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AGNIESZKA MROZIK[1], ŻANETA SWĘDZIOŁ[1], SEWERYN MIGA[2]

COMPARATIVE STUDY OF PHENOL DEGRADATION WITH A WILD-TYPE AND GENETICALLY MODIFIED *P. VESICULARIS* (pBR322). PLASMID STABILITY AND FAME PROFILING

It was evidenced that *P. vesicularis* is an efficient degrader of phenol but does not have a reporter system for monitoring bacterial survival in the environment. Therefore, *P. vesicularis* (pBR322) has been constructed. In this study we experimentally confirmed that introduction of plasmid pBR322 into *P. vesicularis* did not change its ability to degrade phenol in liquid media and after its inoculation into sterile soil. Moreover, it has been shown that plasmid pBR322 was stable in *P. vesicularis* during all experiments. Additionally, the pattern of fatty acid methyl esters for *P. vesicularis* (pBR322) looked similar to that of *P. vesicularis* under phenol exposure. Some fatty acids, especially branched and cyclopropane ones were sensitive markers of phenol utilization. These findings indicate that *P. vesicularis* (pBR322) due to the presence of plasmid could be used instead of *P. vesicularis* in bioaugmentation of phenol-contaminated areas.

1. INTRODUCTION

Phenolic compounds are a large group of low-molecular weight aromatic hydrocarbons which enter the environment from natural and anthropogenic sources. They are present in red fruits and carrots, onions, peanuts, coffee, tea and are known as plant secondary metabolites. Other natural sources of these compounds are volcanic eruptions, conflagration of forests and meadows. However, considerably higher amount of phenol and its derivatives is released to the environment as a result of human industrial activities. They are used in dyes, pesticides, pharmaceuticals, textile, polymers production, pulp mill and wood processing [1]. Phenolic compounds are also common

[1]Department of Biochemistry, University of Silesia, ul. Jagiellońska 28, 40-032 Katowice, Poland, corresponding author A. Mrozik, e-mail: agnieszka.mrozik@us.edu.pl

[2]Institute of Materials Science, University of Silesia, ul. 75 Pułku Piechoty 1A, 41-500 Chorzów, Poland.

constituents of crude petroleum and its products such as gasoline and diesel fuel [2]. The presence of phenols in the environment is currently of great ecological concern due to their toxicity to living organisms even at low concentrations.

There are many methods for the removal of phenols from soil. As an alternative to physicochemical methods, which are very expensive and not always effective, biological approaches seem to be very promising. Among biological treatments, microbial degradation is one of the most effective ways of phenol removal from polluted areas. A wide variety of microorganisms are known to be capable of using phenol as a carbon and energy source under aerobic conditions. They convert aromatic ring of phenol to non-toxic end products [2].

One of the relatively new methods for phenol removal from contaminated soil is bioaugmentation. It is defined as a technique for improvement of the degradative capacity of polluted areas by introduction of specific component bacterial strains or consortia of microorganisms [3]. One difficulty with bioaugmentation approach is ensuring that the introduced microorganisms are capable of survival under harsh environmental conditions long enough to perform their intended clean-up task. For this reason, for tagging bacteria and monitoring their activity and survival in a given environment many biomarkers have been developed. They include bacterial luciferase genes (*luxAB*), eukaryotic luciferase gene (*luc*), green fluorescent protein gene (*gfp*), chromogenic marker genes (e.g. *lacZY*), heavy metal resistance genes (e.g. *mer*) and antibiotic resistance genes (e.g. *nptII*) [4]. The choice of biomarker and monitoring system depends on the nature of contaminated site, properties of the strain and sensitivity and specificity of detection required.

For estimation of physiological status of phenol-degrading bacteria in contaminated environments and monitoring changes in bacterial community structure under chemical stress many traditional and molecular techniques are used. Among them analysis of fatty acid methyl esters (MIDI-FAME) was successfully applied in many experiments [5, 6]. As a response to presence of phenols, bacteria have developed many different adaptive mechanisms such as *de novo* synthesis of fatty acids, *cis* to *trans* isomerization, the increase of branched fatty acid content and the formation of cyclopropane ring in acyl chain. Rapid adaptation to changes in the environment is essential for the survival of bacteria under extreme conditions such as chemical stress.

The aim of this study was to test plasmid pBR322 stability in *P. vesicularis* cells during phenol degradation in liquid media and after introduction into sterile soils. To check the possibility of using *P. vesicularis* (pBR322) with easy detectable antibiotic resistance genes instead of *P. vesicularis* in environmental studies, their growth in liquid media, dynamics of phenol consumption, catechol dioxygenase activities and whole-cell fatty acid composition were compared. Next, in the soil samples the number of introduced and survived bacteria, the rate of phenol degradation and FAME profiles were analysed.

2. MATERIAL AND METHODS

The experiments were performed using the wild-type strain of *Pseudomonas vesicularis* and *P. vesicularis* (pBR322) tagged with selectable antibiotic markers. *P. vesicularis* was obtained from the Polish Collection of Microorganisms of Institute of Immunology and Experimental Therapy, Polish Academy of Science, Wrocław, Poland. It is known as an efficient degrader of phenol and other aromatic compounds such as *p*-cresol, sodium benzoate and sodium salicylate [7] but it does not have any marker system for monitoring its survival and activity in contaminated environment. For this reason, *Pseudomonas vesicularis* (pBR322) was constructed. It was generated by transferring plasmid pBR322 from *E. coli* DH5α into *P. vesicularis* cells as was described in detail elsewhere [8].

P. vesicularis and *P. vesicularis* (pBR322) were grown in minimal medium (3.78 g of $Na_2HPO_4 \cdot 12H_2O$, 0.5 g of K_2HPO_4, 5.0 g of NH_4Cl, 0.2 g of $MgSO_4 \cdot 7H_2O$ and 0.1 g of yeast extract in 1000 cm^3 of deionized water (final pH 7.1–7.3) containing phenol at the concentration of 376 mg/dm^3 in a rotary shaker (130 rev/min) at 28 °C.

Soil samples were collected from the top layer (0–15 cm) at two sites in Sosnowiec (Upper Silesia, Poland). Physical and chemical properties of each soil are presented in Table 1.

