



Original article

Bioremediation potential of hydrocarbon degrading bacteria: isolation, characterization, and assessment

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ABSTRACT

Oil contamination is a worldwide concern now. However, oil contaminated environment is enriched with microorganisms that can utilize petroleum oil and use hydrocarbon for their growth, nutrition and metabolic activities. In the present study, bacteria present in the oil contaminated soil were isolated by enrichment culture technique using Minimal Salt (MS) media supplemented with diesel oil and burned engine oil as a sole carbon source. The isolated bacteria were characterized by morphological and biochemical tests and identified by molecular tool through cycle sequencing method. Three isolates were morphologically characterized as gram-negative, cocci shaped and 16S rRNA sequence analysis revealed that the isolates are closely related to *Pseudomonas* sp., *Acinetobacter* sp., and *Enterobacter* sp. respectively. Growth condition was optimized at pH 7.0 and temperature 37 °C. All the isolates were susceptible to several antibiotics and they have no antagonistic effect with soil beneficial bacteria. Three isolates were grown in two different concentrations of diesel oil and burned engine oil (4% v/v and 8%, v/v) respectively. Study revealed that with increasing the concentration of diesel oil in the media the growth rate of all the isolates were decreased. In contrast, the growth rates of all the three isolates were increased, with increasing concentration of burned engine oil. In our study, all the isolates showed their degradation efficacy in 4% v/v diesel oil and in 8% v/v burned engine oil. So, our research clearly shows that the isolates could be potentially used for bioremediation purposes for cleaning up petroleum polluted area.

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1. Introduction

Use of petroleum products has increased massively for the last few decades. Petroleum products are mainly derived from crude oil and are the world's most widely used primary energy resources of biological origin. The developing countries with weak environmental law practices is facing severe oil spillage problem especially in the marine environment. Environmental pollution is increasing day by day due to wrong channeling and discharging of used engine oil in open ocean. That frequently results in huge disorder of the

ecosystems for both the biotic and abiotic environment (Ezeonu et al., 2012). Leaks and accidental spills become regular issue which can occur during the exploration, production refining, transport and storage of petroleum and petroleum products (Holliger et al., 1997). These hydrocarbons contaminations are hazardous to the health of plants as well as they have carcinogenic, mutagenic and potent immuno-toxicants effects on human and animal health (Liebeg and Cutright, 1999; Ting et al., 1999; Vasudevan and Rajaram, 2001; Zhou and Crawford, 1995). Soil contamination with hydrocarbons has a great impact on the local system because the accumulation of pollutant in animals and plant tissue may cause death or mutations (Alvarez and Vogel, 1991). The release of such petroleum in the environment can cause a permanent damage if they are not treated at time. Contaminated areas are rich with local microorganisms which can take part in biodegradation. Biodegradation provide an effective and efficient strategy to accelerate the clean up processes to remediate the contaminated environment (HAMZAH et al., 2011). Each contaminated area has its native microorganisms

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(Geetha et al., 2013) and they take part in the process of oil biodegradation in favorable environment.

Many bacterial isolates can degrade hydrocarbon and enable them to be a source of carbon and energy (Venosa and Zhu, 2003). It is also reported that to reduce hydrocarbon, biological methods are cost effective than the physical and chemical methods (Erdogan et al., 2012). Hydrocarbon degrading bacteria produce surface active agents such as biosurfactants which can emulsify hydrocarbons in solution (Bredholt et al., 1998; Desai and Banat, 1997; Hommel, 1997; Neu, 1996). Biosurfactants increase bioavailability and subsequent biodegradation of hydrocarbons by direct cell (Banat et al., 2000; Deleu et al., 1999). Long chain hydrocarbons are important for microbes because biosurfactants make the molecules more accessible to microbial enzyme system for utilization (Banat et al., 2000; Hommel, 1997).

