BIOLOGICAL DEGRADATION OF MOTOR OIL IN WATER

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Abstract

The biodegradability of petroleum hydrocarbons from motor oil (Proton Avant, Petrol, Slovenia) was studied in aqueous media using different bacterial strains isolated from native catchments in Štajerska and Prekmurje. An unidentified bacterial strain *AL-12* from the culture of cyanobacteria was found as the most successful in degrading alkanes from n-C_{15} to n-C_{40} present in the fresh motor oil in a quantity of 98%. Small aliquots of motor oil were incubated aerobically for a period of up to 50 days, following the hydrocarbon content by GC/MSD analysis. Within 5 days of incubation up to 70% of n-alkanes n-C_{15}-n-C_{22}, up to 45% of n-C_{22}-n-C_{30} and up to 20% of n-C_{30}-n-C_{40} were biodegraded. As expected, abiotic losses were smaller with increasing alkane chain length, but increased with incubation time.

Introduction

Environmental pollution with petroleum and petrochemical products (complex mixtures of hydrocarbons) has been recognized as one of the most serious current problems, especially when associated with accidental spills on the large scale. If this occurs, hydrocarbons may reach the water table before becoming immobilized in the soil. They spread horizontally on the ground-water surface and continue to partition into ground water, soil pore space air, and to the surfaces of soil particles. Bioremediation has become an alternative way of remediation of oil polluted sites, where the addition of specific microorganisms (bacteria, cyanobacteria, algae, fungi, protozoa) or enhancement of microorganisms already present can improve biodegradation efficiency in both *in-situ* and/or *ex-situ* (in reactors) procedures.[1,2]

Hydrocarbon-degrading microorganisms play an important role in the aquatic environment. Several investigation have shown that the biodegradation of the fractions making up heavy oils occurs according to the following priority scale:

aliphatics>aromatics>polars>asphaltenes.[3] Under ideal conditions, the hydrocarbons are completely mineralized to carbon dioxide and water, with some biomass production. More often biodegradation is not complete. Biodegradation efficiency depends on microorganisms, capable of producing enzymes that will degrade the target compound. Factor such as temperature, pH, and nutrient status are of importance as moderators. Oxygen availability is usually the rate-limiting factor in aerobic degradation of hydrocarbons in groundwater.[4]

Aerobic biodegradation of aliphatic hydrocarbons with bacterial strains depends on biological (enzymatic activity, steric hidrance-diffusion into the cells) and physico-chemical parameters (solubility, emulsion effect, surface tension).[3] As the most chemicals, intimate contact between the microbial cell surface and hydrocarbons appears necessary for high degradation rates. Bacteria are frequently attached to alkane droplets. The first step in the aerobic degradation of hydrocarbons is the incorporation of molecular oxygen in the hydrocarbon.[1] The most common pathway of alkane biodegradation is oxidation at the terminal methyl group.[1] The alkane is oxidized first to an alcohol and then to the corresponding fatty acid. After formation of a carboxyl group the oxidation proceeds by successive removal of two carbon units through β-oxidation, which is universal to most, living systems. Under β-oxidation, the beta methylene group is oxidized to a ketone group followed by the removal of a two-carbon fragment from the compound.[1]

The degradation potential of alkanes is a function of carbon chain length. It was found that the chain compounds shorter than C_9 except methane are more difficult to degrade than longer chains.[1] Under aerobic conditions methane is readily used as the sole carbon source by methanotrophs. For instance, successful degradation of ethane, propane, and butane by *Pseudomonas methani*c, require cometabolism where methane serves as a primary substratum.[1] Short chain liquid aliphatics of less than 10 carbon atoms tend to be toxic for the most bacteria but not for other organisms.[1] Toxicity is a function of solubility in water. The high concentration partly causes damage of bacterial cell membrane and destroys the function of proteins for transport and oxidation.