Table 1

Characteristics of soils

Property	Clay (L1)	Sandy (L2)	Method/source
Sand content, %	59	96	PN-R-04032:1998
Silt content, %	31	4	PN-R-04032:1998
Clay content, %	10	0	PN-R-04032:1998
Density, g/cm^3	0.57	1.17	PN-88/B-04481
pH	6.02	6.89	PN-ISO 10390:1997
Organic matter, % d.w.	29.5	1.9	combustion
Total organic carbon, % d.w.	7.9	0.61	PN-Z-15011-3
Chum[a], % d.w.	1.38	0.24	[9]
CEC, cmol/kg)	13.7	1.9	ISO 23470:2007
P_2O_5, mg/100 g	0.05	0.06	PN-R-04023:1996
K_2O, mg/100 g	41.5	5.0	PN-R-04022:1996
Conductivity, μS/cm	99.3	30.3	PN-ISO 11265+AC1:1997

[a]Total humic and fulvic acids.

Prior to analysis, soil samples were sieved (2 mm), transferred into glass pots (150 g) and sterilized four times by autoclaving (121 °C, 40 min) with 1-day intervals. Next triplicate sterile soil portions were contaminated with phenol at the concentration of 1.7 mg/g soil and pre-incubated for one day. For soil inoculation, both strains were

grown in 250 cm^3 of nutrient broth medium for 6 h (to reach mid-logarithmic growth phase) on a rotary shaker (130 rev/min) at 28 °C. After centrifugation (8000 g) pellets were re-suspended in 13 cm^3 of 0.85% NaCl and poured into sterile soils with the initial number $4.45 \cdot 10^7$/g and $4.93 \cdot 10^7$/g soil of *P. vesicularis* and *P. vesicularis* (pBR322), respectively. Phenol-treated but non-inoculated soils were used as a control of phenol concentration, whereas non-contaminated but inoculated soils as a control of inoculant survival. The final water content of each soil was adjusted to 50% of the maximum water folding capacity. On day 1, 4, 12 and next every 8 days soil samples were taken to determine phenol concentration and to count the number of *P. vesicularis* and *P. vesicularis* (pBR322) cells. All soils samples were kept at room temperature (22 °C).

To determine the metabolic pathway of phenol degradation by bacteria, the first product *cis*,*cis*-muconate of the *ortho* ring cleavage indicating 1,2-dioxygenase activity and 2-hydroxymuconic semialdehyde of the *meta* ring cleavage indicating 2,3-dioxygenase activity were measured at the wavelength of 260 and 375 nm, respectively. The protein content in crude extract was calculated with bovine albumin as a standard. Catechol 1,2- and 2,3-dioxygenase activities were expressed as mU/mg of protein.

Phenol concentrations in liquid media and in soil after extraction with methanol were measured by the colorimetric method with diazoate *p*-nitroaniline at the wavelength of 550 nm. To 1 cm^3 of bacterial suspension and 1 cm^3 of methanol fraction obtained from 5 g of soil following chemicals: diazoate *p*-nitroaniline (1 cm^3), 10% Na_2CO_3 (0.5 cm^3), 10% NaOH (1 cm^3) were added. Next all samples were filled up with deionized water to the final volume of 10 cm^3 and left for 10 min for color stabilization.

Liquid cultures of both strains and soil samples were withdrawn periodically to assess the extent of bacterial growth. The cell density (OD) in liquid media was determined spectrophotometrically by meauring the absorbance of the suspension at the wavelength of 600 nm. Bacteria introduced into soil were counted by the dilution plate-count technique. For this purpose, 5 g of soil was placed into Erlenmeyer flasks containing 45 cm^3 of 0.85% NaCl for shaking (30 min, 125 rev/min) and preparing serial 10-fold dilution. *P. vesicularis* was plated on LB agar plate (10 g of tryptone, 10 g of NaCl, 5 g of yeast extract and 14 g of agar-agar in 1000 cm^3 of deionized water), whereas *P. vesicularis* (pBR322) on LB agar with tetracycline at the concentration of 10 $\mu g/cm^3$. The cell number was expressed as log CFU/g soil.

Extraction of fatty acids from both bacterial strains growing in liquid media with and without phenol was carried out on 0, 24, 48 and 72 h of incubation. Bacteria were harvested by centrifugation (5000 g) at 4 °C for 30 min. Next, the cell pellets were washed twice with 0.9% NaCl to remove residue of culture medium. Fatty acids from soils were isolated on day 1 and 4, and next every 8 days of experiment. FAMEs were analysed using a gas chromatograph (Hewlett-Packard 6890, USA) with a capillary column Ultra 2-HP and flame ionization detector (FID). Peaks from chromatograms

were identified using MIDI software (the Sherlock aerobe method and TSBA library version 6.1).

The determination of phenol concentration, protein concentration, optical density (OD), number of bacteria, enzyme activity and FAME extraction were carried out according to methods described previously [10, 11].

Plasmid pBR322 was isolated from bacteria grown in liquid media with and without phenol and from bacteria re-isolated from phenol-contaminated and control soils at the beginning and the end of the biodegradation experiments. For plasmid extraction, alkaline lysis method was used [12]. Plasmid DNA was visualized in UV light and electrophoretic pattern was photographed in the Vilber Lourmat system using the BioCapt software.

All obtained data were statistically analysed using the Statistica 8.0 StatSoft Inc. For FAME analysis, the non-parametric Wilcoxon test was used.

3. RESULTS

3.1. CELLS GROWTH AND PHENOL BIODEGRADATION IN LIQUID MEDIA

P. vesicularis and *P. vesicularis* (pBR322) were grown in minimal medium with phenol at the concentration of 376 mg/dm^3 served as a sole carbon and energy source. Both strains metabolized phenol completely during 72 h (Fig. 1).

