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Isolation and Biochemical Identification of Soil Bacteria from North Eastern Hill University, Shillong

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Abstract: One of the richest habitats for microorganisms is soil due to its complex composition and availability of nutrients which has allowed for the greatest microbial diversity. This diversity is a blessing and a curse, as along with beneficial and non pathogenic microorganisms, it is also a hub for pathogenic microbes to thrive. In this diverse microflora, a plethora of microorganisms are yet to be discovered and understood. Due to the ever-changing climate conditions, global warming, pollution, erosion etc, there may be change in the microbial diversity in soil of a particular area and may lead to infection and spread diseases. 4 different isolates were obtained from soil sample collected from the North Eastern Hill University campus, Shillong, Meghalaya and biochemically identified. 3 out of 4 isolates were identified to be pathogenic in nature. This paper aims to highlight the importance of estimating the soil microorganisms, understand their functions and make aware about the lurking dangers in our surroundings.

Keywords:- *Soil, pathogen, microorganism.*

I. INTRODUCTION

Soil is a rich habitat for various organisms. One of the most important habitants of soil is the microbial community. The greatest microbial diversity is found in soil out of any other environment. The diversity of microbes in a particular region and its activity is mainly regulated by the climate, vegetation and soil properties (Jha *et al*., 1992). The combined action of colonization of geologic material by micro flora and weathering results in soil formation. It is also a favourable habitat for microbe proliferation and growth. Typically, the number of micro-organism in soil habitats is10^6 to 10^9/g and is usually much greater compared to that in fresh water or marine habitats (Atlas, 1998).

The availability of nutrients corresponds to the diversity in microbes and their concentration (Willey *et al*., 2008). The biological balance in the life of our planet is maintained by the presence of microorganisms. Depending on soil conditions, bacteria, fungi and viruses can be found in varying amounts. The relative abundance of microbes is dependent on the degree of acidity and the types of residue added. The bacterial concentration determines the fertility of soil and organic matter accumulation within a short span of time (Kummerer, 2004).

Microbial primary and secondary metabolites in soil are extremely beneficial to increase the nutrient contents in soil, promote plant growth and play a very important role in nutritional chains (Paul and Clerk, 1966; Kummerer, 2004). For sustainable development of the biosphere and balancing biogeochemical cycles, microorganisms play a crucial role (Diaz, 2004).They have also been reported to produces and consume three major naturally occurring green house gases that distinctly influence agriculture (Levine et al., 2011)

Many disease causing microorganisms or their vectors live in or spend part of their life cycle in soil. This often occurs in disturbed soil ecosystems where pathogens directly or indirectly (i.e., through direct contact or vectors) enter the human system and cause disease. The ever increasing land use ,felling of trees, changing climates, and the current coronavirus-2 (SARS-CoV-2) pandemic, one may ponder the origin of the next pandemic and what role soil might play (Lal R, 2020, Lal R et al., 2020, Gomiero T ,2016)

A quantitative and qualitative estimation of soil microorganisms is therefore necessary to know the role of different microbes and to detect pathogenic organisms. In this study, soil sample was collected from the North Eastern Hill University campus, Shillong, Meghalaya. Four different bacteria were isolated and biochemically identified.

II. STUDY AREA

East Khasi Hills is an administrative district in the state of Meghalaya in India. The district headquarter is located at Shillong. The district occupies an area of 2752 km² and has a population of 825,922 (as of 2011). East Khasi Hills District forms a central part of Meghalaya and covers a total geographical area of 2,748 km^2. It lies approximately between 25°07" & 25°41" N Latitude and 91°21" & 92°09" E Longitude. The present study considered the isolation and characterization of bacteria from soil collected from North Eastern Hill University, Shillong.

III. METHODOLOGY

A. Sampling

Soil sample was collected from the North Eastern Hill University campus using sterile disposable gloves and autoclaved sampling bottles. Samples were transferred to a sterile flask and standard method was followed for isolation of bacteria.

