Microbiology, Vol. 70, No. 3, 2001, pp. 263–269. Translated from Mikrobiologiya, Vol. 70, No. 3, 2001, pp. 313–320.
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EXPERIMENTAL ARTICLES

Effect of Butyric Acid on the Physiological Activity of Hydrocarbon-Oxidizing Rhodococci

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Received May 15, 2000; in final form, January 31, 2001

Abstract—Laboratory experiments showed that butyric acid not only fails to meet the trophic requirements of hydrocarbon-oxidizing microorganisms, but even specifically inhibits their assimilatory and dissimilatory activity. Therefore, butyric acid can be referred to as growth inhibitors. The combined mineralization of carbohydrates and hydrocarbons can be described as follows. Plants polymers are converted to monosugars by heterotrophic soil microorganisms. As the concentration of the monosugars grows and oxygen becomes deficient, the monosugars are no longer oxidized completely but are fermented. As a result, glucose transforms to butyric acid, which inhibits hydrocarbon-oxidizing bacteria. It is concluded that, to be efficient, the cleanup of oil-contaminated soils must include measures to intensify the mineralization of carbohydrates and to inhibit their fermentation.

Key words: soil, glucose, butyric acid, hydrocarbon-oxidizing microorganisms, trophic substrate, growth inhibitor, dormancy.

Crude petroleum and petroleum products are major environmental pollutants [1]. In view of this, the decontamination of oil-polluted soils remains one of the most challenging problems, whose solution is impossible without knowledge of the specifics of the microbial utilization of oil hydrocarbons in situ. The ecological aspects of this problem were considered in a number of publications [2, 3]; however, there are still many points to be elucidated. As the microbial utilization of hydrocarbons in oil-contaminated soils occurs along with the degradation of dead leaves, which are composed predominantly of carbohydrates, the relationship between these processes is of great interest.

The most abundant natural carbohydrate polymer is cellulose [4], which is utilized by microorganisms after its degradation to glucose. As was shown earlier by Guzev *et al.* [5], glucose added in amounts up to 1 mg/g soil considerably augments the population density of hydrocarbon-oxidizing bacteria and enhances the degradation of hydrocarbons, whereas higher glucose concentrations inhibit, and even may stop, this process. On the other hand, Gusev *et al.* [6] showed that the presence of glucose in the cultivation medium does not affect the utilization of hydrocarbons. These contradictory results obtained during the in vitro and in situ investigation of the effect of glucose on hydrocarbon-oxidizing microorganisms can be explained by the property of glucose to act on soil microorganisms indirectly, via an intermediate compound. It should be noted in this regard that the addition of high concentrations of glucose to soil is accompanied by the formation of butyric acid, which has a specific odor.

Taking into account that butyric acid inhibits some hydrolytic enzymes [7], the aim of the present work was to evaluate the possible adverse effect of this compound on hydrocarbon-oxidizing microorganisms.

MATERIALS AND METHODS

Soil. Studies were performed using a sample of weakly loamy gray soil taken from a depth of 0–25 cm in the Samarkand region (Uzbekistan). The humus content of the soil was 2%. The sample was thoroughly mixed, air-dried, rid of roots, ground in a mortar, and sieved through a screen with 1-mm mesh size.

Microorganisms. Two strains of rhodococci, *Rhodococcus ruber* 114 and *Rh. maris* 367-2, were obtained, respectively, from the collection of microorganisms at the Department of Soil Biology of the Faculty of Soil Science of the Moscow State University and from the Laboratory of Petroleum Microbiology of the Institute of Microbiology of the Russian Academy of Sciences. These strains were isolated from oil-contaminated soil and were found to possess high hydrocarbon-oxidizing activity. The strains were maintained on nutrient agar. Before the experiments, they were adapted to hydrocarbons by cultivating them in a hexadecane-containing medium.

