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Isolation and characterization of indigenous Bacteria from contaminated soil samples of Kamrup District, Assam

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Abstract

Soil contains a various number of microorganisms in it. Some of these microorganisms that reside in soil are harmful, while some are useful. The present investigation was carried out to isolate and characterization of bacteria from polluted soil samples. Selected bacterial isolates were evaluated by morphological, and biochemical test. Gram staining of the bacterial isolates was also performed. Based on Gram's staining, 5 bacterial isolates were Gram Positive, while the remaining 3 isolates were Gram Negative. According to Biochemical test (Urease test, Starch Hydrolysis test, Catalase test) done on isolates, Isolate 3 and Isolate 6 showed Negative on all the tests, while Isolate 7 showed positive result only on catalase test. Isolate 1, Isolate 2, Isolate 4, and Isolate 5 showed negative result only on Urease test. While Isolate 8 showed negative result only on catalase test. Out of eight selected bacterial isolates, only Isolate 8 showed positive for Urease test. Antibiotic sensitivity test also showed significant result for the selected isolates. The isolated bacterial strains will be helpful for possible application of bioremediation of contaminated sites.

Key words : Soil pollution, biochemical test, antibiotic sensitivity, Bioremediation.

Soil is a surface material that covers most land, it consist of both organic and inorganic matters. Soil is a home to many microorganisms. It also provides structural support to plants. Microorganisms such as bacteria, fungi, virus, etc. are found everywhere. They are found on water, soil, plants, humans, etc. They can be found in both polluted and unpolluted sites. Some microorganisms can adapt to their environment hence they are found everywhere, even in high temperature sites, and polluted sites, etc [1,29.32].

In earlier studies in microbial ecology

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indicated that number of microorganisms decreased with increasing depth[23,34]. But in recent years it is believed that micro environments are actively inhabited by microorganisms throughout the subsurface[22,30]. Both gram positive bacteria and gram negative bacteria can be found in some polluted areas. Most bacteria are important as they are responsible for various important processes such as breakdown of organic matters, breakdown of environmental toxins, nutrient cycling, etc. While some bacteria are pathogenic, infecting both plants and animals[16,17]. The factors involved in regulating the activity and diversification of soil microorganisms are biotic and abiotic factors. Microbe's presence in soil is based on the existence of ambient conditions provided by the types of vegetation, the texture and chemical nature of the soil, nutrients availability, pH, moisture content, climate, and temperature[18,20,26].

Bioremediation has been recognized as a potential tool by the significant role of bacteria in contaminated soil. Various factors of contaminated soil characterize influence bacterial biodegradation such as pH, electrical conductivity, total nitrogen and heavy metal which are important indicators of soil quality, fertility, and productivity[5,27]. Bacteria degrading hydrocarbons were detected as *Alcaligen* sp, *Bacillus* sp, *Chromobacterium* sp, *Corynebacterium* sp, *Pseudomonas* sp, *Aeromonas* sp, *Serratia* sp, and *Flavobacterium* sp[10].

Microorganisms such as plant growth promoting (PGP) microorganisms which are inhabitants of rhizosphere, root surface and root inner tissues are known for their beneficial effects on plant growth[15,21]. Various types of bacteria are found everywhere. Soil bacteria found in polluted sites may be pathogenic or may not cause any harm. One of the most important method to isolate soil bacteria to know the living cells present in soils is the serial dilution method[3]. A study to isolate bacteria for bioremediation of petroleum soils. They collected soil samples from crude oil and gas oil contaminated soil, and from uncontaminated soil. The bacterial isolate that was identified was mostly *Escherichia coli, Bacillus subtilis, Klebsiealla pneumonia, Pseudomonas aeruginosa,* etc. [11,31].

A study on isolation and characterization of bacteria from Brazilian gold mining area with a capacity of arsenic bioaccumulation. Extraction of gold mineral produces wastes that contribute to environmental contamination by arsenic. They isolated 38 bacterial strains and determined minimum inhibitory concentration (MIC) in solid medium for each strain. Three bacterial strains isolated from the sites were resistant to 3000 mg/L of arsenite. These bacterial strains were identified as *Bacillus cereus* and *Lysinibacillus boronitolerans* by the analysis of 16s rDNA gene sequences[2].

