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SCIENTIFIC PAPER

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EVALUATION OF ASSAYS FOR SCREENING POLYCYCLIC AROMATIC HYDROCARBON--DEGRADING POTENTIAL OF BACTERIA

Article Highlights

- Assays can be used reliably for strain selection with a high potential for bioremediation procedures
- Dehydrogenase activity assay correlated positively with the hydrocarbon growth assay
- Dehydrogenase activity of Rhodococcus RNP05 was significantly higher than Planomicrobium RNP01
- Planomicrobium RNP01 had the lowest ability of growth on pyrene
- Rhodococcus RNP05 had the highest ability of growth on dibenzothiophene

Abstract

Within a 30-day incubation laboratory study, the polycyclic aromatic hydrocarbon (PAH) degradation profile of two bacteria, Planomicrobium sp. RNP01 and Rhodococcus sp. RNP05 were studied by three microtiter plate assays to reveal the combination of certain biological and biochemical characteristics which are reliable indicators in evaluation of bacterial biodegradation abilities. The three assays, which are hydrocarbon growth assay, 2,6-DCPIP assay and dehydrogenase activity assay revealed that Rhodococcus sp. RNP05 exhibited better potential for PAH degradation than Planomicrobium sp. RNP01. Differences between initial and final optical density and specific growth rate constants were significantly higher ($r = 0.995$, $P < 0.05$) in case of Rhodococcus sp. RNP05 on all tested substrates, as compared to Planomicrobium sp. RNP01. This was confirmed by GC-FID analyses. Dehydrogenase activity of Rhodococcus sp. RNP05 was higher ($r = 0.9995$, $P < 0.05$) than Planomicrobium sp. RNP01 and correlated positively with the hydrocarbon growth assay ($r = 0.999$, $P < 0.05$, for Rhodococcus sp. RNP05, $r = 0.986$, $P < 0.05$ for Planomicrobium sp. RNP01). This study has shown that the combination of these assays could be used for determining the bioremediation potential of PAHs in petroleum contaminated soil with the ability of screening a large number of bacterial strains.

Keywords: hydrocarbon growth, PAHs, screening assays, dehydrogenase activity, Planomicrobium, Rhodococcus.

Hydrocarbons contamination in the environment is caused by exploitation, transport, processing, storage and use of petroleum and its derivatives, as well as from combustion plants, motor vehicles and other gasoline-powered equipment. Polycyclic aromatic hydrocarbons (PAHs) are of special concern since these compounds are considered potential health and environmental risks [1], and many of them have toxic, mutagenic and/or carcinogenic properties [2,3]. Sixteen PAHs have been specified as priority pollutants by the US Environmental Protection Agency as well as the European Commission [4,5]. Soil contaminated with hydrocarbons is classified as hazardous waste according to the European Waste Catalogue under index number 17 05 03* (soil and stones containing hazardous substances) and require remediation [6].

Because of their molecular stability and low solubility, bioavailability of PAH compounds could be decreased, and the time to reach acceptable end-

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points for bioremediation treatments could be extended [7-9]. One of the important factors for PAH biodegradation is the presence and activity of PAH--degrading microorganisms [10,11].

To show that a bioremediation procedure will be effective, it is important to first demonstrate the bioavailability of substrate and ability of microorganisms to enhance the rate of hydrocarbon degradation in controlled conditions [12]. Thus, the assessment of bioremediation potential of bacterial communities is an important step when deciding the appropriate bioremediation strategy.

Turbidity and colorimetric measurements are low-cost and rapid procedures to detect the occurrence of microbial metabolism in both aerobic and anaerobic conditions. When used in microtiter plates, besides simplicity, the main advantages of these methods are rapid screening of large numbers of bacterial isolates and the fact they can be used with hydrocarbon-degrading nonculturable bacteria.

2,6-Dichlorophenolindophenol (2,6-DCPIP) assay has a sufficient sensitivity to detect primary oxidation of hydrocarbons in the first four weeks of the biodegradation process and it can be used as a quick screening method [13]. Moreover, this method revealed two extremely potent bacteria for degradation of high molecular weight PAH, *Planomicrobium sp*. RNP01 and *Rhodococcus sp.* RNP5, which was confirmed by gas chromatography analysis of biodegradation of the mixture of high molecular weight PAHs [13].

