Determination of petroleum biodegradation by bacteria isolated from drilling fluid, waste mud pit and crude oil
Sondaj çamuru, atık çamur çukuru ve petrolden izole edilen bakteriler ile petrol biyoyıkımının belirlenmesi

Abstract

Objective: In this study, the aim was to isolate and identify bacterial strains in crude oil, drilling fluid and waste mud pit samples collected from the same oil field, determine the petroleum biodegradation and find the most effective bacteria in the samples in petroleum biodegradation.

Methods: The contents of crude oil, drilling fluid and waste mud pit samples were enriched in appropriate conditions. Upon identification of the isolated bacteria, the incubations in petroleum containing media were performed at 150 rpm at 30°C for 7 days. Petroleum biodegradations by bacteria were measured by using colorimetric, spectrophotometric and gravimetric methods.

Results: Bacillus cereus, Proteus mirabilis, Bacillus subtilis, Enterococcus faecalis were isolated from the crude oil sample; Bacillus subtilis subsp. spizizenii, Bacillus cereus, Enterococcus faecalis were isolated from the waste mud pit; and Proteus mirabilis, Klebsiella pneumoniae, Bacillus tequilensis, Bacillus axarquiensis and Enterobacter cloacae were isolated from the drilling fluid. Klebsiella pneumoniae was found to degrade 60% of the petroleum in the media and Bacillus axarquiensis degraded 51%.

Conclusion: In literature, no study was encountered showing biodegradation efficiencies of Bacillus axarquiensis which was isolated from the drilling fluid in our study. The use of Bacillus axarquiensis can contribute to advanced bioremediation studies.

Keywords: Petroleum; Bacteria; Biodegradation; Waste mud pit; Drilling fluid.
Introduction

Petroleum, along with natural gas, consists of a heterogeneous mixture of hydrocarbons such as aliphatic, aromatic, resins (C5/C9 aromatic hydrocarbons) and asphalted fractions. The hydrocarbon compounds in petroleum are composed of nitrogen, sulfur and oxygen [1]. Although petroleum and petroleum based products are one of the major sources of environmental pollution, they may remain the most significant energy source for decades [2]. Crude oil production processes themselves, spills, leaks from pipelines and oil wells, abandoned refinery sites, incomplete combustion of fossil fuels and misconducted waste disposal of oil are significant causes of environmental contamination.

The contaminations caused by crude oil lead to various toxic, mutagenic and carcinogenic hazardous effects on living organisms [3, 4]. But the effectiveness of physical, mechanical and chemical clean-up techniques is limited and very expensive. Therefore, bioremediation appears to have advantages over the traditional treatment techniques. Bioremediation is defined as a process of complete mineralization of complex organic pollutants into carbon dioxide, water, inorganic compounds and cell protein or transformation of toxic organic contaminants to non-toxic compounds. But some essential parameters such as the physical and chemical composition of hydrocarbons, temperature, oxygen, nutrients, pH, water, salinity, pressure, bioavailability and microbial population could effect of the bioremediation. Bioremediation is also environmentally friendly, it does not produce waste products and is cost effective. It can be combined with other technologies and naturally occurring process when the conditions are suitable for the growth of microorganisms [5–8]. It is known that there are more than 175 genera of bacteria, which biodegrade hydrocarbons solely or in consortia [9]. Aeromonas, Alcaligenes, Acinetobacter, Arthrobacter, Bacillus, Brevibacterium, Mycobacterium, Pseudomonas, Rhodococcus, Sphingomonas and Xanthomonas are known as potential hydrocarbon degraders [1]. Microorganisms with potentials for oil degradation are widespread in nature. These can be isolated from oil and oil contaminated sites for bioremediation purposes [10]. Regarding that, in this study petroleum degrading bacteria isolated from drilling fluid, waste mud pit and oil samples were investigated in terms of their petroleum degradation performances.