Six bacterial isolates were identified which can degrade diesels belong to *Pseudomonas, Providencia, Roseomonas, Stenotrophomonas, Achromobacter,* and *Bacillus.* Among those six bacterial isolates, based on gravimetric analysis, the three potent isolates showed high diesel degradation efficiency. The most potent bacterial isolated strains were identified by 16s rRNA gene sequences, two of them were revealed as *Pseudomonas aeruginosa,* and other one as *Bacillus subtilis.* They demonstrated in their

study that bacterial isolates from hydrocarbon-contaminated or uncontaminated environments could be optimized as potential bioremediation agents for diesel degradation[4].

Description of the study area :

The study area is located in Guwahati city, Kamrup Metropolitan District, Assam. Kamrup Metropolitan district occupies an area of 1528 sq kilometers. Guwahati city is located at Latitude 26.1158° N and Longitude 91.7086° E. The minimum temperatures are normally around 11.1° C, while the maximum temperature is around 38.4° C. Soil samples were collected from Guwahati city area such as Beltola, Ganeshguri (Figure. 1).

A. Collection of soil samples :

Contaminated soil samples (100-250 grams) were collected from sites from a depth of 0-15 cm from the surface of the soil from Guwahati city (Beltola, Ganeshguri), Assam during the month of December, 2023. Soil samples are denoted by Sample A and Sample B.

B. Cultural and morphological features of the bacteria isolates :

The serial dilution technique is followed for the isolation and enumeration of bacteria. The cultural and morphological features falls under the phenotypic characterisation, which were studied by adopting standard methods[12]. Morphology of colonies on plates is a characteristic feature of bacterium, which is quite useful in preliminary identification procedure. Different colony features such as configuration, elevation, margin, texture, consistency etc. were noted down by using a hand lens.

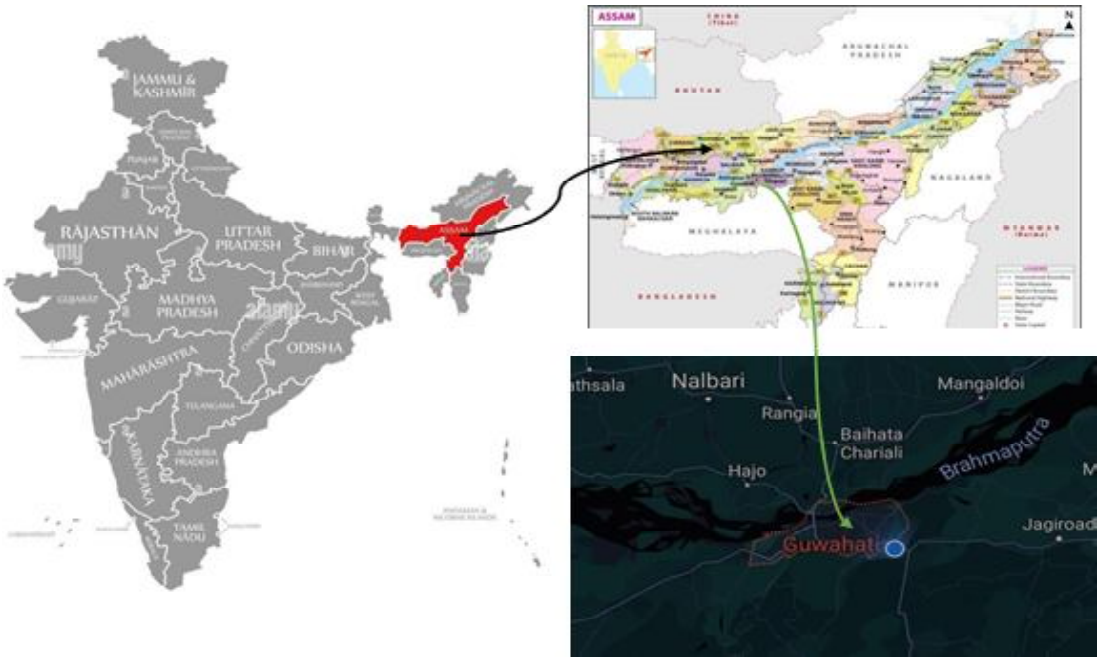


Figure 1: Study site Photo courtesy: Google Source

C. Gram staining :

Gram staining, a differential staining technique separates bacteria into two groups, Gram positive and Gram negative. A thin smear of the bacterial culture was made on a glass slide, after heat fixing it was stained with crystal violet for one minute. After rinsing with water Grams iodine solution was added and kept for one minute. It was washed with water and then decolourised with 95% ethyl alcohol for 20 seconds. Rinsed with water and then counter stained with safranin for one minute. Rinsed with water blotted dried and then observed under the microscope. The nature of Gram's reaction was observed and morphological details were noted down. Cells were identified by the colour observed purple for Gram positive and pink or red for Gram negative cells.

