

Effectiveness of Biodegradation of Petroleum Products by Mixed Bacterial Populations in Liquid Medium at Different pH Values

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Abstract

The possibility of using mineral oils as a carbon source by bacteria adapted to high oil concentrations was tested in liquid media with different pH values (pH = 5, 7 and 9). Two types of inocula were tested: inoculum I consisted of selected strains used in the bioremediation of oil-contaminated soils and inoculum II contained bacteria isolated from soil samples previously bioremediated at pH = 5, 7 and 9. Biodegradation was observed in all the investigated media independently of initial pH value and type of inoculum used. After 21 days of cultivation the reduction of oil content reached 60–70% in medium with pH = 5 and 80–90% in medium with pH = 7 and 9, respectively. Inoculum I consisted of strains of *Arthrobacter*, *Pseudomonas*, *Agrobacter*, *Xanthomonas* spp. After 21 days of incubation the elimination of some strains was observed. In cultures conducted at pH = 5 *Agrobacter* strain was no longer found, at pH 9 – the *Pseudomonas* strain was lost. In cultures maintained at pH = 7 all the introduced strains survived. Prolonged incubation in liquid medium at pH = 5 of strains isolated from bioremediated soils (type II inoculum) leads to the elimination of *Bacillus* from initial consortium of *Arthrobacter*, *Bacillus* and *Pseudomonas*. In cultures containing bacteria of type II inoculum (*Arthrobacter*, *Bacillus*, *Achromobacter*, *Agrobacter*, *Alcaligenes*, *Pseudomonas*, *Xanthomonas*, *Micrococcus*) conducted in liquid media at pH = 9 the *Micrococcus* strain was no longer present. In liquid cultures incubated at pH = 7 all introduced strains were recovered (*Arthrobacter*, *Bacillus*, *Achromobacter*).

Key words: biodegradation, petroleum products, influence of pH

Introduction

Petroleum-derived products are a considerable threat to the natural environment and for human health. Studies on biological and physico-chemical methods for their removal are constantly being pursued. Microbiological processes of the degradation of these compounds are still not far from being elucidated, even though there is no doubt as concerns their effectiveness.

There are numerous microorganisms in soils and waters that are capable of degrading petroleum products. They can be found among both the *Prokaryota* and *Eukaryota* – hydrocarbons are degraded by bacteria, including the actinomycetes, microscopic fungi. In aquatic biosystems biodegradation is carried out mostly by bacteria and microscopic fungi, in the soil by bacteria and molds (Balba *et al.*, 1998; Bonde, 1977; Bossert and Bartha, 1984; Boszczyk-Maleszak *et al.*, 2003).

In the degradation of hydrocarbons a dominating role is played by microscopic fungi, molds and bacteria; mainly of the genera *Achromobacter*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Flavobacterium*, *Pseudomonas*, *Mycobacterium* and *Nocardia* which account for approximately 20% of all organisms capable of degrading hydrocarbons (Leahy and Colwell, 1990). The biodegradation of petroleum products in the environment depends on a host of physical and chemical factors. One of these that is of prime importance in determining the course and effectiveness of the process is pH. Most heterotrophic bacteria and fungi prefer a pH that

is close to neutral (fungi in general are more tolerant to acidic pH). Most aquatic ecosystems are characterized by neutral or slightly alkaline pH, whereas the pH of soils can be within a broad range of values (pH 2.5–11) (Bossert and Bartha, 1984).

Experimental

Materials and Methods

Bacteria. The studies embraced 12 strains of bacteria from the strain collection of the Institute of Microbiology, Warsaw University, adapted to the degradation of petroleum products in high concentration, isolated from crude oil-contaminated soil and 18 strains isolated from soil with pH values of 5, 7 and 9 (following bioremediation) (Boszczyk-Maleszak *et al.*, 2003).

