Evaluation of Antibiotic Resistant and Metal Tolerances Capability of Petroleum (hydrocarbon) Degrading Bacteria

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Abstract. Diesel oil is considered one of the important products of crude oil which constitute a vital source of pollution in the environment. Biodegradation of complex hydrocarbon requires cooperation of more than a single species. This is true in case of pollutants made up of crude oil or petroleum. Soil contains varieties of microorganism that can be established in any natural environment. The bacterial isolates were characterized biochemically by indole test, methyl red test, citrate utilization Test, voges proskauer test, starch hydrolysis, catalase production test, nitrate reduction test, antagonism test, oligodynamic susceptibility and antibiotic sensitivity tests.

Keywords: Hydrocarbon, Biodegradation, susceptibility, oligodynamic, Antagonism

1. Introduction

Petroleum products are worldwide use as a major automobile fuel of them diesel oil is considered one of the crucial products of crude oil which constitute a vital source of pollution in the environment. Diesel oil can go into the environment via wrecks of oil tankers carrying diesel oil, cleaning of diesel tanks by merchants, war ships carrying diesel oil and motor mechanics. Diesel oil spills on agricultural land inhibits plant growth. Major factors responsible for the reduced plant growth in diesel oil contaminated soils range from direct toxic effect on plants. Reduced germination (Udo and Fayemi, 1975) due to inappropriate soil condition and insufficient aeration of the soil because diesel oil displaces air from the space among the soil particles. Degradation progression facilitated by microorganisms helps in removal of spilled oil from the environment by various physical and chemical methods (Ijah and Okang, 1993). Microorganisms have enzyme systems to degrade and utilize diesel oil as a source of carbon and energy (Ezeji et al. 2005). Monooxygenases play crucial role in microbial degradation of oil, chlorinated hydrocarbons and other compounds. Biochemical tests are used to differentiate such bacteria. The characteristics showed the physiology of the test organisms. This study was designed to isolate, characterize and identify diesel oil-degrading microorganisms.

2. Material and Methods

Biochemical test such as indole test, methyl red test, citrate utilization Test, voges proskauer test, starch hydrolysis, catalase production test, nitrate reduction test, antagonism test, oligodynamic susceptibility
and antibiotic sensitivity tests were carried out to find the enzymatic activity of isolated organism. Protocol used was followed as given by Dubey and Maheshwari, 2004.

2.1. Antibiotic sensitivity

Isolated halophilic bacteria were spread over nutrient agar plates. Wells were made in the nutrient agar plates for antibiotic sensitivity testing. Different concentrations of streptomycin (HiMedia, Mumbai, India) and tetracycline (HiMedia, Mumbai, India) (5 mg/ml, 10 mg/ml, 15 mg/ml, 20 mg/ml, and 30 mg/ml) were prepared and the wells were flooded with these antibiotics. Later, plates were incubated at 37°C for 24-48 hours. This method is designed to determine the smallest amount of the antibiotics needed to inhibit the growth of a microorganism. The concentration of antibody required for effective inhibition of microbial growth is called minimal inhibitory concentration (MIC), which was determined by measuring the diameter of growth inhibition (clear) zone surrounding the antibiotics containing wells.

2.2. Oligodynamic Susceptibility

Isolated bacteria were spread over the nutrient agar plates (HiMedia, Mumbai, India), various metals (Fe, Cu, Zn, Al, Hg) were used to evaluate its effect on microbial growth. All plates were incubated at 37°C for 24-48 hours. Effects of metals on the growth of microorganisms can be both stimulatory and inhibitory. Synergistic or antagonistic effects were observed on the growth of organisms in the plates around the metal ion.

2.3. Antagonism

In nature microbes grow in various associations ranging from antibiosis, symbiosis, symbions, and synergism to commensalisms. This association may exist within or between different groups of microbes. In procedure, the bottoms of nutrient agar plates (HiMedia, Mumbai, India) were divided into sectors. Loop full cultures of all organisms were streaked perpendicular to each other without touching. Plates were incubated at 37°C for 24 hrs.

