CHAPTER-III

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Material and methods

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3.1 Sampling available

Sites

Total 4 regions were selected from desert Rajasthan i.e. 1. Marwar region (Barmere, Jaisalmer, Jodhapur and Nagaur), 2. Bikaner region (Bikaner, Shri Ganganagar and Hanumangarh), 3. Shekhawati Region (Churu, Jhunjhunu and Sikar) and 4. Ajmer region (Jaipur, Ajmer and Pali).

Soil samples were collected from every district (13 Districts) and 65 samples of soil were collected.

3.2 Procedure of sampling

Soil samples

Surface soil (30 cm x 30 cm x 10 cm) was collected randomly from five to seven places at each site after removing the surface debris. These were mixed thoroughly to prepare a composite sample. After pulverization and drying in shade, samples were preserved in polythene bags at low temperature (4°C) for further investigation. While sampling, the vegetation cover, encrustation and visible coarseness of texture were also noted.
3.3 Physicochemical attributes

a. pH

pH of soil suspension (prepared by mixing soil and distilled water in the ratio of 1:2.5 (soil:water) and shaking intermittently for 1 h) was measured with Elico Water Quality Analyzer PE 136.

b. Electrical conductivity

Electrical conductivity of saturation extract of soil samples was recorded with Elico Water Quality Analyzer PE 136. Potassium chloride (0.01N) was used as standard reference solution that had an electric conductivity of $1411.8 \times 10^{-6}$ mho cm$^{-1}$ at 25°C.

c. Total nitrogen

Total nitrogen content of soil samples was estimated using Kjeldahl method as modified by Jackson (1962). Pelican KEL- PLUS Version 0.1 was used for digestion and distillation.

d. Available Phosphorus

Soil and water in the ratio of (1:5) were taken in conical flask and kept for mixing on shaker for an hour. The suspension was centrifuged at 5000 rpm for 20 min. Supernatant was collected and passed through Whatman filter paper No. 1. Ten ml of filtrate was collected in a test tube. Available phosphorus was then estimated after Maiti (2001).
e. Available organic carbon

Available organic carbon content of soil was estimated using dichromate method after Walkley and Black (1934).

3.4 Bacterial studies

a. Morphotypic diversity

Diversity of total and in normal conditions of soil samples was determined by enrichment culture technique as described below:

i. Sterilization

The glassware were rinsed with chromic acid and washed in running tap water. Before use, these were rinsed twice with tap water and thrice with distilled water and were dry sterilized at 160° C for 2 h in a hot air oven. Culture medium was sterilized at 15 lb pressure (121° C) for 20 min. in an autoclave.

ii. Enrichment

Bacteria were isolated from soil samples (collected from arid region of Rajasthan). A soil sample was suspended in 1 ml of distilled water. This
suspension was diluted 1:1000 with distilled water and 10 ml of this diluted suspension was inoculated onto a nutrient agar medium (NA Medium) agar plate. The NA agar plate was incubated at 37°C until some colonies had formed. Bacteria that formed colonies were isolated. After single-colony isolation, these bacteria were preserved at 4°C.

iii. Incubation and Identification

The cultures were incubated till the appearance of good growth (24-48 h. in NA medium. Bacterial morphotypes appearing in the enrichment cultures were examined by Gram staining under microscope and identified using taxonomic keys after Smith (1950), Prescott (1982) and Desikachary (1959) with biochemical and molecular techniques. Table 3.2 shows the composition of NA medium.
Table 3.1 Composition of NA medium (Pelzare et al., 1986)

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Ingredients</th>
<th>Gms/Litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Beef Extract</td>
<td>10.0</td>
</tr>
<tr>
<td>2</td>
<td>Peptone</td>
<td>5.0</td>
</tr>
<tr>
<td>3</td>
<td>Sodium Chloride</td>
<td>5.0</td>
</tr>
<tr>
<td>4</td>
<td>Agar</td>
<td>15</td>
</tr>
<tr>
<td>5</td>
<td>Final pH (at 25°C)</td>
<td>7±2</td>
</tr>
</tbody>
</table>
b. Isolation and purification of bacterial strains

For isolation, serial diluted samples of bacteria from enrichment culture were taken and inoculate on sterilized NA medium in a petri plates. The petriplates were incubated in incubator at 37°C for 24 h. Plates were examined periodically for the appearance of bacterial colonies. Discrete and morphologically different colonies were picked up with the help of stereomicroscope and were streaked on fresh agar plates for further purification. This was repeated thrice to obtain pure bacterial cultures. After isolation, the identity of the culture was further established using taxonomic keys.

c. Maintenance of cultures

Cultures were maintained and periodically sub cultured on agar slants in screw capped culture tubes under aseptic conditions, incubated at 37°C and then preserved in refrigerator at 5 ± 1°C.