Cultivation. Material for inoculation was grown on an agar medium for rhodococci [8] containing (g/l) casein hydrolysate, 1; peptone, 2; chalk, 5; yeast

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extract, 1; and agar, 20. The medium was prepared using tap water and contained D-glucose (5 g/l) as the carbon source. In survival experiments, chemically pure hexadecane (5 ml/l) was substituted for glucose. Colonies were counted after five days of growth on agar plates taken in triplicate.

The effects of butyric acid taken at concentrations of 0, 0.5, 1, and 2 mg/ml, and glucose taken at concentrations ranging from 0 to 20 mg/ml, were studied using cultures grown in a liquid mineral medium recommended for the isolation of lipolytic microorganisms [9], in which hexadecane was substituted for olive oil and carbonates were omitted because of their interference with nephelometric measurements. The medium contained (g/l) $(NH_4)_2SO_4$, 1.0; K_2HPO_4, 2.5; $MgSO_4 \cdot 7H_2O$, 1.0; and yeast extract, 0.15 in 1 l of tap water. Cultivation was performed at 27°C in duplicate 500-ml flasks with 200 ml of the growth medium on a shaker (180 rpm). The media were sterilized at 0.5 atm. The growth substrates were sterilized separately at 1 atm (hexadecane and butyric acid) or 0.5 atm (glucose).

Experimental conditions. The effects of glucose and butyric acid on the accumulation of bacterial biomass and carbon dioxide were studied in 12-day experiments. The effects of butyric acid and phosphate–citrate buffer on the evolution of carbon dioxide was studied by incubating 7-day-old cultures with these substances for 1 h in triplicate 30-ml serum vials with 10 ml of the culture liquid. The pH of the medium was measured immediately after the addition of the reagents.

The activity of cultures was evaluated by measuring the cell respiration and changes in the culture turbidity. The turbidity was measured at 540 nm with a Spekol-11 spectrophotometer (Germany) for 12 days at 1- to 3-day intervals. The increase in the content of carbon dioxide in the gas phase was measured at the same time intervals using an Infralit-4 gas analyzer (Germany). The biomass and the production of carbon dioxide were determined at the beginning of the stationary growth phase.

Cell viability was evaluated by counting colonies grown on agar plates inoculated with the fifth, sixth, and seventh tenfold culture dilutions prepared 1 h, as well as 1, 3, and 5 days, after the addition of the substance tested. The results were expressed in colony-forming units (CFU).

Seed germination experiments. Nonsterile soil was wetted to a field moisture content, kneaded to a pasty consistency, supplemented with glucose at concentrations ranging from 1 to 10 mg/g soil, and placed in petri dishes 4-cm in diameter. The thus prepared soil plates were incubated in a humid chamber at 25°C for 1 day, after which 20 cress seeds were placed on the surface of each of the plates and incubated for the next 4–5 days. Then the percentage of germinated seeds was determined. These experiments were performed in triplicate.

The results obtained were statistically processed using the Statistica software program. Graphs were constructed with the aid of the Harvard Graphics program.

RESULTS

The cultural and physiological characteristics of the rhodococci under study were different. For instance, when grown on agar medium with hexadecane, *Rh. maris* produced wet, slimy, red colonies, and *Rh. ruber* produced dry, rough, yellow–orange colonies. When grown in liquid medium, *Rh. ruber* produced cell aggregates and *Rh. maris* produced a viscous culture. It should be noted that the results of the growth experiments greatly varied; nevertheless, tendencies in the action of the substances tested were reproduced well, irrespective of the bacterial state at the beginning of the experiment.

Assimilatory and dissimilatory activities. In this set of experiments, we performed a comparative investigation of the effects of glucose and butyric acid on cell respiration and the biomass of the hydrocarbon-oxidizing rhodococci (Figs. 1–6). In general, changes in the culture turbidity followed a typical growth curve of batch cultures: after a lag phase, the culture turbidity showed a rapid increase and, after 8–10 days of growth, the culture entered the stationary phase (Fig. 1). Using the biomass of the stationary-phase cultures as an index of their assimilatory activity, we could reveal the following regularities.