Preparation of bacterial inoculum. Bacterial strains were plated out on nutrient agar. After 24 hours of incubation the grown colonies were washed off with 0.9% NaCl saline. The obtained suspension served as an inoculum in further studies. **Inoculum I.** The inoculum contained 12 strains representing the following genera: *Arthrobacter* – 3 strains, *Pseudomonas* – 7, *Agrobacter* – 1, *Xanthomonas* – 1 strain. **Inoculum II.** The inoculum composed of bacteria isolated from soil with pH 5.0 following bioremediation contained 7 strains representing the genera: *Arthrobacter* – 5 strains, *Bacillus* – 1 strain, *Pseudomonas* – 1 strain. In the case of soil with pH 7.0, the strains were *Arthrobacter* (2 strains), *Bacillus* (5) and *Achromobacter* (1) and for soil with pH 9.0 – *Arthrobacter* (1 strain), *Bacillus* (2), *Achromobacter* (1), *Agrobacter* (1), *Alcaligenes* (1), *Pseudomonas* (1), *Xanthomonas* (1) and *Micrococcus* (1).

Petroleum fraction. Oil being a mixture of light and heavy hydrocarbons collected from a mechanical purification plant treating petroleum refining wastewaters was used.

Media. The following types of media were used: 1) – mineral medium containing: K_2HPO_4 – 7 g, KH_2PO_4 – 3 g, $MgSO_4 \times 7H_2O$ – 0.1 g, $(NH_4)_2SO_4$ – 1 g, H_2O – 1L. The medium was supplemented with 3% petroleum fraction, 2) – nutrient agar.

Identification of bacteria. Classification of bacteria was based on the following diagnostic tests: Gram stain, presence or absence of L-alanine aminopeptidase, microscopic observations of strains, API-20NE, Kovac's cytochrome oxidase test, Hugh-Leifson test. Based on the results the bacterial strains were identified according to the scheme of Bonde (Fritzsche, 1994) and API 20NE code book.

Culture conditions. Flasks containing 50 mL of mineral medium were inoculated with 7.5 mL bacterial inoculum. Each culture was supplemented with oil to a final concentration of 3%. The cultures, including the controls, were adjusted to three different pH values, 5, 7 and 9 and were maintained for 21 days on orbital shaker at room temperature.

Determination of number of bacteria. The number of bacteria was determined by the plate method on nutrient agar.

Determination of petroleum products. The content of petroleum products was determined by weight method following extraction with petroleum ether.

Results and Discussion

The aim of the study was to investigate the use of petroleum oils as sole carbon source in liquid media with different pH value (5, 7 and 9) by selected strains of microorganisms adapted to the degradation of petroleum products.

The studies were carried out at three different pH values (5, 7, 9) in two stages: 1) with the use of inoculum prepared from bacterial strains that was employed for the bioremediation of oil-contaminated soil (inoculum I); 2) with the use of inoculum containing strains of bacteria isolated from soils previously subjected to bioremediation processes at various pH values (inoculum II).

The studies embraced 12 strains of bacteria adapted to high concentrations of petroleum products from the collection of the Department of General Microbiology. In earlier investigations the bacteria were identified based on API 20NE test and Bonde's scheme and it was found that they represented the following genera: *Arthrobacter* – 3 strains, *Agrobacterium* – 1 strain, *Pseudomonas* – 7 strains, *Xanthomonas* – 1 strain. A mixture of these strains was used to inoculate oil-contaminated soil with different pH values as a bioremediation measure (Boszczyk-Maleszak *et al.*, 2003).

To check the ability of the above-mentioned mixture of bacteria used for the bioremediation of soil to use hydrocarbons as sole carbon and energy source at different pH values, liquid cultures were set up. Mineral medium with appropriate pH value, containing 3% oil, was inoculated with bacterial suspension (inoculum I). At the same time control cultures (non-inoculated) were set up. Every 7 days the amount of petroleum products was determined and the number of bacteria was estimated at the beginning and at the end of each culture. The results are presented in Figures 1 and 2.