3. Results and Discussion

3.1. Biochemical analysis

Isolates responded variably through the various parameters of biochemical analysis. Table 1 shows the results of test such as; gelatin hydrolysis, urea hydrolysis, citric acid utilization, starch hydrolysis, MR, VP, nitrate reduction, indole, casein hydrolysis, phenyl deaminase test and carbohydrate fermentation. For tests such as urea hydrolysis, citric acid utilization, starch hydrolysis, MR, VP, indole production, casein hydrolysis and phenyl deaminase test, all isolates (i.e. I1 (Pseudomonas rhodesiae), I2 (Caulobacter henricii), I3 (Cupriavidus gilardii) and I4 (Phenylobacterium koreense) responded equally (Table 1)

| Biochemical Tests           | Bacterial isolates |
|-----------------------------|--------------------|
|                             | T1     | T2     | T3     | T4     |

Table 1: Biochemical identification of isolates
3.2. Oligodynamic Susceptibility

Table 1.2 shows susceptibility for the isolates to various metals such as; Cu, Zn, Al and Fe. Result showed that besides Zn, all other metals such as Cu, Al and Fe were not susceptible to isolates (I1, I2, I3 and I4). Isolates such as; I1, I3 and I4 were found susceptible to Zn. The only isolate I2 was not susceptible to any of the metal used in this study (Table 2).

Table 2: Oligodynamic susceptibility test of isolates

| Oligodynamic susceptibility Test | Different Metals | Cu | Zn | Al | Fe |
|---------------------------------|------------------|----|----|----|----|
|                                 | T1 NS | NS | NS | NS | NS |
|                                 | T2 NS | NS | S  | NS | NS |
|                                 | T3 NS | NS | NS | NS | NS |
|                                 | T4 NS | NS | NS | NS | NS |

Note: NS = Not Susceptible, S = Susceptible

3.3. Antibiotic sensitivity

All four isolates were examined for antibiotic sensitivity against streptomycin, ampicillin and tetracycline. For streptomycin isolates i.e. I1 and I4 were found susceptible except for I2 and I3. Antibiotic effect of ampicillin was mostly resistant in isolates I2, I3 and I4, however, for I1 it showed susceptibility. Furthermore, for tetracycline I2 and I4 showed susceptibility whereas, I1 and I3 noted with limited susceptibility (Figure 1, Table 3).

Table 3: Antibiotic susceptibility test of isolates

| Antibiotic sensitivity and Concentration of streptomycin |
|----------------------------------------------------------|
|               5ug/ml  | 10ug/ml  | 20ug/ml  | 30ug/ml  | 40ug/ml  |
|----------------------------------------------------------|
3.4. Antagonism

All isolates i.e. I1, I2, I3 and I4 were found negative for examination of antagonism. No associations such as; antibiosis, symbiosis, symbions, and synergism to commensalisms were observed (Figure 2).
4. Discussion

In this study, biochemical analysis showed positive results for gelatin hydrolysis, citric acid utilization, starch hydrolysis, casein hydrolysis and phenyl deaminase test. One of the first studies on Caulobacter sp. reports that when isolated for the first time organism failed to grow on gelatin, but once adapted to culture media, it produced surface growth and filiform growth along the line of puncture in gelatin stabs (Bowers et al., 1954). Same study further conducted on Caulobacter sp explains about colony morphology which mentions that surface agar colonies are up to 5 mm in diameter after 96 hrs of incubation, round, smooth, slightly raised, glistening, finely granular in the center, and grayish white with the center and the reverse side becoming brownish-yellow. Subsurface colonies are dense, brownish-yellow, and lenticular, up to 0.5 mm in diameter and one mm in length (Bowers et al., 1954). The above observation on colony morphology went partially in accordance with our study which noted light yellowish colony in color, irregular form, elevation umbonate, margins are irregular and undulate.

For other biochemical markers a parallel study was missing or not found, therefore, present study was conducted exclusively for this investigation.

