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# BIODEGRADATION OF BENZO[a]PYRENE AND NAPHTHALENE BY BACTERIA ISOLATED FROM THE OŁAWA RIVER

Efficiency of benzo[a]pyrene (B[a]p) and naphthalene degradation by bacteria isolated from the Oława river water was studied. Biodegradation was carried out by mixed populations of bacteria. Loss of B[a]p reached 98.3% and that of naphthalene – 79.1%. During the experiment, several strains were eliminated from the mixed populations. It was found that products of B[a]p degradation had potential mutagenic properties, whereas naphthalene biodegradation products did not exhibit such properties.

## 1. INTRODUCTION

Due to industrialization the environment is affected by a lot of chemical pollutants. They include, among others, polycyclic aromatic hydrocarbons (PAHs) which are hazardous to human health [1]. Numerous epidemiological studies have shown that some PAHs exhibit mutagenic and carcinogenic activities which manifest themselves as early as at the moment when the trace quantitites of these compounds are absorbed by human organism [2]. PAHs of mutagenic and carcinogenic properties include: benzo[a]pyrene, 7-12-dimethylbenzo[a]anthracene, 7-12-benzo[a]phenanthrene, 3-methylcholanthrene, 2-benzo[ah]pyrene, 2-benzo[ai]pyrene and benzo[a]anthracene [1], [3]. PAHs introduced to the environment undergo changes leading to their degradation. They are removed by photodegradation, oxidation, hydrolysis, volatilization and microbial processes [2], [4]–[7]. Products of these transformations are sometimes more dangerous for human health than the parent substances [8]. Therefore understanding of the mechanisms of PAHs degradation and properties of metabolites produced is very important.

Contamination with PAHs of water used for municipal purposes has direct influence on human health. The Oława river is the source of drinking water for

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Wrocław. It was found in previous studies that water from this river contained PAHs [9], [10]. Therefore it seemed likely that the microbial community in the Oława river water would be already adapted to utilization of these compounds as sole sources of carbon and energy.

The objectives of the current study were: isolation of bacteria able to degrade chosen PAHs, determination of biodegradation efficiency and determination of mutagenic and carcinogenic properties of metabolites produced. Two hydrocarbons were selected for the study, one of mutagenic and carcinogenic activity (B[a]p) and one lacking such properties (naphthalene).

## 2. MATERIALS AND METHODS

### 2.1. ISOLATION OF MICROORGANISMS

Mixed population of bacteria from the Oława river water was used for the study. Water samples taken directly above the bottom sediment were transferred aseptically to sterile Pasteur tubes.

Bacteria were cultured in the Šiškina–Trocenko medium [11]. 100 $cm^3$ of water from the Oława river was mixed directly with 100 $cm^3$ of liquid culture medium containing 143 $\mu g/dm^3$ of B[a]p or 7 $mg/dm^3$ of naphthalene. Samples were incubated for 7 days at 20 °C in the dark in order to select and multiply bacteria which are able to utilize the introduced PAHs as sole sources of carbon and energy.

Pure strains were then isolated from the culture on solidified medium of analogous composition.

### 2.2. IDENTIFICATION OF MICROORGANISMS

Identification of isolated strains was based on the analysis of the following features determined on standard diagnostic media:

a) morphological characteristics:
- cell shape,
- Gram stain,
- motility,
- capsules,
- formation of spores,

b) characteristics of growth on solid and liquid media,

c) physiological characteristics:
- optimum growth temperature,
- oxygen requirements,

d) biochemical characteristics:

– catalase activity,
– oxidase activity,
– utilization of carbohydrates (xylose, glucose, lactose, mannitol, arabinose, saccharose, raffinose, starch),
– utilization of citrate,
– nitrate reduction,
– urea hydrolysis,
– gelatin liquefaction,
– indole production,
– $H_2S$ production,
– acetoin production,
– MR reaction,
– phenylalanine deamination,
– arginine dihydrolase production,
– lysine decarboxylation,
– pyocyanine and fluorescein production.

The strains were identified according to Bergey's and Steel's identification manuals [12], [13].

Strains constituting the mixed populations were then used to study the biodegradation of B[a]p and naphthalene.

