Chapter 2

Materials and Methods
2.0 MATERIALS

2.0.1 Sesbania Cultivars

Seeds of *S. aculeata* H-1(DG-1) was obtained for the experiments from National Bureau of Plant Genetic Resources (IARI campus), New Delhi. The seeds of *S. aculeata* were sown in well prepared soil to grow the plants and the parts of plants of different age groups were used in various experiments.

2.0.2 Bacterial Strains

*Sinorhizobium* sp. (*saheli*) strain SB2 (Accession No. AY903255) isolated from root nodule of Sesbania plant was grown at our institute garden.

Mutants of *Sinorhizobium* sp. (*saheli*) strain: SB2M1, SB2M2, SB2M3, SB2M4 and SB2M5 were obtained by Tn5 mutagenesis of parent strain SB2.

*Bradyrhizobium* sp. (*Arachis*) strains NC92, IGR92, GN17; *Bradyrhizobium japonicum* SB16; *Mesorhizobium* sp. (*Cicer*) Rcd301 and *Rhizobium leguminosarum* P-14 were obtained from Indian Agricultural Research Institute (IARI), Pusa, Delhi, India.

*E.coli* (WA803) was purchased from NCCB, the Netherlands Culture Collection of Bacteria (formerly, LMD and Phabagen Collection).

Mutants of *Bradyrhizobium* sp. (GN17) strain, M28 and M19; mutants of *Mesorhizobium* (*Ciceri*) Rcd301 strain LM, HT, and AT were obtained from our laboratory.

2.0.3 List of Chemicals and Reagents

The following chemicals and reagents were used for different experiments:

Trizma base [tris(hydroxymethyl) aminomethane], ethidium bromide (EtBr), sodium dodecyl sulphate (SDS), bromophenol blue (BPB), xylene cyanole, acrylamide, N, N'-methylene bis acrylamide, phenylmethylsulfonylfluoride (PMSF), deoxycholic acid (DOC), orcinol, tween 20, streptavidin peroxidase, 4-chloro-
naphthol, standard sugars (arabinose, D-glucose, N-acetyl-D glucosamine, D-galactose, N-acetyl galactosamine, cellulbiose, fructose, D-mannose, L-fucose, raffinose, ribose, maltose and xylose), tetra methyl ethylenediamine (TEMED), ammonium persulphate (APS), β-mercapto ethanol (βME), agarose type II, sodium acetate, antibiotics (streptomycin, neomycin, kanamycin, ampicillin, gentamycin and nalidixic acid), RNase A, DNase I, magnesium chloride, sodium lauryl sarcosyl, coomassie brilliant blue R-250, dialysis tubings, cetyl dimethyl ethylammonium bromide (cTAB), biotin (long arm), dimethyl sulfoxide, 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide (EDC), N-hydrosulfo succinimide (NHS). All these reagents were obtained from Sigma Chemical, USA.

Standard protein markers, urea, glycine, mineral oil, 3/10 ampholytes, 5/7 ampholyte, TEMED, Triton X-100, CHAPS, glycerol, N,N’-methylenbisacrylamide, acrylamide, ready-made IPG strips pH 3/10 and pH 4/7 of length 7 cm and 18 cm were obtained from Bio-Rad, USA.

Detoxi-Gel for LPS purification was obtained Pierce Chemical Co., USA. Plates for ELISA were procured from NUNC (Denmark).

Taq polymerase, dNTPs and DNA molecular weight markers were procured from Bangalore Genei/New England Biolabs, USA. Nylon membrane (Hybond N+) and Nitrocellulose membrane were obtained from Amersham.

QIAquick gel extraction kit for purification of DNA was obtained from Qiagen, USA.

Hydrochloric acid, NaOH pellets, chloroform, methanol CaHPO₄, K₂HPO₄, NaCl, MgSO₄, FeCl₃, H₃BO₃, MnSO₄ 4H₂O, ZnSO₄ 7H₂O, CuSO₄ 5H₂O, H₂MoO₄, FeCl₃, EDTA, MgSO₄ 7H₂O, KH₂PO₄, CaSO₄ were obtained from E-Merck, Germany. Sephadex G-50 was purchased from Pharmacia, Sweden.

All other reagents and chemicals were of analytical grade and procured from S.D. Fine-Chemicals Ltd. and Qualigens Fine Chemicals, India.

2.0.4 Growth Media

The following different bacterial growth media (with or without agar) were prepared according to following composition and were autoclaved and cooled at (RT)
temperature before use. The Yeast Extract, Mannitol, Bactotryptone, Luria Broth
Congo Red and agar-agar were purchased from Hi-media, India.