3.5 Characterization of isolates from soil

Pure cultures of bacterial colonies were characterized by the help of microcopy for colony morphology of bacterial cells (color, shape and texture) and Gram’s staining with help of microscopy. Single isolated colonies were picked for the examination of morphological characters of bacterial colonies.
Isolated bacterial colonies were tested further biochemical characterized by Voges proskauer, Indole, methyl red, Triple sugar Iron Agar (TSI) test, Catalase test, Urease test and citrate utilization test (IMViC test).

**Analytical techniques**

### 3.6 Biopolymer production

Bacterial samples were maintained by frequent sub culturing on nutrient agar slants under refrigerated conditions. Biopolymer was extracted from these bacterial samples (Almeida et al 2004, Wang et al 2011)

Subculture organism in nutrient broth medium

↓

Inoculate the 1ml. culture in sugar containing medium

↓

Allow it to overgrown
Homogenize

Take 1 ml sample

Estimate intracellular protein content for supernatant

Add pre-chilled acetone (3:1)

Freeze overnight
Biopolymer well be precipitate
And centrifuge

Take precipitate and
dissolve in 1ml. D/W

Take 5 ml. samples
and estimate total
carbohydrates

Take 0.5 ml. sample
and estimate total
protein
(dilute 10 times)
3.7 Estimation of Total protein (Lowry et al., 1951)

Reagents:

a) Alkaline sodium carbonate solution: - 10.0 g of sodium carbonate was dissolved in little quantity of distilled water. After adding 2.0 g of sodium hydroxide to it, the volume was made up to 500 ml.

b) Copper sulfate solution: - 5.0 gms of copper sulfate (CuSO$_4$.2H$_2$O) was dissolve’d in 100 ml distilled water.

c) Sodium potassium tartarate solution: - 10.00 gms. of sodium-potassium tartarate was dissolved in 100 ml distilled water.

d) Copper sulfate-sodium potassium tartarate solution:-One part of each of the reagent 'b' and reagent 'c' were mixed and diluted with eight parts of distilled water. The solution was freshly prepared and used.

e) Alkaline reagent:-Prepared by mixing 50 ml reagent 'a' and 1 ml of reagent 'd'. This was also prepared a fresh before use.

f) Folin-Ciocalteau reagent:-The reagent was a mixture of sodium tungstate(NaWO$_4$.2H$_2$O) and sodium molybdate (Na$_2$MoO$_4$.2H$_2$O) in phosphoric acid and sulfuric acid. The reagent was diluted with distilled water to 1 N strength and confirmed by titration against 1 N sodium hydroxide to a phenolphthalein end point.

g) Trichloro acetic acid: - 6 % W/V
h) Standard protein solution: Bovine serum albumin (BSA) stock (1 mg/ml) stored in a freezer.

In 1 ml of homogenized bacterial suspension proteins were precipitated by suspending the culture in 10 ml of 6% hot (60°C) TCA for 1 min. TCA was removed by filtering the suspension through a sintered filter by suction and the filtrate discarded. Protein thus precipitated was dissolved in 4.5 ml of hot (55°C) reagent 'e' and allowed to stand for 3 min. After the reaction, the filtrate was collected in a test tube by suction and volume made up to 5 ml by adding reagent 'e'. Then 0.5 ml of reagent 'f' was added, mixed rapidly and allowed to stand for 30 min at room temperature. Absorbance was read at 660 nm using a mixture of reagent 'e' and 'f' as blank. Total protein content was estimated from a standard curve prepared by using graded concentration (20-200 μg) of standard bovine serum albumin solution.

3.8 Estimation of Carbohydrate (Roe, 1955)

Reagents:

(a) Anthrone reagent: 100 mg of anthrone and 1 g thiourea were dissolved in 100 ml of 75 % sulfuric acid. The mixture was kept on a water bath at 85°C to dissolve the ingredients completely. The reagent was prepared fresh.

(b) Standard glucose solution: 100 mg glucose dissolved in 100 ml of distilled water to achieve 100 g ml⁻¹. This solution was used to prepare graded concentrations of glucose standard curve.
In 1 ml of homogenized algal/cyanobacterial suspension were mixed with 4 ml of anthrone reagent to each tube and shaken gently. Kept the test tubes in boiling water bath for 15 min. with an aluminium foil wrapped on the mouth of each tube to prevent evaporation. Then tubes were cooled in running tap water. Absorbance was read at 620 nm using a mixture of distilled water and anthrone as blank. Total carbohydrate content was estimated from the help of standard curve, prepared by using graded concentrations (10-100 μg) of standard glucose solution.