Standard plate count method was used to enumerate the bacterial cultures. The colonies formed after the incubation were counted. The number of bacterial colonies in each was referred to as a colony forming units (CFU). Colonies exhibition good variable growth was selected for further streaking on fresh plates. Further purification and multiplications of isolates were done by streaking on fresh plates. The CFU were determined by the following formula:

CFU/g= Average no. of colonies/ Inoculation volume plated (ml) x Dilution factor

D. Biochemical characterization of bacterial isolates :

(i) Urease Test :

The urease test was performed by inoculating the bacterial isolates into Urease broth. Urea was sterilized by ultraviolet irradiation before adding sterilized medium containing in test tubes and observed after incubation. The indicator's colour changes from clear to pink indicate a positive result due to the accumulation of ammonia which raises the pH of the medium.

(ii) Starch Hydrolysis :

This test is used to differentiate based on their ability to hydrolyze starch with the enzyme alpha-amylase or oligo-1, 6-glucosidase by breaking the glycosidic linkages between the sugar subunits. The bacterial cultures were streaked on the starch agar plates, and incubated at 30^{o} C for 24-48 hours. After incubation, iodine solution was flooded on the petri plates. Iodine reacts with starch and produces a blue or dark brown colour; therefore any microbial growth hydrolysis will be revealed as a clear zone surrounding the growth. The formation of the clear zone around the colony was taken as positivetest.

(iii) Catalase Test :

The enzyme catalase present in some microorganisms breaks down hydrogen peroxide to water and oxygen which helps them in their survival. The bacterial isolates were inoculated into Nutrient Agar (NA) and then incubated for 48 hours at 30+-2.0^{o}C. And after proper growth, catalase production was determined by introducing 5-6 drops of H_2O_2 (20%) into each slides. Release of free oxygen gas (O_2) bubbles indicated a positive catalase test.

(iv) Determination of Antibiotic Resistance :

To determine the antibiotic sensitivity

of the bacterial isolates, antibiotic discs (Hi-media) were placed on freshly prepared lawns of each isolates on Mueller- Hilton Agar plates. The isolates were tested for antibiotic sensitivity according to Kirby-Bauer disc diffusion method to 4 antibiotics. The selected antibiotics were placed on the plates and incubated at 37° C for 24 hours. The diameter of the inhibition zones were measured to the nearest mm and the isolates were classified as resistant (R), intermediate (I), and susceptible (S) following the standard antibiotic disc sensitivity testing method. Discs containing the following antibiotics are used- Gentamicin (50 mcg), Penicillin-G (1 unit), Streptomycin (25 mcg), and Vancomycin (10 mcg).

A total of eight bacterial isolates were selected and purified for further characterization and identification. Colony forming unit (c.f.u) is a measure of viable bacterial colonies. It is calculated by the following formula:CFU/g= Average no. of colonies/ Inoculation volume plated (ml) x Dilution factor.

Colony Morphology :

The bacterial colonies' colour, form, margin, elevation were observed in the culture plates with nutrient agar and recorded (Table-1).

Table-1. Colony morphology of isolated bacterial strains

Soil Samples	Isolate No.	Colony colour	Form	Margin	Elevation
Sample A	Isolate 1	White	Irregular	Lobate	Raised
	Isolate 2	White	Circular	Undulate	Flat
	Isolate 3	Yellow	Irregular	Entire	Convex
	Isolate 4	White	Punctiform	Erose	Raised
Sample B	Isolate 5	White	Irregular	Entire	Flat
	Isolate 6	Pink	Irregular	Undulate	Raised
	Isolate 7	Opaque	Rhizoid	Undulate	Pulvinate
	Isolate 8	Yellow	Circular	Entire	Convex

Table-2: Biochemical characteristics of isolated bacterial strains

Samples	Isolates	Gram staining	Urease test	Starch Hydrolysis	Catalase Test
Sample A	Isolate 1	+ (Rod-shaped)	-	+	+
	Isolate 2	+ (Rod-shaped)	-	+	+
	Isolate 3	- (Cocci)	-	-	-
	Isolate 4	- (Cocci)	-	+	+
Sample B	Isolate 5	- (Cocci)	-	+	+
	Isolate 6	+ (Rod-shaped)	-	-	-
	Isolate 7	+ (Rod-shaped)	-	-	+
	Isolate 8	+ (Rod-shaped)	+	+	-

Gram staining were done on bacterial isolates for the morphological identification. It was found that 5 of the isolates were Gram Positive, while rest of the 3 isolates was Gram Negative (Table-2). Based on Gram Staining Test, the bacterial isolates were identified as Gram Positive and Gram Negative. For further characterization, different biochemical test were performed to identify the bacterial isolates. The results of the bacterial isolates are given in the table below. Bacterial strains Isolate 1, Isolate 2, Isolate 4 and Isolate 5 showed negative result on urease test, and positive for Catalase Test and Starch hydrolysis. Isolate 3 and isolate 6 showed negative on all tests. Only Isolate 8 showed positive test for Urease test (Table-2).

