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# Biotechnological Reclamation of Oil-Polluted Soils

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**ABSTRACT**
The aim of the paper was to determine the efficiency of petroleum hydrocarbons (PHs) degradation by developed bacterial consortium during bioremediation of oil-contaminated soils caused by accidental oil spills. The soil samples were collected from three different areas near the Bugruvate field of the Dnieper-Donets oil and gas region, Sumy region, Ukraine. The total petroleum hydrocarbon was determined by conducting measurements using a gravimetric method. Gas chromatographic analysis was performed for determination of polycyclic aromatic hydrocarbons. The level of oil contamination follows an increasing preferential order: Sample 1 < Sample 2 < Sample 3 (5, 10 and 15 g·kg$^{-1}$, respectively). The soil samples comprised different concentrations of PHs including n-alkanes, fluorine, anthracene, phenanthrene, pyrene, toluene, xylene, benzene and other PHs. The results of research indicated that the maximum oil degradation rate at the level of 80% was set at $C_{in}$ within 4–8 g·kg$^{-1}$ and τ = 70 days, under natural condition. In order to improve the efficiency of bioremediation of oil-contaminated soils, bioaugmentation was performed using the developed preparation of such bacteria and fungi strains as *Pseudoxanthomonas spadix, Pseudomonas aeruginosa, Rhodococcus opacus, Acinetobacter baumannii, Bacillus cereus, Actinomyces sp., Mycobacterium flavescens*. The results showed 100% of oil concentration was assimilated after 20, 25 and 35 days for the soil samples with initial hydrocarbon concentrations at the level 5, 10 and 15 g·kg$^{-1}$, respectively. The bacterial consortium application (bioaugmentation) exhibited high efficiency compared to the indigenous microflora in the oil biodegradation. The optimal growth condition for the bacteria in this study can be set as follows: pH = 3–11, wide temperature range 0–35°C.

**Keywords:** bioremediation, oil biodegradation, oil-destructive microorganisms, oil spills, soil pollution.

## INTRODUCTION

Soil pollution by oil and oil products, i.e. different petroleum hydrocarbons (PHs), is a global environmental problem, in particular for the oil producing countries. Nevertheless, numerous oil spills increasing every year affect the natural resources all over the world; in recent years, thousands hectares of soil were contaminated as a result of hundreds oil spills, majority of which were caused by uncontrolled illegal connections.

In order to eliminate accidental oil spills after mechanical and physicochemical stages, a biotechnological approach is used, which has such strengths as the involvement of indigenous microflora in the process of hydrocarbon degradation and a high degree of destruction of oil products into safe substances. The efficiency of bioremediation is mainly determined by such abiotic factors as temperature, nutrients, chemical composition of petroleum hydrocarbons, solubility, bioavailability, physical and chemical properties of the soil, oxygen, soil moisture, acidity and alkalinity.

The oil contamination of soils violates the physical and chemical properties, has a harmful effect on living beings, and destroys the natural balance of ecosystems [Alhassan and Fagge, 2013; Nasehi et al., 2016]. Biochemical technologies based on biodynamic and biotechnological schemes are the most useful tools to solve the problem of oil decontamination [Bachmann, et al., 2014]. All of these processes take place under natural conditions and involve many trophic chains at different levels. In the case of the optimal range of abiotic environmental factors, PHs are degraded by an indigenous microflora consisting of oil-destructive bacteria strains, lower fungi [Nozari, et al., 2018] and microalgae [Younes, et al., 2011].

The processes of bioremediation are enhanced due to the application of biosurfactants by means of emulsification (improved by high molar mass), solubilization and mobilization (promoted low-molar mass) [Usman, et al., 2016]. A number of bacteria and yeast yielded vast amount of phospholipids and fatty acids surfactants when growing on n-alkanes through microbial oxidations [Vijayakumar and Saravanan, 2015]. When oil enters the soil, uneven dynamics of enzymatic activity is noted: an increase in the number of specific enzymes (catalase, peroxidase, polyphenol oxidase) and carbon dioxide emission on the 3rd day, provided that the oil dose is not more than 5%, the initial inhibition of enzymes at an oil concentration exceeding 5% [Suleymanov and Shorina, 2012].

The phosphatase activity levels could contribute to the understanding of P-cycling during aerobic degradation processes, which could allow more efficient use of P fertilizer in agricultural systems [Dindar, et al., 2015]. The measured enzyme activities appeared to be generally lower in crude oil contaminated soils. These lower levels of enzyme activities can be explained by the low viscosity of crude oil resulting in a more widespread contact of soil and the pollutant. In the case of waste engine oil pollution, the pollutant has caused the formation of oily pellets in soil.

The results of numerical investigations confirmed the efficiency of oil destruction by more than ten main bacteria genera including *Pseudomonas* sp. [Panda et al., 2013], *Rhodococcus* sp., *Bacillus* sp. and others. The efficiency of oil destruction by fungal strains such as *Acremonium sp.*, *Alternaria sp.*, *Aspergillus terreus* and *Penicillium sp.* was proven at the level of approximately 10 % [Mohsenzadeh, et al., 2012], while *Aspergillus niger* is capable of decreasing the oil content in the soil by 30% [Büyükgüngör and Kurnaz, 2016]. The results of research [You, et al., 2018] showed the difference between the degradation ability of the *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* strains, as *Pseudomonas aeruginosa* had a higher diesel degradation rate (58% on 14th day), diesel utilization capacity (86%) and faster growth in diesel medium, compared to *Klebsiella pneumoniae*.