Materials and methods

Sample collection

For the isolation of petroleum degrading bacteria; 1 L drilling fluid, 1 L waste mud pit and 2.5 L crude oil samples were collected from an oil field which is located in Batman, Turkey. These samples were transported with sterile bottles to Hacettepe University laboratory for analysis. All samples were stored at room temperature in the dark.

Enrichment and isolation of bacteria

Enrichment and isolation of bacteria were carried out in Bushnell Hass Mineral Salt (BHMS) medium, composed of 0.2 g/L MgSO4, 0.02 g/L CaCl2, 1 g/L KH2PO4, 1 g/L KH2PO4, 1 g/L K2HPO4, 0.05 g/L FeCl3 (Sigma-Aldrich). For each sample, 50 mL BHMS medium transferred to 250 mL Erlenmeyer flasks and sterilized by autoclaving at 121°C for 15 min. Followed by the sterilization, the medium was enriched with 1% (500 μL) crude oil. One millilitre of each sample (drilling fluid, waste mud pit, crude oil) were added in flasks separately and incubated at 150 rpm at 30°C for 3 days (Certomad BS-1; Sartorius, Tokyo, Japan). At the end of the 3 days of incubation, 1 mL aliquots were transferred to fresh BHMS enriched with crude oil. This procedure was repeated twice.

Identification of isolates

For characterization of bacterial isolates, 0.1 mL of enriched samples were inoculated onto nutrient agar and incubated at 37°C for 24 h. Phenotypic identification was performed according to Bergey’s Manual of Systematic Bacteriology [11]. For the taxonomic characterization of the isolated strains, 16SrRNA gene analysis was carried out by Refgen Gene Research and Biotechnology Limited Company, Ankara, Turkey. 533F GTG CCA GCM GCC GCG GTA A; 907R CCG TCA ATT CCT TTR AGT TT primers were used. All identified strains were inoculated on Nutrient Agar (NA) and pure cultures of these strains were maintained in glycerol: Brain-Heart Infusion Broth (BHI) (1:10) at −20°C. All bacterial strains were inoculated in fresh BHI Glycerol medium.

Preparation of inoculum

Bacterial inoculation was prepared by overnight incubation of pure cultures in nutrient broth (NB) at 150 rpm at
30°C for 18 h (Certomad BS-1; Sartorius, Tokyo, Japan). The individual bacterial strains from the overnight culture were centrifuged at 4000 rpm for 20 min (Eppendorf 5810R). The supernatants were removed. Cell pellets were washed twice with sterile saline solution (0.9% NaCl, pH 7) and suspended into the same optical density (0.8) at OD₆₀₀ (Shimadzu-UV 1700, Kyoto, Japan).

**Primary screening of petroleum degrading bacteria by colorimetric analysis**

Two hundred and fifty millilitre Erlenmeyer flasks containing 50 mL BHMS with 1% (v/v) crude oil and 1% (v/v) redox indicator were prepared. Redox indicator 2,6-dichlorophenolindophenol (DCPIP) (Fluka) was prepared by dissolving 1 g of DCPIP powder in 1 L distilled water. The flasks were inoculated with bacterial strains and incubated at 150 rpm at 30°C for 7 days. After the incubation period, the whole broth was centrifuged at 4000 rpm for 20 min (Eppendorf 5810R). Cell pellets were removed. The absorbance of the supernatant was measured at 600 nm to detect the color changes against a blank (Shimadzu-UV 1700, Kyoto, Japan) [12, 13]. The experiments were performed in triplicate.

**Biodegradation assays**

Biodegradation assay was carried out in 250 mL Erlenmeyer flasks containing 50 mL BHMS and 1% (v/v) crude oil as a sole carbon and energy source. BHMS medium containing 1% Triton X-100 was sterilized by autoclaving at 121°C for 15 min. After the sterilization period, the medium was enriched with 1% crude oil which was sterilized with 0.22 μm syringe filter (Millipore; Sartorius). All bacterial cultures which were set to equal growth density were inoculated in prepared BHMS medium containing 1% crude oil. Un-inoculated flask was served as control. The flasks were incubated at 150 rpm at 30°C for 7 days [14].

