Polish Journal of Microbiology
2004, Vol. 53, No 3, 183–187

# Biodegradation of Petroleum Products by Microorganisms Adapted to High Crude Oil Concentration in Presence of Easy Assimilated Carbon Source

HANKA BOSZCZYK-MALESZAK, ANNA PRZESTRZELSKA,
EWA BIESZKIEWICZ and MACIEJ HOROCH

Department of Environmental Microbiology, Institute of Microbiology, Warsaw Univesity
Miecznikowa 1, 02-096 Warsaw, Poland

Received in revised form 21 June 2004

Abstract

The studies focused on the effect of the addition of low concentrations of glucose – a substrate that is readily subject to microbiological degradation, on the rate of biodegradation of petroleum products. Glucose in concentration 1% was introduced into mineral medium containing 3% crude oil, with or without bacterial inoculum. The rate of degradation of crude oil in the individual cultures was determined for 3 weeks. The degradation of petroleum products in liquid mineral medium reached 56% after 21 days of growth in the presence of 1% concentration of glucose. The introduction of bacteria that had been cultured earlier in the presence of petroleum products had an effect on hydrocarbon removal efficiency – in both the presence and absence of glucose, the percent reduction of crude oil was high, reaching 76 and 86, respectively, after 21 days.

K e y w o r d s: petroleum products, biodegradation, co-oxidation

## Introduction

Crude oil and its derivatives are among very significant and dangerous sources of ecosystem contaminants (Donderski and Wódkowska, 1997; Łebkowska, 1997) that reach the environment from refining-petrochemical plants, engineering industry, during the mining and transport of crude oil, during spills to the soil being the result of damage to pipelines (Corseuil and Alvarez, 1996; Kao and Wang, 2000). Crude oil derivatives that contaminate the soil are a threat to human health as well as a hazard to all living beings (Heitkamp and Cerniglia, 1988). Hydrocarbons from contaminated ecosystems may be removed as a result of photodegradation, oxidation, hydrolysis, volatilization and microbiological processes. The most important of the mentioned transformations are those that involve microbiological processes (Maliszewska-Kordybach, 1987; 1993). The main organisms contributing to the degradation of hydrocarbons in the soil environment are bacteria and fungi (Balba *et al.*, 1998). However, it is thought that the dominating role in this process is played by bacteria. Bacteria carrying out the degradation of hydrocarbons belong to the genera: *Achromobacter*, *Alcaligenes*, *Arthrobacter*, *Bacillus, Flavobacterium*, *Micrococcus*, *Mycobacterium*, *Nocardia* and *Pseudomonas* (Leahy and Coldwell, 1990; Bossert and Bartha, 1995).

The elaboration of methods for removing crude-oil-derivative contamination of the natural environment is one of the more important problems related to the protection (Łebkowska *et al.*, 1997). Such methods make use of microorganisms inhabiting the natural environment which utilize hydrocarbons as a source of energy and carbon (Corseuil and Alvarez, 1996).

The aim of the presented studies was to determine the effect of low concentrations of glucose – a substrate that is readily subject to microbiological degradation – on the rate of biodegradation of petroleum products.

## Experimental

### Materials and Methods

**Bacterial strains.** The studies embraced 11 strains from the collection of the Department of Environmental Microbiology, University of Warsaw, and eight strains isolated from crude oil contaminated soil.

**Crude oil fraction.** The crude oil fraction used in the studies was taken from the upper layer of a reservoir of a mechanical purification unit treating wastewater from a petrochemical plant.

**Media.** The mineral medium used contained: $K_2HPO_4$ anh. – 7 g, $KH_2PO_4$ anh. – 3 g, $MgSO_4 \times 7H_2O$ – 0,1 g, $(NH_4)_2SO_4$ – 1 g, $H_2O$ dist. – 1 L. The medium was supplemented with crude oil fraction in concentration 3%. Nutrient agar was also used and contained enriched broth – 10 g, agar – 15 g, distilled water – 1 L.

**Isolation of bacteria.** Bacteria were isolated from garden soil that had been previously contaminated with crude oil. Dilutions were made in saline and spread on nutrient agar. Single colonies grown after incubation were transferred to agar slants.

