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RESEARCH ARTICLE

Isolation and Identification for Some Bacteria from Polluted Soil with Diesel around Powered Generators.

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Abstract

Research work deals with two aspects; the one thing is the collection of diesel polluted soil from various locations around the powered generators, and the other aspect it deals with the laboratory work which includes culturing and sub-culturing to get pure culture of microorganisms from soil sample, and study the characterization. Gram staining , sugar fermentation, biochemical test, sodium chloride test and bacteria spore stain were carried out on isolated microorganisms which revealed the isolated microorganisms were from *Staphylococcus spp*., *Micrococcus spp*., *Klebsiella spp., Bacillus spp*.

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INTRODUCTION

As we dig deeper into the modern industrial age of technologies, several aspects of human life change. People benefit largely from life development and many live in prosperity, but prosperity has a price. This price is paid by our environment that suffers daily from all kinds of pollutants and destruction. People now have to find ways to cure this destruction.

Soil pollution by petroleum products is a widespread problem. Several bacterial species are capable of oil hydrocarbon degradation because hydrocarbons are naturally produced by plants and microbes (1).

Diesel engine oil, which is one of the major products of crude oil, constitutes a major source of pollution in our environment. With the combined dependence on diesel engine oil by some vehicles and generators, greater quantities are being transported over long distances.

Day by day the use of petrol and diesel is increasing, with the use, its transportation; disposal is also increasing which is leading to increase in its spillage during transportation and over soil during disposal causing soil pollution ultimately leading to environment pollution.(2)

Microorganisms have enzyme systems to degrade and utilize diesel oil as a source of carbon and energy (3, 4, and 5).

Soil contamination with hydrocarbons causes extensive damage of local ecosystems since accumulation of pollutants in animals and plants tissues, may cause progeny's death or mutation (6)

In Mexico, an endless number of contaminated sites exist as a result of more than 60 years of oil petroleum activity; in recent years this problem has motivated researches to recover these contaminated sites (7).

Microorganisms survive in contaminated habitat because they are metabolically capable of utilizing its resources and can occupy a suitable niche. Contaminants are often potential energy sources for microorganisms (8).

The ability of microorganisms to utilize hydrocarbons in oil contaminated environments has been documented (9, 10, and 11). Microbial degradation process aids the elimination of spilled oil from the environment after critical

removal of large amount of oil by various physical and chemical methods (12). This is possible because microorganisms have enzyme system to degrade and utilize diesel oil as a source of carbon and energy (13).

The present work has been focused on this approach, aiming to isolate bacterial strains capable of petroleum hydrocarbon degradation in situ conditions. In this study, we report isolates capable of degrading a wide spectrum of hydrocarbons efficiently. Degradation studies to be carried out with different isolates at varying interval of time will help to find out the most potent hydrocarbon degrading strains, which can be used for any bioaugmentation studies during bioremediation.

Materials & methods

1. Sampling Sites

The soil samples for this study were collected from different locations of diesel powered generators, Soil samples were taken from nearby of the generators and disposal areas contaminated with diesel oil waste. Five soil samples each from surface (0-5 cm depth) and deep soil (15-20cm depth) were collected from each power house at 5m distance apart. The samples were taken consecutively after tilling with a sterile scoop and transferred into sterile polythene bags for microbiological determination. 1gram of each soil sample was aseptically transferred to 9ml of sterile water for serial dilution process and then cultured on NA for bacterial isolates. Sub-culturing was done until pure isolates were obtained which were later stored on slants in the refrigerator at $4^{o}C$ for identification.

2. Isolation and Identification of Bacteria

The identification of hydrocarbon utilizing bacterial was based on biochemical characterization such as sugar and alcohol sugars fermentation tests, citrate, catalase, indole, methylred, voges-prauskauer, starch hydrolyses, oxidase etc.

3. Staining Reaction

Gram's Staining Reaction

Smears of 24hour old isolates were on slides and heat fixed. Crystal violet was first applied and allowed to remain for 1 minute and washed off with water. Grams iodine was added. This stayed for another 1 minute and was later washed off. The smear was decolorized with alcohol, washed with water and counter stained with 1% Safranin. Smear was them blot dried with clean filter paper and observed under (X100) oil immersion lens of the microscope.

4. Biochemical Tests

The isolates were made to undergo a number of biochemical tests as follows.

1**. Catalase Test**

This is a test to detect the presence or absence of catalase enzyme. The catalase enzyme catalyses the breakdown of hydrogen peroxides to release free oxygen gas and the formation of water. A few drops of 3% H202 were added to a 24 hour old culture of isolates on slide. Evolution of gas white froth indicated a catalase positive reaction while the absence of the effervescence or white froth showed negative reaction.

2. **Voges-Prauskauer Test**

This is a test carried out to know if the isolates can produce acetymethylcarbinol from glucose. The medium used is glucose phosphate broth. The sterilized medium was distributed into test tubes in 5ml volumes and inoculated with the isolates, an uninoculated tube served as control.

3. **Methyl-red Test**

This test was used to determine whether the production of acid glucose has lowered and help pH at about 4.2 or below. The medium used is glucose-phosphate broth. This was distributed into screw caps and was sterilized at 1210C for 15 minutes. Isolated were grown in the medium for two days after which methyl-red test reagent was added. Uninoculated tubes served as control. Development of yellow colour was recorded as negative result.