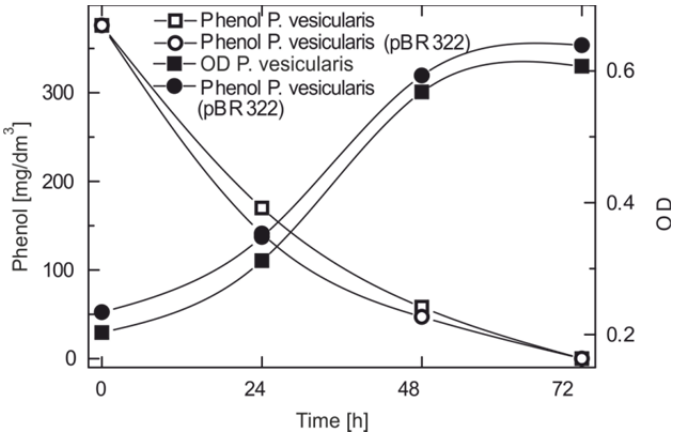

Fig. 1. The rate of phenol degradation and growth curves of *P. vesicularis* and *P. vesicularis* (pBR322)

The highest percentage of phenol removal was observed within the first 24 h of culturing when 57.5% and 62.8% of the initial phenol dose was degraded by *P. vesicularis* and *P. vesicularis* (pBR322), respectively. In successive 2 days phenol biodegradation proceeded slower. The number of bacteria during the first 24 h of culturing was not

accompanied with the highest phenol degradation rate. The OD values increased slightly during the first 24 h, whereas the highest cell growth was observed between 24 and 48 h of incubation (Fig. 1). The number of bacteria increased from initial $3.40 \cdot 10^6$ and $3.30 \cdot 10^6/cm^3$ to $9.30 \cdot 10^6$ and $1.07 \cdot 10^7/cm^3$ at 24 h and to $3.10 \cdot 10^8$ and $4.40 \cdot 10^8/cm^3$ at 48 h of *P. vesicularis* and *P. vesicularis* (pBR322), respectively. For the quantitative comparison of phenol degradation rate of *P. vesicularis* (pBR322) and *P. vesicularis*, the correlation coefficients R were calculated. $R = 0.997$ for the rate of phenol degradation and $R = 1$ for OD pointed to very well correlated features of tested bacteria.

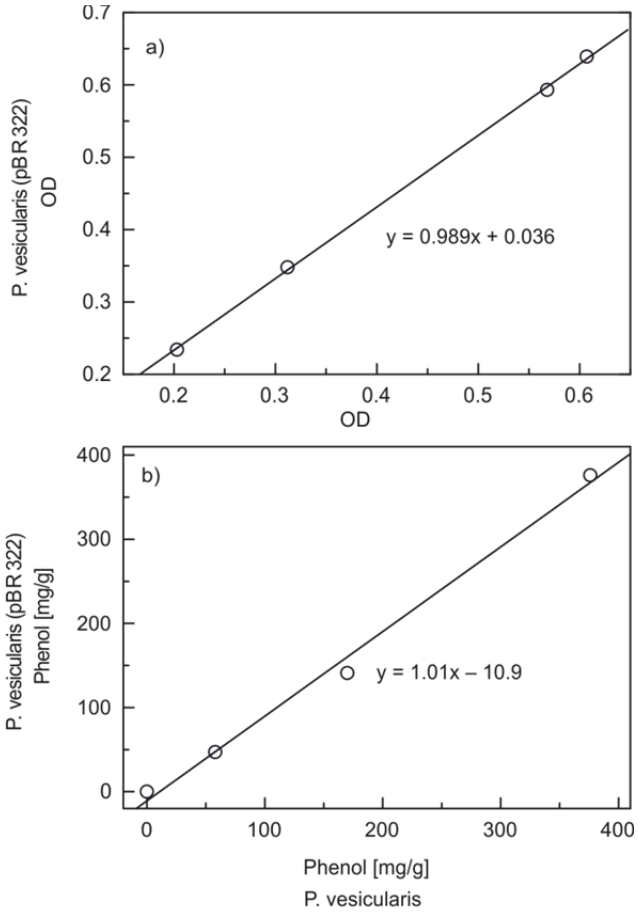

Fig. 2. Rate of phenol degradation (a) and OD (b) of *P. vesicularis* (pBR322) versus *P. vesicularis*. Solid lines – best fit to linear regression

Figure 2 shows the degradation rate of phenol and OD of *P. vesicularis* (pBR322) versus *P. vesicularis*. For OD and phenol degradation rate, the regression coefficients a were 0.989±0.015 and 1.01±0.06, respectively. Within the range of uncertainty, the

obtained coefficients were equal to unity, therefore the presence of plasmid pBR322 in *P. vesicularis* cells did not influence their degradative ability.

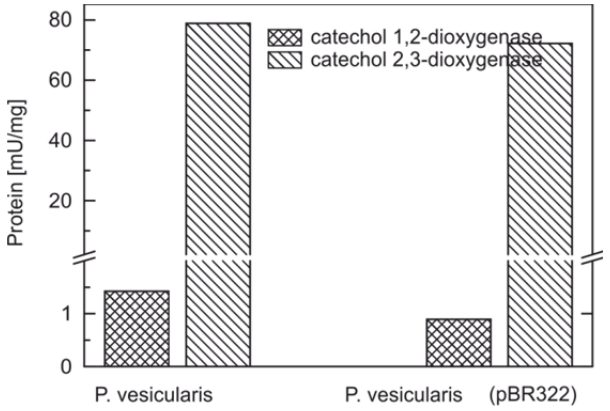

Fig. 3. Enzyme activities in cell-free extracts from *P. vesicularis* and *P. vesicularis* (pBR322) grown on phenol

In parallel to phenol biodegradation studies, the activities of enzymes involved in aromatic ring cleavage were measured. Interestingly, as shown in Fig. 3, *P. vesicularis* as well as *P. vesicularis* (pBR322) synthesized both catechol 1,2- and 2,3-dioxygenase, however activities of the enzymes were definitely different. In bacterial cells growing on phenol, the activity of catechol 2,3-dioxygenase was significantly higher compared to that of catechol 1,2- dioxygenase and reached the value of 78.97 mU/mg and 72.30 mU/ mg of protein for *P. vesicularis* and *P. vesicularis* (pBR322), respectively. In contrast, catechol 1,2 dioxygenase activity in both tested strains was minimal and had average value of 1.12 mU/mg of protein.

3.2. PHENOL DEGRADATION AND NUMBER OF BACTERIA IN SOIL

Results of our studies indicate that both strains degraded phenol most effectively in clay soil (L1) with high amount of organic matter (29.5% d.w.) compared to sandy soil (L2) containing small amount of the organic matter (1.9% d.w.). Phenol biodegradation by both inoculants in L1 soil proceeded within 12 days. The highest percentage of phenol removal was observed during the first 4 days of the experiment, when 92% of the initial dose of phenol was degraded. From that day to the end of the experiment, the degradation rate was lower (Fig. 4a1, a2). In contrast, phenol biodegradation in L2 soil by both strains proceeded much longer and lasted 28 days. The highest rate of phenol utilization was also observed during the first 4 days of experiment. On the day 4 phenol loss reached the value of 70% of the initial dose. Interestingly, from that day

to day 20 phenol concentration did not change significantly but in the next 8 days both strains degraded phenol completely (Fig. 4a1, a2).