B. Isolation of bacteria

Serial dilution of the samples was done using sterile test tubes which were labelled 10^{-1} to 10^{-6} dilutions. 9 ml of 0.85% NaCl were then measured into the seven test tubes. 1 ml of sample solution was introduced into the first test tube labelled 10^{-1} and mixed thoroughly and 1 ml was taken from the first test tube and transferred to the second test tube labelled 10-2. This was continued until the 10^{-6} dilution was

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obtained. 0.1ml of samples from 10-1 and 10-2 dilutions were inoculated on each nutrient agar plates by the spread plate technique. The plates were then incubated at 37°C for 18-24 h. 1g of soil was dried in the hot air oven for 24 hours and measured. The growing colonies on the plates were recorded as colony forming unit (CFU) (Brown, 2009) and the number of bacteria in the samples was expressed as CFU/g which was calculated as CFU X Dilution factor/ Volume of sample X Dry weight of soil.

C. Pure culture generation

Based on the colony morphology, 4 of the most prominent bacterial colonies from sample were isolated using sterile loop and streaked into nutrient agar plates. Resultant single distinct colonies were sub-cultured in slants and maintained at 4°C for further studies.

D. Differential staining using Gram's stain

Gram's staining technique was used to find out the Gram staining nature of the bacterial isolates. A loopful of fresh overnight grown bacterial culture was smeared on a clean slide and then allowed to air dry. The smear was then heat fixed by passing the glass slide over a flame. Few drops of primary stain i.e., crystal violet was added and left for 60seconds and then washed with distilled water. Gram's iodine, a mordant was added for 1 minute and the slide was washed away with Gram's decolourizer (ethyl alcohol 95%). Finally, safranin, counter stain was added for 1 minute which was then washed with distilled water. The glass slide was left to air dry and examined under the microscope.

E. Biochemical characterization

a) Catalase test:
This is a test to ascertain the ability of bacteria to produce catalase that reduces hydrogen peroxide to water and oxygen. Growth of samples were scraped with wired loop. It was then suspended in a drop of 3% H_2 O_2 on a slide, then examined for bubble formation. If effervescence occurs, it is confirmatory positive test for catalase production, but if it does not occur it is negative test for catalase production.

b) Oxidase test:
This test depends on presence of certain oxidases in bacteria that will catalyse the transport of electrons between electron donors in the bacteria and a redox dye- tetraethyl p-phenylene-diamine which is reduced to a deep purple if positive. A strip of filter paper was moistened with freshly prepared 1% solution of the reagent. Immediately, a speck of culture was rubbed on it with a loop. Positive test is indicated with an intense deep purple blue within 10-60 seconds. No colour change after 60 seconds indicates a negative result.

c) Citrate Utilization test:
Citrate is acted upon by enzyme citrase which produces oxaloacetic acid and acetate. These are enzymatically converted to pyruvate and CO_2. During reaction, the medium becomes alkaline as the CO_2 combines with Na and H_2 O to form sodium carbonate which is alkaline. Simmon's citrate medium slants were prepared. Samples were inoculated into 86 agar slants and incubated for 24-48 hrs at 37°. Positive result is indicated by blue colour slope and no colour change indicates a negative result.

d) Triple Sugar Iron (TSI) test:
This test depends on ability of bacteria to ferment lactose, sucrose and glucose and the production of hydrogen sulphide. TSI agar medium was prepared, dispensed in test tubes, sterilized and allowed to set as slopes. Slants were inoculated with samples and incubated for 18-24 hours at 37°C. Yellow butt, red slant indicates positive glucose fermentation. Yellow butt, yellow slant indicates positive lactose and/or sucrose fermenting. Red butt, red slant indicates neither glucose, lactose, sucrose fermenting. Black precipitate at bottom of slant indicates H_2 S production.