**Yeast Extract Mannitol Agar Medium (YEMA)**

Mannitol 10.0 g
Yeast extract 1.0 g
K$_2$HPO$_4$ 0.2 g
KH$_2$PO$_4$ 0.2 g
MgSO$_4$.7H$_2$O 0.1 g
CaCl$_2$.2H$_2$O 0.05 g

Adjust pH to 7.2 and add water to make 1.0 L volume
1.5% -2% agar was added to make solid medium

**Tryptone Yeast Extract Medium (TY)**

Bactotryptone 5.0 g
Yeast extract 3.0 g
CaCl$_2$.2H$_2$O 0.12 g
DW 900 ml

pH adjusted to 7.2 and added water to make the volume 1.0 L
1.5% to 2% agar was added to make solid medium

**Jensen’s Medium used for Plate Making**

Sucrose 20.0 g
K$_2$HPO$_4$ 1.0 g
MgSO$_4$ 0.5 g
NaCl 0.1 g
FeSO$_4$ 0.005 g
Na$_2$MoO$_4$ 2.0 g
CaCO$_3$ 15.0 g

DW 900 ml

Added water to make the volume 1.0 L
1.5% -2% agar was added to make solid medium
Jensen's Medium used for Plant Growth

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaHPO₄</td>
<td>1.0 g</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>0.2 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.2 g</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>0.2 g</td>
</tr>
<tr>
<td>FeCl₃</td>
<td>0.1 g</td>
</tr>
<tr>
<td>DW</td>
<td>900 ml</td>
</tr>
</tbody>
</table>

pH adjusted to 7.0 and added water to make the volume 1.0 L

Mekanight's Media (MKS)

Solution A

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₃BO₃</td>
<td>286.0 mg</td>
</tr>
<tr>
<td>MnSO₄ 4H₂O</td>
<td>154.0 mg</td>
</tr>
<tr>
<td>ZnSO₄ 7H₂O</td>
<td>22.0 mg</td>
</tr>
<tr>
<td>CuSO₄ 5H₂O</td>
<td>8.0 mg</td>
</tr>
<tr>
<td>H₂MoO₄</td>
<td>9.0 mg</td>
</tr>
<tr>
<td>DW</td>
<td>100.0 ml</td>
</tr>
</tbody>
</table>

Solution B

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeCl₃</td>
<td>1.68 mg</td>
</tr>
<tr>
<td>EDTA</td>
<td>200.0 mg</td>
</tr>
<tr>
<td>DW</td>
<td>100.0 ml</td>
</tr>
</tbody>
</table>

Solution C

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgSO₄ 7 H₂O</td>
<td>2.0 mg</td>
</tr>
<tr>
<td>DW</td>
<td>100.0 ml</td>
</tr>
</tbody>
</table>

Solution D

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH₂PO₄</td>
<td>20.0 gm</td>
</tr>
<tr>
<td>DW</td>
<td>100.0 ml</td>
</tr>
</tbody>
</table>

Solution E

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>30.0 gm</td>
</tr>
<tr>
<td>DW</td>
<td>100.0 ml</td>
</tr>
</tbody>
</table>

36
To prepare MKS, 1.25 g of CaSO₄ was dissolved in 200.0 ml of DW by boiling in 500.0 ml flask. The above stock solution A to E (1.0 ml of each) was added into the resulting suspension. Then DW was added to make final volume to 1.0 L. The pH was adjusted to 6.8 using 0.1 N NaOH.

**Luria Bertani medium (LB)**

Luria broth 20.0 g  
DW 900 ml  
Adjusted pH to 7.2 and DW was added to make the volume 1.0 L  
1.5% to 2.0% agar was added to make the medium solid.

**Congo Red (CR)**

Congo red 31.83 g  
DW 900 ml  
Adjusted pH to 6.8 and added water to make the volume 1.0 L

### 2.0.5 Stock Solutions

Autoclaved double-distilled (ADW) water was used to prepare various buffers and solutions. Volumes of solutions were adjusted after complete dissolution of component(s). Solutions which were not autoclavable were filter sterilized.

**Acrylamide (30%)**  
Acrylamide 29.2 g and bis-acrylamide 0.8 g were dissolved in 100.0 ml of ADW.

**Separating gel buffer**  
1.5 M Tris-HCl was prepared by dissolving 18.15 g Tris in 100.0 ml of ADW. The pH was adjusted to 8.8.

**Stacking gel buffer**  
0.5 M Tris-HCl was prepared by dissolving 6.05 g Tris in 100.0 ml of DW and the pH was adjusted to 6.8.