### 2.3. BACTERIAL CULTURES

Bacteria were cultured in 2 $dm^3$ bottles containing 700 $cm^3$ of mineral medium (Šiškina–Trocenko [11]) supplemented with B[a]p (143 $\mu g/dm^3$) or naphthalene (7 $mg/dm^3$) as the sole sources of carbon. Two control samples were also prepared. One of them was sterile Šiškina–Trocenko medium containing the studied PAHs incubated under the same conditions as the other cultures. These control samples allowed us to determine nonenzymatic loss of PAHs during incubation.

Inoculum was prepared from bacterial culture which was centrifuged and suspended in physiological solution of NaCl. Optical density of the suspension at 650 nm was 0.34. Bacterial cultures and controls were incubated for 28 days at 20 °C, in the dark, with constant mixing.

Growth of the population was determined on the basis of viable cell count according to the colony-counting technique on agar plates with 5 $g/dm^3$ of glucose.

### 2.4. DETERMINATION OF PAHs REMOVAL

Loss of PAHs in the bacterial cultures and the control samples lacking bacteria was determined after 7, 14 and 21 days of incubation. Biomass was separated from supernatant by centrifugation at 4000 rpm, for 40 min, at the temperature of 4 °C.

PAHs were then extracted both from the supernatant and from biomass by cyclohexane p.a. Extraction was carried out three times for 30 min with 100 $cm^3$ portions of cyclohexane. Combined extracts were dried with anhydrous $Na_2SO_4$, filtered and vacuum evaporated. The residue was then dissolved in methanol and the PAHs content was determined with HPLC. The analysis was carried out under the following conditions: column – Wydac 201 TP5, column dimensions – $15 \times 4.6$ mm; eluating agents – methanol and water, gradient from 70 to 100% (v/v) of methanol; flow rate – 1 $cm^3$/min; injection volume – 0.02 $cm^3$; detection – 254 nm.

### 2.5. BIOINDICATION OF MUTAGENIC AND CARCINOGENIC METABOLITES

Mutagenic and carcinogenic properties of metabolites produced were evaluated on the basis of Ames test [14] with the strains of *Salmonella typhimurium* LT2 TA98 and TA100. Rat liver microsomal S9 fraction was added for effective detection of carcinogens which require metabolic activation. Samples were prepared as for analytical studies, but the residue after evaporation was dissolved in DMSO.

## 3. RESULTS

In the first phase of experiment, the mixed populations consisting of several strains of bacteria and growing in the presence of PAHs as sole sources of carbon and energy were established. The population isolated from the medium containing B[a]p was composed of four strains. In all features studied they were identical with strains described as: *Moraxella osloensis* – 1B, *Alcaligenes faecalis* – 2B, *Pseudomonas eutrophus* – 3B and *Pseudomonas diminuta* – 4B. These strains were then used to test the biodegradation of B[a]p. The population utilizing naphthalene as a sole carbon source consisted of five strains, whose characteristics were in accordance with descriptions for the following species: *Chromobacterium lividum* – 1N, *Citrobacter freundii* – 2N, *Pseudomonas diminuta* – 3N, *Pseudomonas eutrophus* – 4N and *Acinetobacter calcoaceticus* – 5N. Mixed population composed of the strains 1N, 2N, 3N, 4N and 5N was used for further studies of naphthalene degradation.

Results of chromatographic analysis used to determine the loss of B[a]p in all samples are given in table 1. In the bacterial culture incubated in the presence of B[a]p, a gradual decrease in the content of this compound was noted. Most B[a]p remained in the supernatant, less was determined in biomass. Loss of B[a]p during the first two weeks was 59.1%, compared to the control sample. As a result of B[a]p degradation, three peaks indicating the appearance of metabolites are visible in the chromatograms (figure 1). Amount of these metabolites decreased in the second week