The degradation of n-alkanes higher than C_9 increases with the chain length.[3] The longer-chain aliphatic hydrocarbons is readily degraded by a wide variety of

microorganisms under aerobic conditions. For liquid n-alkanes, C_{12}-C_{16}, low solid n-alkanes, C_{17}-C_{28}, and high solid alkanes, above C_{28}, a different degradation pattern can be proposed.[3] Liquid and low solid n-alkanes which are less soluble in water and therefore less toxic to microorganisms, degrade more readily than short chains ($<C_9$) while degradation of n-alkanes above C_{28} is a function of the hydrocarbon chain lenght.[3] About 95% of low molecular weight alkanes (smaller than C_{30} to C_{40}) are converted to CO_2 and water in a few months, alkanes larger than C_{40} can last for many years.[5]

Bioremediation efficiency demands a basic knowledge of the degradation rate of specific chemicals which can only be obtained from laboratory experiments by detailed characterization of biological parameters and physicochemical properties (temperature, pH, organic matter, nutrients).[3,4] *In-vitro* biodegradation studies are often criticized for being over-simplified, that is, applying single bacterial strains with either unknown or poorly defined histories in unrealistic conditions, but data obtained in laboratory experiments should help in making rapid decisions in cases of ecological accidents, such as fuel spills.[6] For complex mixtures of petrochemical hydrocarbons, laboratory studies in aqueous media is the first step in the controlled screening of potentially useful bacteria species for obtaining fundamental data on biodegradation.

In-vitro experimental petroleum hydrocarbon degradation studies besides mixed cultures isolated from contaminated sites, various pure bacterial have been performed.[7] In our laboratory, biodegradation of acyclic and polyaromatic hydrocarbons from some petrochemical derivatives was investigated.[8] The highest biodegradability of aliphatic hydrocarbons has been demonstrated by a single bacterial species of *Pseudomonas fluorescens* Texaco applied for degradation of diesel oil in *in-vitro* studies in aqueous media.[9]

The present study concentrates on testing new mixed cultures associated with indigenous soil microorganisms and with pure bacterial strains isolated from Štajerska and Prekmurje native catchments in Slovenia. The aim of this study was to elucidate the microbial ability of different bacterial strains on the biodegradation of motor oil (Proton Avant, Petrol, Slovenia) used in petrol and diesel engines. The main goal of this work was to establish the biodegradation and abiotic losses for aliphatic hydrocarbons from motor oil in aqueous media.

Experimental

Rapid biodegradation screening test

Chemical analysis of Proton Avant motor oil demonstrated a high content of aliphatic hydrocarbons from C_{14} to C_{40} (up to 98%) with a small addition of the parent and substituted aromatic and polyaromatic hydrocarbons (<2% of phenols, naphthalenes and anthracenes).

Numerous pure and mixed bacterial strains and some incompletely identified bacterial strains from the collection at the National Institute of Biology (Ljubljana, Slovenia) were tested for use in our biodegradability study of the complex motor oil hydrocarbon mixture.

For screening, 34 pure bacteria strains were taken (*Azospirillium brasilense, Achromobacter cycloclastes, Bacillus cereus, Bacillus megaterium, Bacillus subtilis,* 7 *Escherichia coli*, *Flavobacterium* sp., 3 *Pseudomonas fluorescens*, 2 *Pseudomonas putida, 2 Pseudomonas stutzeri, Pseudomonas aeruginosa, 4 Pseudomonas sp., Pseudomonas paucimobilus, Pseudomonas maltophilia, Serratia marcescens,* 3 *Salmonella typhimurium*, *Mycobacterium* sp*., Alcaligenes* sp*., Pasteurella* sp.) and 11 not identified bacterial species. The bacterial species originated from different locations; some of them were also isolated from an area contaminated with petroleum and petrochemical products.

The unidentified bacterial strains were isolated from the cultures of cyanobacteria from the various lowland lakes of NE Slovenia. The minimal agar plates[10] with spread oil emulsion were inoculated with the sample of cyanobacterial culture and successfully growing bacterial colonies were picked out for the further analysis of biodegradability.

