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EFFECT OF DIFFERENT CONCENTRATION OF SODIUM CHLORIDE ON THE GROWTH OF BACTERIAL FORMS ISOLATED FROM PETROLEUM PRODUCT

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Petroleum hydrocarbon contamination in soil is the major problems resulting from the activities related to petroleum industry, automobile garage/shops, and accidental release in soil. Soil samples from petroleum-contaminated soil were collected from petroleum storage tanks in Ajmer district of Rajasthan. In this work 28 oil degrading bacteria were isolated, identified and characterized by colony morphology, staining and biochemical characteristics based on "Bergey's Manual of Determinative Bacteriology" and "Bergey's Manual Of systematic Bacteriology". Salt tolerance NaCl ability of bacteria isolates to various concentration were studies. The result indicated that sodium chloride appeared to be favourable for majority of bacterial forms at various concentrations. The range of salt tolerance by the bacteria isolates were studied using different concentration of the salt sodium chloride NaCl -2.0% to 13.0%.

Key Words: Degradation; Hydrocarbon; Petroleum products; Salt tolerance.

Introduction

Bacteria are omnipresent. They have been reported from all environments wherever the human being could lay his hand. From the time of discovery till date the knowledge on bacteria is increasing.

Petroleum-based products are the major source of energy for industry and daily life. Leaks and accidental spills occur regularly during the exploration, production, refining, transport, and storage of petroleum and petroleum products. The amount of natural crude oil seepage was estimated to be 600,000 metric tons per year with a range of uncertainty of 200,000 metric tons per year. Release of hydrocarbons into the environment whether accidentally or due to human activities is a main cause of water and soil pollution.

Hydrocarbon utilizing microorganisms are common in oil fields. The rapid disappearance of crude oil, spilled on the ground can be noted which is activity of microbes. During the world wall thousands of tons of petroleum ware lost from torpedoed tankers. The oil on the beaches as well as on the open ocean disappeared, probably due to microbial action.

Bacteria are the most active agents in petroleum degradation, and they work as primary degraders of spilled oil in environment. Several bacteria are even known to feed exclusively on hydrocarbons.

Petroleum hydrocarbons cause damages to the surrounding ecosystems so considered as major contaminants in the environment. Soil contaminated with diesel and engine oil is the major environmental problem today which caused due to increase in the consumption of petroleum hydrocarbon products. Soil contamination by oil leads to loss in its useful properties such as fertility, permeability, water holding capacity and binding capacity.

The MPD-M culture has immobilized on polypropylene fibers and showed that the culture was able to degrade crude oil at much higher salinity up to 18% NaCl[1]. Some investigators were able to show the degradation of diesel fuel in the presence of salt up to 17.5% by microbial communities extracted from Argentinean saline soils. In addition, these investigators isolated several halotolerant bacteria of the genera *Cellulomonas*, *Bacillus*, *Dietzia* and *Halomonas* with the ability to degrade crude oil as the carbon source[2]. Similarly, many other investigators have isolated pure cultures including *Halomonasshengliensis*[3] *Halomonas* sp. strain C2SS100[4], *Marinobacter quaeolei* [5],*Streptomycesalbiaxialis* [6], *Rhodococcus erythropolis*, and *Dietzia maris* [7]from oilfields, production water, and other saline environments that degrade crude oil as the source of carbon in the presence of 0–30% salt. They reported the isolation of several strains of hydrocarbon-oxidizing bacteria representing the genera *Rhodococcus, Gordonia, Dietzia*, and *Pseudomonas* from oil and stratal waters of Tatarstan, western Siberia, and Vietnam oilfields[8]. All these strains oxidized *n*-alkane fraction of crude oil in a medium containing 15% NaCl. A *Bacillus* sp. strain DHT, isolated from oil contaminated soil, grew and produced biosurfactant when cultured in the presence of variety of hydrocarbons including crude oil, diesel oil, hexadecane, naphthalene, pyrene, dibenzothiophene, salicylate, catechol, and phenanthrene as the sole sources of carbon in the presence of 0–10% salinity and at 30–45°C. However, no growth occurred on toluene, phenol, 2-hydroxyquinoline and carbazole [9]. The growth of identified isolates was checked for saline activity on Nutrient agar medium enriched with 1%, 2%, and 3% NaCl (w/v) concentrations. *Bacillus* and *Streptococcus* showed dense growth at 1% NaCl, moderate growth at 2% NaCl, and low growth at 3% NaCl. *Staphylococcus*, *Micrococcus*, *Corynebacterium*, and *Arthrobacter* showed low growth at 1% NaCl, moderate growth at 2% NaCl and dense growth at 3% NaCl,[10].