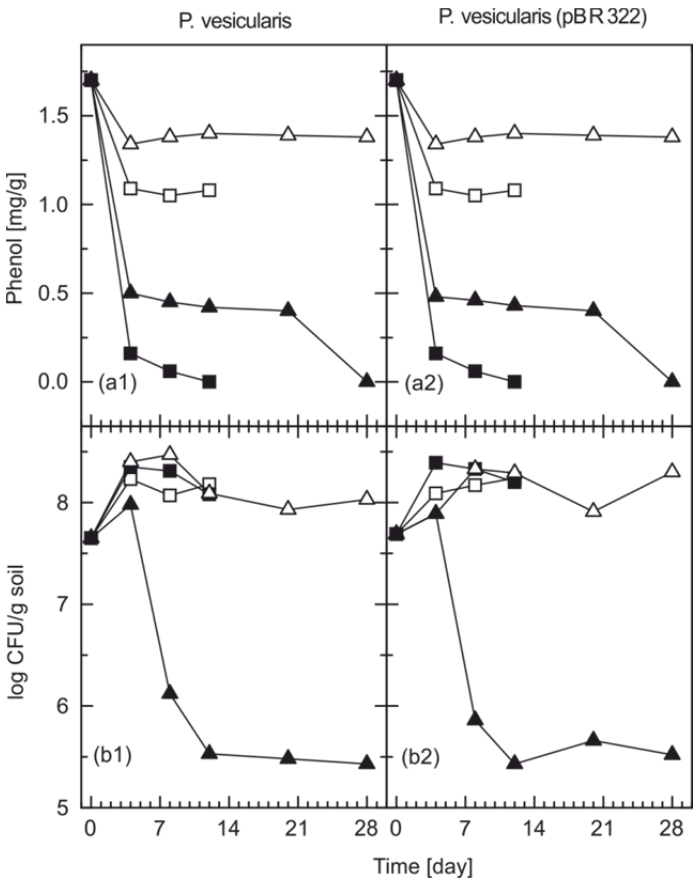

Fig. 4. Dynamics of phenol degradation (a1, a2) and number of bacteria (b1, b2) in L1 (■) and L2 (▲) soil inoculated with *P. vesicularis* (left panel) and *P. vesicularis* (pBR322) (right panel). Control soils: L1 (□) and L2 (Δ)

The correlation coefficients between the rate of phenol degradation by tested strains were $R = 1$ for both L1 and L2 soil. Similar data analysis to OD, resulted in the regression coefficients equal to unity (within range of uncertainty). This is a next confirmation of almost identical degradative capabilities of both strains. Phenol concentration was also measured in control L1 and L2 soils to estimate phenol binding by soil particles. In L1 control soil the amount of bioavailable phenol fraction on day 4 was lower (64% of the total phenol added) than in L2 control soil (79% of phenol initial dosage). From that day to the end of experiment, phenol concentration in control soils remained at the similar level (Fig 4a1, a2).

The survival of *P. vesicularis* and *P. vesicularis* (pBR322) was studied both in phenol-contaminated and non-contaminated L1 and L2 soils. The more effective phenol degradation in L1 than in L2 soil was positively correlated with a survival of inoculated strains. Interestingly, in phenol-polluted L1 soil the number of bacteria increased during the first days of incubation and on day 4 was by ca. 12% higher than in the initial counts. On the same sampling day, a similar increase (ca. 9%) in bacterial counts was observed in L1 control soil. Moreover, during phenol biodegradation, the number of both inoculants in L1 soil did not decrease below the initial counts over experimental period of time (Fig. 4b1, b2). In phenol-contaminated L2 soil the number of inoculated cells increased during the first 4 days about 2–4% in comparison with initial population size but it was lower than in L1 soil. On the next sampling days, the number of introduced cells in L2 soil sharply decreased and on day 12 it comprised 79% and 75% of inoculated *P. vesicularis* and *P. vesicularis* (pBR322) cells, respectively. In turn, in control L2 soil, the number of both inoculants increased ca. 9% between day 4 and 8 in comparison with number of introduced cells and next slightly decreased on day 12 but not below the initial counts (Fig. 4b1, b2). The correlation and regression coefficients between number of *P. vesicularis* and *P. vesicularis* (pBR322) cells in L1 ($R = 0.990$, $a = 0.98 \pm 0.09$) and L2 ($R = 0.991$, $a = 0.97 \pm 0.07$) soil under phenol exposure provided high evidence to their similarity.

3.3. PLASMID STABILITY IN *P. VESICULARIS* (pBR322)

Before biodegradation experiments in the liquid media and in soils, plasmid DNA was isolated from *P. vesicularis* (pBR322) (Fig. 5). To check plasmid stability, it was

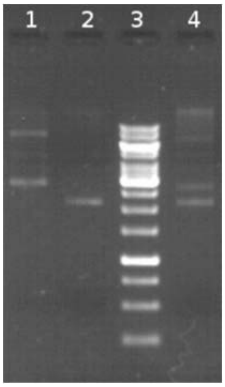

Fig. 5. Agarose gel electrophoresis of plasmid DNA isolated from *E. coli* DH5α (pBR322) (1), *P. vesicularis* (2), standard molecular weight GeneRuler 1000bp DNA Ladder (3), *P. vesicularis* (pBR322) (4)

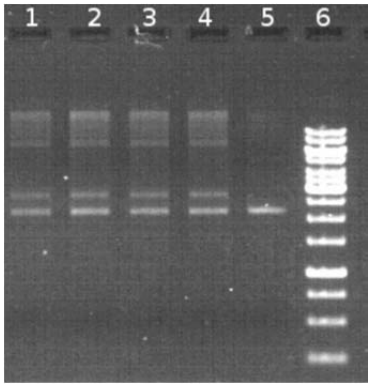

Fig. 6. Agarose gel electrophoresis of plasmid DNA isolated from *P. vesicularis* (pBR322) after phenol degradation in L1 (1) and L2 (2) soil, re-isolated form control soil L1 (3) and L2 (4), *P. vesicularis* (5), standard molecular weight GeneRuler 1000bp DNA Ladder (6)

isolated again from bacteria grown in liquid media with phenol and from bacteria re-isolated from phenol-contaminated and non-contaminated L1 and L2 soils. As shown in Figure 6, plasmid was stable in *P. vesicularis* (pBR322) cells over experimental period of time independently of growth conditions.