e) Methyl red test:
This test detects ability of microbes to oxidise glucose with production and stabilization of high concentration of acid end products. MR-VP broth was prepared, sterilized and inoculated with samples and incubated for 48 hours at 37°C. Following incubation, 5-6 drops of methyl red solution was added. Bright red colour change indicates positive result, red- orange colour indicates a weak positive result and yellow- orange indicates a negative result.

f) Voges-Proskauer test:
The Voges-Proskauer (VP) test is used to determine if an organism produces acetyl methyl carbinol from glucose fermentation. If present, acetyl methyl carbinol is converted to diacetyl in the presence of ∝-naphthol, strong alkali (40% KOH), and atmospheric oxygen. MR-VP broth was prepared and inoculated with samples and incubated for 48 hours at 37 °C. Following inoculation, Barritt's reagent A and Barritt's reagent B was added to the broth in a 3:1 ratio. Tubes were shaken at intervals to ensure maximum aeration. A positive result is indicated by the development of a pink colour in 2.5 minutes, becoming crimson in 30 seconds and no colour change indicates a negative result.

IV. OBSERVATION AND RESULTS

A. CFU calculation:

Serial dilutions of the samples at 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilutions were prepared. Countable colonies were observed only in the 10^{-1} and 10^{-2} plates and considered for bacterial enumeration. Dry weight of soil after 24 hours was found to be .6 grams from the initial 1 gram. The dilution plates and the streak plates are presented in the Fig 1A and Fig 1B respectively. The summary of the CFU obtained is shown in Table 1.

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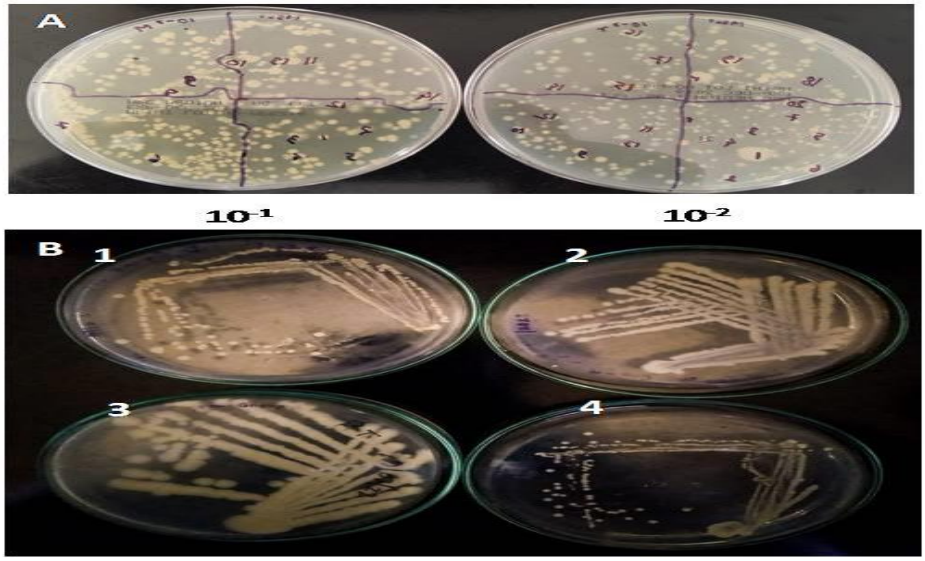

Fig 1: (A) CFU of soil samples (B) Streak plates of isolates

Dilution	Number of colonies
10^{-1}	172
10^{-2}	39
10^{-3}	TFTC
10^{-4}	TFTC
10^{-5}	TFTC
10^{-6}	TFTC
Sample	CFU/g
Soil sample	11,180 CFU/g

Table 1: Summary of CFU obtained for soil sample

B. Gram staining

In this study, out of the total 4 isolates obtained from 1, 2 isolates were found to be Gram negative and isolates 3 and 4 were found to be Gram positive. The colony morphology and the Gram nature of the isolates are shown in Fig 2 and Table 2.