The presence of the aromatic ring hydroxylating dioxygenase genes made it possible for the hydrocarbon-degrading α-and γ-*Proteobacteria* to produce the biosurfactant [Todorova, et al., 2014]. Moreover, a plant-growth-promoting endophytic *Pseudomonas aeruginosa* bacterium L10 has been reported [Wu, et al., 2018] to be an efficient degrader of C10–C26 *n*-alkanes from diesel oil, as well as common polycyclic aromatic hydrocarbons (PAHs) such as naphthalene, phenanthrene, and pyrene.

*Rhodococcus erythropolis*, *Acinetobacter baumanii*, *Burkholderia cepacia* and *Achromobacter xylosoxidans* had a capacity to produce the n-alkane hydroxylase gene necessary for the n-alkane degradation process [Tanase, et al., 2013]. The bacterial consortium of *Pseudomonas putida*, *Rhodococcus erythroplolis* and *Bacillus thermoleovorans* grown on hexadecane has shown the higher biodegradative capability, comparing to the biodegradation of each strain separately. In the case of a mixed culture, 100% of hexadecane was destructed after 8 days. Nevertheless, for individual strains of *Pseudomonas putida*, *Rhodococcus erythroplolis* and *Bacillus thermoleovorans* it took 11-12 days [Abdel-Megeed, et al., 2010]. Microbial consortiums isolated from soil, including *Acinetobacter radioresistence, Bacillus subtilis* and *Pseudomonas aeruginosa* strains were used in bioremediation and provided degradation rate for n-hexadecane and n-dodecane at the level of 17.61% and 28.55%, respectively [Nozari, et al., 2018].

Crude oil, engine oil, kerosene, diesel, cyclohexane, dodecanol, n-dodecane, toluene, phenol, benzene, hexane, naphthalene, anthracene, phenanthrene, fluoranthrene, biphenyl, dibenzothiophene, and 2-chlorobenzoates were tested as the carbon source substrates for gram-negative *Pseudomonas alcaligenes, Pseudomonas luteola*, *Pseudomonas aeruginosa* and gram-positive *Actinomyces sp.* The results have shown decreasing of oil degradation rate in the mentioned above priority of strains from 99.4% to 92.3%, respectively [Agwu, et al., 2013]. Nevertheless, all of these strains have a potential to grow on crude oil, diesel, kerosene, engine oil and cyclohexane (Table 1). The highest crude oil degradation rate at the level of 81.70% was noted by a mixed culture of such bacterial strains as: *Bacillus brevis*, *Pseudomonas aeruginosa*, *Bacillus licheniformis*, and *Bacillus sphaericus*, while this parameter in the case of using individual strains ranges from 75.42% to 63.34%, respectively, for this series [El-Borai, et al., 2016].

**Table 1.** Substrate utilization spectrum of the organisms

| Substrate | Bacterial isolates | Reference |
|---|---|---|
| Crude oil | *Actinomyces sp., Pseudomonas luteola, Pseudomonas alcoligenes, Pseudomonas aeruginosa, Bacillus spp.* | Agwu, et al., 2013<br>Raju, et al., 2017 |
| Diesel | *Actinomyces sp., Pseudomonas luteola, Pseudomonas alcoligenes, Pseudomonas aeruginosa, Cellulosimicrobium cellulans* and *Acinetobacter baumannii, Bacillus* spp. | Agwu, et al., 2013<br>Niazy, et al., 2016<br>Nkem, et al., 2016<br>Raju, et al., 2017 |
| Kerosene | *Actinomyces sp., Pseudomonas luteola, Pseudomonas alcoligenes, Pseudomonas aeruginosa, Enterobacter cloacae, Enterobacter hormaechei, Pseudomonas stutzeri* | Agwu, et al., 2013<br>Mojarad, et al., 2016 |
| Engine oil | *Actinomyces sp., Pseudomonas luteola, Pseudomonas alcoligenes, Pseudomonas aeruginosa* | Agwu, et al., 2013 |
| Cyclohexane | *Actinomyces sp., Pseudomonas luteola, Pseudomonas alcoligenes, Pseudomonas aeruginosa* | Agwu, et al., 2013 |
| Phenol | *Stenotrophomonas, Sphingobium, Pseudomonas, Stenotrophomonas maltophilia* | Wang, et al., 2015<br>Basak, et al., 2014 |
| Toluene | *Bacillus cereus* | Heydarnezhad, et al., 2018 |
| Naphthalene | *Pseudomonas sp., Rhodococcus opacus* | Niepceron, et al., 2013<br>Pathak et al., 2016 |
| Pyrene | *Caulobacter sp., Bacillus fungorum, Mycobacterium flavescens, Polyporus sp.* | Al-Thukair and Malik, 2016<br>Dean-Ross, et al., 2002<br>Hadibarata, et al., 2012 |
| Anthracene | *Rhodococcus sp.* | Dean-Ross, et al., 2002 |
| Phenanthrene | *Actinomyces sp., Pseudomonas luteola, Pseudomonas sp., Sphingobacterium sp., Bacillus cereus, Achromobacter insolitus* | Agwu, et al., 2013<br>Niepceron, et al., 2013<br>Janbandhu and Fulekar, 2011 |
| Fluoranthrene | *Actinomyces sp., Pseudomonas luteola, Mycobacterium flavescens, Rhodococcus sp.* | Agwu, et al., 2013<br>Dean-Ross, et al., 2002 |