The aim of this study was to evaluate and compare the three screening methods in microtiter plates (hydrocarbon growth assay, 2,6-DCPIP assay and dehydrogenase activity assay) in order to reveal reliable indicators in the evaluation of bacterial biodegradation potential. Obtained results could potentially lead to developing of a set of simple microbial assays for quick and reliable PAH bioremediation assessment in laboratory conditions, one of the critical factors in deciding bioremediation strategies.

EXPERIMENTAL

Chemicals

Phenanthrene (PHE) and dibenzotiophene (DBT) were at >96% purity (Sigma-Aldrich, Germany), and pyrene (PYR) was at >97% purity (Fluka, Germany).

Microorganisms

Bacterial strains *Planomicrobium sp*. RNP01 (GenBank Accession No. JN683359) and *Rhodococcus sp.* RNP05 (GenBank Accession No. JQ065876) were isolated from the soil taken from Pančevo Oil Refinery, (Serbia). Isolation and identification of bacterial strains has been described by Milić *et al.* [13].

Screening assays

Isolated pure bacterial strains were grown individually in 100 ml of bacterial culture medium (BCM), which consisted of mineral medium (MM) containing NPK solution (0.1% NH_4NO_3 and 0.025% K_2HPO_4) with 2000 ppm of diesel fuel D2, for 48h on a rotary shaker at 120 rpm and 28 °C. After incubation, the cell cultures were centrifuged at 6000 rpm at 10 °C for 20 min. Bacterial inoculum of each culture was established by suspension of cell pellets (twice washed in saline solution) in sterile MM solution to an optical density of OD_{600} = 1. In all experiments, aliquots of 150 µl of bacterial inoculum were used. Biodegradation tests were performed in polystyrene 24 well microtiter plates in triplicate. This set-up was used in all three methods of assaying *in vivo* microbial degradation of PAHs.

Absorbance data of the control wells were subtracted from the solutions prior to calculations, for all screening methods. Unless otherwise specified, all results reported are averages of triplicate determinations.

Hydrocarbon growth assay

Hydrocarbon growth assay was performed by adding aliquots of inoculum to 2 ml sterile MM solution with individual PAH (PHE, PYR or DBT). PAHs (dissolved in ether) were added to achieve a final concentration of 300 ppm in each microtiter well. The control was prepared using 2 ml of MM solution without individual PAHs. The plates were incubated on a rotary shaker at 120 rpm and 28 °C for 30 days, and optical density was measured at 600 nm on days 0, 5, 10, 15, 20, 25 and 30 of the experiment, using an Elisa reader (LKB 5060-006). Log OD_{600} for each strain was plotted as a function of time. Constant growth rates were calculated by curve fitting using Origin Fit Tool (OriginPro 8, OriginLab software) [13].

2,6-DCPIP assay

It is possible to ascertain the ability of the microorganism to utilize a hydrocarbon substrate by observing the color change of DCPIP from blue (oxidized) to colorless (reduced). This assay is qualitative since absorbance of the blue color (595 nm) suffers from interference from the increasing turbidity of the medium during the incubation time.

The qualitative 2,6-DCPIP assay was performed using the same method as the hydrocarbon growth assay, except that 650 µl of 15.3 % 2,6-DCPIP sol-

ution (153 μg ml^{-1}) was added to every well to achieve a final concentration of 50 μg ml^{-1} [14]. The color change was registered as a + (discoloration) and - (blue color) after 15 and 30 days of growth.

Dehydrogenase activity assay (DHA)

The DHA is measured by a colorimetric method using 2,3,5-triphenyltetrazolium chloride (TTC) as an electron acceptor for many dehydrogenase enzymes [15]. Reduction of this compound by dehydrogenase gives triphenylformazan (TPF) which has a characteristic reddish color. The intensity of color is measured at 485 nm and it is a good indicator of microbial activities [16]. Van der Waarde *et al.* showed the parameter that had the best correlation with hydrocarbon removal and soil respiration was dehydrogenase activity [17].

The bacterial isolates were grown at 28 °C in MM solution with individual PAH (PHE, PYR or DBT, as the sole source of hydrocarbon) for 30 days in microtiter plates, as described in the section "Hydrocarbon growth assay". After 30 days, A 50 μl aliquot of an electron acceptor solution, triphenyl tetrazolium chloride (TTC) solution (0.25 g TTC in 100 ml 100mM tris buffer, pH 7), was added to each well and the plates were incubated at 28 °C for 48 h for color development. The hydrolysis reaction product (TPF) was extracted for 2 h at 30 °C with acetone and absorbance at 490 nm was recorded on the ELISA reader.