**Determination of bacterial growth by spectrophotometric analysis**

After the incubation period, the whole broth was centrifuged at 4000 rpm for 20 min (Eppendorf 5810R). Cell pellets were washed twice with distilled water and centrifuged again at 8000 rpm for 10 min (Eppendorf 5417C). Bacterial growth by utilizing crude oil was detected via measuring the turbidities of microbial suspensions (1 mL) with a UV-visible spectrophotometer at 600 nm against a blank (Shimadzu-UV 1700, Kyoto, Japan). All microbial suspensions were classified as no growth, low growth, medium growth and high growth. The experiments were performed in triplicate.

**Determination of petroleum biodegradation by gravimetric analysis**

For the estimation of crude oil biodegradation by gravimetric analysis, residual crude oil was extracted with dichloromethane (DCM) (Sigma-Aldrich) (1:2) and shaken vigorously. The upper phase of the solution was removed. Flasks with DCM were placed in the hood and DCM was evaporated in a water bath at 80°C for 1 h (Memmert, Schwabach, Germany). The crude oil biodegradation was calculated from the difference between initial and final concentrations of crude oil. The experiments were performed in triplicate.

**Results and discussion**

**Isolation and identification of microorganisms**

Nowadays, petroleum and petroleum hydrocarbons are one of the most significant environmental pollutants and oil spills are great threats to terrestrial and aquatic ecosystems [15]. Although many physical and chemical methods are developed for the removal of environmental pollutants, these methods are not entirely satisfactory for the removal of these organic pollutants from the environment [16]. When bioremediation is compared to other remediation techniques, it is an economical and sustainable method for removal of petroleum based pollutants by microbial cells’ metabolic process. Bacteria can metabolize many of the petroleum based pollutants all the way to CO₂, H₂O and CH₄ [17]. A great number of studies have shown that the most effective bacteria in petroleum biodegradation were isolated from oil contamination sites [18]. Likewise in this study we isolated and identified different groups of bacteria from drilling fluid, waste mud pit and crude oil. We investigated the most effective bacteria in biodegradation of petroleum and determined amount of petroleum that they metabolize in a given period of time in laboratory cultures. Although there is a good deal of information about petroleum degrading bacteria in the literature, this
study reports for the first time the assessment of bacteria isolated from three different sample types which were collected from the same oil field. Followed by the enrichment of the contents of the three different sample types (drilling fluid, waste mud pit and crude oil), all isolated bacterial strains were identified with 16SrRNA gene analysis. Table 1 lists the reference strains where 16SrRNA gene analysis indicated 99% similarity. Phylogenetic tree of isolated strains were shown in Figure 1. Four bacteria were isolated from crude oil sample, three bacteria from waste mud pit sample and five bacteria from the drilling fluid sample.

The isolated bacteria were *Proteus mirabilis* from both crude oil and drilling fluid; *Enterococcus faecalis* from crude oil and a waste mud pit. In the case of bacteria isolated from crude oil, our results were parallel to a similar study in which they reported gram-positive bacteria such as *Bacillus cereus* and *Bacillus subtilis* were predominant [2]. Waste mud pit is used as a storage area for the waste mud and chemicals applied during drilling. In this study, Gram-positive bacteria such as *Bacillus subtilis* subsp. *spizizenii* and *Bacillus cereus* were isolated from waste mud pit (Table 1).