A rapid biodegradation-screening test was performed in order to determine the ability of bacteria to metabolise the samples. To determine which bacteria were useful for degrading hydrocarbons, a bacteria isolation technique called streaking was used.[11] Agar plates (1.5% Difco agar) with no usable carbon substrate, but provided with mineral medium that contains the essential nutrients for the bacteria, were sprayed with

a solution of motor oil (1 g/10 mL in dichloromethane). The sprayed plates were left uncovered till the solvent has evaporated and then streaked with chosen bacteria.

The effectiveness of biodegradability of the motor oil of isolated bacterial strains was tested on the generalized indicator plates[12] with addition of motor oil and bacterial strains were sorted out into three classes (+; ++; +++) on the basis of the intensity of formazan formation.

The bacteria in the samples were shown to be viable prior to analysis by streaking a small loop of the culture onto a nutrient agar plate and incubating for 7 days at 30 ^{o}C.[10] The bacterial activity of *AL-12* during the degradation experiments was checked according to the Miles and Misra drop counts method with serial of dilutions of the sample.[13]

Experimental design

Laboratory studies on selected n-alkanes from the motor oil in the range from n-C_{15} to n-C_{40} were carried out in an aqueous medium (pH = 6.5). Biotic and abiotic samples were incubated aerobically in the absence of light on a reciprocating shaker (30 rpm) at room temperature (22 ^{o}C) for two months. All glassware and mineral media were sterilised by autoclaving (120 ^{o}C, 20 min). The experiments, carried out in duplicate, were performed in Erlenmeyer flasks (250 mL) containing 80 μL of motor oil in mineral media (100 mL of a salt solution containing 5 g NH_4Cl, 1 g NH_4NO_3, 2 g Na_2SO_4, 3 g K_2HPO_4, 1 g KH_2PO_4 and 0.1 g $MgSO_4 \times 7H_2O$ in 1000 mL deionized water with the addition of microelements).[11] Motor oil concentration in mineral media did not exceed the limit of solubility in water.

The washed bacterial suspension by centrifugation and resuspended in mineral medium (1 mL of 1-2 x 10^8 cells mL^{-1}) of *AL-12* was added only to biotic samples. In the case of abiotic samples, an addition of mercury chloride (10 mg, Aldrich, Dorset, UK) was added to ensure that only abiotic conditions prevailed. Control abiotic flasks were incubated under the same conditions to monitor abiological losses (e.g. evaporation). Biotic and abiotic flasks were stoppered with non-adsorbent cotton wool to keep the contact with air. Samples were then incubated aerobically at room temperature in the dark with constant shaking (DOT was not measured).

During incubation two biotic and two abiotic samples were analysed at the same time for bacterial activity. After selected periods of incubation (days: 0, 5, 14, 21, 28, 36 and 50) samples were extracted three times with 10 mL of dichlorometane. The extraction efficiency of liquid-liquid extraction was determined by standard addition of a mixture of n-pentacosane: squalane (1:1; 200 μL; concentration of 5 mg mL^{-1}) to the flasks before extraction. With increasing incubation time, separation between the dichloromethane phase and the bacterial residue became more and more difficult. Therefore, in the case of greater formation of the bacterial residue, an additional extraction with deionized water was applied followed by a repeated organic solvent extraction. The volume of extract was reduced with a gentle stream of nitrogen at room temperature to 1 mL prior to analysis.

Analysis of aliphatic hydrocarbons

Analysis of aliphatic hydrocarbons was performed with a Hewlett-Packard (Waldbroon, Germany) 6890 Series GC coupled to a 5792 A Series MSD using a HP-5MS capillary column (cross linked 5% phenylmethyl silicone, 30m length, 0.25 diameter, 0.25 mm film thickness) in a splitless injection mode. The carrier gas was helium with an average velocity of 38 cm sec^{-1} at a temperature of 120 °C using temperature programming to 280 °C (3 °C/min). Quantification of individual hydrocarbons (n-C_{15} to n-C_{40}) was made on the basis of peak area measurements in samples with respect to internal (n-C_{25}, squalane) and external standards of known concentration (Dro Mix 31214, conc. 10 μg mL^{-1}; Lust 31200, conc. 25 μg mL^{-1}, Restek Co., Bellefonte, USA).