Microcosm assays

Biodegradation of PAH by *Planomicrobium sp.* RNP01 and *Rhodococcus sp*. RNP05 was examined in microcosm assays, simultaneously with screening assays. Microcosm assays were set up in 500 ml Erlenmeyer flasks containing 100 ml MM solution (with PHEN, PYR or DBT in final concentration of 50 ppm) and inoculated with 1 ml of inoculum. The assays were incubated for 30 days, at 28 °C with rotary stirring at 100 rpm.

The remaining PAH was extracted from the whole medium three times with *n*-hexane (50 ml). The organic layer was collected and washed with 50 ml 2% NaCl. After dehydration over anhydrous Na_2SO_4, it was concentrated using a vacuum rotary evaporator until the organic solvent was completely removed. All extracts were dissolved in the same volume of solvent (1 ml), and each extract was analyzed instrumentally by injecting the same volume of the solution (1 μl).

GC-FID analysis

The ability of the bacterial isolates to degrade PAHs was confirmed by gas-chromatography as analytical evidence that they can metabolize these compounds.

A gas chromatograph (Agilent 4890D) with flame ionization detector (FID) and HP-5MS column (30 m×0.25 mm, 0.25 μm film thickness) was used for gas chromatography. The carrier gas was hydrogen with a constant flow rate of 1 ml min^{-1}. Injector temperature was constant (250 °C), as was the detector temperature (300 °C). The following temperature ramping was used: initial temperature 80 °C, and then heating at a rate of 10 °C min^{-1} up to a temperature of 300 °C.

RESULTS AND DISCUSSION

Evaluation of bioremediation potential of bacterial strains for PAHs

Hydrocarbon growth assay

Hydrocarbon growth assay demonstrated that, in term of substrates, both bacterial strains had the high ability of growth on DBT substrate, and low on PYR substrate. *Rhodococcus* sp. RNP05 achieved higher efficiency of degradation on all tested substrates, *i.e.*, PHE, PYR and DBT (A_{600} of 0.320, 0.240 and 0.430, respectively), comparing with *Planomicrobium* sp. RNP01 (A_{600} of 0.219, 0.186 and 0.340, respectively). Difference between initial and final optical density (represented as ΔOD), and specific growth rate constants (K) were significantly enhanced (r = = 0.995, P < 0.05) in case of *Rhodococcus* sp. RNP05 on all tested substrates, as compared to *Planomicrobium* sp. RNP01 (Figure 1).

2,6-DCPIP assay

The biodegradability of petroleum hydrocarbon compounds can be verified using the technique based on the 2,6-dichlorophenol indophenol (2,6-DCPIP) redox indicator [18]. 2,6-DCPIP is a redox indicator that detects oxidation of NADH to NAD+ during the bacterial degradation of hydrocarbons. The principle of this technique is that during the microbial oxidation of hydrocarbons, electrons are transferred to electron acceptors such as oxygen, nitrates and sulphate. This results in the change in color of 2,6-DCPIP from blue (oxidized) to colorless (reduced).

Both bacterial strains changed color of the 2,6--DCPIP solution from blue to colorless, after two weeks of experiment (Table 1), which indicates these strains have the ability to degrade tested PAH compounds, which is in correspondence with earlier published results [13].