The decrease in oil content in the course of the cultivation of the microorganisms in liquid culture is presented in Fig. 1. It was found that the reduction of hydrocarbons in medium with acidic conditions after 7 days was barely 5%, which to some extent probably reflected reduced volatility under low pH conditions. After 14 days the reduction of oils was 59, and after three weeks 70%. In culture with neutral pH the

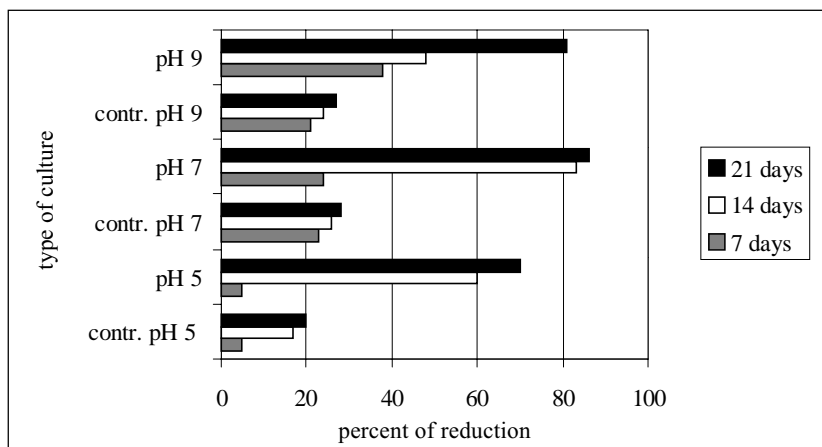


Fig. 1. Oil reduction by bacteria in inoculum previously used for soil bioremediation (inoculum I).

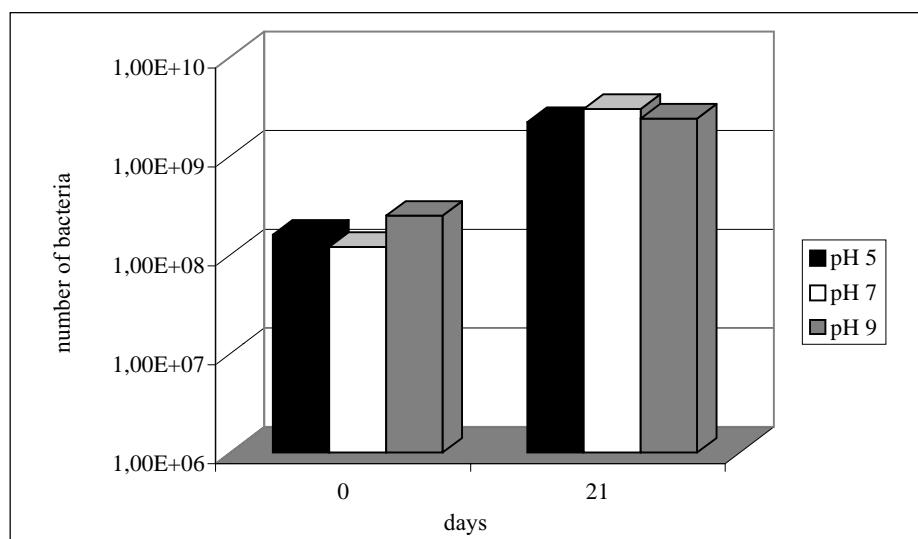


Fig.2. Changes in number of bacteria in cultures with different pH values (inoculum I).

reduction of hydrocarbons after 7 days was 24% and was almost identical to the value obtained for the control (23%) – the loss of oil content during this time was related to the escape of volatile fractions. However, after 14 days of the experiment a reduction in oil content of 86% was observed, which value persisted to the end of the experiment.

In the culture maintained at pH 9, after 7 days 38% reduction of carbohydrates was observed and after 14 days this value slightly increased to 48%. In the third week of the experiment the reduction of oils increased considerably, reaching the value of 81% reduction.

In control cultures maintained at pH 7 and 9 the loss of hydrocarbons was in the 23–28% range (of which 23% reduction was observed in the first week). In control samples (non-inoculated culture) with pH value 5.0 after 7 days only 5% reduction of petroleum products was observed. In the next two weeks this value increased to 20%.