4. **Oxidase Test**

A few drops of the oxidase reagent (1% aqueous tetramethyl-p-phenylene diamine hydrochloride) were added onto an isolated colony from 24 hours old pure culture. A purple coloration produced within five-ten second on clean filter paper indicated oxidase positive cultures. A delayed reaction was recorded as negative.

5. **Sugar Fermentation Test**

The test is used to determine the ability of the bacteria and yeasts to utilize different sugars. This is useful in distinguishing the different strains of organisms. 10% solution of each sugar was separately prepared and 1m1 of each sugar was added to 9mls tubes of phenol red broth base which had been separately sterilized at 1210C for 10 minutes while the phenol broth was sterilized at 1210C for 15mins in screw capped tubes. Each tube was fitted with an inverted Durham tube. The tubes were inoculated with test organisms (bacteria and yeasts cultured) and incubated for 3 days at 350C. Uuninoculated tubes served as controls. Acid production was indicated by a change of colour medium from red to yellow. Production of gas was collected in the inverted Durham's tubes.

6. **Sodium Chloride test**

This test determines whether the microbe can grow in a medium containing 6.5% sodium chloride (NaCl), also known as table salt. The medium used was 6.5% NaCl broth. It is a nutrient broth containing 6.5% sodium chloride, or table salt.An inoculums from a pure culture was transferred aseptically to different sterile test tubes of 6.5% NaCl broth the inoculated tube is incubated at 35-37 C for 24 hours. A positive test is indicated by the presence of turbidity.

7. Bacteria Spore Stain

Some groups of bacteria usually members of bacillus and clostridium produce endospore which was relatively resistant to the common stains however the spore walls can be made permeable to these stains by heating the smear preparation. After preparing the smear, heat fixed the organism to the slide, I added malachite green solution and steamed for 10 and washed off carefully with slightly running tap water and I counterstain with safranin solution for 15 seconds after which I washed off with tap water and examined under the microscope using the oil immersion objective and I put a drop of lmmersion oil. To observe, the spore with green indicates positive while spore with red indicates negative.

Results and discussion

Initially, 4 bacterial strains were isolated from 5 samples of soil originating from different places contaminated with diesel around powered generators. Isolation was carried out using the traditional microbiological technique with petri dishes containing nutrient agar. Result demonstrated from (table 1) that soil sample which showed higher contamination with Gram positive and Gram negative bacteria.

Colony morphology-microscopic observation revealed that the microbial colony was (cocci and rod)

Table 1. Gram stain and colony shape for isolates

isolate	organism	Gram stain	Colony shape
G1S1	1	+ve	cocci
	2	+ve	cocci
	3	-ve	rod
G4S1	1	+ve	rod
G1W1	1	+ve	cocci

	2	+ve	cocci
G2E1	1	+ve	rod
G1E1	1	+ve	cocci

Table 2. Biochemical test for the isolate and identification.

Isolate	organism	Catalase	Voges-Prauskauer	Methyl-red	Oxidase	. Sugar Fermentation	Sodium Chloride	Bacteria Spore	identify
G1s1	**1**	+++	-	-	-	-	+++	-	*Staphylococcus spp.*
	2	+++	-	+++	+++	+++	+++	-	*Micrococcus spp.*
	3	+++	-	+	-	+	-	-	*Klebsiella spp.*
G4S1	**1**	+++	+	+	-	+	+	+	*Bacillus spp.*
G1W1	**1**	+++	-	+	-	+	-	-	*Klebsiella spp.*
	2	+++	-	-	-	-	+++	-	*Staphylococcus spp.*
G2E1	**1**	+++	+	+	-	+	+	+	*Bacillus spp.*
G1e1	**1**	+++	-	+++	+++	+++	+++	-	*Micrococcus spp.*

From Table 2. Biochemical tests performed for the isolation and identification of individual bacteria, site **G1S1** isolated three types of bacteria which are *Staphylococcus spp., Micrococcus spp.,* and *Klebsiella spp.,* and the reason is due to the occurrence of this site soil under the influence of more than generator, *Micrococcus spp.,* has shown significant growth for the rest of the bacteria isolated from the same location and this corresponds with(14), found the mixture of two species of bacteria was better than one for contaminated soil remediation This was probably due to the different enzyme system from two different bacterial isolates that acts on hydrocarbon at a time which proved to be an excellent option to degrade that hydrocarbon if both the bacterial enzyme system posses considerable efficiency to act upon it and to degrade it.

This also applies to the site **G1W1**. While at the other sites was isolated only one type of bacteria, because the degree of contamination was less.

Biochemical tests showed clearly that the growth was strongest for *Micrococcus spp.* and *Bacillus spp.* due to their ability to growth in different mediums, and the diesel oil degradation best when it combine with other species, this proven in many researches before in **2013** in the other study.(15)

Conclusion.

According to the result in our research was found four types of bacteria in soil contaminated with diesel around powered generators. So this study concludes these bacteria had shown ability to grow in soil contaminated with hydrocarbons.

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