The noticeable increase in the culture turbidity of *Rh. maris* in response to the addition of glucose was observed only when the glucose concentration was higher than 2 mg/ml (Fig. 2, curve *1*). In the presence of hexadecane in the growth medium, the effect of glucose was similar (Fig. 2, curve *2*). Poor growth in the medium containing butyric acid as the sole source of carbon was observed only when its concentration did not exceed 0.5 mg/ml (Fig. 2, curve *3*). The addition of butyric acid to the hexadecane-containing medium led to a partial or complete (when the concentration of butyric acid was 1 mg/ml and higher) inhibition of growth (Fig. 2, curve *4*).

Unlike the biomass of *Rh. maris*, that of *Rh. ruber* increased in response to the addition of glucose to the medium only when the concentration of glucose was lower than 1 mg/ml, while it did not change when the glucose concentration was higher (Fig. 3, curve *1*). In the presence of hexadecane in the growth medium, the effect of glucose was similar (Fig. 3, curve *2*). The effect of butyric acid alone on the growth of *Rh. ruber* was the same as in the case of *Rh. maris* (cf. curves *3* in Figs. 2 and 3). The biomass of *Rh. ruber* in the hexadecane-containing medium increased in response to the addition of butyric acid when its concentration did not exceed 0.5 mg/ml and considerably decreased when its concentration was 1 mg/ml or higher (Fig. 3, curve *4*).

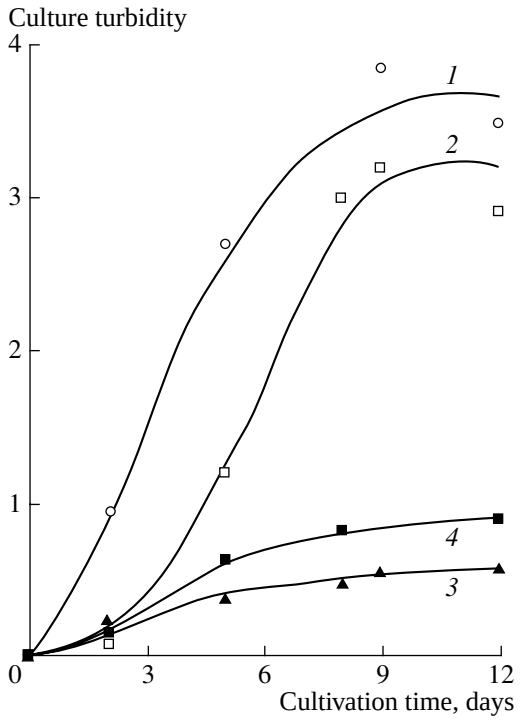

Fig. 1. Growth of (*1* and *2*) *Rh. maris* and (*3* and *4*) *Rh. ruber* in media with (*1* and *3*) hexadecane alone and (*2* and *4*) hexadecane plus butyric acid taken at concentrations of 0.5 mg/ml.

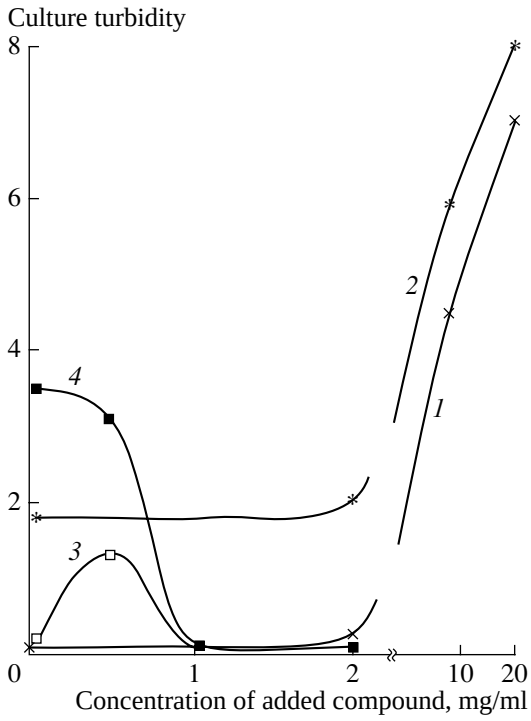

Fig. 2. Effect of (*1* and *2*) glucose and (*3* and *4*) butyric acid on the growth of *Rh. maris* in media (*2* and *4*) with and (*1* and *3*) without hexadecane.