### 3.9 Viscosity

Viscosity was measured by Oswald viscometer in reference of distilled water using following relation:

\[
\frac{\eta_1 \cdot t_1 \cdot d_1}{\eta_2 \cdot t_2 \cdot d_2} = 1
\]

\( \eta_1 = \) viscosity of distilled water

\( \eta_2 = \) viscosity of sample

\( t_1 = \) time of distilled water (sec.)

\( t_2 = \) time of sample

\( d_1 = \) density of distilled water

\( d_2 = \) density of sample
Finally effect of days on growth and polymer production was done using sugar containing media and growth was estimated as intracellular protein content. Biopolymer was characterized for protein, carbohydrate and viscosity of the supernatant was measured. The biopolymer was characterized for composition after derivitization by Gas chromatograph and Viscometer. Effect of growth was studied at days 5, 8, 10, and 12 in sugar containing medium.

3.10 Fatty acid methyl ester (MIDI, 2001)

FAME Profile:-

Preparation of sample (for soil isolates)

Cell paste 40-50mg fresh weight of soil isolates and 200mg dry weight of plant material were taken for fatty acid analysis.

Saponification

Add 40-50 mg of fresh weight/200mg dry weight of cell paste were taken into a screw capped (Teflon lined) GC vial. Add 1 ml of reagent A in GC vials. The pellet was mix well by vortexing. The vials Incubated in a waterbath at 90°C for 45 min with intermittent shaking. Cool the vials to room temperature after incubation.
Methylation

Add 2 ml of reagent B. Seal and mix by end-to-end shaking. Incubate in a water bath at 80°C with intermittent shaking for 10 min. Cool immediately in an ice bath with intermittent end-to-end shaking for 10 min.

Extraction

Add 0.5 ml of freshly prepared reagent C and mix gently. A clear upper layer will form. Allow air bubbles to rupture, then transfer upper layer with a Pasteur pipette to a fresh GC via

I. Base wash

Now add 3 ml of reagent D. Shake end-to-end for 5 min and allow bubbles to settle. Collect 1/3rd of the upper layer with the help of a Pasteur pipette in a separate storage vial.

Gas Chromatography

Gas chromatograph equipped with FI detector and SS column (8’ long, 2mm internal diameter, 1/8” filled with SP 2300+2310 on Ch. W.H.P. support of 100-120 mesh). Set initial oven temperature to 70°C, final oven temperature 230°C, Programming rate 80°C min-1, detector, and injector temperatures to 240°C, attenuation 1X and sensitivity to 1000X. Nitrogen was used as carrier gas at a flow rate of 30 ml min-1. Inject 5.0µl sample.
Reagents:

**Reagent A:** Dissolve 45 gm anhydrous NaOH in 150 ml methanol and then make up the volume to 300 ml with double distilled water.

**Reagent B:** Add 275 ml methanol to 325 ml 6N HCl.

**Reagent C:** Add equval volume of methyl tert butyl ether and n-hexane (Highly volatile. Prepare fresh in glass vials, immediately before use).

**Reagent D:** Add 10.8gm NaOH to 900 ml of double distilled water and make up 1000ml final volume.

### 3.11 Analysis of fatty acids

AIMIL Nucon 5700 GC chromatograph equipped with FID was used to determine fatty acid methyl ester profiles at column temperature 220°C, injector and detector 250°C and oven programmed from 70-230°C with rise of 8°C per min. The column was 1/8", 8’ long, 2 mm internal diameter filled with liquid phase SP 2300+2310 on Ch. W. H. P. support of 100-120 mesh. Nitrogen (carrier gas) and hydrogen for flame were passed at a flow rate of 30 ml min⁻¹.
Cultures were grown on peptone agar for 48 hrs at 37°C.