Table 1

Removal of benzo[a]pyrene and naphthalene by bacteria

| Sample | Time of incubation [days] | PAHs content in the sample without bacteria [μg] | PAHs content in the sample with bacteria [μg] | PAHs removal [%] |
|---|---|---|---|---|
| Mixed population culture with B[a]p | | | | |
| Supernatant | 7 | 71.04 | 24.9 | 37.5 |
| Biomass | 7 | 71.04 | 19.5 | 37.5 |
| Supernatant | 14 | 52.07 | 11.5 | 59.1 |
| Biomass | 14 | 52.07 | 9.8 | 59.1 |
| Supernatant and biomass | 21 | 39.00 | 0.66 | 98.3 |
| Single strain cultures with B[a]p | | | | |
| *Moraxella osloensis* 1B | 21 | 37.5 | 37.5 | 0.0 |
| *Alcaligenes faecalis* 2B | 21 | 37.5 | 34.8 | 7.2 |
| *Pseudomonas eutrophus* 3B | 21 | 37.5 | 34.2 | 9.8 |
| *Pseudomonas diminuta* 4B | 21 | 37.5 | 37.5 | 0.0 |
| Mixed population culture with naphthalene | | | | |
| Supernatant | 14 | 0.47 mg | 0.1 mg | 79.1 |
| Biomass | 14 | 0.47 mg | trace | 79.1 |

of incubation, and the amount of metabolites associated with biomass was particularly low. After 21 days of incubation 95.8% of B[a]p was removed. At this time metabolites were determined. They differed radically from those formed at the beginning of the experiment (figure 1E).

The curve representing the mixed population growth in the medium with B[a]p is shown in figure 2. The most intensive growth was observed during the first 10 days of incubation. Then the number of bacteria decreased gradually until the 18th day. After this time, the number of cells increased again, but never reached the level from the first 10 days.

Species composition of the mixed population was analyzed again after 21 days of incubation. Only two strains – 1B and 2B – survived in the culture. The other two strains were eliminated.

In order to examine the ability of single strains constituting the mixed population to degrade B[a]p, the experiment was repeated using pure cultures of *Moraxella*

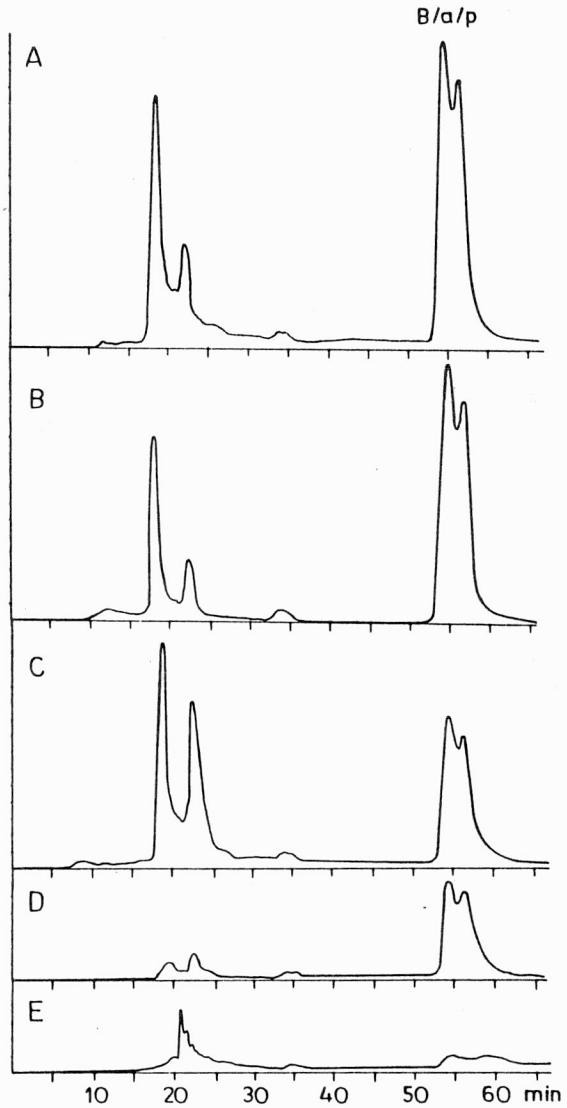


Fig. 1. HPLC chromatograms of extracts from the mixed culture with benzo[a]pyrene
A – supernatant, 7 days: B – biomass, 7 days; C – supernatant, 14 days; D – biomass, 14 days; E – supernatant and biomass, 21 days

*osloensis* – 1B, *Alcaligenes faecalis* – 2B, *Pseudomonas eutrophus* – 3B and *Pseudomonas diminuta* – 4B. No significant removal of B[a]p was observed in any culture (table 1). Removal of B[a]p after three weeks of incubation did not exceed 10%. It was equal to 0.0%, 7.2%, 9.8% and 0.0%, respectively. During the incubation no increase in the number of cells was noted (figure 3). The number of bacteria was constant throughout the experiment.