**Identification of bacteria.** The following physiological tests were made: staining with the Gram method, observations of motility and cell morphology and the arrangements of cells, test for the presence of L-alaninoaminopeptidase, API 20NE test, Kovacs test for the presence of cytochrome oxidase, Hugh-Leifson test for ability to degrade glucose under aerobic and anaerobic conditions. Identification was based on Bonde's scheme (Bonde, 1977) and API 20NE test.

**Preparation of bacterial inoculum.** Strains of bacteria stored on agar slants were spread on nutrient agar plates to form a "lawn". After 24 hour incubation at 27°C the bacterial growth was washed off the plates with saline and after being thoroughly mixed, was used to inoculate soil samples or was added directly to liquid culture.

**Maintenance of culture.** Liquid culture were set up in mineral medium in flasks containing 50 ml mineral medium and 3% crude oil. The experiment was carried out in the following variants: 1) mineral medium + bacterial inoculum prepared from strains from the collection + 1% glucose, 2) as above, but without glucose, 3) mineral medium + inoculum prepared from bacteria isolated from garden soil after 3 weeks of growth of culture in soil supplemented with crude oil + 1% glucose, 4) as above, but without glucose.

**Determination of bacterial number.** The number of bacteria was determined by the plate method and at the end of the experiment. Dilutions of liquid culture were plated on nutrient agar plates. After 24 h incubation the grown colonies were scored and the number of bacteria in 1 ml of mineral medium was calculated.

**Determination of amount of crude oil.** The amount of petroleum products in the soil and liquid medium was assayed following the extraction of the hydrocarbons with petroleum ether. Measurements were made every 7 days.

## Results and Discussion

The studies embraced 3 stages: 1) isolation and identification of bacterial strains, 2) studies on the degradation of crude oil fraction in mineral medium in the presence of 1% glucose and absence of glucose, by a mixture of strains, 3) studies on the degradation of crude oil fraction in the presence of 1% glucose and absence of glucose with the use of an inoculum prepared from bacterial strains isolated from garden soil contaminated with crude oil (following 3-week bioremediation).

**Isolation and identification.** The studies embraced 11 strains from the collection of the Department of Environmental Microbiology and 8 strains isolated from garden soil contaminated with crude oil (following 3-week bioremediation). Gram-negative bacteria were identified with the use of API 20NE strips, gram-positive strains were identified using Bonde's scheme. The results obtained indicated that of the 11 strains taken from the collection 6 belong to the genera *Pseudomonas*, 4 to *Bacillus*, and 1 to *Aeromonas*. All the strains were characterized by good growth in medium with crude oil and were able to ferment glucose.

Eight strains isolated from garden soil contaminated with crude oil following 3-week bioremediation belonging to the genera *Flavobacterium, Arthrobacter, Micrococcus, Achromobacter*, *Pseudomonas* (1 strain each)*, Bacillus* ( 2 strains) were isolated, as well as the yeast *Candid*a (1 strain). Many authors (*e.g.* Austin *et al.* 1977, Leahy and Colwell, 1990) claimed strains belonging to the above-mentioned genera are capable of degrading hydrocarbons.

### Degradation of crude oil in mineral medium in the presence of 1% glucose

The aim of the experiment was to check whether the degradation of crude oil fraction is influenced by the type of medium including the presence of glucose. Two types of cultures in liquid mineral medium with 3% crude oil and bacterial inoculum (prepared from strains in our collection), without glucose or with the addition of 1% glucose. The control was mineral medium with 3% crude oil without glucose and without inoculum. The culture was incubated at room temperature for 21 days. The crude oil content was determined every 7 days, and the number of bacteria was assayed at the beginning and end of the experiment. The results are presented in Figs. 1 and 2.

It was found that on day 21 of the experiment, the reduction of crude oil in the presence of 1% glucose was 56%, compared to 65% in the cultures without glucose. During the same time the reduction of crude oil