**SDS (10%)**  
10.0 g of SDS was dissolved in 100.0 ml of ADW.

**APS (10%)**  
Ammonium persulfate (0.5 g) was dissolved in 5.0 ml of ADW. It was always prepared fresh.
| **SDS gel loading** | 0.5 M Tris-HCl (pH 6.8) 1.25 ml, 10% SDS 2.0 ml, glycerol 1.2 ml. Made the volume to 5.0 ml and add bromophenol blue 0.1%, β- mercaptoethanol 2.5% (v/v) by ADW. |
| **buffer 2X** |  |
| **Electrophoresis buffer** | Glycine 14.4 g, Trizma base 3.0 g, 10% SDS 10.0 ml, per litre. |
| **(SDS-PAGE)** |  |
| **Destaining solution** | Methanol : acetic acid : water in ratio of 40:10:50. |
| **Staining solution** | 1% Coomassie Brilliant Blue R-250 prepared in destaining solution. |
| **Lysis buffer** | Urea 19.2 g, CHAPS’ 1.6 g and Tris Base 0.194 g were dissolved in 40.0 ml of ADW. |
| **Rehydration stock solution** | Urea 12.0 g, CHAPS’ 0.5 g, 3/10 Ampholyte 500.0 μl, few grains of Bromophenol blue dissolved in 25.0 ml of ADW. |
| **SDS equilibration buffer** | 1.5 M Tris-Cl, pH 8.8 6.7 ml, Urea 72.07 g, Glycerol 69.0 ml, SDS 4.0 g and few grains of Bromophenol blue dissolved in 200.0 ml of ADW. |
| **Agarose sealing solution** | Agarose 0.5 g and few grains of Bromophenol blue dissolved in 100.0 ml of ADW. |
| **1M Tris HCl, pH 8.0** | 121.14 g of Trizma base dissolved in distilled water, pH adjusted to 8.0 using 6 N HCl and volume made up to 1.0 L with ADW. |
| **0.5 M EDTA, pH 8.0** | 186.1 g of disodium salt of ethylene diamine tetra acetic acid (EDTA) was dissolved in 500.0 ml of ADW, pH was adjusted to 8.0 by 1.0 M NaOH. |
| **Ethidium Bromide** | 10.0 mg/ml solution in ADW. |
| **50X Tris-Acetate Buffer (TAE)** | 242.0 g of Trizma base was dissolved in 57.1 ml of glacial acetic acid and 100.0 ml of 0.5 M EDTA (pH 8.0), the volume made upto 1.0 L. |
TE buffer 10.0 mM Tris HCl (pH 8.0), 1.0 mM EDTA (pH 8.0).

GTE buffer (solution I) 50.0 mM glucose, 25.0 mM Tris HCl (pH 8.0), 10.0 mM EDTA.

Lysis buffer (solution II) 0.2 N NaOH (freshly diluted from a 10 N stock), 1% SDS (freshly diluted from 10% stock).

Neutralization solution (solution III) 5.0 M Potassium acetate: glacial acetic acid: distilled water; 60.0 ml: 11.5 ml: 28.5 ml, pH 4.8.

Equilibrated phenol Distilled phenol containing 8-hydroxyquinoline (0.1% final concentration) was neutralized with 10.0 N NaOH or 500.0 mM Trizma base, stirred over a magnetic base with 2/3 volume of TE buffer for 10 to 15 min. Aqueous phase was aspirated. Equilibration was repeated 4-5 times until the pH of the phenol phase (yellow color) reached to pH 8.0. Stored in a dark colored bottle at 4°C.

DNA loading buffer (10X) 2.5% bromophenol blue, 2.5% xylene cyanol FF, 25% glycerol.

PCR reaction buffer (10X) 200.0 mM Tris HCl (pH 8.8), 100.0 mM KCl, 100.0 mM (NH_{4})_{2}SO_{4}, 1% Triton X-100, 15.0 mM MgCl_{2}.

Sodium phosphate buffer (PBS) 137.0 mM NaCl, 2.7 mM KCl, 4.3 mM Na_{2}HPO_{4} 7H_{2}O, 1.4 mM KH_{2}PO_{4}, pH 7.4

Sodium Tris buffer (TBS) 10.0 mM Tris HCl (pH 7.2), 150.0 mM NaCl.

Phosphate Citrate buffer 0.2 Na_{2}HPO_{4} and 0.1 M citric acid, pH 5.0.

Extraction buffer 10.0 mM Tris HCl, 0.15 M NaCl, 4.0 mM β-mercaptoethanol and 0.1 mM EDTA, containing 0.5 μg/ml PMSF, pH 7.2.

TEN buffer 10.0 mM Tris-HCl, 0.1 mM EDTA, 100.0 mM NaCl, pH 8.0.
**DTT (0.5m)**

1.545 g of DTT in 20.0 ml of 0.01 M sodium acetate, pH 5.2.

**Denaturing solution**

1.5 mM NaCl, 0.5 M NaOH.

**Neutralizing solution**

1.5 mM NaCl, 0.5 mM Tris-HCl, 1 mM EDTA, pH 7.2.

**Sephadex G-50**

5% suspension in ADW, autoclaved for 15 min and equilibrated in TEN buffer, pH 7.6.

### 2.1 METHODS

#### 2.1.1 Lectins of *Sesbania aculeata*

**2.1.1.1 Preparation of Seed Extracts**

The *S. aculeata* seeds (50.0 gm) of cultivar H-1(DG-1) were washed thoroughly with DW and soaked overnight in ADW. The seeds were then crushed in 100.0 ml of TBS (10.0 mM Tris HCL, 0.15 M NaCl) at pH 7.2 containing 0.5 μg/ml of PMSF after removing the seed coats and washing with distilled water. It was then centrifuged at 23,700 x g for 1.0 h at 4°C. The supernatant thus obtained was dialyzed overnight against TBS. The dialyzed extract thus obtained was designated as seed extract.

**2.1.1.2 Seed Germination and Seedling Growth**

For isolation of root, stem and nodule lectin, seeds of *S. aculeata* were germinated. Seeds were first washed with 95% ethanol and then rinsed 2-3 times with DW. The seeds thoroughly washed with sterile water were placed on moist autoclaved filter paper in a petridish for germination. The germinated seeds were sown in the soil properly prepared in the garden of IGIB in the month of July. Plants were allowed to grow under natural condition upto 10 wks. The grown up plants were healthy having hight of approximately 2 feet and weight 150.0 g.