Antibiotic susceptibility is another important marker in distinguishing between species and strains. The first report of antibiotic sensitivity was published in 1980 by Stringfellow et al., who studied the protein synthesis elongation factors Tu and Tu-Ts from Caulobacter crescentus in relation to its sensitivity to kirromycin and activity in Qβ replicase (Stringfellow et al., 1980). Through biochemistry we found that Phenylobacterium koreense were positive for citric acid utilization, starch hydrolysis, nitrate reduction, casein hydrolysis and carbohydrate fermentation. Aslam et al., (2005), also reported few of the above parameters which went in agreement with our result such as, positive result for nitrate reduction and negative for gelatin hydrolysis. It was however also important to notice that for VP our result differed as Aslam et al., reported negative for VP and our result turned out to be positive (Aslam et al., 2005). We also observed positive result for carbohydrate fermentation which confirms presence of acetoin in Phenylobacterium koreense. Various fermentive bacteria use Acetoin which utilizes natural four carbon molecule to store external energy. Acetoin is produced by decarboxylation of alpha-acetolactate, which is a common precursor in metabolism of branched amino acids.

We observed quite interesting results in antibiotic sensitivity tests, which indicated that Phenylobacterium koreense is ampicillin resistant and partial susceptibility to streptomycin and tetracycline. Nevertheless, it was not completely susceptible to any of the antibiotics used in this investigation. There were no parallel results available in any of the Phenylobacterium sp., to confirm our results. As limited susceptibility observed by streptomycin and tetracycline, it is possible that other antibiotic might have greater susceptibility. However it can be concluded that among all four isolate investigated in this study Phenylobacterium koreense tends to differentiate itself from other isolate both biochemically and metabolically.

We observed forming smooth colonies on blood agar, positive for oxidase and catalase, non-users of glucose, incapable of reducing nitrate or nitrite, and susceptible to colistin (Vaneechoutte et al., 2004).
Our results for biochemistry showed accordance to the above reported results, we also observed positive test for gelatin hydrolysis, citric acid utilization, starch hydrolysis, VP, casein hydrolysis and phenyl deaminase test, similarly, negative for nitrate reduction and carbohydrate fermentation.

Through antibiotic susceptibility test it was observed that Cupriavidus gilardii was susceptible to tetracycline and streptomycin, however, resistant to ampicillin. There were not much studied about the particular strain, nevertheless, one reported case of infection by Cupriavidus gilardii was investigated for antimicrobial sensitivity. Limited reports are available on antibiotic sensitivity of Cupriavidus sp., require more work to establish its antibiotic susceptibility/resistant. Oligodynamic action is the tendency of small amounts of heavy metals to exert a lethal effect on bacterial cells. Some metals have the ability to kill bacteria by entering into cells and act as a toxicant in characteristic way. Metals such as silver and copper have been used as a disinfectant for many generations. Oligodynamic metals kills almost all non-spore forming bacteria and viruses (Thurman & Gerba, 1988). According to a study, 1000 time lower concentration than a toxic level, silver can still serve as a disinfectant (Warrington, 1996). Our result for oligodynamic susceptibility test showed that all isolates were not susceptible to copper, aluminum, and iron, however, susceptible to zinc. Zinc is used as a weak antiseptic, and in paints as a white pigment and mold-growth inhibitor (Leikin and Paloucek, 2008). Previous studies reported that zinc chloride is a common ingredient in mouthwashes and deodorants, and zinc pyrithione is an ingredient in antidandruff shampoos. Zinc coated fittings on house hold items obstruct growth of algae. There are many shingles available that are made of Copper and Zinc (Tortora et al., 2010). Likewise, Zinc iodide and zinc sulfate are used as antiseptic (Rohe and Wolf, 2007). The mechanism of action of zinc and copper is still unknown, however, some studies suggest that metal ion denature vital proteins by tagging to reactive groups resulting in precipitation and inactivation of proteins. Therefore, high affinity of cellular protein for the respective metal ions causes’ death of the cells, imbalance of ions are the reason of cellular deaths (Benson, 2002). Since these isolates were exclusively studied for oligodynamic tests, no parallel studies were observed to confirm our results. Why copper, aluminum and iron were not susceptible to isolates was not understood, therefore more studies are required to ascertain any conclusion.