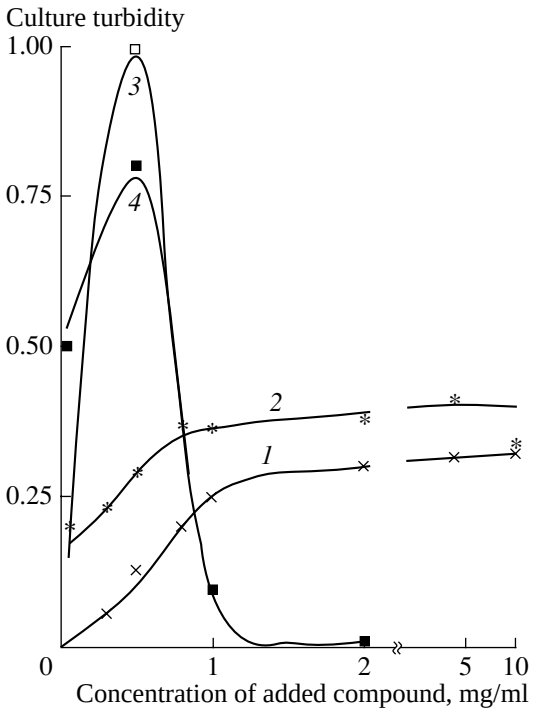

Fig. 3. Effect of (*1* and *2*) glucose and (*3* and *4*) butyric acid on the growth of *Rh. ruber* in media (*2* and *4*) with and (*1* and *3*) without hexadecane.

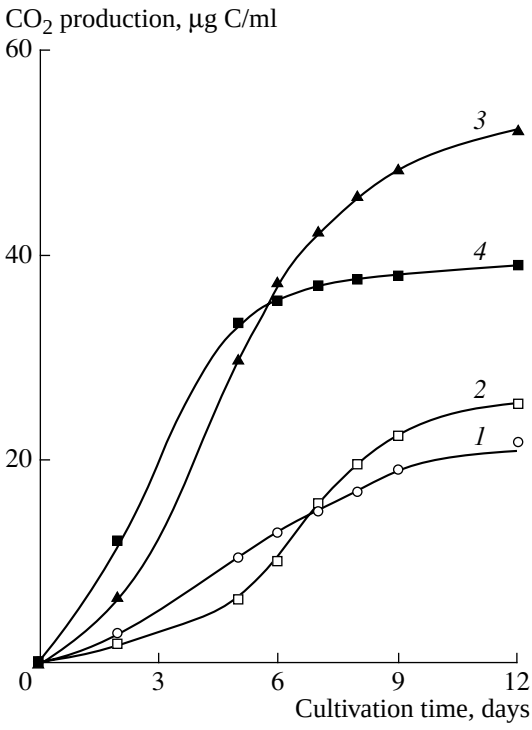

Fig. 4. Production of carbon dioxide by (*1* and *2*) *Rh. maris* and (*3* and *4*) *Rh. ruber* in media with (*1* and *3*) hexadecane alone and (*2* and *4*) hexadecane plus butyric acid taken at concentrations of 0.5 mg/ml.