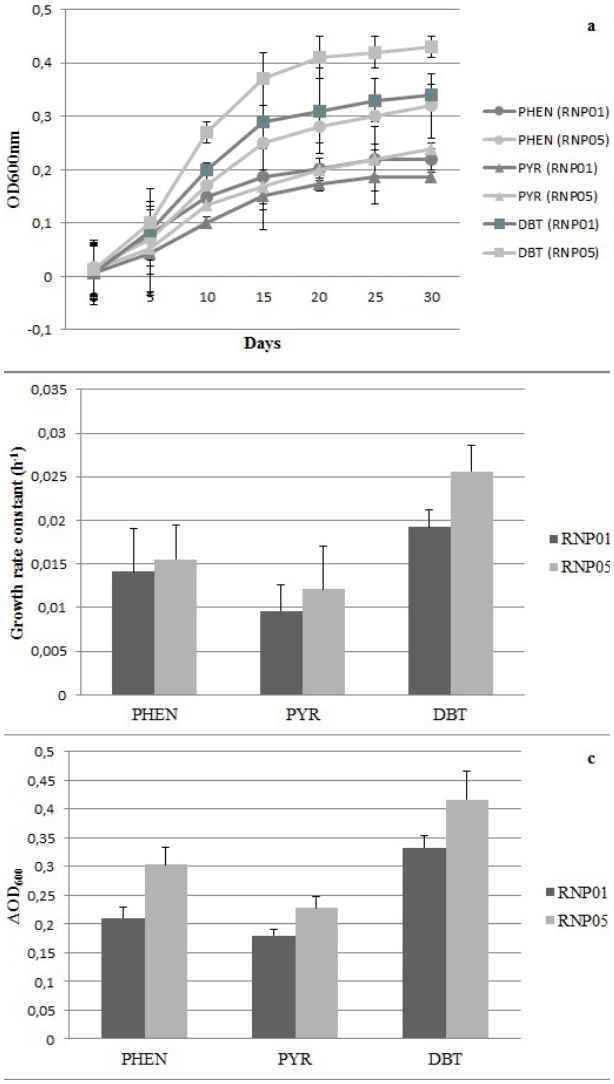

Figure 1. Parameters of: a) phenanthrene-PHEN, b) pyrene--PYR and c) dibenzothiophene-DBT biodegradation by Planomicrobium sp. RNP01 and Rhodococcus sp. RNP05; a) bacterial growth (OD_{600}), b) specific growth rate constant, K (h^{-1}), c) difference between initial and final optical density represented as ΔOD.

Table 1. Bacterial oxidative activity measured using qualitative 2,6-DCPIP assay; blue (-/no growth) colorless (+/growth)

Bacterium	PHE	PYR	DBT
Planomicrobium sp. RNP01	+	+	+
Rhodococcus sp. RNP05	+	+	+

Dehydrogenase activity assay

Dehydrogenase activity (DHA) typically occurs in all intact, viable microbial cells. Oxidation of organic matter by microorganisms assumes the involvement of a dehydrogenase enzymatic system by transferring hydrogen from the organic substrates to the electron acceptor, so that its activity is a good indicator of the microbiological action in contaminated environments, as well as of the bioremediation potential of microorganism and dynamics of bioremediation within a period of time. The very low water solubility of PAHs and their slow mass transfer rates from solid phase may limit their availability to microorganisms, thus hindering natural microbial processes of attenuation. The DHA assay can be used as a simple method to examine the possible inhibitory effect of environmental contaminants on microbial activities.

Both bacterial strains generally had a good dehydrogenase activity on tested PAH compounds after 30 days of biodegradation (Figure 2). However, dehydrogenase activity was the highest during biodegradation test with *Rhodococcus* sp. RNP05 with highest DHA values achieved after growth on DBT and PHEN, with A_{490} up to 0.397 and 0.323, respectively.

Dehydrogenase activity of *Rhodococcus* sp. RNP05 was significantly higher ($r = 0.9995$, $P < 0.05$) than *Planomicrobium* sp. RNP01 and correlated positively with the hydrocarbon growth assay ($r = 0.999$, $P < 0.05$ for *Rhodococcus* sp. RNP05, $r = 0.986$, $P < 0.05$ for *Planomicrobium* sp. RNP01).

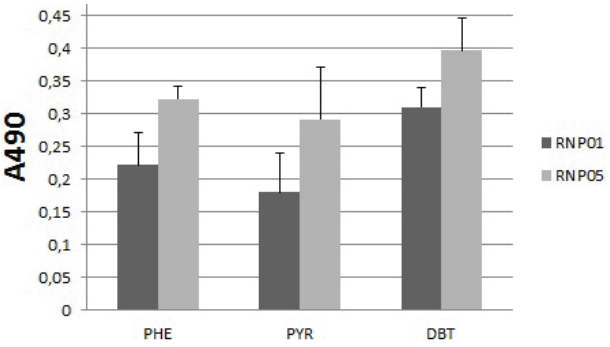

Figure 2. Dehydrogenase activities (A_{490}) of bacteria after growth on PAHs.