Microbial antagonism is a method in which two different strain compete for survival with each other. It is a self-defense mechanism of host culture to protect against foreign strains. In this phenomenon the foreign strain could not thrive in the pressure of existing host and may extinct completely. For example, mouth bacteria which have this in built resistance against other bacteria coming in from inhalation, mouth to mouth contact or food consumption. During infection the act of antagonism can be used to eliminate harmful infectious bacteria (Ledermann, 2013). Ledermann in his study reported three types of antagonism i.e. bacterial killing by other bacteria, virus against bacteria and blockade of cellular receptors by bacterial filtrates. In the first type, the piocianase from Pseudomonas aeruginosa and the activity of Bacillus subtilis over Mycobacterium tuberculosis were the better examples; in the second, the French D’Herelle was a pioneer using bacteriophages against Shigella dysenteriae; and another French, Besredka, headed the third line with his “antivirus thérapie” on Staphylococcus aureus (Ledermann, 2013). Our result for antagonism between these four isolates i.e. Pseudomonas rhodesiae, Caulobacter henricii, Phenyllobacterium koreense and Cupriavidus gilardii were found partially endosymbiotic. Growth of any of the bacteria versus each other seem to unaltered in nutrient media and grew uninterrupted. This was obvious because most of the investigated isolates were surviving together at the site of examination. The theory of endosymbiosis was first articulated in 1905 and 1910 by the Russian botanist Konstantin Mereschkowski, and later advanced by microbiologist Lynn Margulis in a 1967 (Mereschkowsky, 1910; Mereschkowski, 1905; William et al., 2012; Sagan, 1967). According to the theory, endosymbiosis is a theory of selection relying on the phenomenon of symbiosis No parallel study was found to compare these results as these four isolates were exclusively examined for antagonism in this study.
5. Conclusion

If the bacteria are sensitive to the antibiotic, a clear ring, or zone of inhibition, is seen around the wafer indicating poor growth. Antimicrobial susceptibility was tested using Strokes method, antibiotic diffusion tests, Agar and broth dilution methods and minimum inhibitory concentration methods. Through antibiotic susceptibility test is remarkable to notice that Pseudomonas rhodesiae was susceptible for all three antibiotics used in this investigation i.e. Streptomycin, ampicillin and tetracycline. Although this result was exclusive for Pseudomonas rhodesiae it was compared with other Pseudomonas sp. to understand its variation for antibiotic susceptibility whereas, our result for Pseudomonas rhodesiae showed susceptibility to ampicillin at 40 µg/ml.

In microbiology, the fast and simple biochemical tests can identify bacteria. Such as metabolic or enzymatic characteristics, for example the ability to ferment carbohydrate and the pattern in which it is metabolized can dictate the genus and species of bacteria. The other technique is to analyze acids, alcohols and gases when a bacteria is grown on to a selective media. Through biochemical analysis for the first time we came to know that Pseudomonas rhodesiae is positive for starch hydrolysis, gelatin hydrolysis, citric acid utilization, casein hydrolysis and, phenyl deaminase test. It was however, not uniquely present in Pseudomonas rhodesiae but was also found in other Pseudomonas sp. We observed quite interesting results in antibiotic sensitivity tests, which indicated that Phenylbacterium koreense is ampicillin resistant and partial susceptibility to streptomycin and tetracycline it was not completely susceptible to any of the antibiotics used in this investigation. There were no parallel results available in any of the Phenylbacterium sp., to confirm our results. As limited susceptibility observed by streptomycin and tetracycline, it is possible that other antibiotic might have greater susceptibility. However, it can be concluded that among all four isolate investigated in this study Phenylbacterium koreense tends to differentiate itself from other isolate both biochemically and metabolically. Through antibiotic susceptibility test it was observed that Cupriavidus gilardii was susceptible to tetracycline and streptomycin, however, resistant to ampicillin. There were not much studied about the particular strain, nevertheless, one reported case of infection by Cupriavidus gilardii was investigated for antimicrobial sensitivity.

Conflict of interest
None.

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