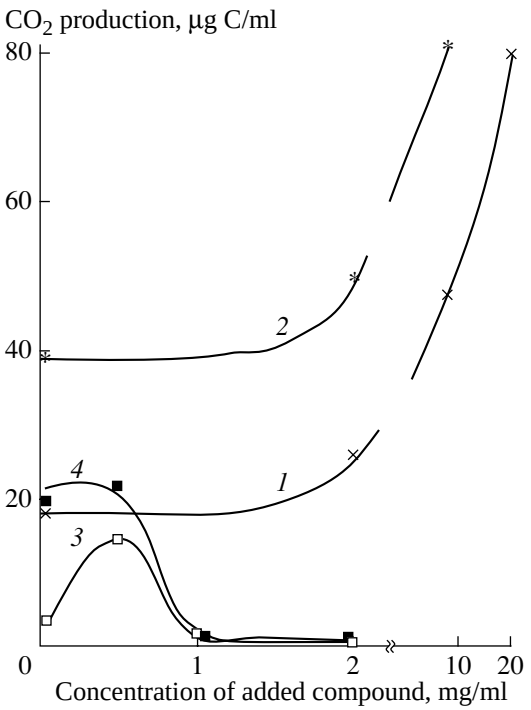

Fig. 5. Production of carbon dioxide by *Rh. maris* in media with (*1*) glucose, (*2*) glucose and hexadecane, (*3*) butyric acid, and (*4*) hexadecane plus butyric acid.

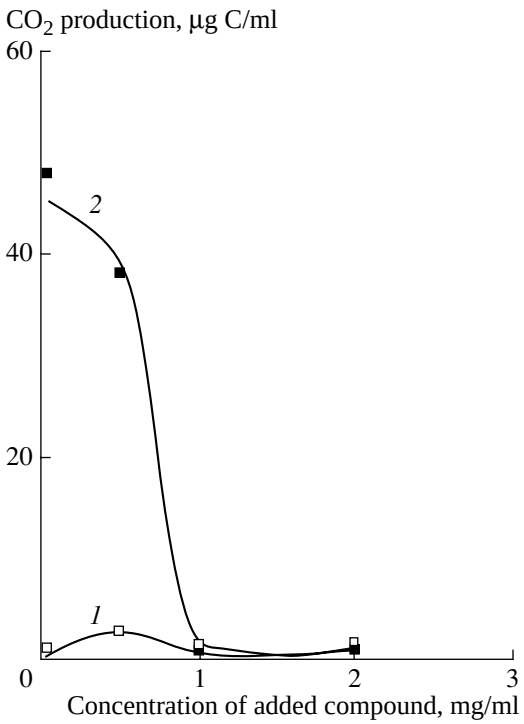

Fig. 6. Production of carbon dioxide by *Rh. ruber* in media with (*1*) butyric acid alone and (*2*) hexadecane plus butyric acid.

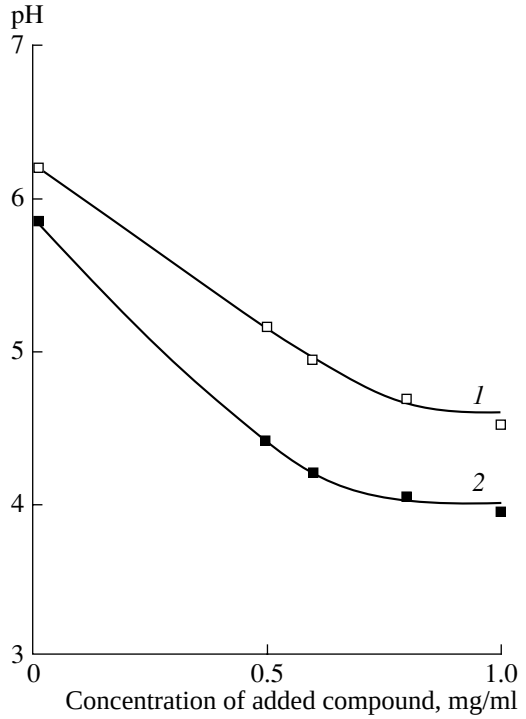

Fig. 7. Effect of the addition of butyric acid to 7-day-old cultures of (*1*) *Rh. maris* and (*2*) *Rh. ruber* on the pH of the culture liquids.