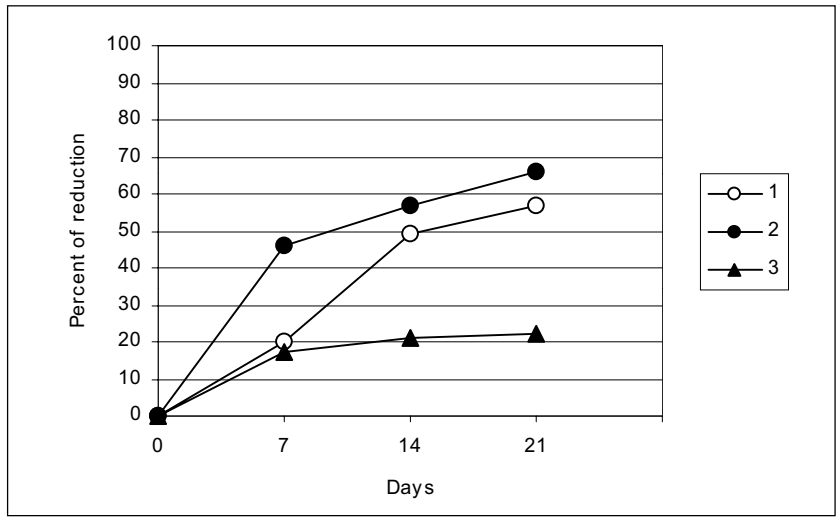


Fig. 1. Utilization of crude oil in liquid mineral with the use of bacterial inoculum in the presence of 1% glucose and without glucose.
1 – with glucose and with inoculum, 2 – without glucose, with inoculum, 3 – without glucose and without inoculum

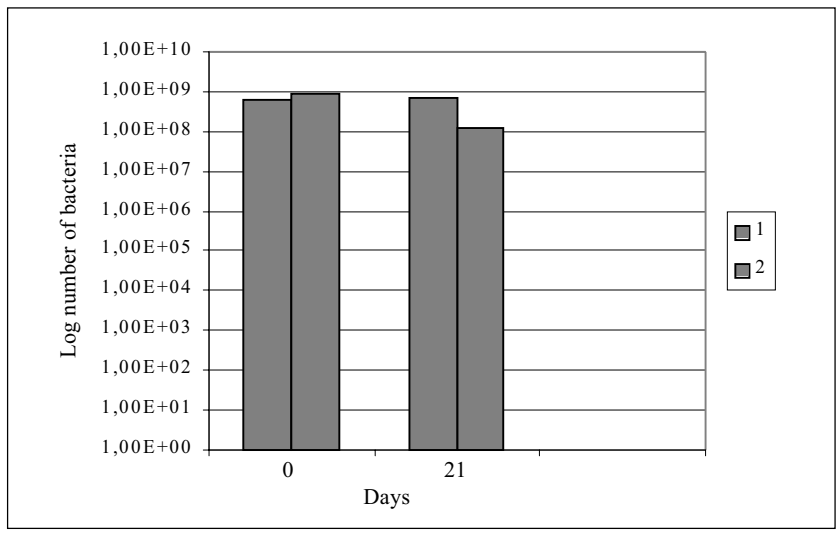


Fig. 2. Changes in the number of bacteria in liquid mineral medium with the use of bacterial inoculum in the presence of 1% glucose and without glucose.
1 – with glucose and with inoculum, 2 – without glucose, with inoculum

in the control was about 20% (Fig. 1). In the medium without glucose very fast rate of degradation of crude oil in the first 7 days (45%) was observed. This high percentage of reduction was the result of the utilization of hydrocarbons as nutrients by the bacteria, as well as by the volatilization of light crude oil fractions. On the other hand, in medium with 1% glucose in the same time (7 days) a low rate of degradation of crude oil was found. The reduction of petroleum compounds was thus about 20% and approximated the result for the control culture, which means it was caused mainly by the volatilization of hydrocarbons, and not by the action of bacteria introduced with the inoculum. Increased utilization of crude oil in the culture with glucose occurred between day 7 and 14 of the culture and approximated 50%, reaching 56% after 3 weeks. The obtained results allow to conclude that in the initial stage of the culture, the bacteria utilized more accessible substrate-glucose and began to utilize hydrocarbons only when this source became depleted. Similar results were previously obtained by Liu *et al.* (1995). The number of bacteria dropped insignificantly from $8.8\times10^8$ to $1.4\times10^8$ cells/ml medium during the course of the experiment in culture without glucose, whereas in culture with glucose, increased during the experiment from $4.5\times10^8$ to $7.0\times10^8$ cells/ml medium (Fig. 2).