**2.1.1.3 Preparation of Root, Stem and Nodule Extract**

Roots and stems of 2, 3 and 4 wks old plants and root nodules from 4 wks old plants were washed thoroughly with tap water followed by ADW, excised, crushed and homogenized in the double volume of TBS (10.0 mM Tris-HCL, 0.15 M NaCl) with respect to weight of plant materials at pH 7.2 containing 0.5 μg/ml of PMSF.
The homogenates were centrifuged at 23,700 x g for 1.0 h at 4°C. These extracts were dialyzed against TBS. The dialyzed extract was designated as root extract, stem extract and nodule extract.

2.1.1.4 Preparation of Rabbit Erythrocytes

Rabbit erythrocytes (RRBC) were used to perform the hemagglutination assays. Rabbit was bled by using sterile blade, 2-3% drops blood was collected in 4% (w/v) sodium citrate saline. Erythrocytes were washed three times with TBS (pH 7.2) by low-speed centrifugation at 19064 x g. The cell pellet thus obtained was finally suspended in the same buffer to make a suspension of 4% RRBC.

2.1.1.5 Hemagglutination Assays (HA)

The HA was performed to determine the hemagglutination activity of extracts prepared from seeds, roots, stem and the nodules of S. aculeata. Each of these tissue extracts (30.0 µl) were serially diluted with TBS (pH 7.2) and mixed with equal volume (30.0 µl) of 4% RRBC suspension. The agglutination was examined after 30 min under microscope. Hemagglutination assay of these extracts at various age (2, 3 and 4 wks) were also carried out in the same manner. The reciprocal of the highest dilution of the samples that gave complete agglutination in 30 min at 25°C was taken as a hemagglutination (HA) titre. One hemagglutination unit (HU) was defined as the minimum amount of protein required for 100% agglutination under the assay conditions. Specific activity was defined as the number of hemagglutination units per mg of protein, at 25°C in 30 min.

2.1.1.6 Sugar Specificities of S. aculeata Extracts

The carbohydrate specificity of S. aculeata extracts was determined by hemagglutination inhibition assays. The inhibition of hemagglutination by various saccharides was performed at 25°C. The aliquots of sugars (30.0 µl) of known concentration were diluted serially (200.0 mM to 50.0 mM) with phosphate buffer in micro titre plates. To each well 30.0 µl of the sample having 4.0 HA units was added. The micro titre plates were then incubated for 20 min at RT and then 60.0 µl of 4% RRBC suspension was added. The agglutination was examined after 30 min microscopically as well as visually. The results were expressed as the minimum amount
of sugar required to give complete inhibition of agglutination of 4% rabbit erythrocytes suspension under the assay condition. The inhibition of agglutination upto the level of 50% was indicated by ‘+’ and upto the level of 90% was indicated by ‘++’.

2.1.2 Purification and Quantification of *S. aculeata* Lectins

Affinity column chromatography was performed to purify the lectins of *S. aculeata* following the method as described by Kalsi *et al.* (1992). Sephadex G-50 column was prepared by soaking the matrix in DW for 2 h. The matrix was then filled in the column (50 x 2.5 cm) and equilibrated with TBS (10.0 mM, pH 7.2). Each of the extracts (seed, root, stem and nodule) was loaded on to the column separately. The column was then washed thoroughly with the same buffer at a flow rate of 12.0 ml/h until the absorbance at 280 nm became zero. The bound protein was eluted as 5.0 ml fraction with the elution buffer (TBS containing 100.0 mM glucose). Protein contents in the fractions were monitored spectrophotometrically at 280 nm. The protein positive fractions were pooled and dialyzed against TBS (0.1 mM). The dialyzed samples were then lyophilized and stored at 4°C. Separate columns were used to purify the lectins from different extracts. The seed, root, stem and nodule lectins thus obtained were designated as *Sesbania* Seed Agglutinin (SSA), *Sesbania* Root Agglutinin (SRA), *Sesbania* Stem Agglutinin (SSTA) and *Sesbania* Nodule Agglutinin (SNA) respectively.

Protein Estimation

The protein concentration of SSA, SRA, SSTA and SNA was assayed by the method of Lowry (Lowry 1951) using commercially available bovine serum albumin (BSA) as the standard.

2.1.3 Characterization of Purified Lectins

2.1.3.1 Lectin Activity

The agglutination activity was performed by taking 3.0 μg (25 μl) purified lectin in 25.0 μl of RRBC on a slide. It was mixed manually for about 30 min at RT and agglutination was observed microscopically as well as visually. The agglutination activity at the level of 50% was represented by ‘+’ and 90% was represented by ‘++’.  