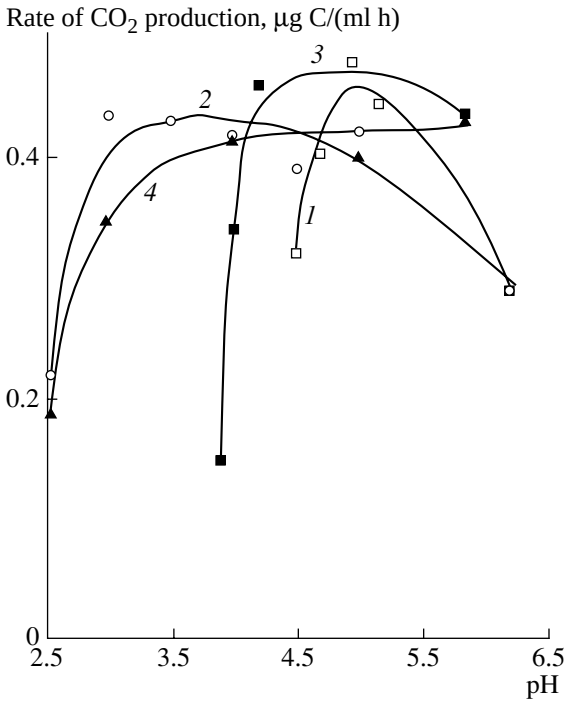

Fig. 8. Effect of the pH of the culture liquid after the addition of (*1* and *3*) butyric acid and (*2* and *4*) phosphate–citrate buffer on the rate of carbon dioxide evolution by (*1* and *2*) *Rh. maris* and (*3* and *4*) *Rh. ruber.*

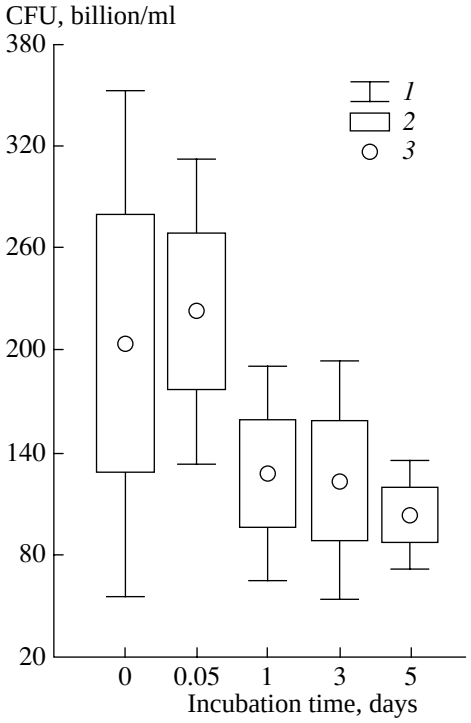

Fig. 9. Effect of butyric acid on the viability of *Rh. ruber*: *1*, confidence interval; *2*, standard error; and *3*, mean value.

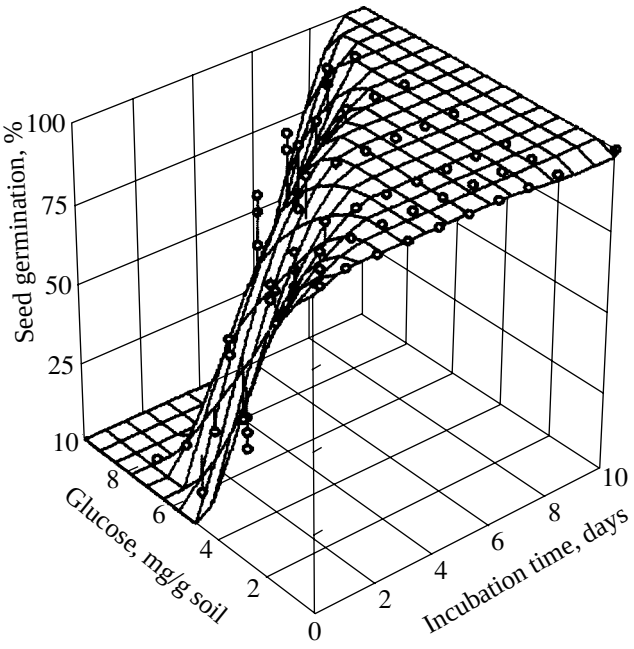

Fig. 10. Effect of different concentrations of glucose present in soil on the germination of cress seeds.