Oil spillage and petroleum pollution are now major causes of environmental vulnerability, and Bangladesh has been hit particularly hard by this. Due to a collision between an oil tanker and a cargo vessel on the Shela River in the Sundarbans on December 9, 2014, Bangladesh experienced its worst oil spill disaster. Some microorganisms such as bacteria, develop resistance against hydrocarbon and can utilize them as carbon source. Those bacterial strains can be utilized in bioremediation of oil contamination. Therefore, focusing on the biodegradation process would be an effective, cost effective and sustainable way for tackling oil spillage or oil contamination in moderate income countries like Bangladesh.

Bangladesh has very few studies on biodegradation of hydrocarbon in spite of having a rich microbial diversity. Therefore, we aimed to identify bacteria capable of degrading the diesel oil and burned engine oil from the contaminated site, then characterization of the isolated bacteria and evaluate their efficacy to degrade hydrocarbon. These isolates can be used in the bioaugmentation process for biodegradation.

2. Materials and methods

2.1. Sample collection

Soil sample was collected from a dumping site of petrol pump at Rangpur, Bangladesh (GPS coordinate: 25°48'57.0"N 89°14'41.5"E) (Fig. 1). The sample was collected in sterile bottle and transported to laboratory. Sample were stored at 4 °C for experiment.

2.2. Enrichment and isolation of oil degrading bacteria

Oil degrading bacteria was isolated in minimal salt media (without any sugar) where diesel and burned engine oil were used as sole

carbon source using standard procedure with some modifications (Ekram et al., 2020). As petroleum oils do not dissolved in water, we used tween 20 in the media to improve solubilization. 0.005% tween 20 (v/v) was used in the medium (Wu et al., 2013). In brief, 1.0 gm of soil added in the sterile 100 ml distilled water in 250 ml conical flask, mixed very well and waited for sedimentation. Then, 1 ml of supernatant added in MS media (K_2HPO_4 , 2.0gm/L; $(NH_4)_2SO_4$, 0.5gm/L; KH_2PO_4 , 0.02gm/L; $MgSO_4$, 0.05gm/L; $FeSO_4 \cdot 4H_2O$, 400 mg/L; $MnSO_4 \cdot 4H_2O$, 400 mg/L; $ZnSO_4 \cdot 7H_2O$, 200 mg/L; $CuSO_4 \cdot 7H_2O$, 40 mg/L; KI , 300 mg/L; $Na_2MoO_4 \cdot 2H_2O$, 50 mg/L; $CoCl_2 \cdot 6H_2O$, 40 mg/L. pH of the media was adjusted to 7.0 with different concentration of diesel and burned engine oil along with 0.005% tween 20 and incubated at 37 °C for 7 days (180 rpm). Obtained enrichment culture was spread on MS agar solidified plate supplemented with diesel oil and burned engine oil (10 μ l/ml). Then morphologically distinct colonies are identified and cultured in MS media with diesel and burned engine oil were finally considered as a pure culture.

2.3. Identification of the isolates by 16S rRNA gene sequence

The genomic DNA of the isolated bacteria was extracted by TIA-Namp Bacterial DNA Kit (Tiangen, Beijing, china, Cat. no. DP302) according to the manufacturer's protocol. 16S rRNA gene that is highly variable region was amplified by the PCR (Polymerase Chain Reaction) technique. PCR was carried out by the universal primer 16S8 F (5'-GAGTTTGATCCTGGCTCAG-3') & 16S806R (5'-GAAAG GAGGTGATCCAGCC-3') (Integrated DNA Technologies Pte. Ltd., Singapore). In PCR initial denaturation was carried out for 5 min at 95 °C, followed by 35 cycles of 94 °C for 30 sec denaturation, at 49 °C for 30 sec annealing and, 72 °C for 1 min of extension. Afterwards, amplicon was cleaned up using Wizard® SV Gel and PCR Clean-Up System (Promega corporation, Madison, Wisconsin, US, Catalog number: A9281). Sequencing of the PCR product was carried out cycle sequencing. Using the BLASTN (<http://www.ncbi.nih.gov/BLAST>) algorithm, the homology of the 16S rRNA gene sequence was compared with the 16S rRNA gene sequences of the other organisms.

2.4. Morphological and biochemical characterization

To characterize the isolated oil degrading bacteria morphological and biochemical characterization was carried out according to Bergey's Manual of Systematic Bacteriology (Holt and Williams, 1989).