Similarly, the isolation of several strains of thermophilic and mesophilic hydrocarbon degrading as well as biosurfactant producing organisms have reported from Tunisian oil fields. Among these, *Pseudomonas* sp. strain C450R and *Halomonas* sp. strain C2SS100 could degrade 93–96% of the aliphatic fraction of crude oil (C_{13}–C_{29}), while producing biosurfactants in the presence of 5–10% NaCl. Such organisms could play important role in the degradation of poorly soluble high molecular weight hydrocarbons in crude oil[11].

Degradation of octane as the sole source of carbon in the presence of 6% salt by several gram positive bacteria including *Rhodococcus* sp, *Arthrobacter* sp, and *Bacillus* sp., isolated from sediment samples from chemical- and salt processing plants reported in Russia[12].

Oil spills in terrestrial and aquatic environments are increasingly common and cause significant ecological damage and civil challenges [13-14]. Due to pipeline leakage, mismanagement, and offshore oil

production, petroleum- and salt-contaminated soils are often found in oilfields and coastal zones[15]. Salinity is a key factor which can inhibit microbial growth and subsequent degradation of petroleum contaminants [16], and high salt concentrations (400 mmol NaCl) can inhibit microbial growth in soils by more than 90% [17]. Some investigators found that salt concentrations of 200 dS/m can decrease oil mineralization in soils by 20–44%[18]. Consequently, bioremediation of petroleum- and salt-contaminated soil requires microbe strains to be salt-tolerant, and the degradation process can be complicated and time-consuming. Saline soil pollution from crude oil presents substantial hazards to coastal ecosystems[19]. For these reasons we propose a study to isolate microbe strains from crude oil polluted saline soils and evaluate each based on their ability to thrive on crude oil as a carbon (C) source. Suitable strain will then be selected and evaluated for salt-tolerance and ability to degrade crude oil contaminants in saline soils. The aim of present work is to find out optimum condition of salt concentration on which petroleum hydrocarbon degrading bacteria can grow efficiently.

Materials and Methods

(i) Collection of soil samples:

Soil samples spilled with petroleum product from the petroleum storage tanks, petrol pumps and service stations located in Ajmer District Rajasthan in India, were collected. These samples collected in sterile containers were brought to the laboratory. Isolation for microorganisms was made on the same day of collection.

(ii) Isolation and purification of bacteria from petroleum spilled soil:

In the laboratory one gram/one ml of sample was transferred into 10 ml sterile water and then further dilutions were made by serial dilution. From the final dilutions 1 ml was transferred into sterile petriplates and media was poured. (All in aseptic conditions) and incubated for 48 to 72 hrs. at 28±2^{0}C. The colonies which appeared were selected and picked up on the basis of their colony characteristics for purification.

The bacteria thus isolated were purified by repeated streaking on isolation media and pure forms were picked up from a well separated colony.

The bacteria isolated were grown on standard bacteriological media (a) common media and (b) selective media/diagnostic media, which provide information for their identification. The following isolation media were used in the present study.

(iii) Isolation media used:

(Number opposite ingredients indicate grams per litre)

Nutrients broth and Nutrient Agar (NB and NA)

Peptone	5.0
Sodium Chloride	3.0
Beef Extract	3.0
Agar	15.0
pH	6.8-7.2

* Do not add agar if nutrient broth is desired.

(iv) Effect of Salt concentration:

The range of salt tolerance by the bacterial isolates was studies using different concentration of the salt Sodium chloride (NaCl) -2.0% to 13.0%.

The salt was mixed in various concentrations to the NA and sterilized by autoclaving at 15 PSI for 15 minutes. After sterilization the media was poured into petriplates (in five replicates) aseptically and were then streaked with the bacterial suspension and incubated for 7 days in B.O.D. incubator at 28±^{0}C. The growth of the bacteria on the media was recorded at the end of incubation period.

In another method the salt was mixed in various concentrations to the nutrient borth, then dispensed in 10ml quantity in tubes and autoclaved. When cooled at room temperature, a loopful of 12 hrs. Old bacterial suspension was added into each tube and incubated over night at 28±^{0}C. The growth of different bacteria was determined in terms of optical density (O.D.)[20].