↓

Took 40 mg of bacterial Biomass in GC Vial

↓

1 ml of reagent A added and mix well

↓

Water bath treatment for 45 min at 85-90°C

↓

Cool it at room temperature

↓

2 ml of reagent B added in the sample

↓

Heat in water bath at 80°C for 10 min

(Temperature and time sensitive step)
Cool in ice bath immediately

Add 0.5 ml of reagent C

Rotate end over end for 10 min

Separate upper organic layer

Add a 5µl of methyl stearate as internal standard (1 mg/ml)

Add 3 ml of reagent D for base wash

Shake for 5 min
Extract upper organic phase by pasture pipette

Store in vials

Use in GC

3.12 Fourier Transform Infrared Spectrometer (FTIR)

Sample preparation: -

For the IR absorbance measurements, bacterial cells from 24 hours-growing cultures were harvested from nutrient agar medium. The bacterial cells were collected by scribing with sterile spatula and placed on sterile petri plate for lyophilization. After lyophilization the dried biomass was collected by gentle scrapping, in powdered form. The FTIR analysis was done by using KBr discs (1:100: biomass: KBr) to obtain a uniform film which is suitable for suitable for
FT-IR measurements. KBr is used to control the humidity and to prevent the instrument from contamination

FT-IR graph were taken using attenuated total reflectance on ABB Boman Fourier Transform Infrared Spectrometer FTLA 2000. The absorption spectrum between 400 to 4000 cm\(^{-1}\) was calculated by co-adding 50 scans and subtracting both the background and atmospheric water. Their adjustment allows the threshold value for a valid identification of an isolate at the functional compound level to be set at a spectral distance of 1.0-1.5, approximately. For comparison, the difficulties arising from unavoidable baseline shifts and to improve the resolution of complex bands, and then go for average (between three spectra of each sample) of spectra.

3.13 Gel Electrophoresis

- Protein profiling will be done using discontinuous and denaturing after SDS-PAGE (Maniatis et al., 1982)
- Check antimicrobial activity of isolated Micrococcus sp. against some medicinal value plants (Dahiya et al., 2012)

- DNA isolation: - DNA was isolated from selected strains and go for 16s-rRNA with universal primer.
a. DNA isolation from bacterial strains

Took 1.5 ml culture in eppendorf

Centrifuge at 10000 rpm for 15 mins.

Pellet

Washed with 1 ml sterile water

Mixed 0.5 ml SET + 1 mg/ml lysozyme in eppendorf and added in sample

Shake well

Incubated for 1 hour

50 l of 10% SDS added
Hand to hand shaking

↓

Incubated for 30 mins.

↓

180µl 5M NaCl added

↓

Equal volume of Phenol: Chloroform (1:1) added

↓

Incubated for 15 mins.

↓

Centrifuged at 8000 rpm for 15 mins.

↓

Upper layer in new eppendorf

↓

Equal volume of Phenol: Chloroform (1:1) added

↓

Centrifuged at 8000 rpm for 15 mins.

↓

Upper layer transferred in new eppendorf
Filled with chilled isopropanol

Placed in freeze over night

Centrifuged at 8000 rpm for 15 mins.

Pellet washed with 70% alcohol

Pellet air dried

Dissolved in 50µl TE
TE Buffer

10 mM tris HCl pH 8.0

1mM EDTA

Isopropanol

Rnase A 10mg/ml

Phenol saturated with TE

b. Removal of RNA

1. To the DNA solution add 2.0 µg/ml DNase free RNase and incubate at 37°C for 1 h.

2. Add 0.1 volume of 3 M sodium acetate pH 5.2.

3. Add an equal volume of phenol: Chloroform mixture (1:1) and mix well by swirling. Centrifuge and collect the aqueous phase.

4. Repeat the above extraction step till no white interface is seen.
5. Precipitate the DNA by adding 2 volumes of absolute ethanol. Recover the DNA by centrifugation, wash it with 70% ethanol, air dry and dissolve in TE buffer.

c. Agarose gel analysis

1. Cast agarose gel (0.8-1.2%) in 1 X TAE.

2. Load DNA sample (2-5 µl) after adding 1/10 volume of 10 X dye.

3. Load a known amount of uncut λ phage DNA (2-3 different dilutions) as control in the adjacent wells.

4. Run the gel at 80 V for 30min.

5. Stain the gel with ethidium bromide (0.5 µg/ml) for 10 min, wash with distilled water and visualize under UV light.

6. Compare the intensity of test DNA with those of standards and score concentration.

7. Presence of a single intact band at the corresponding position to λ phage DNA indicates high molecular weight of the isolated DNA.
Precautions:

1. Plant material should not be subjected to frequent freezing and thawing.

2. Take care not to spill any liquid nitrogen as it causes burns.

3. Phenol must be extra pure and buffered with TE. Phenol is dangerous and causes burns so handle carefully.

4. The DNA pellet should not be over dried, an over dried DNA pellet is difficult to dissolve.

5. All mixing should be done very gently.

d. Determination of DNA concentration

Spectrophotometric analysis:-

The concentration of DNA sample used in the study was determined by the measurement of the optical density (OD) in a UV spectrophotometer (systronics) at 260 and 280 nm. The purity of DNA was ascertained by measuring the ratios at 260 to 280 nm. The concentration and quality of DNA was calculated by the following equivalents as suggested by Sambrook *et al.* (1989).