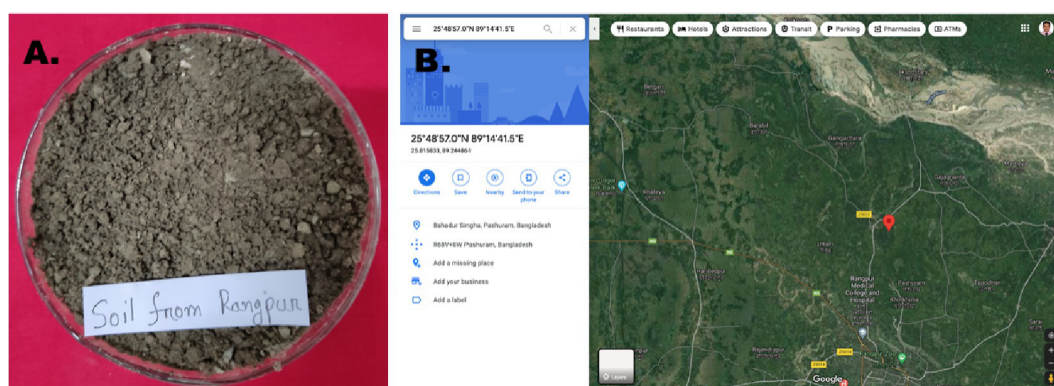


Fig. 1. Figure shows the soil sample. Soil collected from the dumping site of petrol pump.

2.5. Effect of pH and temperature on bacterial growth

To optimize the growth condition of the isolated oil degrading bacteria, we culture the isolates on MS media in presence of diesel oil (10 µl/ml), media was adjusted to different pH viz. pH 5.0, 6.0, 7.0 and 8.0. Simultaneously, to know the optimum temperature for the highest growth of the isolated bacteria, incubation was carried out in different temperature viz. 30 °C, 35 °C, 37 °C and 40 °C. Optical density (OD) taken at 600 nm by spectrophotometer (Genesys 10S UV–VIS Spectrophotometer, Thermo Scientific, MA, USA) for determination of bacterial growth.

2.6. Antibiotic sensitivity test

As the isolates have the potentiality to be used in the field for bioremediation, therefore it is important to check their susceptibility to have the way of remedy for any accidental human infection. In this study, we were used commonly available antibiotics viz., Levofloxacin (5 µg/disc), Doxycycline (30 µg/disc), Neomycin (30 µg/disc), Carbenicillin (100 µg/disc), Ceftazidime (10 µg/disc), Azithromycin (15 µg/disc), Vancomycin (30 µg/disc), Ampicillin (10 µg/disc), Tetracycline (10 µg/disc), Penicillin-G (10 µg/disc), Cefixime (5 µg/disc), Ciprofloxacin (10 µg/disc) to check the resistance or susceptibility of the bacterial isolates. Blank paper disc (6 mm) was used as a negative control. Zone of inhibition were measured on Mueller Hinton agar plates (HIMEDIA, India) and noted after 36 h of incubation at 37 °C.

2.7. Biodegradation efficacy of the isolated bacteria by gravimetric analysis

The biodegradation assay was carried out as earlier described (Saxena, 1990) with minor modifications. In brief, the 100 ml culture of all strains was used for the experiment and taken in conical flasks. According to the acetone and petroleum ether (1:1) mixture, 1 N HCl was mixed to flasks for stopping the bacterial activity and transferred to the selecting funnel and kept for 15 to 20 min. After 20 min, the solution is divided into three layers: the upper layer, which is an organic layer, the middle layer, which is an aqueous layer, and the bottom layer, which includes a cell biome. Sodium sulfate was applied to upper layers. Then, the final weight of the beakers was measured after organic layers were degraded and therefore, the oil degradation activity was observed OD at 600 nm using by UV–Vis spectrophotometer (Thermo Scientific, MA, USA). We used two replications for each bacterial strain in biodegradation assay, and to investigate the degradation ability of the diesel oil (4% v/v) and burned engine oil (8% v/v).