Drilling fluids are used for cleaning of the boreholes that were generated during drilling for oil and gas. Drilling fluids may also contain cellulose, barite and lignosulfonates, which can be a rich source of nutrient for microorganisms. These compounds, which are used for increasing the density of drilling fluid, providing appropriate rheology for hole cleaning or thinning the drilling fluid, are also rich sources of carbon and sulfate for microorganisms [19]. Drilling fluid which contains different organic chemicals can be metabolized by microorganisms and it is important for environment but this situation leads to serious cost problems for the petroleum industry. Metabolization of drilling fluid chemicals leads to deviation of desired drilling fluid parameters and causes extra cost of drilling fluid additives. Identification of microorganisms isolated from different parts of the oil field (crude oil, waste mud pit, drilling fluid) is likely to provide basis for the development of appropriate anti-bacterial agents. This way the economic loss due to the biodegradation of drilling fluid can be minimized. In this study, various gram negative and positive strains were isolated from drilling fluid, different from the ones in crude oil and waste mud pit samples. In addition to *Klebsiella pneumoniae* and *Enterobacter cloacae*, which belong to Enterobacteriaceae family, different *Bacillus* strains such as *Bacillus tequilensis* and *Bacillus axarquensis* were isolated from the drilling fluid as well (Table 1). In a similar study which used drilling fluid as microorganism source, different gram-negative and positive strains, including *Proteus* sp. *Enterobacter* sp., *Klebsiella* sp., *Bacillus* sp., were isolated [20]. When all isolated and identified strains were evaluated it

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**Table 1**: Microorganisms isolated from crude oil, waste mud pit and drilling fluid.

| Strain number | Microorganisms          | Sample origin | Reference strain              |
|---------------|-------------------------|---------------|-------------------------------|
| P1            | *Bacillus cereus*       | Crude oil     | *Bacillus cereus* ATCC 14579  |
| P2            | *Proteus mirabilis*     | Crude oil     | *Proteus mirabilis* ATCC 29906 |
| P3            | *Bacillus subtilis*     | Crude oil     | *Bacillus subtilis* ATCC 6633  |
| P4            | *Enterococcus faecalis* | Crude oil     | *Enterobacter faecalis* ATCC 19433 |
| MP1           | *Bacillus subtilis* subsp. *spizizenii* | Mud pit | *Bacillus subtilis* subsp. *spizizenii* ATCC 19433 |
| MP2           | *Bacillus cereus*       | Mud pit       | *Bacillus cereus* ATCC 14579  |
| MP3           | *Enterococcus faecalis* | Mud pit       | *Enterobacter faecalis* ATCC 19433 |
| DF1           | *Proteus mirabilis*     | Drilling fluid| *Proteus mirabilis* ATCC 29906 |
| DF2           | *Klebsiella pneumoniae* | Drilling fluid| *Klebsiella pneumoniae* ATCC 13883 |
| DF3           | *Bacillus tequilensis*  | Drilling fluid| *Bacillus tequilensis* NR104919 |
| DF4           | *Bacillus axarquensis*  | Drilling fluid| *Bacillus axarquensis* NR115929 |
| DF5           | *Enterobacter cloacae*  | Drilling fluid| *Enterobacter cloacae* NR028912 |

**Figure 1**: The phylogenetic tree of the isolated strains.
was found that Gram positive bacterial populations were more abundant than Gram negative ones. Gram-positive bacteria can better adapt to adverse environmental conditions such as high temperature and osmotic pressure easily with the contribution of their strong cell walls [21]. However, different studies in the literature show that Gram-negative or Gram-positive bacteria can predominate in various petroleum and petroleum products [20].