3.4. FAME PROFILES OF BACTERIA GROWN IN LIQUID MEDIA

To determine the effect of phenol on the whole, cell-derived fatty acid composition of *P. vesicularis* and *P. vesicularis* (pBR322) FAME profiles were analysed. For the data interpretation all fatty acids were grouped into two major classes. The former one contained saturated fatty acids (straight-chain, hydroxylated, cyclopropane and branched) and the latter one comprised all unsaturated fatty acids.

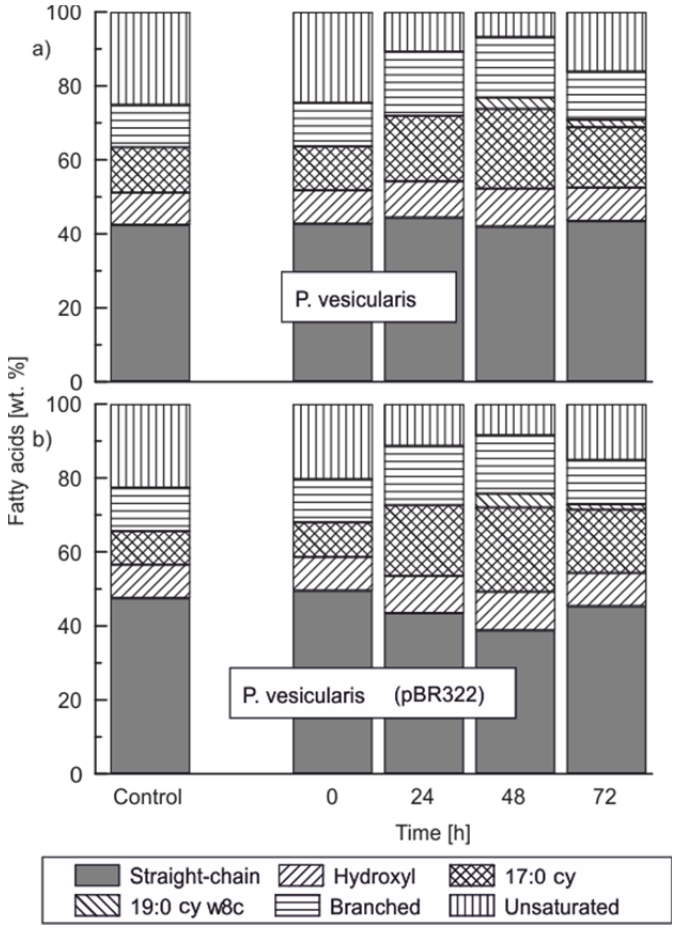

Fig. 7. Percentages of selected fatty acids isolated from *P. vesicularis* (a) and *P. vesicularis* (pBR322) (b) grown in LB (control) and on phenol

It was found that both *P. vesicularis* and *P. vesicularis* (pBR322) strains cultured in liquid medium with phenol at the concentration of 376 mg/dm^3 characterized by higher proportion of saturated fatty acids compared to control sample (bacteria cultured in LB medium without phenol, Fig. 7). The content of these fatty acids comprised 75.45–93.23% of total extracted fatty acids from *P. vesicularis* and 79.71–91.58% from *P. vesicularis* (pBR322) grown on phenol, whereas in control samples their abundance ranged from 74.90 to 77.37%. Under phenol exposure, significant differences in the percentage of 17:0*cy* cyclopropane fatty acid have been observed. Its content depended on phenol concentration in the culture medium. In extracts from *P. vesicularis*, the percentage of 17:0*cy* increased from 11.91% at 0 h to 21.54% at 48 h, whereas in FAME profile obtained from *P. vesicularis* (pBR322) increased from 9.38 to 22.80% in the same period of time. At the last sampling time, the abundance of 17:0*cy* in FAME patterns was lower and reached the value of 16.38% and 17.12% of total fatty acids isolated from *P. vesicularis* and *P. vesicularis* (pBR322), respectively. In this study, the most interesting changes in fatty acid composition of both strains were related to 19:0*cy* ω8*c* cyclopropane fatty acid. Its appearance in FAME profiling was detected between 48 and 72 h under phenol exposure but it was not detected among FAMEs isolated from *P. vesicularis* and *P. vesicularis* (pBR322) cells grown in LB medium (Fig. 7). In the presence of phenol, important changes were also observed in the content of branched fatty acids in comparison with control samples. Their percentages increased from 11.76 to 17.33% and from 11.68 to 16.00% in FAME profiles of *P. vesicularis* and *P. vesicularis* (pBR322), respectively, and the top values reached at 24 h of culturing. The crucial changes in fatty acid composition were observed in the content of unsaturated fatty acids. Their percentages in FAME profiles of *P. vesicularis* and *P. vesicularis* (pBR322) during phenol degradation decreased from 24.55 to 6.77% and from 20.29 to 8.42% during 48 h of experiment, respectively. The amount of unsaturated fatty acids increased again when phenol was completely degraded but on the last sampling time it was slightly lower as compared to control samples (Fig. 7).

3.5. FAME PROFILES OF SOILS

To illustrate FAMEs variability in *P. vesicularis* and *P. vesicularis* (pBR322) cells during phenol utilization fatty acids were extracted directly from polluted and non-polluted L1 and L2 soils. The most distinct differences between FAME profiles obtained from soils with inoculants and control samples were observed between day 1 and 4 after phenol addition. In L1 soil inoculated with *P. vesicularis*, the content of saturated fatty acids under phenol exposure increased from 80.07% on day 1 to 85.81% on day 4, whereas in soil with *P. vesicularis* (pBR322), from 76.54 to 85.54%. Their content decreased on day 12 when reached the value of 80.22% in soil with *P. vesicularis* and 78.02% in soil with *P. vesicularis* (pBR322) (Fig. 8).