2.8. Statistical analysis

Statistical analyses for the antibiotic sensitivity test and antagonistic test were performed using student's *t*-test method. Data are expressed as mean ± SD (n = 3). Level of significance was set at $P < 0.05$ and $P < 0.01$.

3. Results

3.1. Isolation of the oil degrading bacteria

MS (Minimal salt) medium were supplemented with diesel and burned engine oil (10 µl/ml), used for the isolation of the hydrocarbon degrading bacteria. After 4 days of incubation at 37 °C we found that bacteria grown on the medium. Then colonies were separated based on morphological features by streak and spread plate method. Three different isolates (initially names as Isolate-1,

isolate-2 and isolate-3) were identified as potential candidate for utilization in bioremediation. As the isolates were grown on media with diesel and burned engine oil as sole carbon source for their survival, growth and replication, it may be considered that these isolates are able to uptake diesel and burned engine oil as their food and nutrition and thereby are able to break them down.

3.2. Molecular identification of oil degrading bacteria by 16S rRNA gene sequence analysis

To identify the oil degrading bacteria, 16S rRNA gene amplified and sequenced. BLASTN search revealed that Isolate-1 possesses 96.05% identity with the *Pseudomonas aeruginosa*, isolate-2 has 96.56% identity with the *Acinetobacter baumannii* and isolate-3 has 97% identity with *Enterobacter* sp. The sequences were deposited in Genbank and accession numbers were assigned for them.

3.3. Morphological characterization

The bacteria were partially identified based on color and colony morphology. The result was observed after 48 h for both spreading and streaking. Morphologically three colonies have been separately cultured and indicated. One was indicated as yellow colored colony, second is yellow colored small colony and third number is white colored colony. Gram staining showed that all the isolates are gram negative, cocci shaped. Motility test showed that they are motile.

3.4. Biochemical characterization

Biochemical characterization of all the isolated bacterial isolates were carried according to the protocol described in Bergey's Manual of Systematic Bacteriology (Holt and Williams, 1989). Biochemical test results of the three bacterial isolates are presented in Table 1.

3.5. Physiological characterization

To investigate the optimum growth conditions, isolated bacteria were cultured in different pH and temperature and also incubated for different time duration viz. 24 h, 48 h and 72 h. *Pseudomonas* sp., *Acinetobacter* sp. and *Enterobacter* sp. showed maximum growth at pH 7.0 after 48 h of incubation (Fig. 2a–c). To determine optimum temperature for their growth, incubation was carried out at 30 °C, 35 °C, 37 °C and 40 °C. In our study, we found highest cell density after 48 h incubation at 37 °C (180 rpm) (Fig. 2d–f).

3.6. Antibiotic sensitivity test

In the case of *Pseudomonas* sp., we found that most antibiotics are significant at 0.01 percent, ampicillin and vancomycin are significant at 0.05 percent, and vancomycin and penicillin-G are non-significant at 0.05 percent and 0.01 percent, respectively, when compared to control. In the case of *Acinetobacter* sp., however, vancomycin, ampicillin, and penicillin-G are non-significant, whereas neomycin, ceftazidime, and cefixime are significant at 0.05 percent, and the majority of antibiotics are significant at 0.01 percent. Antibiotic sensitivity of *Enterobacter* sp. showed that vancomycin, ampicillin, and penicillin-G are non-significant at 0.05 percent and 0.01 percent, respectively, whereas neomycin and cefixime are significant at 0.05 percent and the rest of the antibiotics are significant at 0.01 percent. (Fig. 3).

Table 1

Summarized results of biochemical tests for three bacterial isolates.