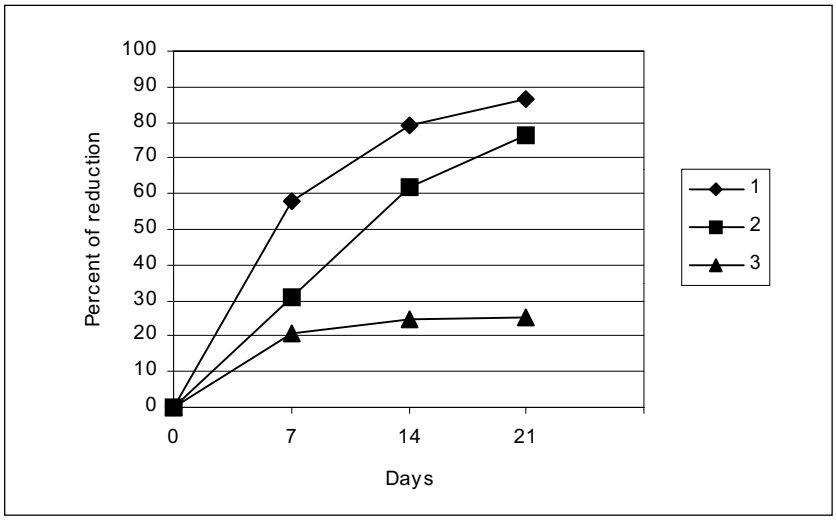


Fig. 3. Utilization of crude oil in liquid mineral medium by bacteria isolated from garden soil (following earlier 3 – week reclamation) in the presence of 1% glucose and without glucose.
1 – without glucose and with inoculum, 2 – with glucose, with inoculum, 3 – without glucose and without inoculum

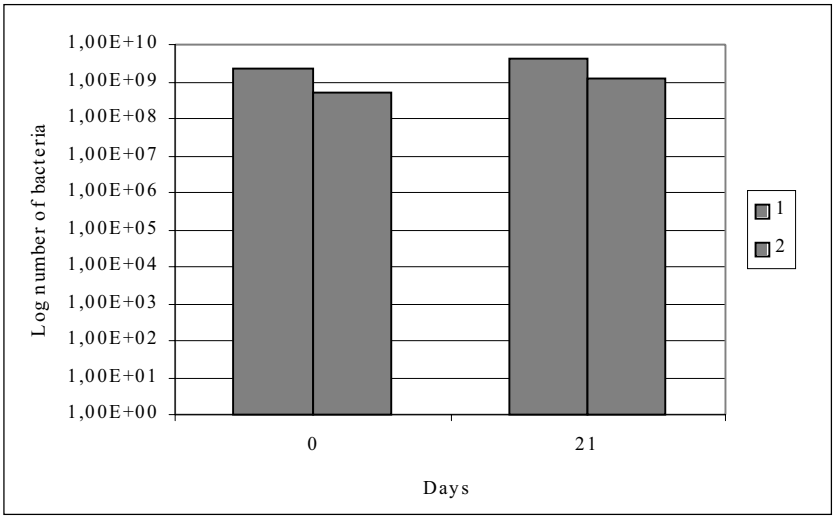


Fig. 4. Changes in number of bacteria isolated from garden soil (following earlier 3-week reclamation) in the presence of 1% glucose and without glucose.
1 – without glucose, with inoculum, 2 – with glucose, with inoculum

The obtained results allow to conclude that reduction of oil in liquid medium was more efficient in the absence of another easy to approach carbon sources.

In the last stage of the experiment, the degradation of crude oil fraction in mineral medium by an inoculum composed of strains of bacteria isolated from crude oil contaminated soil that had earlier been subjected to a 3-week bioremediation, was studied. The experiment was carried out in 2 variants – the bacterial inoculum was introduced into mineral medium containing 3% crude oil, which was supplemented with 1% glucose or not. The control was mineral medium with crude oil and without inoculum as well as without glucose. Cultures were incubated at room temperature on a shaker. The amount of crude oil was determined every 7 days and the number of bacteria was scored on day 0 and 21. The results are presented in Figs. 3 and 4.

It was found that the percent of reduction was high the presence and absence of glucose and reached 76 and 86%, respectively. The amount of crude oil reduced in the control was about 25%. In culture without glucose high reduction of crude oil was observed already on day 7 day of the experiment, reaching about

60% (after 14 days reduction increased to 79%). In the presence of glucose the bacteria began to utilize hydrocarbons present in the medium already in the first week of the culture (loss of crude oil after 7 days was 32%), and after 14 days the reduction of crude oil increased to 62%. The high reduction between day 7 and day 14 of the culture was probably caused by the depletion of glucose in the medium and the beginning of the process of the degradation of hydrocarbons (Fig. 3). The results given above allow to conclude that the introduction of bacteria isolated from crude oil contaminated soil into mineral medium contributed to increased rate of utilization of hydrocarbons.