After 10 days of the experiment a change in the appearance of the culture maintained at a neutral pH was observed – the hydrocarbons that initially appeared in the form of a thick surface layer began to pass to the aqueous phase. In the acidic and alkaline cultures similar changes were observed only towards the end of the experiment. This could be related to the production of surfactants by the bacteria introduced with the inoculum, causing liquefaction of the hydrocarbons and their increased solubility in water. No such changes were observed in the control cultures. Changes in the number of bacteria in the course of the culture are presented in Fig. 2. It was found that in the course of cultivation at all pH values the number of bacteria increased by an order of magnitude. The number of bacteria at the beginning of the experiment was $1.2\text{--}3.2 \times 10^8$ cells/mL, and on the last day of cultivation $2.6\text{--}4.9 \times 10^9$ cells/mL.

After completion of the experiments the bacteria present in the individual cultures were isolated and identified. Five strains belonging to three genera were isolated from culture with pH 5.0: *Arthrobacter* – 2 strains, *Pseudomonas* – 2 strains, *Xanthomonas* – 1 strain. In the case of culture with pH 7.0 six strains were isolated: *Arthrobacter* – 2 strains, *Agrobacterium* – 1 strain, *Pseudomonas* – 1 strain, *Xanthomonas* – 2 strains. A total of seven strains were isolated from culture with pH 9.0, representing: *Arthrobacter* – 2 strains, *Agrobacterium* – 2 strains, *Xanthomonas* – 3 strains.

It was found that in culture with neutral pH all the strains introduced with the inoculum persisted. In the cultures with pH 5.0 and 9.0 bacteria belonging to the genera *Agrobacterium* and *Pseudomonas*, respectively, did not survive.

In earlier studies, following bioremediation 7 strains of bacteria representing the genera: *Arthrobacter* – 5, *Pseudomonas* – 1, *Bacillus* – were isolated. In the case of soil with pH 7.0 eight strains were isolated: *Arthrobacter* – 2, *Achromobacter* – 1, *Bacillus* – 5 strains. In soil with pH 9.0, nine strains belonging to eight genera were found: one strain each of *Arthrobacter*, *Achromobacter*, *Agrobacter*, *Alcaligenes*, *Pseudomonas*, *Xanthomonas* and *Micrococcus* and two *Bacillus* strains (Boszczyk-Maleszak *et al.*, 2003).

Further studies on the ability to utilize hydrocarbons as sole carbon and energy source were carried out with the use of the above-mentioned bacteria comprising inoculum II. The strains were multiplied and introduced into mineral medium (with different pH values) containing 3% oils. The inoculum for the media with values pH 5, 7 and 9 consisted of strains isolated from soil with the same pH values following its bioremediation. The experiments were carried out for 21 days at room temperature using an orbital shaker.

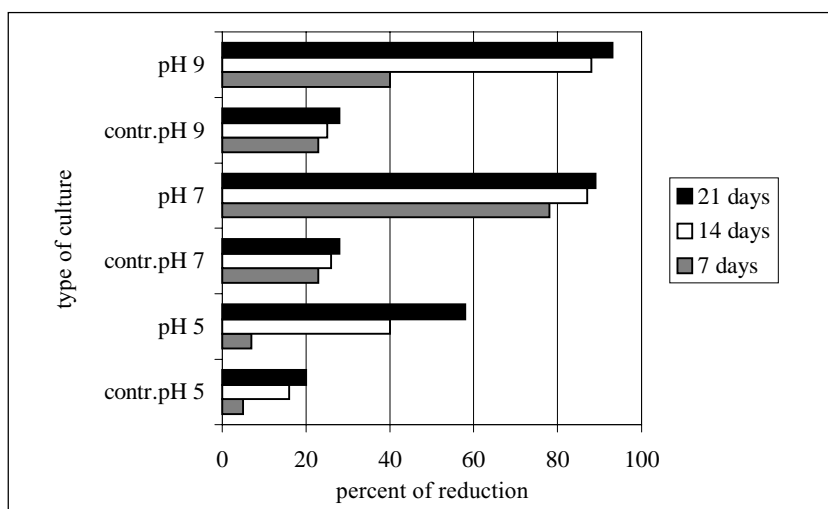


Fig. 3. Oil reduction by bacteria isolated from soil after bioremediation (inoculum II).