The *Enterobacter cloacae*, *Enterobacter hormaechei*, and *Pseudomonas stutzeri* Bacteria strains have been proven as efficient degrader of kerosene due to the presence of a carbon and sulfur source. In particular, the degradation level of 67.43%, 48.48%, and 65.48% of 5% kerosene in seven days, respectively, was reported. Moreover, *Pseudomonas stutzeri* and *Enterobacter hormaechei* could use kerosene as sulfur source and provide the degradation rate equal to 54.14% and 12.98% of 10% kerosene, respectively, at the same time [Mojarad, et al., 2016].

*Stenotrophomonas maltophilia* could totally (100%) devour 500 mg/L initial phenol concentration with 0.0937 qmax and 16.34 mg/L/h substrate consumption rate within a very short time span of 48 h [Basak, et al., 2014]. In the study [Wang, et al., 2015], two nonylphenol-degrading bacteria, designated as the *Stenotrophomonas* strain within the Gammaproteobacteria class and the *Sphingobium* strain within the Alphaproteobacteria class were isolated from soil and river sediment, respectively, and had a high efficiency in nonylphenol degradation. *Polyporus sp.* S133 produces the laccase and 1,2-dioxygenase enzymes that are necessary for pyrene metabolism [Hadibarata, et al., 2012].

Naphthalene was noted to be a potential carbon source for *Proteobacteria*, in particular more than 60% of the bacterial population of the biofilm community was presented by *Betaproteobacteria*. In addition, the presence of *Bacteroidetes* and *Chloroflexi* was observed, which is associated with high carbon source availability. In general, the following bacterial strains that grow on naphthalene have been isolated: *Variovorax paradoxus, Starkeya novella, Xanthobacter polyaromaticivorans*, *Pseudoxanthomonas spadix, Rhizobium naphthalenivorans, Pseudomonas veronii* and *Microbacterium paraoxydans;* among them, the first two strains were dominant [Martirani-Von Abercron, et al., 2017].

Degradation of pyrene by *Caulobacter sp* and *Bacillus fungorum* was established at the rate of 35-59%, respectively, under different environmental conditions such as temperature and pH. For instance, the growth of *Caulobacter sp* does not depend on temperature while the temperature range 25-37ºC was the most optimal for *Bacillus fungorum*. In the case of pH, acidic media was more optimal then alkaline for *Caulobacter sp*., but *Bacillus fungorum* was tolerant to wide pH ranges [Al-Thukair and Malik, 2016]which were previously isolated from oil-contaminated sites and identified via 16S RNA sequences, were tested for their hydrocarbon degrading efficiency. Media spiked with 100 ppm pyrene were incubated at 25 °C and 37 °C. The bacterial isolates' pyrene-degrading capability was assessed in acidic (pH 5.0. The consortium of *Sphingobacterium sp., Bacillus cereus, Achromobacter insolitus* was reported to be capable of phenanthrene utilization and variety of other hydrocarbons for growth [Janbandhu and Fulekar, 2011].

Thus, the use of bioaugmentation, i.e. introduction of bacterial preparations on the basis of the consortium, has a positive effect on the hydrocarbons biodegradation. However, there is no one-size-fits-all consortium, which justifies the purpose and objectives of this study. The present research was focused on the biotechnological approach, aiming to determine the efficiency of petroleum hydrocarbons degradation by developed bacterial consortium during bioremediation of oil-contaminated soils caused by accidentally oil spills. There are the following tasks:

1) To assess the degree of degradation of hydrocarbons depending on their initial content in the soil (contaminated substrate), the time of destruction for given initial data (air temperature, type and physicochemical properties of the soil).
2) To justify the potential of bioaugmentation, i.e. the use of bacterial preparations, in accelerating the process of oil decomposition in comparison with natural conditions.
3) To assess the efficiency of the proposed bacterial consortium in the speed and rate of petroleum hydrocarbons destruction.

## MATERIALS AND METHODOLOGY

### Soil analysis

The soil samples were collected from three different areas near Bugruvate field of Dnieper-Donets oil and gas region (50°11′55″N, 34°58′06″E), Sumy region, Ukraine. The petroleum hydrocarbon contaminations in all cases resulted from oil spills during accident situations. The samples were collected during August. The top 20 cm of soil was collected using a sterile spatula into sterile plastic bags for further transportation and microbiological analysis. The samples were stored at 4°C until further processing. The experimental study was conducted at a temperature of 21°C. The type of soil samples was chernozem typical leached deep low-humus large-cacked-light-argillaceous. The physical and chemical parameters of the soil are shown in Table 2.