No.	Name of the tests	Results		
		Isolate 1	Isolate 2	Isolate 3
01	Methyl red test	–	–	–
02	Catalase test	+	+	+
03	MacConkey agar test	+	+	+
04	Lactose fermentation	+	+	+
05	Mannitol test	+	–	–
06	Urea hydrolysis test	+	+	+
07	Starch hydrolysis test	+	+	+
08	Triple sugar iron test	–	–	–
09	Citrate test	+	+	+
10	Carbohydrate utilization test	+	+	+

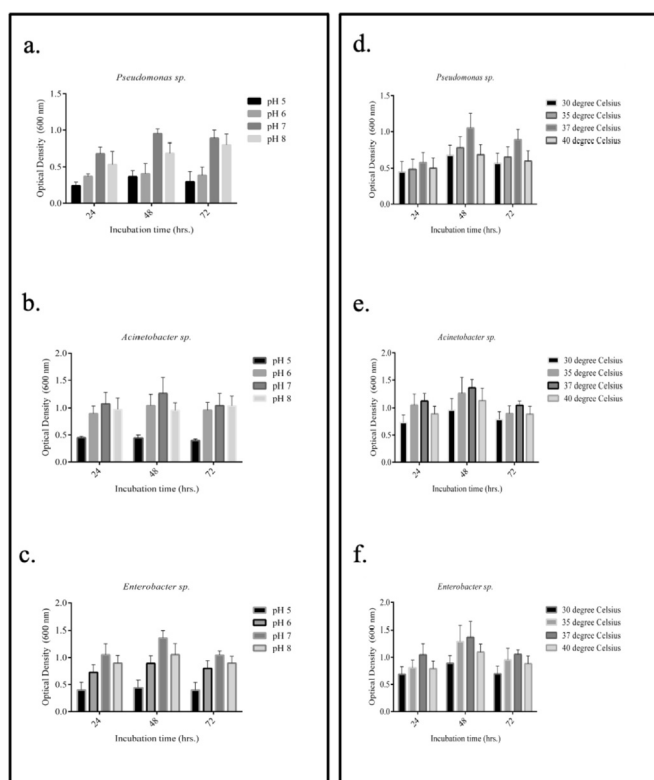


Fig. 2. Physiological characterization of the isolated bacteria. Effects of pH on growth of isolates (a) *Pseudomonas* sp., (b) *Acinetobacter* sp., (c) *Enterobacter* sp. and effects of temperature on growth of isolate (d) *Pseudomonas* sp., (e) *Acinetobacter* sp., (f) *Enterobacter* sp. 10 μ l/ml of diesel oil with 0.005% tween 20 was used for bacterial culture. Optical density of the culture was measured at 600 nm to determine the growth of the isolates after incubation for three different time durations.

3.7. Biodegradation efficacy of the isolated bacteria

By a culture dependent approach, hydrocarbon degrading efficacy of the isolated strains was determined. The maximum biodegradation of oil was done by *Pseudomonas* sp. as degrading percentage was as 14.19% and 11.97% (Fig. 4), and the lowest degradation was done by *Acinetobacter* sp. as degrading percentage found was 8.2% and 6.09% respectively (Fig. 4), for diesel (4% v/v) and burned engine oil (8% v/v) after 7 days of incubation. Isolated *Enterobacter* sp. could degrade 9.55% and 7.46% respectively of diesel (4% v/v) and burned engine oil (8% v/v) (Fig. 4).

4. Discussion

For the last few decades, thousands of hazardous waste sites have been generated worldwide resulting from the accumulation of xenobiotics in soil, water and air. Xenobiotic compounds pollute the environment as some of them are recalcitrant. Petroleum (Hydrocarbons) contamination one of them. Already many microorganisms have been isolated from two major environment like as soil and marine sources which is mostly contaminated by petroleum hydrocarbon (Bossert and Bartha, 1984).

In this study, we isolated the bacteria from soil samples that have been contaminated by petroleum hydrocarbon. In our experiment, we used diesel and burned engine oil as a sole carbon source for the isolated bacteria to test degradation efficacy. Though these isolates have the ability to degrade diesel oil and burned engine oil, but their degradation ability depends on the physical, chemical, and biological conditions.

To identify of these isolates, comparison of the bacterial 16S rRNA gene sequence considered as a best genetic procedure (Claridge, 2004). Our sequencing result of the 16S rRNA gene indicated that the bacterial isolates are *Pseudomonas* sp., *Acinetobacter* sp., and *Enterobacter* sp. respectively.