The evaluation of bioremediation potential of contaminated soil by different bacteria is a fundamental task when deciding correct bioremediation strategies. In order to accomplish this, colorimetric screening methods have many advantages, because they are simple, fast, robust, inexpensive and convenient methods, making them applicable in most laboratories. These methods provide a picture of the metabolic activity of a microorganism growing on the hydrocarbon substrate examined, and take into account the nonculturable bacteria when consortiums from soil extracts are used.

In order to evaluate and compare three screening methods, the bioremediation assessment of two hydrocarbon-degrading bacteria were analyzed using

the hydrocarbon growth assay, 2,6-DCPIP assay, and dehydrogenase activity assay. The hydrocarbon growth assay successfully determined difference in biodegradation potential between two bacterial strains. Furthermore, the results of oxidation activity measured by the 2,6-DCPIP completely corresponded with results of hydrocarbon growth assay, indicating that the 2,6-DCPIP assay can be used to evaluate microbial PAH-degradation abilities in an accurate, sensitive and simple manner. However, even though these results indicate that these assays could be used separately, our recommendation is to use both assays, because the 2,6-DCPIP assay corresponds to hydrocarbon concentration decreases while the increasing turbidity of the suspension in the hydrocarbon growth assay could be a consequence of the increasing bacterial numbers, but also the size of the cells, which could obscure the findings. The 2,6-DCPIP assay has been employed for evaluation of hydrocarbon-degradation abilities in several studies [18-20]. Kubota *et al.* showed that biodegradation profiles analyzed by 2,6-DCPIP assay completely corresponded to GC analysis of hydrocarbon-degradation [14]. Furthermore, Mariano *et al.* [20] valuated the capability of different microorganisms to degrade butanol/gasoline and ethanol/gasoline blends using 2,6-DCPIP assay and respirometry assay, and verified the order of biodegradability as ethanol > butanol > gasoline by both experiments. Bidoia *et al.* qualitatively examined a reducing 2,6-DCPIP color as a result of biodegradation and concluded that 2,6--DCPIP concentrations below 0.03 g L^{-1} could not be measured by this method [21]. In our previous examinations, 2,6-DCPIP assay has been successfully used to evaluate degradation of PAH by microorganisms in 30 days [13], yet the results obtained after 15 and 30-day experiments suggested that the time period of the assay can be set to 15 days.

The third assay, the dehydrogenase activity assay, was used to check if the examined microorganisms just tolerate PAHs or metabolically use them as sole carbon sources. In this assay, the reduction of compounds by dehydrogenase gives triphenylformazan (TPF) which has a characteristic reddish color, and the intensity of color is measured at 485 nm, which is a good indicator of microbial activities [16]. The measurement of the dehydrogenase activity by microorganisms from soil contaminated by petroleum hydrocarbons has the potential to assess an effectiveness of various bioremediation procedures [17]. DHA results revealed that both bacteria were metabolically active on tested PAH substrates and confirmed the results from the previous two assays.

Gas chromatographic analysis of bioremediation efficiency of strains *Planomicrobium* sp. strain RNP01 and *Rhodococcus* sp. strain RNP05

In order to confirm the results of screening assays, *Planomicrobium* sp. RNP01 and *Rhodococcus* sp. RNP05 have been growing in microcosms on PHEN, PYR and DBT as individual substrates. After 30 days of microcosm assay, GC-FID analysis of PAH extracts was used to check the degradation of substrates.

According to GC analysis, both tested microorganisms utilized almost all of the DBT substrate after 30 days of growth. GC analysis revealed lower utilization of PHE and PYR by both microorganisms, where *Rhodoccocus* sp. RNP05 showed higher biodegradation potential for PHE and DBT, compared to *Planomicrobioun* sp. RNP01 (Figures 3 and 4).

To assure the accuracy and sensitivity of screening assays, the results of bioremediation assessment of hydrocarbon-degradation pattern of two strains, *Planomicrobium* sp. strain RNP01 and *Rhodococcus* sp. strain RNP05, was confirmed by the GC-FID analysis. The results from the gas chromatograph analysis confirmed the order of biodegradability which has been indicated by screening assays: DBT> PHE>PYR; this shows that the three screening assays used as microtiter plate method can be used for quick and reliable selection of microorganisms that have a high potential for soil bioremediation procedures.