### Analysis of petroleum hydrocarbons

*Gravimetric analysis of TPH.* The total petroleum hydrocarbon (TPH) was determined by conducting measurements using a gravimetric method, according to RD 52.18.647-2003. For sample preparation and subsequent analysis, a sample weighing 10 g of averaged sample was used. A portion of the soil from the TPH was placed in a conical flat-bottomed flask, chloroform was poured to extract the TPH from the soil, the flask was vigorously shaken and filtered through a blue ribbon filter into a labeled glass at number one, pre-weighed. This procedure was repeated several times until the filtrate was completely discolored. Chloroform was evaporated and the beaker was weighed again. Afterwards, hexane was poured into the flask with the soil in comparison with chloroform, and a similar action was performed to extract the non-polar TPH fractions from the soil. Hexane was evaporated from a glass and weighed again.

The mass fraction of TPH in the sample $X$, $g \cdot kg^{-1}$, was calculated by the formula:

$$X = \frac{M_2 - M_1}{P} \cdot 10^3 \quad (1)$$

**Table 2.** Physical and chemical properties of the soil samples

| Parameters | Units | Uncontaminated soil (control) | Sample 1 | Sample 2 | Sample 3 |
|---|---|---|---|---|---|
| Initial oil content | % | 0 | 5 | 10 | 15 |
| pH | – | 6.6 | 6.5 | 6.7 | 6.9 |
| Moisture content | % | 26.8 | 32.7 | 33.4 | 38.2 |
| Inorganic phosphate content | $mg \cdot kg^{-1}$ | 189 | 111 | 119 | 99 |
| Nitrate content | $mg \cdot kg^{-1}$ | 117 | 73 | 68 | 50 |
| Available potassium | $mg \cdot kg^{-1}$ | 172 | 119 | 116 | 98 |
| Organic carbon | % | 1.00 | 0.55 | 0.47 | 0.32 |

where: $M_2$ is the mass of the second glass with the residue after removal of hexane, g;
$M_1$ is the initial mass of the second glass, g;
$P$ is weight, g.

The arithmetic mean $\bar{X}$ was calculated from the results of parallel determinations of the TPH mass fraction in weights of a single soil sample. The measurement result of $C_X$, g·kg$^{-1}$, are in the formula:

$$C_x = \bar{X} \pm \Delta \quad (2)$$

where: $\bar{X}$ is the arithmetic average mass fraction of TPH in the soil sample, calculated by the formula (1), g·kg$^{-1}$;
$\Delta$ is characteristic of measurement error at $P = 0.95$, g·kg$^{-1}$.

### Gas chromatographic analysis

GC/FID analysis of the TPHs and PHs was performed on a Shimadzu GC-2010 gas chromatograph supplied with a PAL 5000 Autosampler and FID detector coupled with a fused silica capillary column (30×0.32 mm DB-5 (95 metil-5%-fenilpolisiloxane)). The oven temperature was programmed from 40°C (3 min.) to 320°C at rate 15 °C/min. The samples were injected in splitless mode. The injector and detector temperatures were 250°C and 350°C, respectively. Nitrogen was used as the carrier gas at a linear velocity of 38 cm·s$^{-1}$.

### Determination of the oil degradation rate

The first-order kinetics model used is expressed by the following:

$$C_\tau = C_i e^{-k\tau} \quad (3)$$

where: $C_\tau$ is the oil concentration in soil at instant $\tau$, g·kg$^{-1}$;
$C_i$ is the initial concentration of soil, g·kg$^{-1}$;
$k$ is the rate constants of the first order, day$^{-1}$;
$\tau$ is the time, days.

The model estimated the oil degradation rate (*DR*) in soil relative to the treatments applied:

$$DR = \frac{C_i - C_\tau}{C_i} \cdot 100\% \quad (4)$$

where: *DR* is the oil degradation rate, %.

### Data and statistical analyses

The statistical significance of the TPH data from the biodegradation experiments was evaluated by Analysis of Variance (ANOVA). The data were considered to be significantly different if P≤0.05. Systematic error shifts equally all indicators values are monitored during the experiment. This error was determined by measuring the class accuracy of measurement. Random errors served as a confidence interval, the length of which is determined by the confidence level. The center of the confidence interval for the measured value of *Ci* was posed as mean statistical *C*, calculated in the result of a series of measurements of *Ci*. The limits of the confidence interval expressed product of standard deviation and coefficient dimensionless Student, *t* [Ablieieva and Plyatsuk, 2016]. The Statistica, version 13.0.0.0 data analysis software system (TIBCO Software Inc., 2017) was used for all statistical analyses and assay evaluation. Each encoded sample was considered as independent and duplicates were performed.

## RESULTS AND DISCUSSION

### Investigation of the petroleum hydrocarbons degradation under natural conditions

The analysis of soil was carried out using the gravimetric method and gas chromatography. The obtained results indicate the same quality but different quantity content of petroleum hydrocarbons in the three samples of oil-polluted soils (Table 3). The level of oil contamination follows an increasing preferential order: Sample 1 < Sample 2 < Sample 3 (5, 10 and 15 g·kg$^{-1}$, respectively).

Despite the greater content of n-alkanes in all investigated samples, this group of PHs has higher capacity for biodegradation. Nevertheless, the group of polycyclic aromatic hydrocarbons including fluorene, anthracene, phenanthrene and pyrene is more difficult to destruct due to their complex chemical structure, high toxicity and low bioavailability level for mostly microorganisms. Aromatic compounds, i.e. benzene, toluene and xylene (known as BTX) have been determined in the half mass of the total PHs which requires specific microbiota in the bacteria consortium.