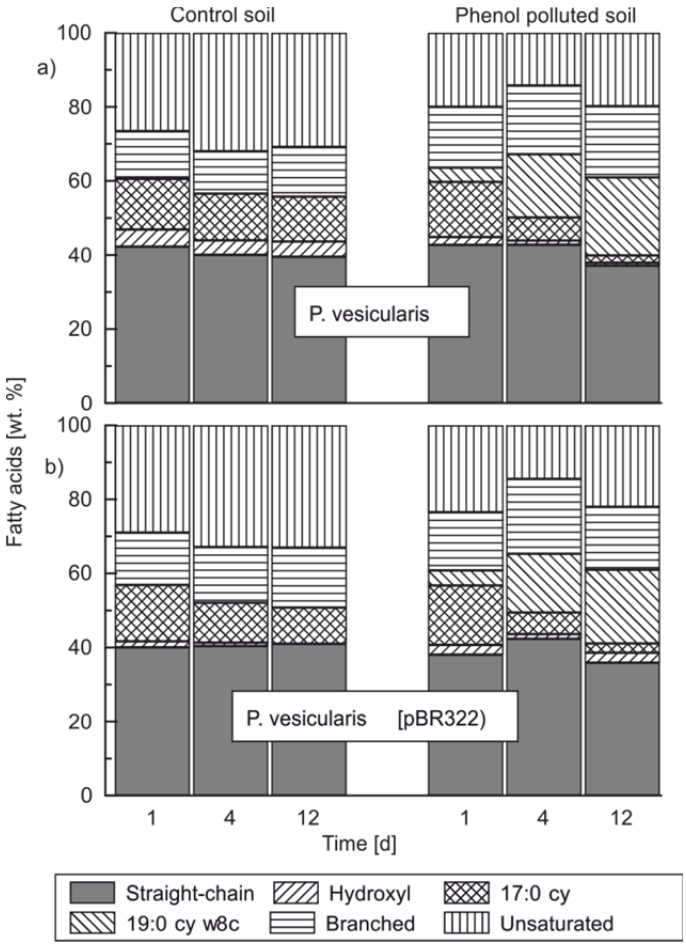

Fig. 8. Percentages of selected fatty acids isolated from L1 soil contaminated with phenol and from control soil inoculated with *P. vesicularis* (a) and *P. vesicularis* (pBR322) (b)

In L1 soil with *P. vesicularis*, the decrease in percentage of hydroxylated fatty acids was observed from 2.10% on day 1 to 0.78% on day 12. Similar tendency was noticed in phenol-contaminated soil with *P. vesicularis* (pBR322) but only during the first 4 days. On day 12, their content slightly increased in FAME profiles. In comparison, in L1 control soil inoculated with the wild-type strain of *P. vesicularis*, the content of hydroxylated fatty acids was higher than in L1 phenol-polluted and inoculated soil and ranged from 3.90 to 4.64%. In turn, in L1 soil inoculated with *P. vesicularis* (pBR322), the amount of hydroxylated fatty acids decreased from 1.57% on day 1 to 0.93% on day 4 and on the last sampling day they were not detectable (Fig. 8). Another important change in FAMEs extracted from phenol-polluted and inoculated L1 soil was connected with cyclopropane fatty acid content. Their percentage increased from

18.74% on day 1 to 23.35% on day 4 in soil with *P. vesicularis*, whereas in soil with tagged strain from 20.14 to 22.40%. On the last sampling day, the percentage of cyclopropane fatty acids slightly decreased and reached the value of 23.04% in L1 soil inoculated with *P. vesicularis* and 21.76% with *P. vesicularis* (pBR322). In contrast, no significant differences in cyclopropane fatty acid content in control soils were found. The 19:0*cy* ω8*c* cyclopropane fatty acid was detected only in L1 soil under phenol exposure. Its content was increasing continuously during phenol removal and on day 12 reached the value of 21.08% and 19.92% of total fatty acids extracted from soil inoculated with *P. vesicularis* and *P. vesicularis* (pBR322), respectively (Fig. 8). In phenol-amended L1 soil inoculated with *P. vesicularis*, a slight increase of branched fatty acid content was observed from 16.46% on day 1 to 19.24% on day 12 and from 15.69% on day 1 to 20.18% on day 4 in this soil with *P. vesicularis* (pBR322). The crucial changes in the percentage of unsaturated fatty acids in L1 soil under phenol exposure were also observed. Their content decreased during biodegradation experiment from 19.93 to 14.19% and from 23.46 to 14.46% in soil samples inoculated with *P. vesicularis* and *P. vesicularis* (pBR322), respectively (Fig. 8).

The changes in FAME profiles obtained from phenol-contaminated L2 soil and inoculated with *P. vesicularis* or *P. vesicularis* (pBR322) at the successive sampling days were also very similar. Over phenol degradation the content of saturated fatty acids in both soils increased about 10% from day 1 to 20 and next decreased on day 28 (Fig. 9). In comparison, in control soils their content remained at the constant level during experimental period of time. Phenol contamination caused alternations in the amount of hydroxylated fatty acids. Their content in FAME profiles obtained from L2 soil inoculated with *P. vesicularis* decreased from 1.81% on day 1 to 0.23% on day 12, and next they were not detectable. In turn, in L2 soil with *P. vesicularis* (pBR322), their content was decreasing continuously from the beginning to the end of experiment and on day 28 did not exceed 0.5% (Fig. 9). Another significant change in FAME profiles of tested soil was related to the abundance of 17:0*cy* and 19:0*cy* ω8*c* cyclopropane fatty acids. The former one was isolated from both phenol-contaminated and inoculated soils and from control soils, whereas the latter one was extracted from inoculated soils under phenol exposure only. The content of 17:0*cy* strongly decreased from 12.53% on day 1 to 3.92 % on day 20 in phenol-contaminated soil with *P. vesicularis* and in the same sampling days from 12.42 to 2.99% in soil with *P. vesicularis* (pBR322). Next, its abundance slightly increased and on 28 day reached the value of 6.00% in soil inoculated with both strains. The 19:0*cy* ω8*c* cyclic fatty acid appeared in L2 soil on day 4. The highest percentages of this fatty acid composed 15.37% and 14.03% of total fatty acids were detected on day 20 in soils inoculated with *P. vesicularis* and *P. vesicularis* (pBR322), respectively. Next, its abundance slightly decreased (Fig. 9). Important changes in FAME patterns were also connected with branched fatty acid contents. Their abundance slightly changed in control soils and ranged from 9.02–11.81% and from 9.36–10.62% in soil with *P. vesicularis* and

P. vesicularis (pBR322), respectively. However, in phenol-polluted soils their content increased till 20 day of experiment from 12.62 to 17.26% in soil with *P. vesicularis* and from 12.70 to 18.66% in soil with *P. vesicularis* (pBR322). On the last sampling day, their abundance in both inoculated soils slightly decreased and reached the value of 16%. The crucial alterations in hydroxylated, cyclopropane and branched fatty acid contents in the presence of phenol were correlated with the decrease in unsaturated fatty acid abundance. In L2 soil with both inoculants, their content decreased about 10% during 20 days, whereas in non-polluted and inoculated control soils varied in the range 22.36–28.93% (Fig. 9).