To show that a bioremediation procedure is potentially useful, it is important to demonstrate the bioavailability of substrate as well as the ability of selected microorganisms to enhance the rate of hydrocarbon degradation in controlled conditions [12]. Measuring the success of bioremediation of petroleum-contaminated soil is based on several parameters, among which is the degradation of polycyclic aromatic hydrocarbons.

The potential of microorganisms to metabolize hydrophobic substrates under the defined conditions is used as a screening parameter for choosing the strains with multiplicity of catabolic pathways for hydrocarbon compounds because bioremediation procedures are strongly dependent on process duration and biological efficiency of bacterial communities [11]. Thus, the isolation, characterization and profile of specific bacteria for hydrocarbon degradation are important when deciding the correct bioremediation strategy.

Turbidity and colorimetric measurements are low-cost and rapid procedures to detect the occurrence of microbial metabolism, in both aerobic and

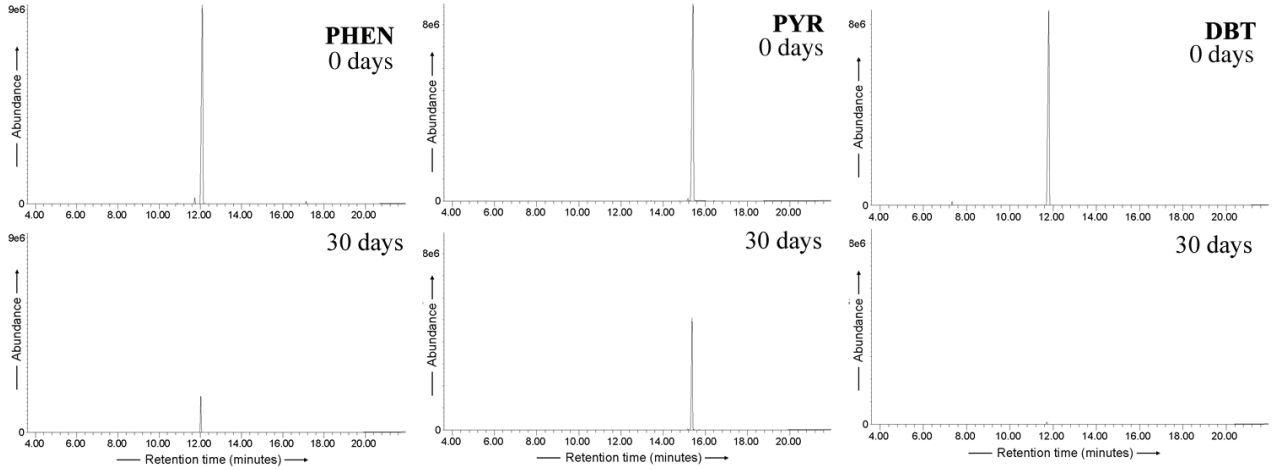

Figure 3. GC-FID chromatograms of PAH extracts before (0 day) and after (30 days) biodegradation by Planomicrobium sp. strain RNP01.

