produced oxygen in the oil will create conditions that are favorable for the aerobic component of the microbial cenosis.

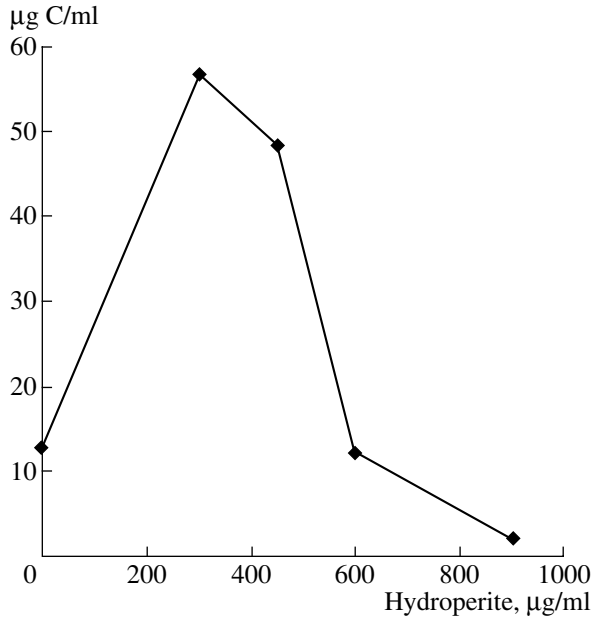

Fig. 7. Growth of *P. aeruginosa* 202 at different concentrations of hydroperite.

The use of hydrogen peroxide as an aqueous solution has certain advantages to pumping air into the layer. It does not require high-pressure compressors spending energy on compressing air, which consists mostly of an inactive component (nitrogen). Active oxygen can effectively neutralize such toxic compounds as hydrogen sulfide, thus creating favorable conditions for the aerobic stage of the microbial technology for enhanced oil recovery.

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