Pre-evaluation of petroleum biodegradation by colorimetric analysis

2,6-Dichlorophenol indophenol (DCPIP) is a quantitative and qualitative redox indicator [22]. The color change or decolorization of DCPIP redox indicator in petroleum containing medium indicates bacteria's ability to metabolize petroleum [12]. In similar studies, DCPIP redox indicator was used for investigating the biodegradation of petroleum and petroleum hydrocarbons [13, 22]. Similarly, DCPIP redox indicator was used in this study for the pre-investigation of petroleum biodegradation by the bacteria from crude oil, waste mud pit and drilling fluid. At the end of the 7 days incubation in BHMS medium, the color change of blue redox indicator was measured with spectrophotometer and potential strains that can be used in petroleum biodegradation were presented in Table 2. Percent biodegradations were observed as 31% for Klebsiella pneumoniae (DF2), Enterococcus faecalis (P4, MP3), Bacillus tequilensis (DF3), Bacillus axarquiensis (DF4) and Enterobacter cloacae (DF5) have significantly higher growth intensities than other species (Table 3). In similar studies dealing with the determination of growth abilities of bacteria spectrophotometrically, petroleum hydrocarbon degradation capabilities of bacteria were reported [23, 24]. In the related literature, it was suggested that the growth of microorganisms in petroleum containing conditions indicates biodegradation ability of microorganisms [17].

In this study, the effectiveness of bacteria isolated from drilling fluid Klebsiella pneumoniae (DF2), Bacillus tequilensis (DF3), Bacillus axarquiensis (DF4) and Enterobacter cloacae (DF5) was determined via redox indicator in biodegradation of petroleum. Furthermore, these bacteria had higher growth intensities. The growth of pure cultures of microorganisms with only one substrate such as petroleum shows the individual degradation ability of the microorganisms [25]. Accordingly, high growth intensities of, Klebsiella pneumoniae (DF2), Bacillus tequilensis

Table 2: Primary screening of petroleum degrading bacteria by colorimetric analysis.

| Decolorization of redox indicator DCPIP | Strains | %a |
|---|---|---|
| High | P4, MP3, DF2, DF3, DF4, DF5 | 30%–50% |
| Low | P2, P3, MP1, DF1 | 0%–10% |
| None | P1, MP2 | 0% |

aP1, Bacillus cereus; P2, Proteus mirabilis; P3, Bacillus subtilis; P4, Enterococcus faecalis; MP1, Bacillus subtilis subsp. spizizeni; MP2, Bacillus cereus; MP3, Enterococcus faecalis; DF1, Proteus mirabilis; DF2, Klebsiella pneumoniae; DF3, Bacillus tequilensis; DF4, Bacillus axarquiensis; DF5, Enterobacter cloacae. % Biodegradations of all bacterial strains. Growth of bacterial strains was conducted in incubator which was set to 30°C and 150 rpm for 7 days. Results are average of three measurements.

Table 3: Determination of bacterial growth by spectrophotometric analysis.

| Growth | OD (600 nm) | Strains | %a |
|---|---|---|---|
| High | 1.0 | P4, MP3, DF2, DF3, DF4, DF5 | 50% |
| Medium | 0.5–1.0 | MP1 | 8.3% |
| Low | 0.1–0.5 | P2, P3, DF1 | 25% |
| None | 0 | P1, MP2 | 16.6% |

aP1, Bacillus cereus; P2, Proteus mirabilis; P3, Bacillus subtilis; P4, Enterococcus faecalis; MP1, Bacillus subtilis subsp. spizizeni; MP2, Bacillus cereus; MP3, Enterococcus faecalis; DF1, Proteus mirabilis; DF2, Klebsiella pneumoniae; DF3, Bacillus tequilensis; DF4, Bacillus axarquiensis; DF5, Enterobacter cloacae. % Growth ratios of all bacterial strains. Results are average of three measurements.
(DF3), Bacillus axarquiensis (DF4) and Enterobacter cloacae (DF5) isolated only from drilling fluid, Enterococcus faecalis (P4, MP3) isolated from petroleum and waste mud pit implies that these strains have high effectiveness in biodegradation of petroleum (Table 3).