Observation

The effect of different concentration of sodium chloride on the growth of different bacterial isolates was studied.

Table 1. Effect of concentration (sodium chloride) on growth of bacterial isolates

S. No.	Isolate. No.	Concentration of NaCl in molars and %									
		2	3	4	5	6	7	8	9	10	11
1	I	0.00	0.00	0.04	0.12	0.60	0.00	0.00	0.00	-	-
2	II	-	0.00	0.04	0.06	0.06	0.00	0.00	0.00	-	-
3	III	0.01	0.01	0.04	0.08	0.06	0.01	0.01	0.01	0.01	-
4	IV	-	0.00	0.05	0.12	0.05	0.01	0.01	0.00	-	-
5	V	0.00	0.01	0.02	0.06	0.03	0.01	0.01	-	-	-
6	VI	0.00	0.02	0.03	0.08	0.01	0.00	0.00	-	-	-
7	VII	0.00	0.00	0.02	0.06	0.04	0.01	0.00	0.00	-	-
8	VIII	0.03	0.02	0.01	0.02	0.00	-	-	-	-	-
9	IX	0.01	0.01	0.01	0.06	0.03	0.01	0.00	-	-	-
10	X	0.00	0.02	0.05	0.12	0.05	0.02	0.01	-	-	-
11	XI	0.02	0.02	0.02	0.06	0.02	-	-	-	-	-
12	XII	0.01	0.02	0.02	0.06	0.02	0.01	0.01	-	-	-
13	XIII	0.00	0.05	0.08	0.13	0.09	0.04	0.03	0.01	-	-
14	XIV	0.00	0.00	0.06	0.14	0.06	0.02	0.00	0.00	-	-
15	XV	0.00	0.02	0.04	0.08	0.06	0.02	0.01	0.00	-	-
16	XVI	0.00	0.01	0.03	0.07	0.06	0.04	0.03	0.00	-	-
17	XVII	0.01	0.02	0.04	0.18	0.06	0.04	0.03	0.01	-	-
18	XVIII	0.00	0.01	0.02	0.06	0.04	0.01	0.01	0.00	-	-
19	XIX	0.00	0.04	0.08	0.12	0.04	0.01	0.01	-	-	-
20	XX	0.01	0.06	0.12	0.17	0.06	0.01	0.01	0.00	-	-
21	XXI	0.00	0.03	0.10	0.15	0.06	0.01	0.01	0.01	0.01	-
22	XXII	0.00	0.00	0.03	0.06	0.05	0.01	0.01	0.00	-	-
23	XXIII	-	0.00	0.06	0.06	0.05	0.02	0.01	0.01	-	-
24	XXIV	0.01	0.01	0.02	0.06	0.05	0.01	0.01	0.01	0.01	-
25	XXV	0.00	0.00	0.02	0.06	0.18	0.05	0.05	0.02	0.01	-
26	XXVI	0.00	0.05	0.14	0.08	0.04	0.05	0.01	0.04	-	-
27	XXVII	0.00	0.00	0.00	0.01	0.06	0.05	0.03	0.01	0.01	-
28	XXVIII	0.00	0.01	0.03	0.08	0.04	0.04	0.03	0.00	-	-

Reading Indicate Optical Density Of Medium ;
O.D. Range:- 0.00-0.05 = Little 0.06-0.11 = Moderate 0.12 and above = Luxuriant - = No Growth

Bacteria differed in their abilities to grow and utilize various concentration of sodium chloride. Some isolates exhibit growth even at high concentration of salt (10%) whereas other exhibited moderate to little growth in different salt concentrations.

All the bacterial isolates showed good to moderate growth up to 6% of sodium chloride. Five bacterial isolates namely *Bacillus lecheniformis, Clostridium acetobutylicum, Arthrobacter globiformis, Pseudomonas cepacia and cellulomonas* sp.1 showed growth up to 10% of sodium chloride. *Bacillus mycoides, Flavobacterium ferrugineum, Flavobacterium sp. 1, Vibrio sp., Corynebacterium sp., Pseudomonas avenae, Erwinia rubrifaciens, Pseudomonas pseudomallei, Serriata fonticola, Cellulomonas sp.1, Pseudomonas putida, Bacillus megatherium, Bacillus lecheniformis* tolerated sodium chloride up to 9% some other isolates (*Micrococcus conglomerates, Pseudomonas aeruginosa, Corynebacterium cylitidis, Serriarta proteamaculans, Bacillus subtilis, Escherichia coli, Pseudomonas stutezeri and Arthrobacter flavescens*) were able to tolerate sodium chloride up to 7%.