42
2.1.3.2 Sugar Specificity

The lectin activity of purified lectins SSA and SRA from S. aculeata were carried out by hemagglutination inhibition assay. The purified SSA and SRA (3.0 μg) in 25.0 μl were mixed with 25.0 μl of various sugars (D-Glucose, D-Mannose, α-Methyl-D mannopyronaside and Maltose) in 50.0 mM and 100.0 mM concentration. The sample was incubated for 20 min at RT. The control sample was incubated with phosphate buffer for the same time. The RRBC (50.0 μl) was added and after the incubation time (30 min) wells were observed under microscope for inhibition of hemagglutination. The results were expressed as given in 2.1.1.6.

2.1.3.3 Native Polyacrylamide Gel Electrophoresis (Native-PAGE)

Polyacrylamide slab gel electrophoresis was performed as per the method of Davis (1964) using 8% gel with Tris glycine (pH 8.5) electrode buffer. Stacking gel was of 5% polyacrylamide concentration. The gel was run at constant current of 20.0 mA. For estimation of molecular weights, the gel was calibrated with non-denatured marker proteins. It was then fixed in a mixture of methanol:acetic acid:water (40:10:50) for 30 min, followed by staining at 37°C for 10 min in the same solvent system containing 1% coomassie brilliant blue R-250. The gel was destained at 37°C with destaining solution and analyzed for the protein band.

2.1.3.4 SDS-PAGE

Electrophoresis in polyacrylamide slab gels was performed using the discontinuous system of Laemmli (1970). The separating gel 12% and the stacking gel 5% containing Tris glycine buffer (pH 8.53) with 0.1% SDS (w/v) were prepared as described by Sambrook et al. (1989). The samples (SSA, SRA, SStA and SNA) were dissolved in the SDS gel loading sample buffer and heated to 100°C for 5 min before loading on to the gel. Electrophoresis was performed at constant current of 30.0 mA. For estimation of molecular weights, the gel was calibrated with denatured marker proteins. The gel was fixed in a mixture of methanol:acetic acid:water (40:10:50) for 30 min. It was followed by staining at 60°C for 10 min in the same solvent system containing 1% coomassie brilliant blue R-250. The gel was again destained at 37°C with destaining solution and
stored in 7% glacial acetic acid. The gels were analyzed for the protein band profile.

2.1.3.5 Effect of Metal Ions on Hemagglutination Activity of S. aculeata Lectins

An aliquot of 3.0 µg (25.0 µl) of purified lectin (SSA, SRA, SStA and SNA) was incubated at RT for 20 min with different concentrations (0.06 mM to 2.0 mM) of MgCl₂, CaCl₂, MnCl₂. To this 25.0 µl of 4% (v/v) RRBC was mixed. The hemagglutinating activity was then measured after 30 min under the microscope for inhibition of hemagglutination, if any. The hemagglutination activity at the level of 90% was represented by ‘++’.

2.1.3.6 Two-D Gel Electrophoresis

Two dimensional gel electrophoresis for the purified protein samples was carried out using basic method of Farrell (1975) and Klose (1975). It was found to be a powerful and widely used method for the analysis of complex protein mixtures extracted from cells, tissues or other biological samples. This technique sorts proteins according to two independent properties in two discrete steps. The first dimension step was isoelectric focusing (IEF), separates proteins according to their isoelectric points (pI). The second dimension step was SDS polyacrylamide gel electrophoresis (SDS-PAGE) separates proteins according to their molecular weight (MW). Each spot on the resulting two dimensional array corresponds to a single protein species in the sample.

2.1.3.6.1 Isoelectric Focusing

The PROTEAN IEF cell, BIO-RAD, US was used to perform the first dimension IEF. It was a flatbed system run at very high voltages with active temperature control. The procedure for the first dimension involved immobilized pH gradient (IPG) strip rehydration, sample application and isoelectric focusing. Ready-made IPG strips of various pH range were obtained from BIO-RAD, US. The following steps were performed to carry out the first dimension IEF.

(i) Preparation of samples for 2-D gel electrophoresis

Samples were prepared according to BIO-RAD instruction manual. The purified lectin samples were incubated with sample buffer for 2 h at RT. The sample was then centrifuged at 19,064 x g for 30 min. The supernatant was then concentrated
by lyophilization in Bench Top Freeze Dryer, Virtis. The lyophilized proteins were stored at -20°C till use.

(ii) First-dimension IEF

The following steps were followed

(a) 100.0 μg of protein was dissolved in 125.0 μl of rehydration buffer for 7 cm, 3/10 ready-made IPG strips.

(b) 7.0 mg DTT per 2.5 ml rehydration buffer was added and mixed just before adding protein sample into the rehydration buffer and kept at RT for 1 h.

(c) Each sample was then put into the separate reservoir slots of immobiline dry strip reswelling tray.

(d) The protective cover from the IPG strip was then removed and the strip was gently slid into the sample solution in the reservoir slot keeping the gel side down towards the surface of the reservoir plate.

(e) It was then overlayed with mineral oil and incubated overnight at 28°C.

(f) The strips were then rinsed with deionized water to remove excess rehydration solution and was placed on the tissue paper to soak excess of moisture.

(g) The strips were then placed on the reservoir of focusing tray keeping the positive side of the strip towards anode and negative side of the strip towards the cathode.

(h) The focusing tray was then placed in the IEF cell and then each strip was overlayed with mineral oil.