Morphological and biochemical characterization was carried out to characterize the isolates. Morphological test results indicate that, all the isolated bacteria were gram negative, cocci shaped and motile. Biochemical results indicate that, isolates are able to detoxify hydrogen peroxide and also able to utilize citrate as the carbon sources. Sugar utilization test also confirmed that they can use a wide range of carbohydrates such as glucose, sucrose and lactose as the source of nutrient. All the isolates are lactose fermenting. In addition, they are able to hydrolyze urea and starch.

In this study, all these isolates can grow in MS media supplemented with diesel oil and burned engine oil as a sole carbon source. We also optimized the growth parameters such as pH and temperature for maximum growth of these isolates. The highest growth of all the isolates were found at pH 7.0. We also recorded that, 37 °C temperature is optimum for the growth of the isolated bacteria. The goal of the optimization was to identify the most favorable growth condition for the isolates, so that we can obtain highest bacterial growth with minimal cost for use in contaminated site.

A concern for us to control of the isolated bacteria, in case of accidental human pathogenicity. So, antibiotic sensitivity tests of the isolated bacteria against different antibiotics were checked. We can definitely use levofloxacin, doxycycline, carbenicillin, azithromycin, tetracycline, and ciprofloxacin to control any severe situation because they are significant at 0.01 percent in all isolates.

Among the three bacteria, as *Enterobacter* sp., was susceptible to levofloxacin, tetracycline, and ciprofloxacin we can control human pathogenicity by these antibiotics. In case of *Acinetobacter* sp. We