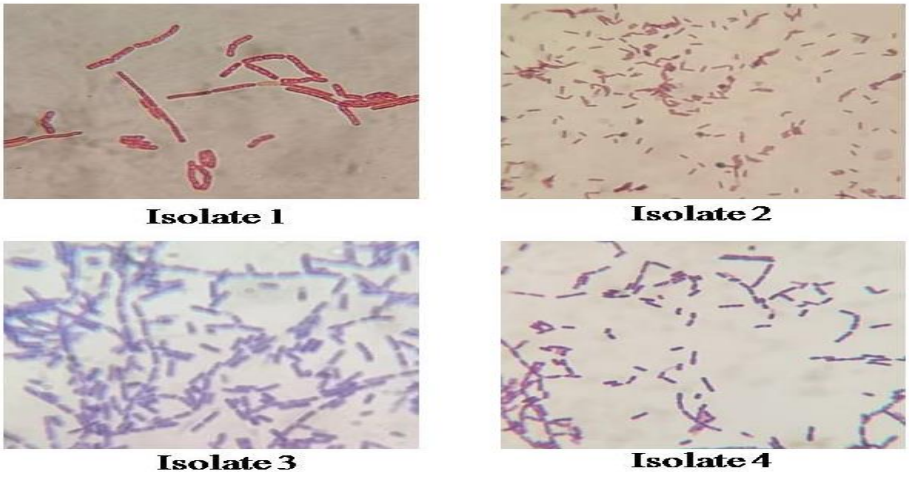

Fig 2: Colony morphology and Gram staining of the isolates

Isolate	Gram positive	Gram negative	Morphology
1	-	+	Bacilli
2	-	+	Bacilli
3	+	-	Bacilli
4	+	-	Bacilli

Table 2: Colony morphology and Gram nature of the isolates

C. TSI test:

The samples were observed after incubation for 18-20 hours at 37 ° C. The isolates 1 and 3 showed yellow butt-red slant and are glucose fermenting. Yellow butt-yellow slant was observed for isolates 2 and 4 indicating lactose or sucrose fermenting. All 4 isolates were found to be TSI positive.

D. Methyl Red Test:

After incubation at 37o C for 48 hours, methyl red was added into each inoculation. The isolates 1, 3 and 4 gave bright red colour that indicated a pH of 4.2 or less and hence the samples are methyl red positive. The isolate 2 showed yellowish orange colour indicating they were methyl red negative.

E. VP Test:

The Voges-Proskauer test detects the presence of acetoin, a precursor of 2,3 butanediol. No isolates indicated colour change colour that indicated the absence of acetoin and hence were VP negative.

F. Catalase and oxidase assay:

All 4 isolates gave positive results that indicated these bacteria producing catalase enzyme that rapidly degraded H_2O_2. Isolates 1, 3 and 4 gave oxidase positive results. This indicated that these samples produced cytochrome oxidase enzyme that catalysed the oxidation of reduced cytochrome by molecular oxygen forming H_2O or H_2O_2.

G. Citrate Assay:

In absence of glucose or lactose, some microbes use citrate as carbon source which depends on presence of citrate permease enzyme. All 4 samples containing isolates appeared blue, indicating these microbes were citrate positive due to their ability to produce citrate permease enzyme.

The summary of the results of biochemical test carried out for the isolates are presented in Table 3 and Fig 3.

Sample	Catalase test	Oxidase test	TSI assay	Methyl red test	Citrate assay	VP test	Probable Bacteria
1	Positive	Positive	Positive	Positive	Positive	Negative	*Salmonella enterica*
2	Positive	Negative	Positive	Negative	Positive	Negative	*Klebsiella pneumoniae*
3	Positive	Positive	Positive	Positive	Positive	Negative	*Bacillus smithii*
4	Positive	Positive	Positive	Positive	Positive	Negative	*Paenibacillus konsidensis*