Indigenous microflora under natural conditions using different hydrocarbons as sole carbon sources, provide the growth capacity and oil biodegradation. The kinetics of this process must be dependent on the time and tolerance to different concentrations of PHs in oil-contaminated soils.

**Table 3.** Results of soil sample analysis on the PHs content

| Substance | Value of content (±standard deviation), g · kg$^{-1}$ | | |
|---|---|---|---|
| | Sample 1 | Sample 2 | Sample 3 |
| n-alkanes | 1.787 ± 0.123 | 3.574 ± 0.246 | 5.361 ± 0.369 |
| Fluorene | 0.134 ± 0.017 | 0.267 ± 0.034 | 0.400 ± 0.051 |
| Anthracene | 0.126 ± 0.016 | 0.252 ± 0.032 | 0.378 ± 0.048 |
| Phenanthrene | 0.119 ± 0.009 | 0.238 ± 0.018 | 0.357 ± 0.027 |
| Pyrene | 0.123 ± 0.015 | 0.246 ± 0.030 | 0.370 ± 0.045 |
| Toluene | 0.543 ± 0.078 | 1.087 ± 0.156 | 1.630 ± 0.234 |
| Xylene | 0.721 ± 0.098 | 1.442 ± 0.196 | 2.163 ± 0.294 |
| Benzene | 1.015 ± 0.113 | 2.029 ± 0.226 | 3.044 ± 0.339 |
| Other PHs | 0.434 ± 0.059 | 0.868 ± 0.118 | 1.302 ± 0.177 |
| **Total** | **5.002** | **10.003** | **15.005** |

Figure 1 shows the results of multivariate analysis, reflecting the dependence of the oil degradation rate from exposure time τ and initial concentration of oil $C_i$.

The influence of these factors on oil degradation rate can be approximated by the regression equation:

$$Y = -11.8961 + 11.8395 \cdot X_1 + 0.9027 \cdot X_2 + \\ + 0.7914 \cdot X_1^2 + 0.0187 \cdot X_1 \cdot X_2 - 0.0079 \cdot X_2^2 \quad (5)$$

where: $Y$ is oil degradation rate $DR$, %;
$X_1$ is exposure time $\tau$, days;
$X_2$ is initial concentration of oil $C_i$, g · kg$^{-1}$.

The maximum oil degradation rate at the level of 80% is set at $C_i$ within 4–8 g·kg$^{-1}$ and $\tau$ = 70 days. However, this indicator does not reach 100%, which is most likely due to the presence of hard-to-decompose polycyclic aromatic hydrocarbons. The results of the study necessitated a more in-depth study of the biodegradation mechanisms of PAH in order to correctly determine the composition of the bacterial consortium.

## Substantiation of the bioaugmentation effectiveness in the case of oil spill response

Various strains of microorganisms have the ability to oxidize petroleum hydrocarbons, which leads to their destruction, and therefore to a decrease in the concentration of oil pollution in the soil. Such properties of bacteria, archaea and some lower fungi are explained by the presence of the corresponding enzymatic systems.

The mechanism of bacterial transformation of aliphatic hydrocarbons with the linear structure is the most clearly presented and thoroughly studied [Brzeszcz and Kaszycki, 2018]. The general view of the process of oxidative destruction of alkanes can be submitted in the form of such a scheme of successive transformations (Fig. 2).

The biochemical conversion of aliphatic hydrocarbons proceeds according to the following