(i) The IEF was conducted in different phase such as 500 V for 25 min, 1000 V for 1 h, 4000 V for 4 h. The IEF was continued till its Vh reached to 10,000 Vh.

As isoelectric focusing was proceeded, the bromophenol blue tracking dye migrated towards the anode.

2.1.3.6.2 Second-Dimension SDS-PAGE

The second dimension SDS-PAGE was performed by using Mini-PROTEAN II slab cell, vertical system from BIO-RAD,US. It consists of four steps:
(i) Resolving gels (12%) were prepared as described by Sambrook et al. (1989).

(ii) The strips were equilibrated in the following equilibrium solution. The first equilibrium step was done by adding 100.0 mg DTT per 10.0 ml SDS equilibrium buffer. The equilibrium buffer (3.0 to 4.0 ml) was overlayed on 1st dimension strip gel in the reservoir slots of rehydration tray and it was kept for 15 min incubation at RT. Similarly, second equilibrium step was performed by adding 250.0 mg iodoacetamide per 10.0 ml of SDS equilibration buffer for 15 min incubation at RT.

(iii) The equilibrated IPG strips were placed on the filter paper to remove excess equilibration solution. The strips were then placed on the 12% vertical second-dimension slab gels and sealed with agarose gel solution.

(iv) Electrophoresis in polyacrylamide slab gels was performed using the discontinuous system of Laemmli (1970) for about 6 h at 20°C. After electrophoresis, the gel were stained with coomassie blue solution, with repeated changes of the staining solution with continuous shaking on a platform shaker. The background stain was removed with repeated change of destaining solution.

2.1.3.6.3 Mass Spectrometry Compatible Silver Staining

For protein identification by MALDI TOFF, the gels were first fixed in 5% methanol and 1% acetic acid in DDW for 15 min followed by three washing of 10 min each. The gels were then sensitized with sodium thiosulphate for 90 sec followed by three washing of 30 sec each with DDW. Silver staining was done in 200.0 mg/100.0 ml silver nitrate for 30 min followed by 60 sec washing thrice with DDW. The gel was developed with sodium carbonate 6.0 g/100.0 ml containing 50.0 μl formaldehyde and 2.0 ml sodium thiosulphate. Over development was stopped with 6 % acetic acid in DDW.

2.1.3.6.4 Identification of Purified Lectin by MALDI TOF

The identification of the purified lectin was performed by MALDI TOF spectrum analysis. The samples were prepared by cutting the required single spot from the SDS gel with a sterile blade. The MALDI TOF spectrum analysis of the gel pieces was done at TCGA, New Delhi.
2.1.4 Bacterial Cultures

Rhizobial cultures were grown routinely in YEMA or TY broth at 28°C whereas *E. coli* cultures were grown in LB broth at 37°C in an orbital shaker (New Brunswick Scientific, USA).

The stocks of original rhizobial cultures were prepared and stored at -70°C in 20% glycerol. These were streaked on YEMA (yeast mannitol with 1.8% agar) plates and sub cultured after every 15 days. Single colonies were picked and used for inoculating and routinely maintaining the liquid cultures.

The purity of rhizobial strains was checked by growing them on Jensen’s and Congo red media. A nitrogen free Jensen’s medium allowed the growth of those colonies, which were able to grow by fixing atmospheric nitrogen and, therefore, was a suitable medium for examination of rhizobia. Purity of the culture was further checked by growing the rhizobial culture in Congo Red medium in which bacterium produced slimy white colonies which otherwise were red in colour. This medium was used routinely through out the course of the work. Cell density measurements were done spectrophotometrically by taking OD at 620 nm using Perkin Elmer (Lambda Bio 20) spectrometer.

2.1.5 Isolation of Sesbania Specific Rhizobium

The seeds of *S. aculeata* H-1(DG-1) was soaked overnight in DW at RT. The soaked seeds were sown and watered in the well prepared soil in the IGIB garden, Mall Road, Delhi, India. The seeds were allowed to grow for 4 wks in natural condition by watering them everyday. Well developed and matured nodules appeared in the roots of plant after 4 wks of growth. The nodules were pink in color. Two to three nodules were selected and detached from the roots by sterile blade under aseptic condition. Detached nodules were first washed with DW than with 70% alcohol followed by washing with ADW. The nodules were then crushed in YEM medium. The suspension of the crushed nodules were sprayed over Jensen’s medium agar plate and incubated at 28°C for 2-3 days. The white colony appeared over Jensen’s medium agar plate on 3\(^{rd}\) day of incubation. A single white and slimy colony was selected out of many colonies and striked over Congo Red medium plate. The appearance of white colony was indicative of rhizobia when grown in Congo Red medium. The single white colony which does
not take the red stain of Congo Red medium were picked up and grown in YEM medium for 3 days at 28°C. The culture was centrifuged and the cell pellet was washed with PBS. Genomic DNA was isolated from these cells. The 16S rRNA gene PCR was carried out for verification of *Sesbania* rhizobia. The PCR product was then sequenced and analyzed for identification.