Table 3: Summary of biochemical tests performed and identified bacteria

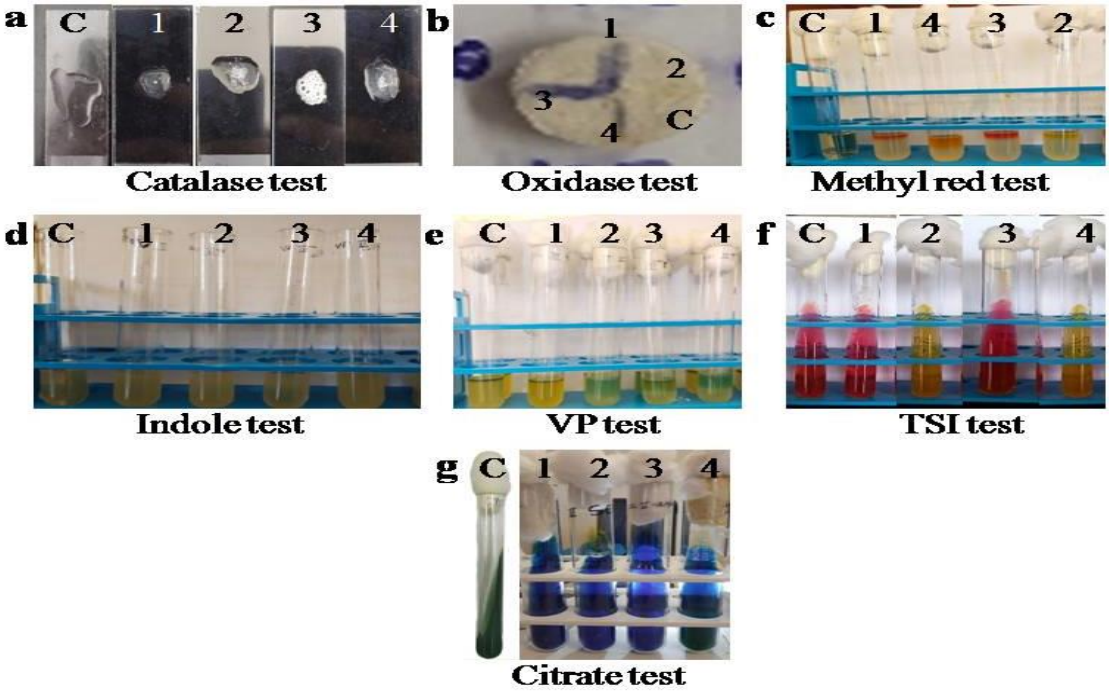

Fig 3: Representative pictures of the biochemical tests (C) control (1) Isolate 1 (2) Isolate 2 (3) Isolate 3 (4) Isolate 4

Bacteria	**Possible diseases**
Salmonella typhi	Salmonellosis, typhoid fever
Klebsiella pneumoniae	Urinary tract infection, pneumonia, meningitis, bloodstream infection
Paenibacillus konsidensis	Bacteremia

Table 4: Pathogenic isolates identified biochemically and their probable diseases

V. DISCUSSION

Soil is a rich habitat for various microorganisms, both beneficial and harmful. Primary and secondary metabolites released by bacteria in soil are beneficial to increase the nutrient contents in soil, promote plant growth and also play a major role in nutritional chains. While pathogenic strains are commonly less present as compared to non pathogenic strains, disturbance of the microfloral habitat by human actions such as littering, defecation by animals, climate change etc may lead to accumulation of infectious microorganisms. Out of the 4 isolates, 3 were identified as *Salmonella typhi*, *Klebsiella pneumonia* and *Paenibacillus konsidensis* which have the capability of causing diseases in humans. The possible disease these strains may cause is highlighted in Table 4.

This paper aims to highlight the importance of estimating the microbial community of an area, to understand their function and to detect emergence of pathogenic strains in the vicinities of educational institutes where hundreds of students, faculty and staff alike may come into contact with these microbes and may unknowingly transmit the disease around the campus.

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