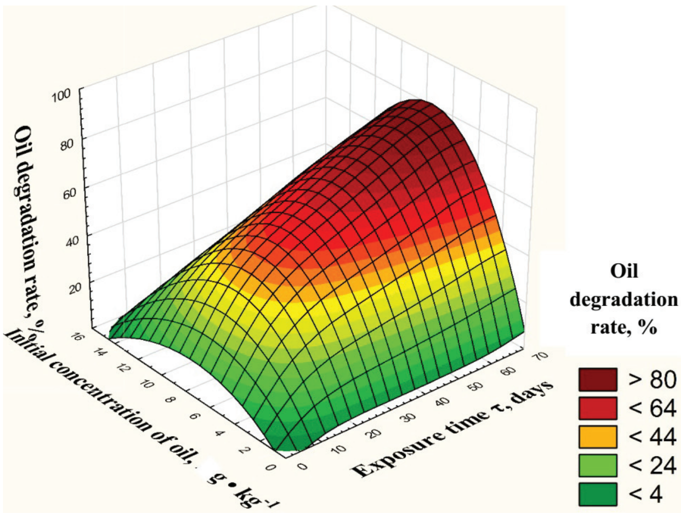


**Figure 1.** Dependence of oil degradation rate *DR* from exposure time τ and initial concentration of oil $C_i$.

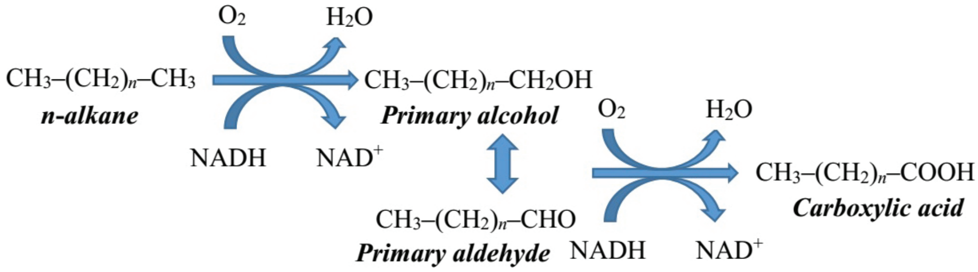


**Figure 2.** Enzymatic reactions involved in the processes of aliphatic hydrocarbons degradation

mechanism: alkanes → alcohols → aldehydes → carboxylic acids. In the case of alkenes and alkynes, the process differs due to the presence and different arrangement of double and triple bonds. Carboxylic acids are used by bacteria as a source of carboxylate groups ($RCOO^-$), participating in the initial stages of the Krebs cycle or tricarboxylic acids (TCA) cycle.

Aliphatic hydrocarbons are most easily amenable to biooxidation [Wu, et al., 2017]. Cyclic and aromatic hydrocarbons such as phenanthrene, anthracene and others, on the contrary, are very heavily involved in the biodegradation processes due to the strength of the benzene ring, but strains of microorganisms that include these substances in metabolic processes are known for today [Spini, et al., 2018]. The enzymatic reactions involved in the processes of hydrocarbons degradation are shown in Figure 3. They are updated and modified from [Das and Chandran, 2011] by adding a naphthalene degradation and catechol transformation into pyruvate and acetyl-CoA that are successfully involved in the TCA cycle.

The polycyclic aromatic compounds identified in the contaminated soil samples have different reaction modules of biochemical catabolism. In general, according to the reaction modules (Table 4) final substances of one module can be an initial substance for other (highlighted with the same fill color). It should be emphasized that all these transformations involve different enzymatic systems and, consequently, different strains of microorganisms, which justifies the effectiveness of consortium.

Most of these metabolic pathways after activation (primary oxidation reactions using ring-hydroxylating oxygenase and dihydrodiol dehydrogenase enzymatic systems) and dearomatization reactions based on meta- ($O_2$ oxidation) or ortho-ring cleavage (ring-cleavage dioxygenase) are reduced to the formation of pyruvate-CoA, acetyl-CoA or succinyl-CoA during lower pathways (Fig. 4).

The last CoAs involved in bacteria TCA cycle are used in ring cleavage and energy production.

## Discussion of the oil degradation using bacterial consortium

On the basis of the previous investigations [Ablieieva, 2020] and data obtained by other researchers, bacterial consortium has a higher potential to oil degradation and soil bioremediation due to the diversity of metabolic pathways and

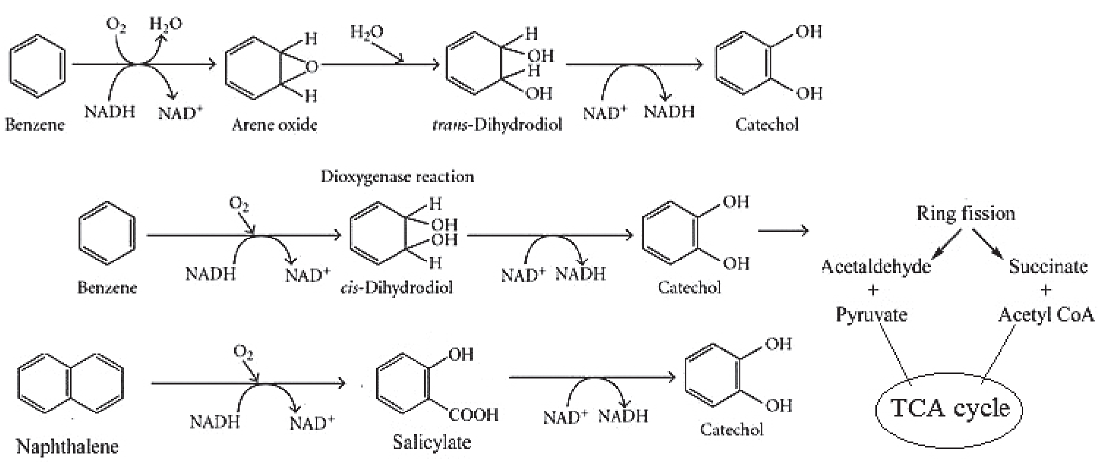


**Figure 3.** Enzymatic reactions involved in the processes of hydrocarbons degradation

**Table 4.** Possible modules for polycyclic aromatic hydrocarbon degradation

| Module | Initial substance | Final substance | Ring cleavage and energy production |
|---|---|---|---|
| Methane oxidation | methane | formaldehyde | |
| Biphenyl degradation | biphenyl | 2-oxopent-4-enoate + benzoate | |
| Xylene degradation | xylene | methylbenzoate | |
| Terephthalate degradation | terephthalate | 3,4-dihydroxybenzoate | |
| Benzoate degradation | benzoate | catechol | |
| Naphthalene degradation | naphthalene | catechol | |
| Catechol degradation | catechol | pyruvate/acetyl-CoA/succinyl-CoA | |
| Trans-cinnamate degradation | trans-cinnamate | acetyl-CoA | |
| Catechol meta-cleavage | acetyl-CoA | propanoyl-CoA | |
| Benzene degradation | benzene | benzoyl-CoA | |
| Toluene degradation | toluene | benzoyl-CoA | |
| Benzoyl-CoA degradation | benzoyl-CoA | 3-hydroxypimeloyl-CoA | |
| Phthalate degradation | phthalate | protocatechuate | |
| Pyrene degradation | pyrene | 1-hydroxy-2-naphthoic acid | |