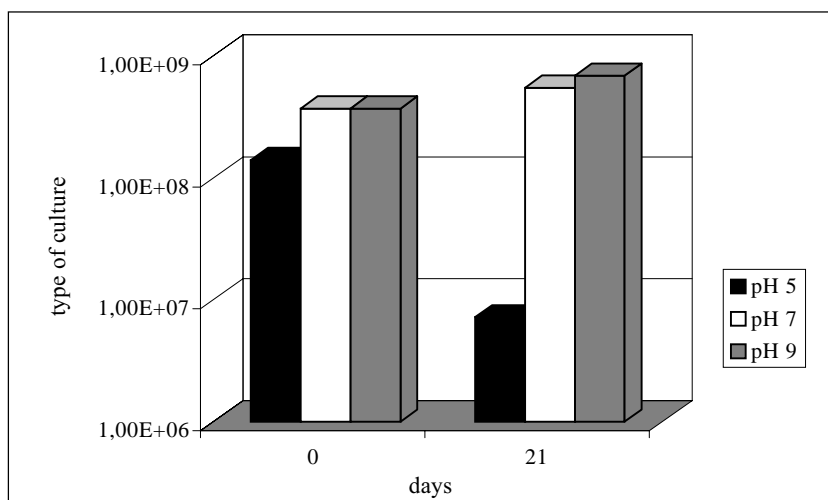


Fig. 4. Changes in the number of bacteria in cultures with different pH (inoculum II).

Parallel control cultures, not inoculated with bacteria, were set up. Every 7 days determinations of petroleum products were made and the number of bacteria in it was estimated at the beginning of the culture and its end. The results obtained are presented in Figures 3 and 4.

Changes in the amounts of petroleum products during the cultivation of bacteria are illustrated in Fig. 3. It was found that in culture with pH value 5, after 7 days of the experiments the loss of hydrocarbons was 7% (like in the control). After 2 weeks the reduction of the oils attained the value of 40%, and after 3 weeks reached 58%. The percent reduction of the oils on the last day of the experiment in the control culture was 20%. In the culture in which a neutral pH was maintained after 7 days 78% reduction of petroleum products was observed and after 2 and 3 weeks of the experiment the respective values were 87 and 89%. In the control culture reduction of hydrocarbons persisted at 23–28% throughout the experiment. In the case of the culture with pH 9, after 7 days 40% reduction of oils was found, with 88% and 93% of the compounds being removed after 2 and 3 weeks, respectively. The loss of hydrocarbons in the control culture, similarly as at pH 7.0, was lower and ranged from 23 to 28%. The appearance of all the cultures changed similarly in the course of the experiment as described in the first part of this study, this being most probably caused by changes in the solubility of the hydrocarbons. Changes in the number of bacteria in the course of the experiment are presented in Fig. 4. In the culture with pH 5 a visible drop in the number of bacteria was observed, from 1.4×10^8 on the day the culture was set up to 7.2×10^6 cells/mL on the last day of the experiment. The number of bacteria in the cultures with pH 7 and 9 showed a slight increase – from the initial value of 3.7×10^8 (identical for both cultures) to 5.5×10^8 (pH 7) and 6.9×10^8 cells/mL (pH 9). After completion of the experiment the bacteria were isolated and identified. It was found that in the cultures with pH 5 and 7 all strains introduced with the inoculum persisted throughout the experiment. In the case of culture with pH 9 only the bacteria belonging to the genus *Micrococcus*, introduced with the inoculum, were no longer present. The results obtained allow to conclude that the biodegradation of petroleum products is the poorest at acidic pH. This is probably related to the observed lower number of bacteria and possibly also with poorer solubility of hydrocarbons at low pH values.

Strains of bacteria originating from oil-contaminated soil earlier subjected to bioremediation, retain their ability to remove effectively petroleum products. Originated strains of bacteria and from the Collection were able to degraded similar concentration of petroleum products. Originated strains existed in environment contaminated of petroleum products, and bacteria from Collection were adapted to high concentration of petroleum products. This situation could influence the activity of tested bacteria.

Compared to the results published earlier (Sorkhoh *et al.*, 1995) it can be said that hydrocarbons are removed faster in aqueous environments, which is probably a result of the better access of microorganisms to the substrates.

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