The number of bacteria at the beginning of the experiment was $7.6\times10^8$ cells/ml in the culture with glucose, and $2.1\times10^9$ cells/ml in the culture without glucose. After 3 weeks of the experiment the number of bacteria in both cultures showed a slight increase (Fig. 4).

The obtained results allow to conclude that the crude oil concentration of 3% was not toxic for bacteria isolated from crude oil contaminated soil. Similar results were obtained by Piekarska *et al.* (2000), who found that bacteria are able to survive in an environment considerably contaminated with crude oil. Earlier exposure of microorganisms to crude oil contaminats resulted in enhanced reduction of petroleum products (Bauer and Capone, 1988; Leahy and Colwell, 1990). High effectiveness of removal of crude oil in oxygen conditions can be achieved with the use of microorganisms that have previously been adapted to the degradation of hydrocarbons in the absence in the medium easy assimilated another carbon sources.

## Literature


Austin B., J.J. Calomiris, J.D. Walker and R.R. Colwell. 1977. Numerical taxonomy and ecology of petroleum-degrading bacteria. *Appl. Environ. Microbiol*. **34**: 60–68

Balba M.T., N. Al.-Awadhi and A. Al.-Daher. 1998. Bioremediation of oil-contaminated soil: microbiological methods for feasibility assessment and field evaluation. *J. Microbiol. Methods*. **32**: 155–164.

Bauer J.E. and D.G. Capone. 1988. Effect of co-occurring aromatic hydrocarbons in marine sediment slurries. *Appl. Environ. Microbiol.* **54**: 1649–1655.

Bonde G.J. 1977. Bacterial indication of pollution. In: Advances in aquatic microbiology. Drop M.R. and Jannash W.W. (ed). 1:273. Academic Press, London.

Bossert I. and R. Bartha. 1984. The fate of petroleum in soil ecosystems. p. 434–476. In: R.M. Atlas (ed.), Petroleum Microbiology. Macmillan Publishing Co., New York.

Corseuil H.X. and P.J.J. Alvarez. 1996. Natural bioremediation perspective for BTX-contaminated groundwater in Brazil: effect of ethanol. *Wat. Sci. Tech*. **34**: 311–318.

Donderski W. and A. Wódkowska. 1997. Occurrence of aquatic and soil bacteria degrading engine oils ( in Polish ). V Ogólnopolskie Symp. Nauk.-Tech. „Biotechnologia Środowiskowa", Ustroń-Jaszowiec.

Heitkamp M.A. and C.E. Cerniglia. 1988. Mineralization of polycyclic aromatic hydrocarbons by a bacterium isolated from sediment belowan oil field. *Appl. Environ. Pollution*. **107**: 245–254.

Kao C.M. and C.C. Wang. 2000. Control of BTEX migration by intrinsic bioremediation at a gasoline spill site. *Wat. Res.* **34**: 3413–3423.

Leahy J.G. and R.R. Colwell. 1990. Microbial degradation of hydrocarbons in the environment. *Microbiol. Rev*. **54**: 305–315.

Liu Z., A.M. Jacobson and R.G. Luthy. 1995. Biodegradation of naphthalene in aqueous nonionic surfactant systems. *Appl. Environ. Micobiol.* **61**: 145–151.

Łebkowska M., E. Karwowska and E. Miaśkiewicz. 1995. Isolation and identification of bacteria from petroleum derivatives contaminated soil. *Acta Microbiol. Pol*. **44**: 297–303.

Maliszewska-Kordybach B. 1987. Microbiological transformation of polycyclic hydrocarbons in the soil environment (in Polish ). *Post. Mikrobiol*. **26** : 233–247.

Maliszewska-Kordybach B. 1993. The effect of temperature on the rate of disappearance of polycyclic aromatic hydrocarbons from soil. *Environ. Pollution.* **79**: 15–20

Piekarska K., B. Kołwzan, T. Traczewska. 2000. The adaptation of biological methods to prognosis of biodegradation of petroleum products in the soils (in Polish). *Inżynieria środowiskowa.* **45:** 89–98