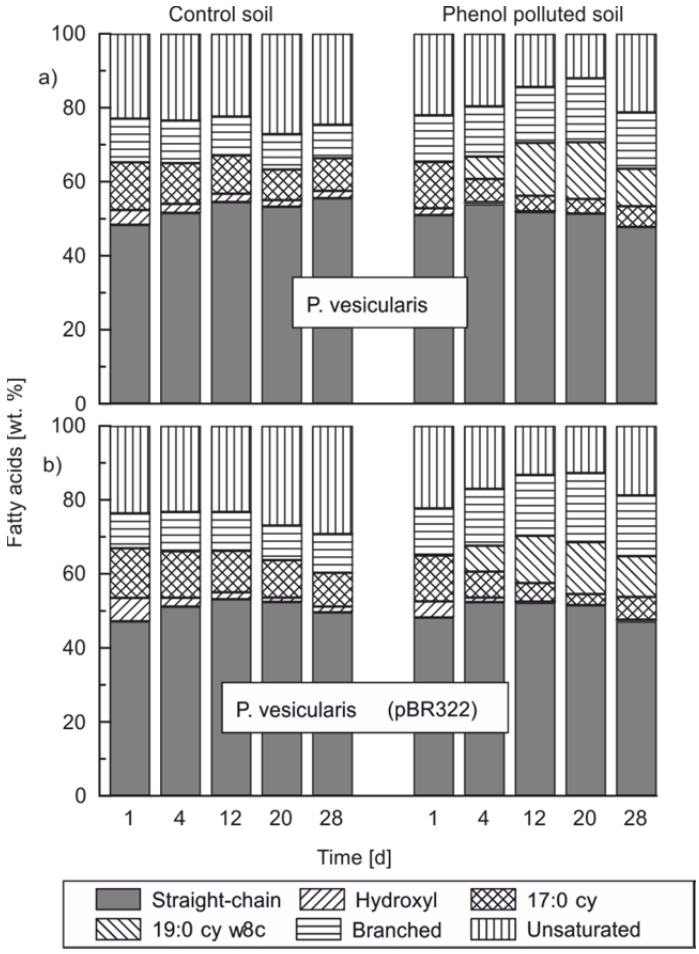

Fig. 9. Percentages of selected fatty acids isolated from L2 soil contaminated with phenol and from control soil inoculated with *P. vesicularis* (a) and *P. vesicularis* (pBR322) (b)

The results of the Wilcoxon test obtained for all dependent samples shown $p > 0.05$, that indicated no statistical differences between fatty acid composition of *P. vesicularis* and *P. vesicularis* (pBR322) grown in liquid media and introduced into soils.

4. DISCUSSION

The reported results confirmed strong ability of the wild-type strain of *Pseudomonas vesicularis* and antibiotic-resistant *P. vesicularis* (pBR322) to degrade phenol in the liquid media and after introduction into sterile soils. In this study, we demonstrated that antibiotic resistance genes reside on plasmid pBR322 were stable in *P. vesicularis* (pBR322) cells independently of growth conditions and did not influence its ability to phenol degradation. For this reason, these resistance genes could be used for selection of plasmid carrying cells among microorganisms originally present in soil. Until now phenol-degrading *P. vesicularis* was not known as a potential host of the plasmid pBR322, although other species referred to this genus were used in transformation experiments. For example, Fukumoto et al. [13] showed that *Pseudomonas avenae* K1, the pathogen of leaf blight disease in cereals, was transformed with efficiency 3.0×10^6, 1.5×10^6 and 1.3×10^6 with pBR322, pBR325 and pBR328, respectively. Moreover, all plasmids were stable in bacterial cells during incubation time and after subcultures, what indicated a low frequency of plasmid loss. In other study, Trevors et al. [14] investigated the plasmid pBR322 stability in *Klebsiella aerogenes* NCTC 418 in sterile and non-sterile agricultural drainage water. They showed that plasmid pBR322 was not stably maintained in *K. aerogenes* NCTC 418 under all conditions in experiment.

The biodegradation studies showed that *P. vesicularis* and *P. vesicularis* (pBR322) were able to degrade similar doses of phenol in various experimental systems and did not significantly differ in their degradative activity. Both tested strains utilized phenol at the concentration of 376 mg/dm^3 in liquid media within 72 h and their growth curves during phenol biodegradation were similar. Phenol removal in liquid medium was also tested by Khleifat [15] who stated that *Ewingella americana* degraded phenol at the concentration of 300 mg/dm^3 in M9 medium during 72 h and the highest phenol removal occurred within the first 12 h of incubation. He also observed that different initial (inocula) cell densities did not affect the time of phenol degradation. Other strains of bacteria *Burkholderia cepacia* PW3 and *Pseudomonas aeruginosa* AT2 with high catabolic potential and high tolerance towards phenol were isolated from a coking plant by El-Sayed et al. [16]. Both strains were able to degrade phenol at the concentration of 100 mg/dm^3 in basal mineral medium during ca. 200 h. Moreover, they showed a high tolerance towards phenol and were still viable in the presence of phenol at the concentration of 3 g/dm^3.

It was demonstrated that *P. vesicularis* and *P. vesicularis* (pBR322) growing on phenol in liquid media synthesized both catechol 1,2- and 2,3-dioxygenase. The activity of 2,3-dioxygenase in cell-free extracts was significantly higher in comparison with catechol 1,2-dioxygenase and reached the average value of 75 mU/mg of protein. These results indicated that in the presence of phenol tested strains induced both *ortho* and *meta* cleavage pathways. It was in accordance with results obtained by Mrozik and Łabużek [7] and Mrozik et al. [10] who established that *P. vesicularis* degraded phenol at the same time *via meta* and *ortho* pathways, however *meta* cleavage was the major in the opening of aromatic ring of catechol.