The results of naphthalene biodegradation are presented in table 1. After 14 days of incubation, the substrate loss was 79.1%. The majority of naphthalene remained in the supernatant; it was not associated with bacterial biomass (figure 4). Growth of the

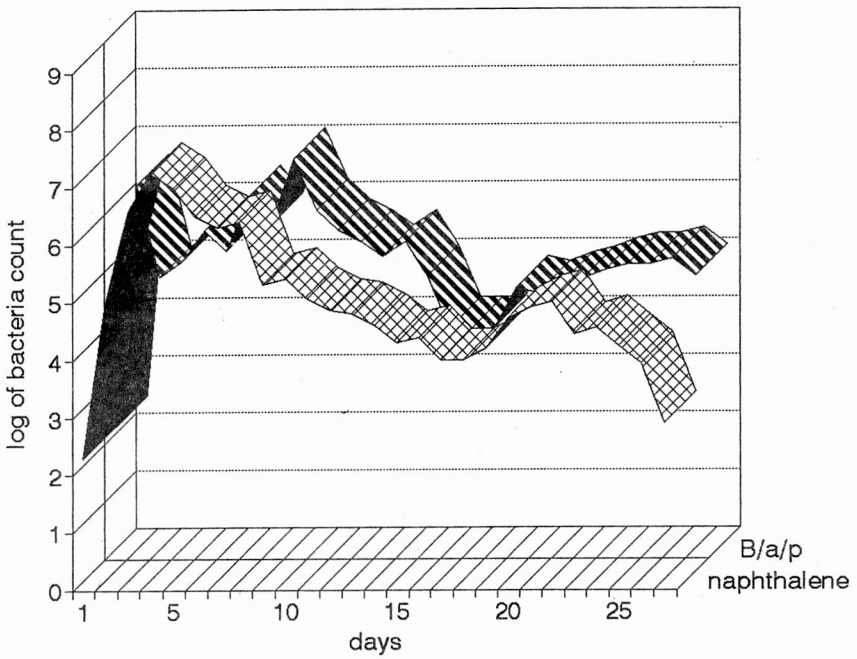


Fig. 2. Growth of the mixed populations in the medium with benzo[a]pyrene and naphthalene as sole carbon sources

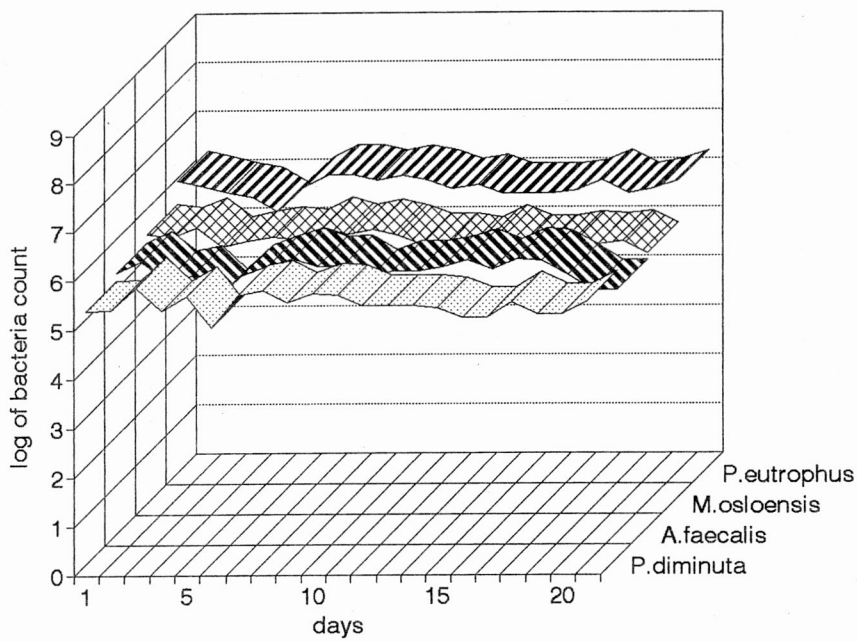


Fig. 3. Growth of single strains in the medium with benzo[a]pyrene as a sole carbon source