Results and Discussion

Bacteria differed in their abilities to grow and utilize various concentration of sodium chloride. Sodium chloride appeared to be very favourable for the growth of various bacterial forms.

Table 2. Generic & specific names of bacterial isolate

Isolate no.	Identified Bacteria
I	*Bacillus mycoides*
II	*Bacillus megatherium*
III	*Bacillus lecheniformes*
IV	*Micrococcus conglomeratus*
V	*Pseudomonas aeruginosa*
VI	*Corynebacterium cyslitidis*
VII	*Serriata proteamaculans*
VIII	*Bacillus subtilis*
IX	*Escherichia coli*
X	*Pseudomonas stutezeri*
XI	*Arthrobacter flavescens*
XII	*Xanthomonas fragariae*
XIII	*Xanthomonas campestris*
XIV	*Vibrio sp.*
XV	*Staphylococcus sp.*
XVI	*Corynebacterium sp. 1*
XVII	*Flavobacterium ferrugineum*
XVIII	*Flavobacterium sp. 1*
XIX	*Pseudomonas avenae*
XX	*Erwinia rubrifaciens*
XXI	*Clostridium acetobutylicum*
XXII	*Pseudomonas pseudomallei*
XXIII	*Serriata fonticola*
XXIV	*Pseudomonas cepacia*
XXV	*Arthrobacter globiformis*
XXVI	*Cellulomonas flavigeno*
XXVII	*Cellulomonas sp. 1*
XXVIII	*Pseudomonas putida*

According to some investgaters very little attention was paid on salt tolerance studies of bacteria. They considered sodium chloride tolarance of Pseudomonads as a determanitative character and observed that some species of Pseudomonas are characteristically inhibited by 2% NaCl[21]. A similar result was reported by them from China[22]. In the present study *Pseudomonas stutezeri, Pseudomonas pseudomsllai* and *Pseudomonas putida* tolerated sodium chloride upto 9% concentration indicating that these isolates ware different from those which were isolated by them[21] &[22].They

also noted that no one isolate among xanthomonads grows in 5% sodium chloride broth. In the present study it was observed that *Xanthomonas sp*. tolerated upto 5% concentration. This isolate appear to be different from those xanthomonads reported by them[21.]

Studies have revealed that many organisms are capable of degrading a mixture of hydrocarbons in widely fluctuating salinities and some produce surfactants and also some fix nitrogen thus underscoring the importance of such microbes in the cleanup of contaminated sites. Though appreciable progress has been made recently in understanding diversity of microorganisms responsible for hydrocarbon degradation under aerobic conditions, similar information under anaerobic condition is lacking.

All bacterial isolates showed good to moderate growth upto 6% of sodium chloride *Bacillus lecheniformis, Clostridium acetobutylicum, Pseudomonas cepacia, Arthrobacter globiformis* and *Cellulomonas* sp.1 exhibited growth upto 10% NaCI concentration. It was interesting to note that the bacteria of the soil and water associated with petroleum products tolerated high concentration of NaCI. This may be because of the adaptation, since sodium chloride is an important constituent of vegetable and animal matter. Its presence in high quantities in the environment cannot be ruled out. Therefore the high tolerance abilities are on expected lines.

Conclusion

Lots of microorganisms adapt to petroleum contaminated soil. During this research soil samples were collected from petroleum filling stations and storage tanks, petrol pump and service stations in Ajmer (Rajasthan). A total of 28 bacterial genera were isolated. These genera were – 4 *Bacillus* ,1 *Micrococcus,* 6 *Pseudomonas,* 2 *Corynebacterium,* 2 *Arthrobacter*, 2 *Cellulomonas*, 2 *Serriate,* 1 Vibrio, 2 Flavobacterium, 1 Erwinia, 2 Xanthomonas, 1 Escherichia, 1 Staphylococcus, 1 *Clostridium* . During our research it was found that most of the microorganisms showed optimum growth in saline environments, indicating that they tolerate high salt Concentration.

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