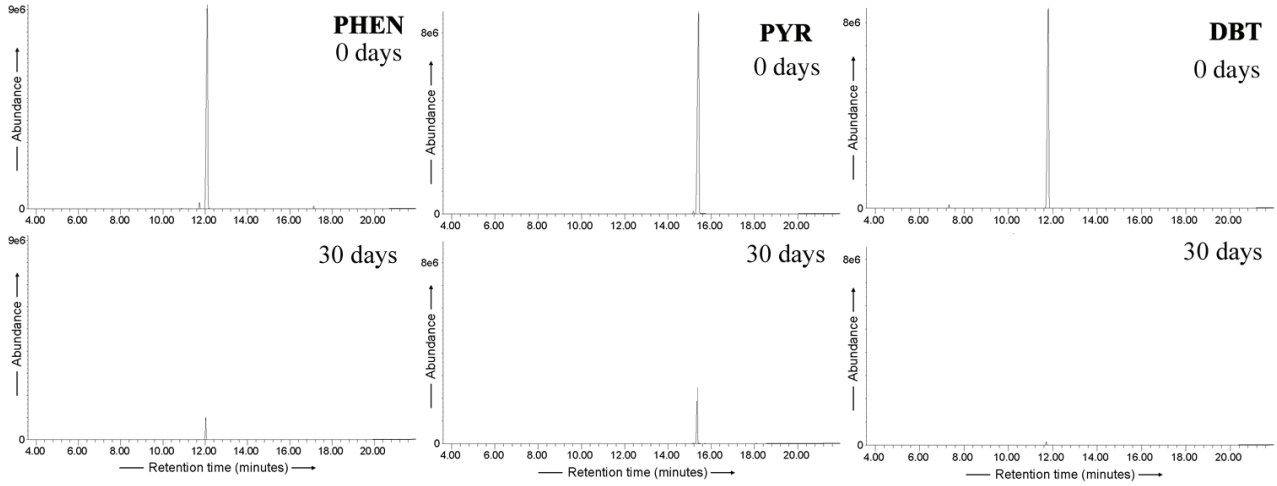

Figure 4. GC-FID chromatograms of PAH extracts before (0 day) and after (30 days) biodegradation by Rhodococcus sp. strain RNP05.

anaerobic conditions. The main advantages of these methods are rapid screening of large numbers of bacterial isolates, simplicity and the fact that they can be used with hydrocarbon-degrading nonculturable bacteria. The use of these measurements in the form of microtiter plates contributes to faster screening of a large number of microorganisms, and better selection of the most potent strains.

CONCLUSIONS

In this work, a set of three simple screening methods have been used to evaluate the bioremediation potential for PAH of bacterial isolates obtained from a soil contaminated with petroleum. In all three assays, *Rhodococcus* sp. strain RNP05 showed better potential than *Planomicrobium* sp. strain RNP01 for utilizing all the examined hydrocarbon substrates (phenanthrene, pyrene and DBT), which was confirmed by GC-FID analysis. Even though these results indicate that the assays could be used separately, our recommendation is to combine all three assays for determination and comparison of bacterial bioremediation potential for PAHs, because they correspond to different parameters of bacterial metabolic activity.

To the best of our knowledge, this is the first report demonstrating that a combination of these simple screening methods is effective for determining the bioremediation potential for PAHs. Using all three methods to assess bioremediation potential for PAHs appears to be suitable for practical work and assures that the best microbial candidates for soil bioremediation are chosen.

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NAUČNI RAD

PROCENA SKRINING TESTOVA ZA ODREĐIVANJE BAKTERIJSKOG POTENCIJALA ZA DEGRADACIJU POLICIKLIČNIH AROMATIČNIH UGLJOVODONIKA

Degradacija policikličnih aromatičnih ugljovodonika (polycyclic aromatic hydrocarbons - PAH) ispitivana je testovima u mikrotitar pločama pomoću dva bakterijska soja Planomicrobium sp. RNP01 i Rhodococcus sp. RNP05, u periodu od 30 dana. Rezultati ispitivanja su ukazali da se kombinacijom određenih bioloških i biohemijskih karakteristika mogu kreirati dobri indikatori u proceni bakterijskog degradacionog potencijala. Tri testa, 2,6-DCPIP test, test dehidrogenazne aktivnosti i test rasta na ugljovodonicima pokazali su da Rhodococcus sp. RNP05 ima veći potencijal za degradaciju PAH jednjenja u odnosu na Planomicrobium sp. RNP01. Razlike u početnoj i krajnjoj optičkoj gustini i specifične konstante rasta bile su značajno više ($r = 0,995$, $P < 0,05$) u testu sa Rhodococcus sp. RNP05 na svim testiranim supstratima, u poređenju sa Planomicrobium sp. RNP01, a dobijeni rezultati su potvrđeni gasno-hromatogafskom-FID analizom. Dehidrogenazna aktivnost soja Rhodococcus sp. RNP05 bila je viša u odnosu na Planomicrobium sp. RNP01 ($r = 0,9995$, $P < 0,05$) i u pozitivnoj korelaciji sa testom rasta na ugljovodonicima ($r = 0,999$, $P < 0,05$, za Rhodococcus sp. RNP05, $r = 0,986$, $P < 0,05$ za Planomicrobium sp. RNP01). Rezultati prikazanog istraživanja ukazuju na to da se kombinacija ovih testova može koristiti za određivanje bioremedijacionog potencijala za razgradnju PAH jedinjenja u zemljištu zagađenim naftom, pri čemu se dobija i mogućnost testiranja velikog broja bakterijskih sojeva.

Ključne reči: rast na ugljovodonicima, PAH, skrining testovi, dehidrogenazna aktivnost, Planomicrobium, Rhodococcus.