APPENDIX

REAGENTS

1. Reagents for P-solubilization:
a) **SnCl₂**: Stannous chloride (2.5 g) was dissolved in 10 mL of concentrated HCl and volume was made to 100 mL with distilled H₂O.
b) **NH₄MoO₄ solution**: Ammonium molybdate (15 g) was dissolved in 300 mL warm distilled water and 342 mL concentrated HCl was added. The volume was made to 1 L with distilled water.
c) **Phosphate standard solution**: Analytical grade KH₂PO₄ (0.22 g) was dissolved in 100 mL distilled water and diluted to 1 L. This solution contains 50 µg P mL⁻¹ (50 ppm).

2. Salkowsky’s Reagent
1 mL of 0.5M FeCl₃ was added in 50 mL of 35% HClO₄ with continuous stirring.

3. 1% Picric Acid
1 g of Picric acid was added in 100 mL of distilled water.

3. Reagent for Siderophore Assay
a) **Chrome Azurol S Dye**
Dissolve 60.5 mg CAS in 50 mL MQ water. Add 72.9 mg of HDTMA on 40 mL of MQ water. Mix CAS solution and HDTMA solution. Add 10 mL of 1mM FeCl₃ solution slowly by stirring without foaming till the color turns brown to blue.

b) **CAS Assay Solution**
Add 6 mL of 10 mM HDTMA in 100 mL volumetric flask and dilute with water. Add 1.5 mL of 1mM FeCl₃. 6H₂O prepared in 10 mM HCl. Slowly add 7.5 mL of 2mM aqueous CAS dye solution by stirring. Separately prepare 4.307 g anhydrous piperazine solution in water and make its pH up to 5.6. Add 6.25mL of HCL (12M) to get a buffer solution. Add this to above volumetric flask and make volume upto 100mL with double distilled water to give CAS assay solution.
4. Nitrite Molybdate Reagent
10 g of sodium nitrite and 10 g of sodium molybdate were added in 100 mL of distilled to give 100 mL of nitrite molybdate reagent.

5. Gram Staining Reagents
Crystal violet
Solution A
2.0 g Crystal violet (90% dye content) was dissolved in 20.0 mL ethyl alchol (95%)
Solution B
0.8 g Ammonium oxalate was dissolved in 80.0 mL distilled water
Solution A and B were mixed.
Gram’s iodine
1.0 gm of sublimed iodine crystals and 2.0 g of potassium iodide were mixed. A little of 30 % acetic acid was added in mixture. Stir thoroughly till crystals dissolve. Add rest of the acetic acid to make 100 mL solution.
Ethyl alcohol (95%)
95.0 mL ethyl alcohol (100%) was added in 5.0 mL distilled water.
Safranin
10.0 mL Safranin (2.5% solution in 95% ethyl alcohol) was dissolved in 100.0 mL distilled water.

6. Endospore stain:
5.0 g Malachite Green was added in 100 mL distilled water.

7. Kovac’s Reagent for Oxidase Test (Bailey and Scott, 1966)
10.0 g of p-Dimethyl amino benzaldehyde was dissolved in the 150 mL amyl alcohol, 25.0 mL concentrated HCl was then added and kept in a Glass stopper bottle and stored at 4 °C.

8. Kovac’s Reagent for Indole Test (Bailey and Scott, 1966):
Tetramethyl p-Phenylene diamine dihydrochloride (0.05 g) dissolved in 50 mL of distilled water.
9. **Methyl Red Solution**
Suspend 1 g copper sulfate in 40 mL concentrated ammonia and add 690 mL of an approximately 10% potassium hydroxide solution.

10. **Nitrate Test (Pelczar et al., 1957)**
Solution A
1 gm of sulphanilic acid was added in 100 mL of 30% acetic acid.
Solution B
0.3 g of 1-Naphthylamine was added in 100 mL of 30 % acetic acid.
Solution A and Solution B were mixed

11. **Barrit’s reagent**
Suspend 5 g naphthol in 100 mL absolute ethanol.