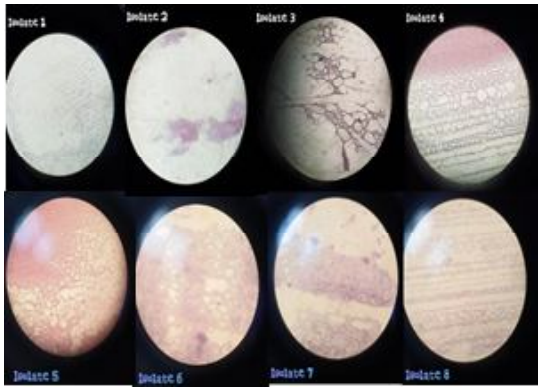
Antibiotic Sensitivity Test :

The bacterial isolates are tested for Antibiotic sensitivity. Most of the bacterial strains isolated are resistant to Streptomycin and Gentamicin. On the other hand, almost all the isolate showed no inhibition to Penicillin. The antibiotic zones were evaluated by Antibiotic zones scale and recorded. The recorded inhibition zones were measured to identify the susceptible, intermediate and resistance nature of all bacterial isolates (Table 3).

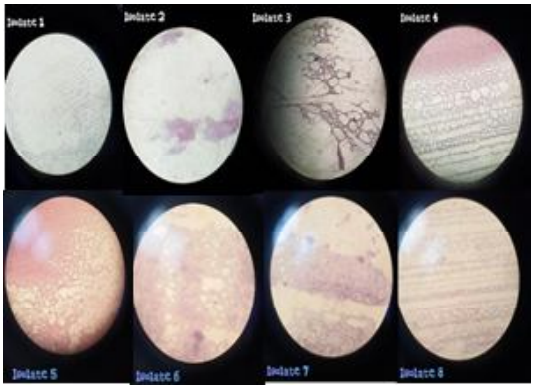
Table-3: Antibiotic sensitivity test of isolated bacterial strains

Samples	Isolates	Gentamicin	Penicillin	Streptomycin	Vancomycin
Sample A	Isolate 1	22 mm (I)	11 mm (S)	20 mm (R)	18 mm (I)
	Isolate 2	20 mm (I)	NI	18 mm (R)	17 mm (I)
	Isolate 3	24 mm (R)	NI	23 mm (R)	21 mm (R)
	Isolate 4	24 mm (R)	NI	24 mm (R)	20 mm (R)
Sample B	Isolate 5	23 mm (R)	NI	17mm (I)	10 mm (S)
	Isolate 6	22 mm (I)	NI	18 mm (I)	11 mm (S)
	Isolate 7	22 mm (I)	NI	14 mm (I)	NI
	Isolate 8	23 mm (R)	NI	16 mm (I)	NI

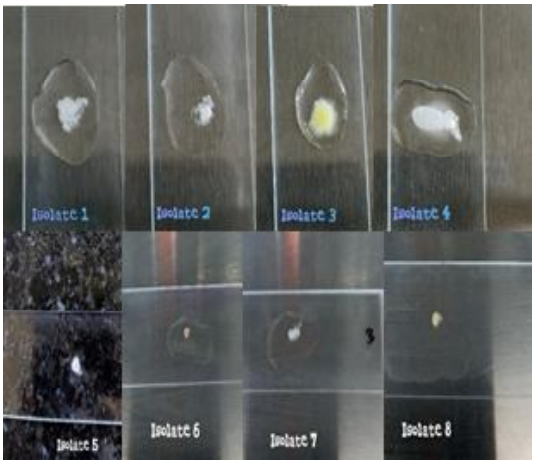
NI=No Inhibition; S= Susceptible; I= Intermediate; R=Resistant