### 2.1.6 Isolation of DNA

#### 2.1.6.1 Isolation of Genomic DNA

Rhizobial cultures (5.0 ml) were grown in YEM media till late log phase and spun at 2,050 x g for 5 min using eppendorf centrifuge. The pellet was washed with TE buffer (10.0 mM Tris, 1.0 mM EDTA, pH 8.0). A volume of 500.0µl of 4.0 M GIT solution was added to the cells and it was then kept at 60°C for 2 h for lysis. The samples were then centrifuged at 18,500 x g for 15 min and clear supernatant was taken for extraction of DNA. The samples were extracted with equal volume of phenol: chloroform followed by extracting twice with equal volume of chloroform. The aqueous phase containing DNA was carefully separated. The DNA was precipitated with either 100% chilled absolute alcohol or with 0.6% isopropanol and pelleted by centrifugation at 13900 x g for 15 min. The DNA pellet was dissolved in appropriate amount of TE buffer and stored at -20°C. The purity of the DNA was verified by checking the OD at 260 and 280 nm.

#### 2.1.6.2 Plasmid DNA Isolation

Isolation of plasmid DNA from *E. coli* cultures was performed by the alkaline lysis method as described in Sambrook *et al.* (1989). Overnight cultures (25.0 ml) were pelleted by centrifugation at 9,900 x g for 5 min. The pellet was washed with Tris-EDTA. The pellet was resuspended in 25.0 ml of glucose-Tris-EDTA buffer (solution I) by gentle pipetting and was incubated in ice for 10 min. Double volume of solution II was added and mixed gently by swirling the tube and was incubated at RT for 10 min. Solution III was added with the amount equal to half the volume of solution I and solution II. The sample was then mixed by inversion and incubated in ice for 10 min. The mixture was then centrifuged at 13,900 x g for 15 min. The supernatant was decanted in a fresh tube. Equal volume of isopropanol was added to the supernatant for precipitating the DNA, incubated at RT for 10 min and pelleted by centrifugation at 13,900 x g for 15 min. The
DNA pellet was washed with 70% ethanol, centrifuged as before and air dried. The pellet was dissolved in an appropriate amount of TE buffer. RNase A (5.0 mg/ml) was then added and incubated at 37°C for 1 h. The digest was extracted once with an equal volume of MOS (mixture of organic solvents; phenol: chloroform: isoamylalcohol in ratio of 24:24:1) and once with 0.5 volume of chloroform and then precipitated with an equal volume of chilled isopropanol. The DNA was pelleted by centrifugation at 13,900 x g for 15 min at 4°C, washed sequentially with 70% (v/v) and 100% (v/v) alcohol and air dried. The DNA pellet was dissolved in TE buffer. The purity of the DNA was verified by checking the OD at 260 and 280 nm.

2.1.7 Agarose Gel Electrophoresis

The DNA samples were analyzed by agarose gel electrophoresis in 1X TAE following the method described by Sambrook (1989). Agarose (1% w/v Sigma, Type II) gel was used for analysis and electrophoresis was carried out at constant voltage of 5 V/cm in a horizontal gel electrophoresis apparatus. Electrophoresis was stopped after the bromophenol blue tracking dye had migrated close to the end of the gel. The DNA was stained with ethidium bromide solution and the gel was visualized over the UV transilluminator. The photographs were taken using Fotodyne with Polaroid type 667. The size of the PCR product was estimated by relating with the sizes of the standard marker 100 bp DNA ladder.

2.1.8 PCR Amplification of 16S rRNA Gene

Verification of Sesbania rhizobia was carried out by 16S rRNA gene amplification by PCR followed by sequence analysis of the PCR product. The genomic DNA (100 ng) as a template and universal primers designed for 16S rRNA gene (Acc. No. AY 217118) were used to perform the PCR (Saiki et al.1988). The amplification was carried out by a Gradient Cycler (MJ Research, USA) for 30 cycles using a pair of oligonucleotide primers. FP2: 5'-AGAGTTGATCATGGGCTCAG-3' and RP2: 5'-TA GGCTACCTTGTACG -3'
The reaction setup was as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template DNA</td>
<td>8.0μl (100 ng)</td>
</tr>
<tr>
<td>Forward primer</td>
<td>2.5 μl (5 pM)</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>2.5 μl (5 pM)</td>
</tr>
<tr>
<td>DNTP's mix</td>
<td>10.0 μl (2 mM stock)</td>
</tr>
<tr>
<td>Buffer (10X)</td>
<td>10.0 μl</td>
</tr>
<tr>
<td>Taq Polymerase</td>
<td>2.0 μl</td>
</tr>
<tr>
<td>ADW</td>
<td>65.0 μl</td>
</tr>
<tr>
<td>Total volume</td>
<td>100 μl</td>
</tr>
</tbody>
</table>

The following conditions were used in the amplification reaction:

(i) Initial denaturation at 95°C for 5 min.
(ii) Denaturation of the template DNA at 95°C for 1 min.
(iii) Annealing of the primers to the template DNA at 56°C for 1 min.
(iv) Extension of the annealed primer at 72°C for 1 min.
(v) Blocking temperature was kept at 72°C for 7 min.

The block was finally allowed to cool down to 4°C.