12. **Solutions and buffer for polymerase chain reaction (PCR):**
All chemicals were obtained either from Ferments, U.K. or Banglore Genei Pvt. Ltd., Bangalore, India.

a) **Tris Borate EDTA (TBE) buffer (5X Stock):** Tris HCl (54 g), Boric acid (27.5 g) and 20 mL of 0.5 M of EDTA (pH 8.0) were dissolved in 500 mL of sterile MilliQ water and final volume was made up to one litre with sterile MilliQ water.

b) **TBE buffer (0.5X):** Hundred mL of 5X stock TBE buffer was taken in a volumetric flask and volume was made up to one litre with sterile MilliQ water.

c) **Tracking Dye (6X):** Prepare 0.25% Bromophenol blue in 40% sucrose (w/v).

d) **Ethidium bromide stock:** A stock solution of EtBr containing 5 mg mL⁻¹ was prepared in sterile MilliQ water.

e) **DNA molecular weight marker:** DNA ladder of 1 kb and 100 bp (Banglore Genei Pvt. Ltd.) were used as molecular weight marker for visualizing PCR product.

f) **Agarose (0.8%):** Agarose (0.8 g) was added to 100 mL of 0.5X TBE buffer in a 250 mL sterile Erlenmeyer flask and dissolved to homogeneity by melting in a microwave oven. When temperature of the solution is about 50-55 °C add 3 μL of Ethidium bromide (5 mg mL⁻¹) and pour into the gel casting tray with combs in place. The agarose was allowed to polymerize for about half an hour.
g) **Polymerase reaction buffer (10X):** The Polymerase reaction buffer commercially provided contains 100 mM Tris-HCl (pH 9.0); 500 mM KCl, 15 mM MgCl₂ and 0.1% gelatin.

h) **Taq Polymerase:** Taq DNA polymerase was 5U μL⁻¹ in a storage buffer consisting of 20 mM Tris-HCl (pH 8.0), 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5% Tween-20 (v/v), 0.5% Igepal and 50% Glycerol (v/v).

i) **dNTP mix:** A stock of dNTP mix (dATP, dCTP, dGTP and dTTP) has 2.5 mM concentration of each dNTP.

### 13. DNA isolation solutions

a) **Tris: HCl buffer (1 M):** 121.1 g of Tris HCl was dissolve in 75 mL of sterile double distill water. Adjust the pH 7.5 and final volume was made upto 100 mL with double distilled water.

b) **Ethylene di amine tetra acetic acid (EDTA) (0.5 M):** EDTA (Disodium salt dihydrate) (18.61 g) was dissolved in 40 mL of sterile double distill water in a pre sterilized reagent bottle. The bottle was placed on a magnetic stirrer for uniform shaking and slowly 40% NaOH solution was added through the wall of reagent bottle till pH 8 was attained. The final volume was made up to 100 mL with sterile distilled water.

c) **Sodium dodecyl sulphate (SDS) solution (25%):** Sodium dodecyl sulphate (Sodium lauryl sulphate) (25 g) was added in 75 mL distill water in a clean, pre sterilized reagent bottle and dissolved by stirring slowly avoiding formation of froth. Finally the volume was made upto 100 mL.

d) **Lysozyme:** Lysozyme powder 0.2 g was dissolved in 10 mL of sterile milliQ water and stored at -20°C for future use.

e) **Ammonium acetate solution (7.5 M):** Ammonium acetate (57.81 g) was added in 50 mL sterile double distill water in a pre-sterilized reagent bottle and stirred till homogeneous, transparent solution is obtained and the final volume was made up to 100 mL.

f) **Tris-EDTA buffer (T₁₀ E₂₅):** To prepare 100 mL of T10E25 buffer, add 2 mL of Tris-HCl stock (1M) and 5 mL of 0.5 mM EDTA stock and was mixed. Finally the volume was made upto 100 mL.
g) **Tris-EDTA buffer (T10 E1):** For preparing 100 mL of T10 E1 buffer, add 2 mL of 1M Tris-HCl stock and 0.2 mL of 0.5 mM EDTA stock was mixed together and volume was made upto 100 mL.
STANDARD METHODS