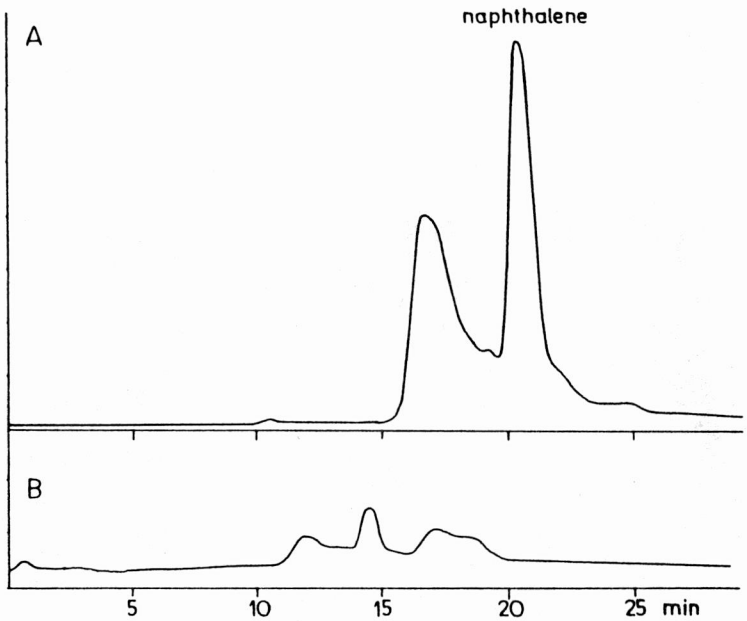


Fig. 4. HPLC chromatograms of extracts from the mixed culture with naphthalene
A – supernatant, 14 days; B – biomass, 14 days

Table 2

Results of the Ames test*

| Sample | Mutation ratio | |
|---|---|---|
| | *Salmonella typhimurium* TA 98 | *Salmonella typhimurium* TA 100 |
| | TA 98 | TA 100 |
| Spontaneous reversion | 1.0 | 1.0 |
| Negative control (solvent) | | |
| Cyclohexane | 1.1 | 1.1 |
| DMSO | 1.0 | 1.0 |
| Positive control | | |
| Aminofluorene | 207.3 | 20.5 |
| Products of PAH degradation by the mixed population of bacteria | | |
| Products of B[a]p biodegradation | 9.6 | 2.7 |
| Products of naphthalene biodegradation | 1.2 | 1.1 |

*A sample is considered mutagenic when the mutation ratio is higher than 2.

$$\text{mutation ratio} = \frac{\text{number of revertants per test plate}}{\text{number of spontaneous revertants per plate}}$$

mixed population of bacteria on the medium supplemented with naphthalene was intensive in the first two days of incubation (figure 2). Later the number of bacteria slightly changed, and on the 27th day of incubation it started to decline. After two weeks of incubation, when removal of PAHs reached almost 80%, three strains of bacteria, i.e. 2N, 3N and 5N, were isolated. Other strains were eliminated.

Bioindication studies were carried out on extracts from those cultures in which the compounds of interest were almost completely degraded. These were extracts from whole cultures (supernatant and biomass) of the mixed population incubated in the presence of B[a]p for 21 days and in the presence of naphthalene for 14 days. The results of the Ames test are shown in table 2. A sample is considered mutagenic when the mutation ratio (MR) is greater than 2. The MR of the products resulting from B[a]p degradation is equal to 9.6 and 2.7 for *Salmonella typhimurium* TA 98 and *S. typhimurium* TA 100, respectively. Products of naphthalene biodegradation did not induce mutations in the TA 98 and TA 100 strains.