The dissimilatory activities of both species were described by S-shaped curves with different saturation levels (Fig. 4). The stimulatory effect of glucose and the inhibitory effect of butyric acid on the growth of *Rh. maris* (Fig. 2) were in agreement with data on the production of carbon dioxide by this species (Fig. 5). The inhibitory action of butyric acid at a concentration of 1 mg/ml or higher on the production of carbon dioxide by *Rh. ruber* was observed only when the growth medium contained hexadecane (Fig. 6). When butyric acid was the sole source of carbon, the respiration of *Rh. ruber* was almost independent of its concentration.

Butyric acid and pH. The addition of various concentrations of butyric acid to 7-day-old cultures of rhodococci led to an acidification of the culture liquid and to a change in the carbon dioxide evolution rate. The pH of the culture liquid dropped from 6.2 to 4.5 in the case of *Rh. maris* and from 5.8 to 3.9 in the case of *Rh. ruber* (Fig. 7). The difference in the pH drop in response to the addition of butyric acid can be explained by the difference in the buffer capacities of the culture liquids of rhodococci due to the accumulation of different extracellular metabolites and/or their different amounts. The effect of butyric acid on the respiration of rhodococci can be described as follows. At concentrations below 0.6 mg/ml, butyric acid slightly stimulated respiration, whereas it inhibited it at higher concentrations (Fig. 8, curves *1* and *3*). When the culture liquid was acidified to a pH of about 4 by the addition of phosphate–citrate buffer, the respiration of rhodococci either did not change (*Rh. ruber*) or slightly increased (*Rh. maris*). Only when the buffer was added in amounts such that the pH of the culture liquid dropped below 3.0, the respiration of rhodococci decreased (Fig. 8, curves *2* and *4*).

Culture viability. When the time of the incubation of rhodococci with butyric acid was extended, the number of viable cells estimated in CFU/ml decreased almost twofold (Fig. 9). However, the statistical processing of the experimental results showed that the respective 95% confidence intervals overlapped, indicating that the experimental difference may be accidental and, hence, there are no grounds to believe that butyric acid affects the viability of rhodococci.

Effect of glucose on seed germination. The germination of cress seeds in the soil and on filter paper impregnated with solutions containing up to 20 mg/ml glucose was close to 100%. When glucose was added to the soil in amounts below 2 mg/g soil, seed germination almost did not change (Fig. 10). However, when added at a concentration of 4 mg/g soil or higher, glucose caused soil swelling. In this case, soil had the specific odor of butyric acid and the percentage of germinated cress seeds decreased, tending to zero at a glucose concentration of 6 mg/g soil. In the course of the soil incubation, the odor of butyric acid gradually disappeared and the percentage of germinated seeds increased, returning to the initial value after 8 days of incubation.

DISCUSSION

The experimental results presented in Fig. 10 confirmed the wide-spread opinion that butyric acid is the major product of the carbohydrate conversion in soil under oxygen deficiency [10].

Hydrocarbon-oxidizing rhodococci are able to utilize glucose as a cosubstrate: they can grow on this monosaccharide as a sole source of carbon and respond to the addition of glucose to hexadecane-containing medium by increasing the culture turbidity and enhancing the production of carbon dioxide. These data agree well with the data of Gusev *et al.* [6], who showed that glucose is utilized by hydrocarbon-oxidizing bacteria together with the hydrocarbon, and of Poindexter [11], who found that microorganisms fall into trophic groups with respect to the utilization of glucose. *Rh. ruber*, whose growth is stimulated by glucose concentrations below 1 mg/ml (Fig. 3, curve *3*), can be considered an oligotroph, whereas *Rh. maris*, whose growth is stimulated by glucose concentrations above 2 mg/ml (curves *1* in Figs. 2 and 5), can be considered a copiotroph. This suggestion is confirmed by the cultural properties of the rhodococci under study, bearing in mind that a copiotrophic microorganism accumulates the high biomass, produces slimy colonies on agar media, and synthesizes extracellular proteins when grown in liquid media.