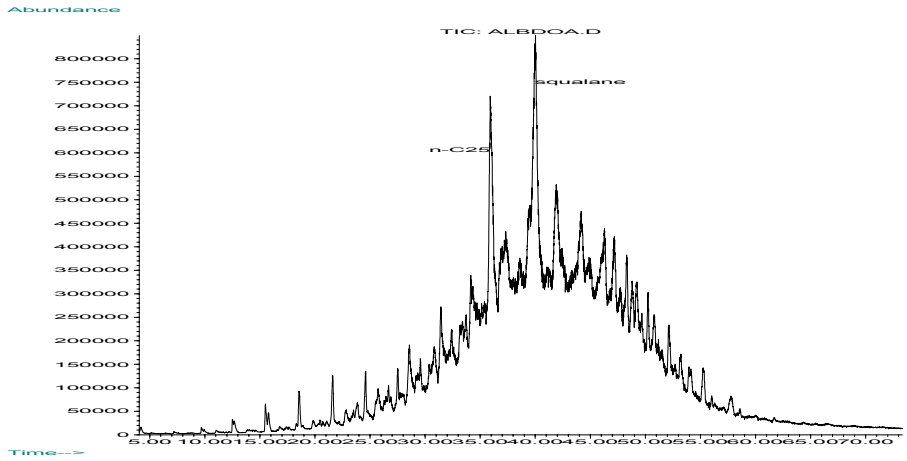
The recovery of the hydrocarbons was determined by spiking the samples with standards at a high (1 mg L^{-1}) and low (0.01 mg L^{-1}) concentrations. Replicate analyses gave an error of less than ± 5% for abiotic and less than ± 10% for biotic ones. Biological degradation was calculated from the differences between the concentrations of selected compounds in abiotic and biotic samples analysed on the same day of incubation.

Results and discussion

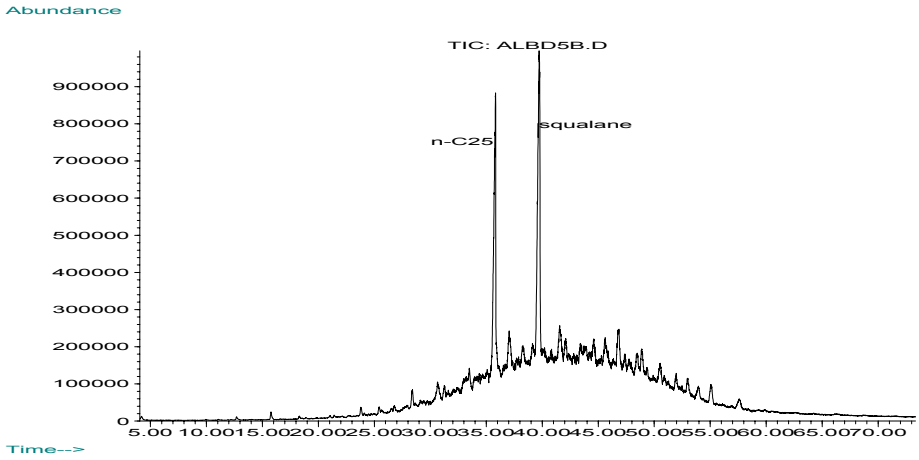
From the rapid biodegradation screening method of the bacterial strains it was observed that among 45 bacteria tested only 9 showed satisfied biodegradation potential for n-alkanes, as follows: *Azospirillium brasilense, Achromobacter cycloclastes, Bacillus cereus, Pseudomonas fluorescens,* Texaco, *Salmonella typhimurium, Mycobacterium* sp. and 4 unidentified strains. Two of unidentified bacteria strain isolated from cyanobacterial cultures from Štajerska and Prekmurje native catchment showed enhanced abilities to degrade petroleum hydrocarbons in the shortest time. One bacterial strain, named *AL-12*, was the most successful and therefore chosen for the further biodegradation study.