## 4. DISCUSSION

The studies described above and carried out on the bacterial populations from the Oława river water allowed isolation of such mixed populations which were capable of degrading B[a]p and naphthalene without earlier adaptation. Mixed populations degraded PAHs efficiently. Removal of B[a]p after 14 days of incubation was 59.1%, and that of naphthalene – as high as 79.1%. Naphthalene was more susceptible to microbial degradation than B[a]p. The above results confirmed the possibility of biodegradation of PAHs in water environment.

Formation of B[a]p metabolites different from those found after the first and second weeks of incubation indicates that further transformation of metabolites took place in the culture in the third week. The above conclusion was confirmed by the characteristic shape of the mixed population growth curve. Renewed increase in the number of bacteria was probably associated with utilization of metabolites produced at the beginning of biodegradation.

It was found that degradation of B[a]p took place only when all strains constituting the mixed population were cultured together. No strain could degrade B[a]p when grown separately. This indicated that during biodegradation a metabolic cooperation between the strains was developed. In the period of prolonged incubation of bacteria with PAHs, several strains were eliminated. Detailed knowledge about the course of this process requires further investigations.

It is known that changes of PAH chemical structures can alter their biological activities. Therefore it was interesting to learn whether metabolites produced during microbial degradation of carcinogenic and non-carcinogenic hydrocarbons (B[a]p and naphthalene, respectively) also exhibit such activities. Bioindicator studies

showed that products of microbial degradation of B[a]p had potential mutagenic and carcinogenic properties, whereas those of naphthalene did not show such properties. This confirms the possibility of contamination of surface waters with metabolites of B[a]p, hazardous for human health, as a result of microbial activity.

To conclude, it must be stated that, depending on the composition of the water bacterial community, metabolites deteriorating the water quality can be formed. Thus biodegradation is not always a beneficial phenomenon.

## 5. CONCLUSIONS

1. Mixed populations of bacteria isolated from the Oława river successfully degraded benzo[a]pyrene and naphthalene as sole sources of carbon.

2. B[a]p was degraded only by the mixed population of bacteria which was confirmed by the inability of single strains to degrade this compound.

3. Metabolites formed as a result of enzymatic degradation of B[a]p by the mixed population of bacteria had potential mutagenic properties.

4. Metabolites of naphthalene enzymatic degradation carried out by the mixed population of bacteria did not have mutagenic properties.

5. During the growth of bacteria in the media with B[a]p and naphthalene, elimination of some species from mixed populations occurred.

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## BIODEGRADACJA BENZO[a]PIRENU I NAFTALENU PRZEZ BAKTERIE WYIZOLOWANE Z WODY RZEKI OŁAWY

Badano wydajność rozkładu benzo[a]pirenu (B[a]p) i naftalenu przez bakterie wyizolowane z wody rzeki Oławy. Biodegradacji dokonywały mieszane populacje bakterii. Maksymalny ubytek B[a]p wynosił 98,3%, a naftalenu – 79,1%. W czasie trwania eksperymentu następowała eliminacja niektórych gatunków wchodzących w skład mieszanych populacji. Stwierdzono, że produkty pobiodegradacyjne B[a]p wykazują potencjalne właściwości mutagenne, natomiast produkty pobiodegradacyjne naftalenu nie mają takich właściwości.

## БИОДЕГРАДАЦИЯ БЕНЗО(а)ПИРЕНА И НАФТАЛЕНА ИЗОЛИРОВАННЫМИ БАКТЕРИЯМИ ИЗ ВОДЫ РЕКИ ОЛАВЫ

Исследована эффективность бензо(а)пирена и нафталена изолированными бактериями из воды реки Олавы. Биодеградацию делали смешанные популяции бактерий. Макцимальная потеря бензо(а)пирена составляла 98,3% а нафталена – 79,1%. Во время эксперимента наступало удаление некоторых штаммов бактерий, входящих в состав популяции. Было установлено, что продукты, образующиеся после биодеградации бензо(а)пирена обнаруживают потенциальные мутагенные свойства, зато продукты, образующиеся после биодеградации нафталена не обладают такими свойствами.