\[
\text{DNA mg/ml} = A_{260} \times \text{dilution} \times 50.0
\]

\[
A_{260} = \text{Absorbance in OD at 260nm.}
\]
1OD = 50µg/ml (double standard DNA) and

1OD = 30µg/ml (single standard DNA)

The primers used in the present investigation have been listed as under. The primers used for the identification of Cyanobacterial and plant at genus and species level were those designed by other investigators by targeting the 16S rRNA gene.

### 3.14 Polymerase Chain Reaction (PCR)

**a. DNA amplification (PCR): Preparation of template DNA** (Salem et al., 2012)

Universal primer used for the identification of Micrococcus at genus and species level.

PCR involves preparation of template DNA, with universal primer and designed of desiccation tolerant primer from NCBI nucleotide database and aligned using DNA star program as well as Clustal W (1.82) Multiple Alignment Program and analysis of PCR product.

**Reagents:**

**SET BUFFER (pH7.5)**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>g/100ml</th>
</tr>
</thead>
</table>

86
75mM NaCl 0.435

25mM EDTA 0.930

20mM Tris 0.242

The above ingredients were dissolved in distilled water and pH was adjusted to 7.5 before autoclaving.

SDS 10%

NaCl (5M) - 29.25gm/100ml of distilled water

All the above reagents were autoclaved at 121°C/15 min.

Isopropanol (ice cold) and Chloroform: Isoamyl alcohol (24:1)

Tris EDTA (TE) buffer (pH8.0)

Ingredients g/100ml

10mM Tris 0.121

1mM EDTA 0.37

The pH was adjusted to 7.5 before autoclaving 121°C/15 min.
b. PCR ASSAYS:-

Before setting up of PCR, all the reagents were thawed except Taq DNA polymerase, Mixed and spinned. Reagents were always kept on ice bath during the period of setting up of the reaction. The reaction mix, comprising of 10 X PCR buffer (containing MgCl₂), dNTPs, and primers was prepared and distributed to reaction tubes according to the requirements. The final volume of the PCR mix was adjusted to 25 µl.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile water</td>
<td>18.3 ml</td>
</tr>
<tr>
<td>Reaction buffer, 10X with MgCl₂</td>
<td>2.5µl</td>
</tr>
<tr>
<td>DNTPs mix</td>
<td>0.7µl</td>
</tr>
<tr>
<td>Primer (forward)</td>
<td>1µl</td>
</tr>
<tr>
<td>Primer (reverse)</td>
<td>1µl</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>0.5µl</td>
</tr>
<tr>
<td>Template DNA</td>
<td>1µl</td>
</tr>
</tbody>
</table>
The PCR conditions used included an initial denaturation of 94°C for 5 min., 94°C for 1 min., 55°C for 1 min., 72°C for 2 min., 72°C for min., 0°C for pause followed by 29 cycles each of varying conditions depending on the primer used which will be discussed. The PCR products were resolved on 1.2% Agarose gel containing Ethidium bromide using 1X TAE buffer (pH 8).

c. Agarose gel electrophoresis

The DNA samples as well as PCR amplified products were electrophoresed on the Agarose gels (0.8-1.2%) by following the standard procedure as given by Sambrook et al., (1989).

Tris Acetate –EDTA (TAE) Buffer, pH (8.0)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>g/1000ml (50X)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>242</td>
</tr>
<tr>
<td>EDTA</td>
<td>8.3</td>
</tr>
</tbody>
</table>

- pH adjusted to 8.0 by using glacial acetic before autoclaving at 121°C/15 min.
Tracking Dye

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>g/100ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05 % Bromophenol blue</td>
<td>0.05</td>
</tr>
<tr>
<td>66 % Glycerol</td>
<td>47.6</td>
</tr>
<tr>
<td>Distilled water</td>
<td>52.4</td>
</tr>
</tbody>
</table>

d. Primer use for PCR

One bacterial Universal primer was used for amplification and further for species level identification. Different nine species were amplified with universal primer.

Universal primer (xselaries 2013) was-

Forward Primer
27f – 5’AGAGTTTGATCMTGGCTCAG3’ [M = A or C]
Reverse primer
U1492R – 5’GGTTACCTTGTTACGACTT3’