In Figure 1 the total ion chromatograms of motor oil (concentration 83 mg ml^{-1}) are presented of biotic samples on day 0 (a) and after 5 days of incubation (b), and of the abiotic sample after 5 days of incubation (c). The majority of n-alkanes were significantly biodegraded in the first 5 days of incubation. The most rapid biodegradation was observed for the n-alkanes n-C_{15}–n-C_{22}. After 5 days of incubation aliphatic hydrocarbons (up to Rt = 37 min.) containing from 15 to 22 carbon atoms were found to disappear completely. During this time 70±5 % of n-alkanes were biodegraded and the losses effected by abiotic processes were up to 30±2%. Within 5 days of incubation up to 45±5% of n-C_{22}-n-C_{30} and up to 20±5% of n-C_{30}-n-C_{40} were biodegraded. Abiotic losses of aliphatic hydrocarbon increased with incubation time because of their low solubility in water, volatilisation and adsorption on the walls of the Erlenmeyer flasks. The lower molecular mass alkanes showed the greatest amount of abiotic losses during incubation. Although abiotic losses were smaller for hydrocarbons of longer alkane chain length, they increased with incubation time, being up to 20±2% for >n-C_{30}.

a



b



c

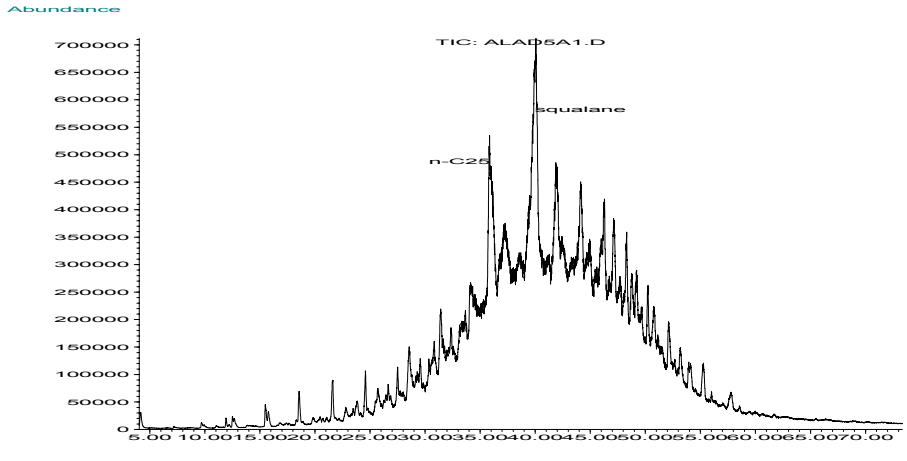


Figure 1. The total ion chromatogram (a) of motor oil (concentration 83 mg mL^{-1}) and of biotic (b) and abiotic (c) samples after 5 days of incubation.

The higher molecular mass alkanes were less affected by abiotic losses but were also less biodegraded.

In the following 2 months, however, biodegradation increased slowly, even though metabolism was high enough to maintain the bacterial activity at the same level. A possible reason was that the bacteria did not become modified to degrade less degradable higher molecular mass compounds. It was also proved that the first 5 days of incubation were the most important for biodegradation for e.g. n-C_{28} (Figure 2), during which the majority of the compound was degraded, and only a slight increase followed in the remaining incubation time (45 days). Unfortunately, abiotic losses increased during incubation time from 5±2% on 5 day to 40±2% on 50 day.