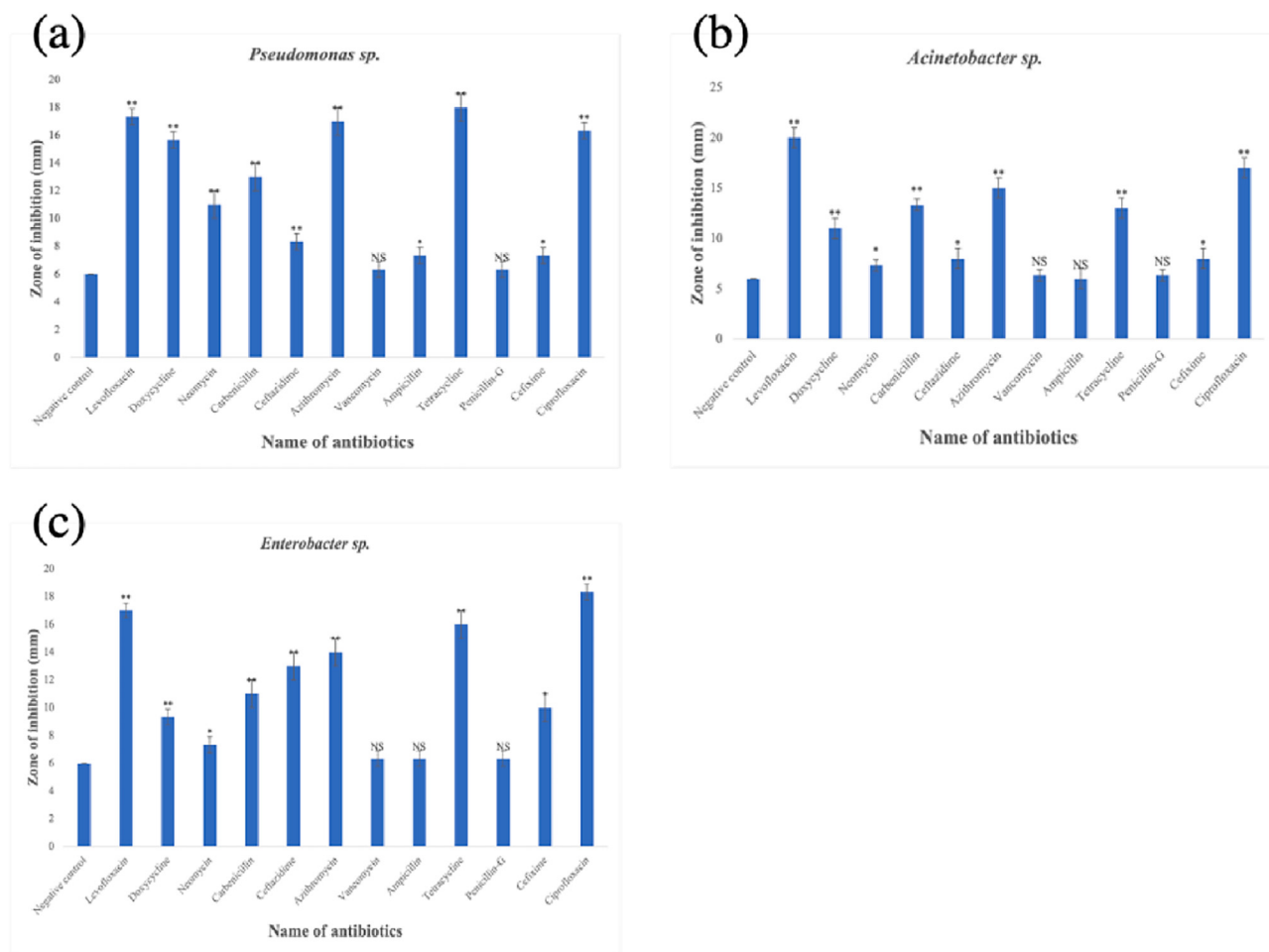


Fig. 3. Antibiotic sensitivity test of the isolated bacteria using disk diffusion assay. Antibiotic sensitivity was determined by measuring the zone of inhibition in millimeter. A sterile blank paper disc (6 mm) was used as a negative control. Incubation was carried out at 37 °C for 36 h.

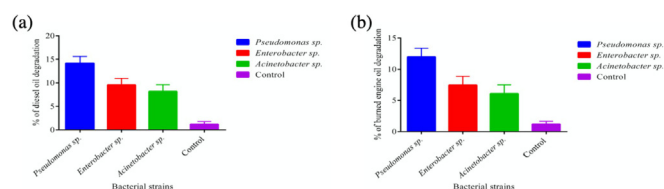


Fig. 4. Oil degradation efficacy of the isolated bacteria by gravimetric analysis. *Pseudomonas sp.* showed the highest oil degradation percentages of 14.19% and 11.97%, respectively, between diesel (4% v/v) and burned engine oil (8% v/v), while *Acinetobacter sp.* showed the lowest oil degradation percentages of 8.2% and 6.09%, respectively, after 7 days incubation at 37 °C.

can use levofloxacin, azithromycin and ciprofloxacin to control pathogenicity. However, *Pseudomonas sp.* can be controlled through susceptible to levofloxacin, doxycycline, azithromycin, tetracycline and ciprofloxacin.

In this study, the removal of diesel oil and burned engine oil by isolates was studied. All the isolates showed highest degradation in 4% (v/v) diesel oil and in 8% burned engine oil MS broth medium. In our study, we observed that growth rates of the isolates decreased with increasing the concentration of diesel oil. In contrast, with increasing the concentration of burned engine oil, growth rates of bacteria were increasing. So, our isolates are able to degrade highest amount of burned engine oil than the diesel oil. So, this is the remarkable findings of our study that burned engine oil that are not further used are accumulating and contaminating environ-

ment can remove from the earth by these isolates. In 2003 Marchal et al., reported that less than 4% diesel oil can degrade in biodegradation process by microorganisms (Marchal et al., 2003). Surprisingly, our isolates degrade more diesel oil and burned engine oil compared with this report.

So, all three isolates are the naturally occurring most potent oil degrading bacteria. This study shows the path for isolation of oil degrading bacteria from the oil contaminated site and also indicated that oil contaminated soil is the primary source for isolation of the petroleum degrading bacteria. We highly recommend that, all these isolates could be potentially used for bioremediation purposes for cleaning up oil from the polluted area.

5. Conclusion

This study reveals that oil degrading bacteria could be isolated from oil contaminated soil and feasible for the bioremediation. This finding suggests that using of these isolates is responsible treatment opportunity for the deduction of oil from the contaminated soil or water as biodegradation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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