Analysis of the effect of butyric acid on the growth of hydrocarbon-oxidizing bacteria and their production of CO_2 showed this compound at low concentrations (no more than 0.5 mg/ml) can be used, to a certain degree, by these bacteria as a sole source of carbon and energy. At higher concentrations (1 mg/ml or more), butyric acid inhibited the growth of rhodococci in all the media studied. Therefore, in a strict sense, butyric acid is not a growth substrate of hydrocarbon-oxidizing rhodococci but acts as a growth inhibitor. Furthermore, butyric acid inhibits the germination of cress seeds and the activity of soil microbiota (Fig. 10). These effects of butyric acid are transient (probably, because this compound is volatile): after 8 days of incubation, the inhibitory effect of butyric acid on the seed germination diminished to zero.

Generally, the effect of pH on the dissimilatory activity of bacteria is described by a dome-shaped curve [12]. The lower limits of the tolerance of rhodococci to the medium acidification caused by butyric acid and phosphate–citrate buffer differ by 1.5–2 pH units (Fig. 8). Therefore, the inhibitory action of butyric acid is stronger than that of phosphate–citrate buffer and probably has a different mechanism related to the chemical nature of this compound.

After incubation with butyric acids, rhodococci retained their viability, although their metabolic activities became low, as was judged from the data on their respiration rate and biomass. This suggests that rhodococcal cells transformed to the state of dormancy [13] or, in the terms of Kalakutskii and Sidyakina [14], of exogenous dormancy. The stimulation of the formation of cysts in *Azotobacter vinelandii* by butyric acid [15] suggests that this compound is involved in the processes of both polysaccharide degradation and nitrogen conversion in soil.

The data presented in this paper show that glucose added to soil at high concentrations undergoes microbial transformation to butyric acid, which inhibits the growth and activity of hydrocarbon-oxidizing microorganisms and promotes their transition to the state of dormancy. Therefore, butyric acid can be considered a growth inhibitor [16].

Under laboratory conditions, the inhibitory concentration of butyric acid with respect to the growth and respiration of rhodococci is about 1 mg/ml. The inhibitory effect of glucose (due to the formation of butyric acid) on the seed germination and the activity of hydrocarbon-oxidizing microorganisms in soil is observed when its concentration is about 4 mg/g soil [5]. Since the field moisture content of soil is about 30% and the degree of the transformation of glucose to butyric acid during fermentation is about 37% [17], the fermentation of 1 mg of glucose present in 1 g of soil may give rise to a concentration of butyric acid of about 1 mg/ml soil suspension, whereas the remaining 3 mg of glucose present in 1 g of soil may be metabolized in an alternative way, particularly by butyric acid–fermenting clostridia. These calculations show that the inhibitory action of glucose on hydrocarbon-oxidizing soil microorganisms is really due to the formation of butyric acid.

The effect of carbohydrates on the utilization of hydrocarbons in soil may be described as follows. Polysaccharides are first transformed to monosugars, which are then utilized by heterotrophic soil microorganisms. Due to the low rate of the polysaccharide transformation, the monosugars accumulate in minor amounts and are utilized through their complete oxidation. As the degradation rate of plant polymers increases, the concentration of the resultant monosugars rises and, provided that the concentration of oxygen is limited, the monosugars begin to undergo fermentation rather than complete oxidation. As a result, glucose is transformed to butyric acid and thus serves as a signal providing a positive feedback between the processes of the mineralization of carbohydrates and hydrocarbons.

Thus, it is clear that, to be efficient, the cleanup of oil-contaminated soils must include measures to intensify the mineralization of carbohydrates and to inhibit their fermentation.

ACKNOWLEDGMENTS

We are grateful to N.S. Panikov for instructive advice on the experiments.

This work was supported by grant no. 00-15-97886 from the Russian Foundation for Basic Research.

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