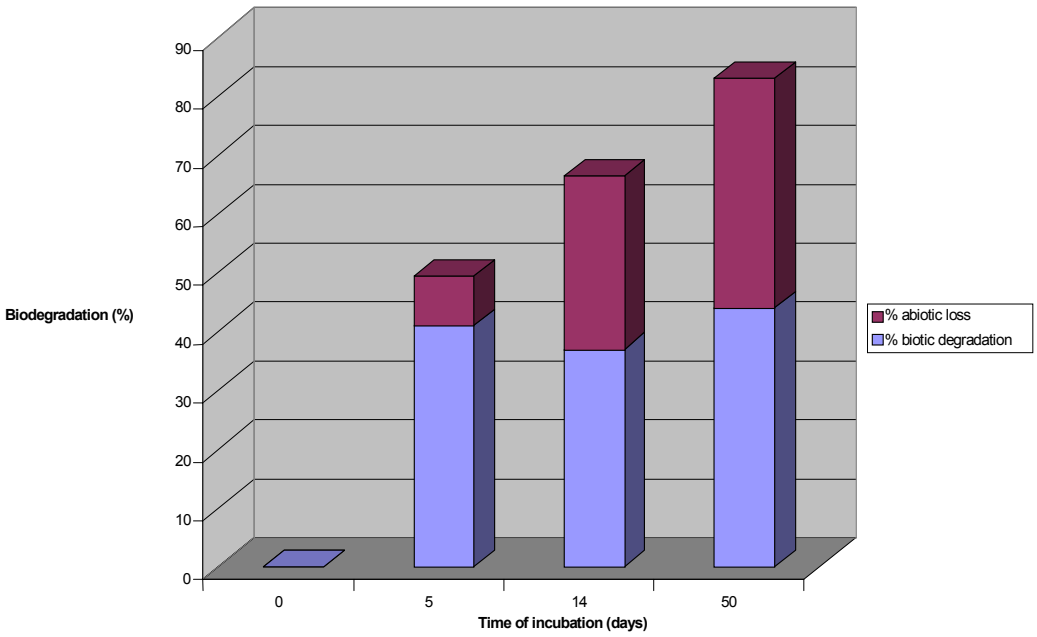


Figure 2: Biodegradation and abiotic losses of n-C_{28} during 50 days of incubation.

After 5 days of incubation of biotic samples the n-alkanes in the range n-C_{15}–n-C_{22} disappeared totally, being degraded (70±5%) and evaporated (30±2%). At the same time some other organic compounds appeared which were not originally present in the motor

oil. These were identified as branched alkanes and substituted phenols whose concentrations increased with incubation time. The origin of these compounds has not yet been clarified but could be explained as the degradation products of motor oil and bacterial cells, or as the presence of accumulated impurities.

Conclusions

This is the first reported biodegradation study of petrochemical products by the bacteria *AL-12* isolated from a Slovenian native catchment. Although a direct comparison of the bacterial activity is not strictly possible when tested on different substrates we demonstrated that *AL-12* was very successful at the beginning of the incubation period. The bacterial strain *Pseudomonas fluorescens,* Texaco used in our previous study of the biodegradation of diesel oil (plinsko olje D-2, Petrol, Slovenia) [9] showed the most rapid biodegradation of up to 65% in 8 days for n-alkanes n-C_{14}–n-C_{18}, while *AL-12* was able to degrade 70% of aliphatic hydrocarbons in the range of n-C_{15}–n-C_{22} from the complex mixture of motor oil within 5 days.

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Povzetek

Preoučevali smo *in-vitro* biodegradacijo motornega olja (Proton Avant, Petrol) v vodnem mediju z različnimi bakterijskimi sevi. Iz različnih kultur cianobakterij s področja Štajerske in Prekmurja smo osamili več sevov bakterij, katere smo testirali na minimalnem gojišču in tako prišli do najbolj učinkovite bakterije *AL-12*. Med 50-dnevno inkubacijo smo v biotičnih in abiotičnih vzorcih spremljali razgradnjo alifatskih ogljikovodikov (n-C_{15}-n-C_{40}), ki so v motornem olju zastopani z 98%. V petih dneh je prišlo do 70% biološke razgradnje alifatskih ogljikovodikov v območju od n-C_{15}do n-C_{22}, do 45% za n-C_{22} do n-C_{30} in do 20% razgradnje n-alkanov v območju od n-C_{22} do n-C_{40}. Za abiotične izgube velja, da so z daljšanjem alkanske verige pričakovano padale, a naraščale z inkubacijsko dobo.