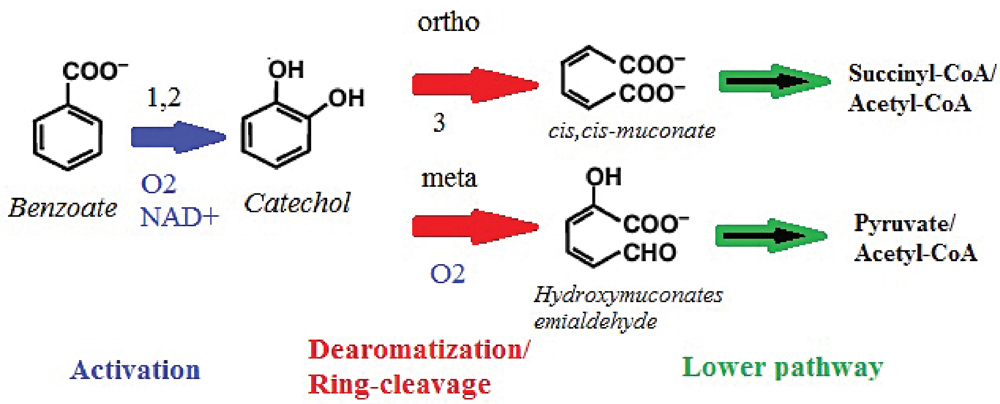


**Figure 4.** Metabolic pathways and enzymatic systems of benzoate degradation by bacteria: 1 – ring-hydroxylating oxygenase; 2 – dihydrodiol dehydrogenase; 3 – ring-cleavage dioxygenase.

involved enzymatic systems. According to the presence of n-alkanes and PAHs in the soil samples (see Table 2), theoretical substantiation of the complex biochemical transformations of hydrocarbons, in which certain enzymes must be involved– capable of producing only certain strains of microorganism's bacterial consortium – has been developed. In order to increase the level of hydrocarbon degradation and, accordingly, to improve the efficiency of bioremediation of oil-contaminated soils, bioaugmentation was performed using the developed preparation, which included 5 strains of such bacteria as *Pseudoxanthomonas spadix, Pseudomonas aeruginosa, Rhodococcus opacus, Acinetobacter baumannii, Bacillus cereus* and 2 strains of lower fungi *Actinomyces sp., Mycobacterium flavescens*.

The research results for three soil samples, which differ in initial hydrocarbon concentrations (5, 10, and 15 $g \cdot kg^{-1}$, respectively), are shown in Figure 5. Numbers 1, 2 and 3 show the curves of changes in the oil concentration in the soil over time for the initial concentrations of 5, 10 and 15 $g \cdot kg^{-1}$, respectively. The numbers 1′, 2′ and 3′ identify the oil degradation rate curves for the same input data.

The obtained experimental results indicate the 100% of oil concentration was assimilated after 20, 25 and 35 days for the soil samples with initial hydrocarbon concentrations at the level 5, 10 and 15 $g \cdot kg^{-1}$, respectively (see Fig. 5, curves 1′, 2′ and 3′). The graph shows the trend lines for the dependence of the level of oil degradation on time, and also provides approximation equations with an indication of the value of the approximation reliability. The margin of error for all curves does not exceed 5% at a given acceptable probability (called significance level α) $\alpha = 0.05 = 5\%$.

The curves of changes in the concentration of oil in the soil with time have the same trend, i.e. all three curves are linear. It was clear that the bacterial consortium application (bioaugmentation) exhibited high efficiency compared to the

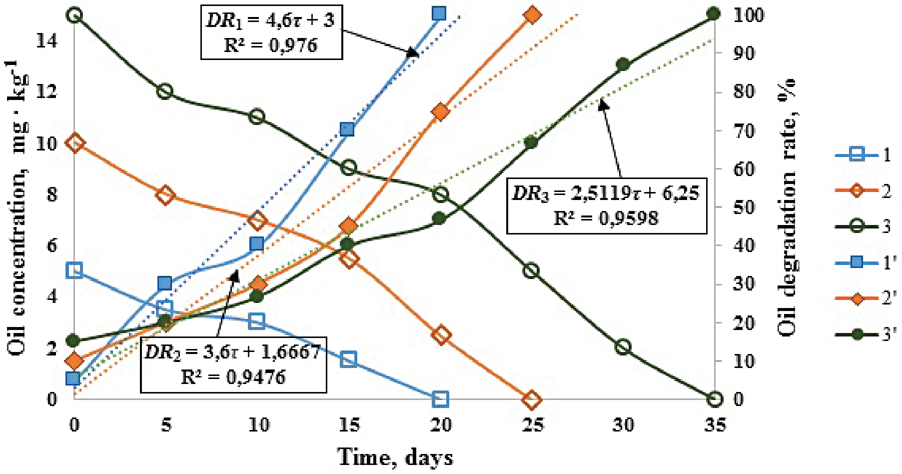


**Figure 5.** Dependence of oil concentration decreasing and oil degradation rate from time for different initial concentration of oil

indigenous microflora in the oil biodegradation (Figures 1 and 5). Such results are due to the effectiveness in soil bioremediation of bacteria and fungi that were included into the introduced bacterial preparation, which is also confirmed by the results of other studies.