Study of phenol degradation by bacteria in soil environment is not an easy task. Many reports indicated that phenol particles are bound to soil organic matter (humic/fulvic and the soil humin fraction) and may not be available for microorganisms [17]. In addition, phenol is considered as one of the major components participating in formation of humus soil fractions. From this follows that decrease of phenol concentration in soil may result not only from catabolic activity of microorganisms but also from the incorporation of phenol particles into the aromatic structure of the humus. In this study, the loss of phenol in sterile soils inoculated with *P. vesicularis* or *P. vesicularis* (pBR322) was strongly correlated with organic matter content. In clay soil (L1), containing high amount of organic matter (29.50%), phenol was completely degraded within 12 days, whereas in sandy soil (L2) with low organic matter content (1.90%), its biodegradation proceeded slower and lasted 28 days. In control sterile, and contaminated L1 and L2 soils, phenol sorption to organic matter was observed during the first 4 days of experiment. In this time, in L1 and L2 soil, 36% and 21% of initial phenol dose was bound to organic matter, respectively. Kim et al. [18] also reported that *Pseudomonas spadix* BD-a59 strain metabolized BTEX significantly faster in the soil containing organic matter than in artificial soil without organic matter. In turn, Chen et al. [19] showed that *Ralstonia taiwanensis* TJ86 was capable of degrading phenol in the concentration of 500 μg/g soil during 9 days in the soil with 4.8% of organic matter. These findings confirmed the protective role of organic matter for bacteria exposed to toxic aromatic compounds.

The more effective phenol degradation at the concentration of 1.7 mg/g soil in L1 soil than in L2 soil was correlated with a high survival rate of both inoculants. In general, the cell number after soil inoculation sharply decreased but we did not observe such effect. During the first 4 days of experiment the number of bacteria significantly increased and on day 4 was about 12% higher than initial cell numbers. In comparison, in L2 soil the number of inoculated cells on day 4 was about 3% higher as compared to initial population size. In the following days the cell number of both inoculants in L1 phenol-contaminated soil slightly decreased but on day 12 was still higher than inoculated cell number. In turn, in L2 phenol-amended soil the cell number of both inoculants significantly decreased and on the last sampling day it constituted 70% of introduced cells. These results indicated that the survival of *P. vesicu-*

laris and *P. vesicularis* (pBR322) in each soil type was similar throughout the experiment and both strains grown better in L1 than in L2 soil. Similar results obtained Haluška et al. [17] who confirmed a strong correlation between the survival of bacteria in soil contaminated with PCBs and the content of organic matter. They reported that number of bacteria *Alcaligenes xylosoxidans* inoculated into PCB-contaminated soil with high content of organic matter increased 4 times on day 7 in comparison with soil containing less organic matter. In other study, Ronen et al. [20] observed that number of inoculated *Achromobacter piechaundii* cells in soil contaminated with tribromophenol at the concentration of 50 μg/g soil decreased 10 times within 15 days.

FAMEs analysis was designed for identification of microorganisms to genus or species level. For example, Martinez et al. [21] used this method for identification of sulfentrazone degrader strains in a Rhodic Hapludox soil. MIDI-FAME analysis can also be used for assessment of the physiological state of microorganisms. It is possible because bacteria alter their membrane fatty acid components in response to environmental factors, and therefore fatty acid composition can change with respect to extracellular stress (temperature, pH, heavy metals, aromatic compounds) [5, 6, 22]. Changes in cellular fatty acid profiles of phenol-degrading *P. vesicularis* strain have been well documented [10]. However, FAME profiling of the new *P. vesicularis* (pBR322) during phenol biodegradation has not been investigated so far.

The presented results showed that FAME profiles of *P. vesicularis* and *P. vesicularis* (pBR322) cultured in liquid media with phenol and inoculated into L1 and L2 soils contaminated with phenol characterized by significantly higher proportion of saturated fatty acids compared to control samples. The highest percentages of saturated fatty acids were determined at this time, when bacteria degraded more than 50% of the initial phenol dosages. Among saturated fatty acids the abundance of branched and cyclopropane fatty acids in phenol-degrading bacterial cells increased, whereas hydroxyl fatty acids decreased. The increase of branched fatty acid content may indicate their participation in the adaptive mechanism of bacteria to the presence of lipophilic aromatic compounds. This hypothesis was confirmed by Tsitko et al. [23] who studied the effects of benzene, phenol and toluene on cellular fatty acid composition of *Rhodococcus opacus*. The high proportion of branched fatty acids in FAME profiles of *Pseudomonas* sp. JS150 during naphthalene degradation was also reported by Mrozik et al. [24].

The most interesting changes in FAME profiles obtained from *P. vesicularis* and *P. vesicularis* (pBR322) cultivated in liquid media with phenol as well as inoculated into the phenol-contaminated soils were related to the abundance of cyclopropane fatty acids, represented by 17:0*cy* and 19:0*cy* ω8*c*. The first one was also detected in FAME profiles obtained from both strains grown on LB medium and introduced into non-contaminated soils. In turn, 19:0*cy* ω8*c* appeared only in FAME profiles obtained from phenol-degrading *P. vesicularis* and *P. vesicularis* (pBR322) after 48 h of culturing in liquid media and after 24 h after soil inoculation. We also found that the increase of 19:0*cy* ω8*c* was correlated with lower 18:1 ω7c amount which may indicate

a post-synthetic modification of this unsaturated fatty acid to cyclic form by cyclopropane synthase. According to Grogan and Cronan [25], cyclopropane ring is formed to protect against oxidation of the double bond and therefore it stabilizes membrane lipids. Similar mechanism described Mrozik et al. [11] who studied the changes in FAME profiles of *Pseudomonas* sp. CF600 under phenol exposure in soil. They reported that the content of cyclopropane acids was different in various soils and depended on phenol degradation rate. In wet meadow soil and forest soil, 19:0*cy* ω8*c* was detected when 60% of initial phenol dosage was degraded, whereas in sandy soil when about 40% of phenol added was utilized. Cyclopropane fatty acids have been known as compounds that stabilize membrane lipids and make it more rigid. Cyclopropanation is believed to play an essential role in the adaptation of bacteria to toxic aromatic compounds.

5. CONCLUSIONS

In conclusion, the wild-type strain of *P. vesicularis* and the antibiotic-resistant *P. vesicularis* (pBR322) demonstrated similar growth rates, degradative capabilities, catechol dioxygenase activities and FAME profiles under phenol exposure in liquid media as well as in different soils. Moreover, plasmid pBR332 was stable in bacterial cells what could make it a reliable tool to predict the fate of bacteria released into phenol-contaminated environment. However, further studies are needed to get information about the spread of pBR322 antibiotic resistance genes between genetically modified strain and autochthonous microorganisms.

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