1. ESTIMATION OF SOLUBLE PHOSPHATES IN PIKOVSKYAYA’S MEDIUM (Gaur, 1990)

REQUIREMENTS:
1. Chloromolybdic Acid
2. Chlorostannous Acid
3. Std Phosphate solution:
   Dissolve 0.4390 g of dried K₂HPO₄ in 400 mL distilled water. Add 25 mL of 7 N H₂SO₄. Make up to 1 L to give a standard stock of (100 ppm).
   Working solution: Dilute 2 mL of the stock solution and make up to 100 mL with distilled water.
1) Transfer different aliquots of the working solution in 50 mL volumetric flasks and make the total volume up to 10 mL with double distilled water.
2) Add 10 mL of Chloromolybdic acid. Dilute the contents in flasks up to 40 mL by adding double distilled water.
3) Add 5 drops of Chlorostannous acid reagent and mix well till blue colour develops. Make the final volume up to 50 mL with double distilled water as quickly as possible.
4) Take absorbance at 600 nm and plot a standard graph so as to get a straight line.
5) To check the amount of phosphate solubilized in the medium, collect 1 mL of supernatant and add 9 mL of double distilled water. Perform the whole procedure mentioned above till blue colour develops and take absorbance at 600 nm. Calculate the total amount of phosphate solubilized and present in 1 mL of supernatant from the standard graph.

2. ESTIMATION OF INDOLE ACETIC ACID (Bric et al., 1991)

REQUIREMENTS:
1. Salkowsky’s reagent
2. Standard IAA: Stock: (1000 μg mL⁻¹)
   Working Solution (10-100 μg mL⁻¹)
1) Prepare different aliquots of Standard IAA solution so as to get final concentration in the range of 10-100 μg mL⁻¹. Make final concentration up to 1 mL by adding MQW.

2) Add 1 mL of Salkowsky’s reagent and keep in dark for incubation for 30 min for pink colour to develop.

3) Measure the optical density at 536 nm and plot standard graph.

4) Take 1 mL of supernatant and add equal amount of Salkowsky’s reagent.

5) Incubate for 30 min and take OD at 536 nm.

6) Estimate the amount of IAA produced from the standard graph.

3. *Chrome Azurol S (CAS) Agar Medium (Schwyn and Neilands, 1987)*

Modified *Chrome Azurol S* Medium was used for detection of siderophores.

**Solution A**

To prepare 1L of CAS medium, 60.5 mg *Chrome Azurol S* (CAS) was dissolved in 50 mL water and mixed with 10 mL Fe (III) solution (1mM FeCl₃.6H₂O, 10mM HCl). This solution was slowly added to 72.9 mg Hexadecyl-trimethyl ammonium bromide (HDTMA) dissolved in 40 mL water. The resultant dark liquid was autoclaved.

**Solution B**

Deferrated 1 M sucrose (30 mL), deferrated 100 mL of 10X basal salt medium containing K₂HPO₄ (1.0 g); NaCl (0.5 g); NaMoO₄ (0.005 g); CaCl₂ (1.0 g); PIPES (30.24 g) (1,4- piperazine di-ethane sulphonylic acid) and Difco agar 15 g in 730 mL water was prepared. The pH of the medium was adjusted to 6.8 by the addition of NaOH (0.01N) before autoclaving. After cooling to 50 °C, 30 mL of deferrated casamino acid (10%) was added as sterile solution. The solution A was finally added to solution B along the Glass wall with enough agitation to achieve maximum mixing without formation of foam. About 30 mL of medium was poured into each plate and stored in refrigerator.

**Deferration**

In studies involving siderophore, contaminating iron should be avoided to get good results, so treating the chemicals and Glassware for removal of iron, therefore becomes essential.
Removal of contaminating iron from Glass ware

Glass is a good ion exchange surface and hence may get contaminated with iron on the surface. All glasswares used for siderophore study were soaked in 2N HCl for 24 h and washed with double distilled water to remove the acid.

Removal of contaminating iron from media components

Media components such as sucrose, K$_2$HPO$_4$, MgSO$_4$.$7$H$_2$O, CaCl$_2$, NaCl and casamino acid were deferrated by extraction with 3% (w/w) 8-hydroxyquinoline in chloroform (Schwyn and Neilands, 1987)
LIST OF PUBLICATIONS

A. Full length paper


B. Abstract published

Kusum and Saharan, B. S. (2013). Isolation and identification of efficient plant growth promoting Rhizobacteria associated with the rhizosphere of *Stevia*: “Present Status and Prospects” organized by Department of Biotechnology, Deenbandhu Chotu Ram University of Science and Technology, Murthal (Sonepat), Haryana, India, 15-16 March, 2013.


C. Poster presented

Kusum and Saharan, B. S. (2013). Poster presentation on “Isolation and identification of Plant Growth Promoting Rhizobacteria associated with Stevia”. “Present Status and Prospects” organized by Department of Biotechnology, Deenbandhu Chotu Ram University of Science and Technology, Murthal (Sonepat), Haryana, India, 15-16 March, 2013.