Biosurfactant-producing *Pseudomonas aeruginosa* strains are capable of degrading crude oil, even in the presence of salinity [Ebadi, et al., 2017] and 91.5% oil of refinery oily sludge may be recovered by a rhamnolipid producing of F-2 strain [Yan, et al., 2012]. The rhamnolipid biosurfactants produced by *P. aeruginosa* IMP67 strain have been reported to have the best physicochemical properties, as well as antimicrobial and antiadhesive activity [Das, et al., 2014]. The results obtained by Yan P et al. suggest that 91.5% oil of refinery oily sludge during the pilot-scale study was recovery by a rhamnolipid producing strain of *Pseudomonas aeruginosa* F-2 [Yan, et al., 2012]. Besides, the *Pseudomonas* strains have been reported to be able to produce polyhydroxyalkanoate using Gachsaran crude oil (2 % v/v) as carbon source [Goudarztalejerdi, et al., 2015].

*Pseudomonas aeruginosa* produces catalase and oxidase enzymes that play important role in diesel degradation [Niazy, et al., 2016]. *Pseudomonas putida* or *Pseudomonas aeruginosa* into oil-contaminated soil samples resulted in pronounced bioaugmentation [Ramadass, et al., 2018]and their bioavailability remains a poorly quantified regulatory factor. In a microcosm study, we used two strains of Pseudomonas, P. putida TPHK-1 and P. aeruginosa TPHK-4, in strategies of bioremediation, viz., natural attenuation, biostimulation and bioaugmentation, for removal of weathered total petroleum hydrocarbons (TPHs. *Mycobacterium flavescens* and *Rhodococcus sp.* have been reported to be capable for fluoranthene degradation in the presence of pyrene and anthracene respectively, although fluoranthene had a negative influence on the growth speed on the mentioned above substrates [Dean-Ross, et al., 2002]. The optimal medium and cultivation conditions for cell growth and toluene degradation by *Bacillus cereus* ATHH39 were found at pH 6.72, 33.16 ºC, and toluene concentration of 824.15 mg/l, under which toluene degradation was reached 64.11% [Heydarnezhad, et al., 2018].degrading bacterial species were isolated from oil-contaminated environments (located in Bandar-Anzali, Guilan, Iran

Thus, the method of bioremediation is advisable to apply for temperate latitudes characterized by the optimal temperature and humidity regime during the year, with the exception of the winter months. On the basis of the bacterial metadata from electronic bioinformatic databases, the optimal growth condition for the bacteria in this study can be set as follows: pH = 3–11, wide temperature range 0–35°C. The problem in the high oil environment can be partially solved by the artificial maintenance of heat at the optimal level, forced aeration, additional introduction of organic and inorganic fertilizers as a source of basic nutrients, soil reclamation. However, such strategy significantly reduces the economic efficiency of the bioremediation.

Further research will be addressed to the biostimulation application and regulation of the optimal external conditions (temperature, humidity, pH etc.). For instance, the studied efficiency of organic/inorganic fertilizer increases with additional use of biochar and biosurfactant, corresponded to the removal of 23% more Total Petroleum Hydrocarbons (TPH) than fertilizer alone, and this treatment has been reported to be able to degrade up to 53% of the total petroleum hydrocarbon in the soil within 16 weeks [Brown, et al., 2017].

## CONCLUSIONS

The biotechnological method of oil-polluted soil decontamination is becoming more and more popular and useful nowadays due to its advantages and positive features over physical and chemical techniques. A high efficiency of petroleum hydrocarbons degradation by different bacteria strains is explained by the capacity of specific living being to include these substances in their metabolic cell processes. Numerical studies show that arenas, naphthenic, paraffin are available practically for the entire indigenous microflora.

The following chemicals were identified in the oil-contaminated soil samples: n-alkanes, fluorine, anthracene, phenanthrene, pyrene, toluene, xylene, benzene, other PHs. The dependence of oil degradation rate *DR* from exposure time $\tau$ and initial concentration of oil $C_i$ was investigated. The results of research indicated that the maximum oil degradation rate at the level of 80% was set at $C_i$ within 4–8 $g \cdot kg^{-1}$ and $\tau = 70$ days.

Polycyclic aromatic compounds identified in the contaminated soil samples have different reaction modules of biochemical catabolism. Most of the investigated transformations involve different enzymatic systems and, consequently, different strains of microorganisms, which justifies the effectiveness of consortium. It was determined that *Pseudoxanthomonas spadix, Pseudomonas aeruginosa, Rhodococcus opacus, Acinetobacter baumannii, Bacillus cereus, Actinomyces sp., Mycobacterium flavescens* belong to the group of the most productive bacteria and fungi in this context.

The experiments for treatment of oil-polluted soils showed an increase in biodegradation by bioaugmentation application. The experimental results indicate the 100% of oil concentration was assimilated after 20, 25 and 35 days for the soil samples with initial hydrocarbon concentrations at the level 5, 10 and 15 $g \cdot kg^{-1}$, respectively.

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