Evaluation of petroleum biodegradation by gravimetric analysis

Figure 2 shows the percent biodegradations by the bacteria determined with gravimetric analysis. The growth intensities of microorganisms isolated from all samples were parallel with petroleum biodegradation and also parallel with the results of a similar study [15]. It was determined in our study that, petroleum biodegradation by Klebsiella pneumoniae (DF2), which was isolated from drilling fluid and having high growth, was 60% after 7 days of incubation. It was the most effective strain in petroleum biodegradation. As it was previously reported, K. pneumoniae metabolizes heavy hydrocarbons as well as long chain n-alkanes [26]. Although Klebsiella sp. is very effective in petroleum biodegradation, it is emphasized that, biodegradation capacity with environmental conditions, chain type and hydrocarbon structure of petroleum [27, 28].

In this study, percent biodegradations of the bacteria isolated from drilling fluid, were found to be 51% for Bacillus axarquiensis (DF4) and 46% for Bacillus tequilensis (DF3) (Figure 2). In a similar former study, the biodegradation efficiency in 2% petroleum containing medium was determined by gravimetric analysis and it was concluded that Bacillus subtilis and Pseudomonas aeruginosa isolated from petroleum contaminated soil, can be used in bioremediation of petroleum-contaminated areas [29]. In other previous studies, the effectiveness of Bacillus sp. in bioremediation of petroleum hydrocarbons was determined [30, 31]. It is crucial that, in bioremediation of organic contaminants such as petroleum and petroleum hydrocarbons, bacteria such as Bacillus isolated from local areas do not generate any risk to the ecosystem [9]. Bacillus sp. that are among endogen microorganisms living in petroleum contaminated soils have an important role in biodegradation process due to its spore production and high durability in extreme conditions [32, 33]. They lead hydrocarbon biodegradation with stimulating various metabolic pathways which are necessary for catabolic enzyme production [21]. Additionally, it was demonstrated that Bacillus subtilis is effective in petroleum biodegradation. However, the results of this study indicated that, petroleum biodegradation efficiencies of Bacillus subtilis, Bacillus cereus, and Bacillus subtilis subsp. spizizenii were lower than Bacillus axarquiensis and Bacillus tequilensis strains (Figure 2). Since biodegradation efficiency varies with the bacterial group, petroleum concentration, nutrients in the environment, carbon and nitrogen proportion, environmental conditions, biosurfactant production capability of microorganisms and metabolic capacities [34], this is perhaps the reason for discrepancy.

In addition to percent biodegradations of bacteria isolated from drilling fluid, 42% biodegradation was found for Enterococcus faecalis isolated from the crude oil and the waste mud pit. Variations in petroleum biodegradation for microorganisms were detected. The variations in percent biodegradations are due to different enzyme systems which play an important role in biodegradation metabolism [21, 35]. Our results implied that the most efficient enzyme system in petroleum biodegradation may belong to the bacteria isolated from the drilling fluid. Microbial populations and biodegradation effectiveness of these populations are supported by a wide variety of hydrocarbons and surfactants constituting the content of the drilling fluid [20]. Therefore, it is likely that the microorganisms isolated from the drilling fluid were more effective in petroleum biodegradation than other bacterial species.

Conclusion

In this study, spectrophotometric and gravimetric methods were used in the determination of the
effectiveness of microorganisms in petroleum biodegradation, which were isolated from drilling fluid, waste mud pit and crude oil samples. Different than the studies found in related literature, which focus on biodegradation by microorganisms isolated from several regions such as petroleum contaminated soils and water, in our study petroleum biodegradation of different bacteria isolated from three different sample types from the same oil field were determined. A total of 12 bacteria, four from petroleum, three from waste mud pit and five from drilling fluid were isolated, identified and analyzed. *Klebsiella pneumoniae* and *Bacillus axarquiensis* were found to be the most effective in petroleum biodegradation and they can be used in further bioremediation studies. Finally, in the literature, another study on the biodegradation efficiencies of *Bacillus axarquiensis* isolated from drilling fluid was not encountered. This study reports for the first time the involvement of this species with the petroleum biodegradation.

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