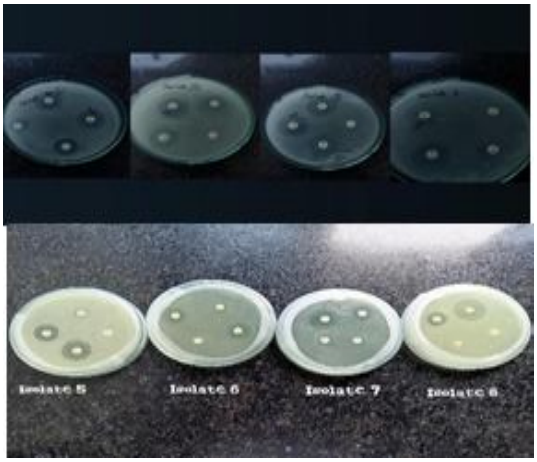
PHOTOPLATE 2: Gram Staining



PHOTOPLATE 3: Starch Hydrolysis test



PHOTOPLATE 4: Catalase Test



PHOTOPLATE 5: Antibiotic sensitivity test

Soil pollution is inevitable in these modern days. Microorganisms play a crucial role in bioremediation process for sustainable development[7,9,13,33]. There are vast numbers of soil pollutants including food wastage, industrial waste, oil spillage, household wastes, etc [19,24].

Numerous studies are being done on bacteria from polluted sites. Morphological, biochemical test are being done to characterize the bacterial strains. Bacteria found in contaminated soils are studied upon for various purposes including biodegradation of kerosene, derivatives of kerosene, their tolerance against heavy metals, bioremediation, etc [8,14,25]. Bacterial strains isolated from oil polluted sites showed the potential of TPHs biodegradation[6,11,28]. Two samples were collected from polluted soil sites: Sample A (Beltola) and Sample B (Ganeshguri). The samples are isolated on Nutrient Agar medium by Serial dilution method, and incubated. After incubation, the CFU of bacterial isolates were counted and recorded (Table-1). From the recorded table, it was observed that Sample B had high bacterial growth. Morphology of isolated colonies are also observed and noted (Table-2), it showed various forms (irregular, circular, punctiform, rhizoid), margins (lobate, undulate, entire, erose), and elevations (raised, flat, convex, pulvinate).

Gram staining of the bacterial isolates showed 5 positive, rod shaped (bacilli), and 3 negative, cocci shaped (Table-3). Isolate 1, Isolate 2, Isolate 6, Isolate 7, and Isolate 8 showed in the result as gram positive (rod - shaped). While Isolate 3, Isolate 4, and Isolate 5 showed gram negative (Cocci) in the tests. Photos of the Gram's staining result showing gram positive and gram negative is also given (Photoplate 2).

Biochemical tests were also performed on the bacterial isolates (Table-3). Isolate 3, and Isolate 6 showed negative result on all the biochemical tests (Urease test, Starch hydrolysis test, Catalase test), which means they do not produce urease and catalase enzyme and does

not contain alpha-amylase. Isolate 1, Isolate 2, Isolate 4, and isolate 5 showed negative result on urease test and showed positive result on starch hydrolysis test and catalase test, which means that these bacteria canproduce catalase enzyme and contain alpha-amylase. Isolate 7 showed positive result on Catalase test, and showed negative result on Urease test and Starch hydrolysis test, which means it can produce catalase enzyme. Isolate 8 is the only bacterial isolate from the selected bacterial isolates that showed positive result on urease test i.e. the bacterial strain can produce urease enzyme. It also showed positive result on starch hydrolysis test; and negative result on Catalase test[4] (Table-3; Photoplate 3).

Antibiotic sensitivity to some antibiotics is also performed on bacterial isolates. Antibiotic disc were placed on freshly prepared lawn of each isolates on Mueller-Hilton Agar plates. The bacterial isolates were tested sensitivity to Gentamicin, Penicillin, Streptomycin, and Vancomycin. The antibiotic sensitivity zone was observed and noted. The zones were calculated by Antibiotic zone scale and noted in table-3, for both samples A and sample B (Photoplate 4). Almost all the bacterial isolate showed no inhibition to Penicillin, only Isolate 1 was susceptible to it. Most of the selected bacterial strains isolated from the soil sample are resistant to Gentamicin and Streptomycin. The isolated bacterial strains will be further analyzed and characterized by 16s RNA method to get the accession number from NCBI.

Soil bacteria found in contaminated soils are mostly pathogenic in nature. There are some bacteria which are useful to us. Some bacteria found in contaminated site can be altered and used in bioremediation, while some bacteria help in biodegradation toxic metals, chemicals, etc. The isolated bacterial strains will be helpful for possible application of bioremediation of contaminated sites.

Declaration of interest :

Authors declare no conflict of interest.

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