2.1.9 Quantification of DNA

The concentration and purity of DNA was determined by measuring the absorbance at 260 and 280 nm in a spectrophotometer. An absorbance of 1 unit at 260 nm in a cuvette with a 1.0 cm path was indicative of double-stranded DNA at a concentration of 50 μg/ml. The ratio of the absorbance at 260 to 280 nm was a useful indication of purity. The ratio of 1.8-1.9 was considered to be of significance for good quality DNA.

2.1.10 Sequencing of PCR Product

The PCR product was sequenced using automated DNA sequencer (Perkin Elmer ABI PRISMS 377 DNA sequencer). The universal primers for 16S rRNA were used to confirm the sequence. The blast homology search was carried out to find the appropriate matches. The analysis of initial results showed that the sequence of _Rhizobium_ isolated from _S. aculeata_ nodules matched 99% with that of _Sinorhizobium saheli_. It was designated as SB2.
2.1.11 Antibiotic Sensitivity of SB2 Isolated from S. aculeata

The strains of SB2 were checked for their resistance to various antibiotics such as kanamycin, ampicillin, neomycin, streptomycin and nalidixic acid upto the concentration of 400.0 µg/ml. The YEM tube of 5.0 ml was made, autoclaved and after cooling the tubes to RT, various concentration of above mentioned antibiotics were added. The tubes were inoculated with SB2 and kept for incubation at 28°C for 48 h. The growth of SB2 observed after 48 h was measured by taking OD at 620 nm. The tubes in which the SB2 showed growth were resistant to that antibiotic. The SB2 sensitive to the particular antibiotic was indicated by ‘−’ and resistant to the particular antibiotic was indicated by ‘+’.

2.1.12 Tn5 Mutagenesis

2.1.12.1 Transconjuration

Tn5 mutagenesis of SB2 was carried out by the method of Selvaraj and Iyer (1983). E. coli WA803 consisting of pGS9 vector neomycin resistant (Neo<sup>5</sup>) and chloramphenicol resistant (Cm<sup>5</sup>) was grown in TY broth to the stationary phase. The recipient rhizobial cells were grown in YEM broth till stationary phase. The mating was performed by mixing 1x10<sup>9</sup> cells of donor and 1x10<sup>8</sup> cells of recipient on YEM agar plates followed by incubation at 30°C for 24 h. After mating, the patched cultures were diluted in YEM broth and dilutions of cell suspension were plated on YEM agar supplemented with neomycin 400 µg/ml and nalidixic acid 50 µg/ml. The neomycin and nalidixic acid resistant colonies were screened after 5 days of incubation at 30°C. These colonies (approximately 300 in number) were called as mutants. These mutants were white in colour and less slimy compared to wild strain SB2. The SB2 was slightly yellowish in colour and was having very slimy appearance.

2.1.12.2 Verification of Tn5 Insertion

Verification of Tn5 insertion into rhizobial cells was carried out using PCR amplification of 820.0 bp product encoded by a region 1533-2350 bp in Tn5. The SB2 genomic DNA was used as a template. As a positive control the plasmid pGS9 was used. The amplification was performed using a Minicycler (MJ Research, USA) for 29.0 cycles using a pair of oligonucleotide primers 5'-GGATGAGGATCGTTTCGCAT -3' and 5'-CCCGCTCAGAAGAACTCGTC -3'.
The reaction setup was as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template DNA</td>
<td>8.0 µl (100 ng)</td>
</tr>
<tr>
<td>Forward primer</td>
<td>2.5 µl (20 pmoles)</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>2.5 µl (20 pmoles)</td>
</tr>
<tr>
<td>dNTP's mix</td>
<td>10.0 µl (2 mM stock)</td>
</tr>
<tr>
<td>Buffer (10x)</td>
<td>10.0 µl (25 mM stock)</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>2.0 µl (6 units)</td>
</tr>
<tr>
<td>ADW</td>
<td>65.0 µl</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>100.0 µl</strong></td>
</tr>
</tbody>
</table>

The following conditions were used in the amplification reaction:

(i) Initial denaturation at 95°C for 5 min.
(ii) Denaturation of the template DNA at 95°C for 1 min.
(iii) Annealing of the primers to the template DNA at 62.4°C for 1 min.
(iv) Extension of the annealed primer at 72°C for 1 min.
(v) Blocking temperature was kept at 72°C for 7 min.

The block was finally allowed to cool down to 4°C.

The PCR product from SB2 was analyzed on 0.8% (w/v) agarose gels for its Tn5 insertion as mentioned above.

2.1.12.3 Screening of Transconjungants

(i) Antibiotic Profile

The transconjungants were checked for its resistance to antibiotic such as, kanamicin, ampicillin, neomycin, streptomycin and nalidixic in a concentration range from 0.0 µg/ml to 400.0 µg/ml. Each transconjungant was plated in YEM patch plate containing above mentioned antibiotics. The colonies which were resistant to neomycin and nalidixic acid were inoculated in a tube containing 5.0 ml YEM medium and grown at 28°C for about 4 days in the presence of 400.0 µg/ml neomycin and 50.0 µg/ml nalidixic acid. The growth of the strains was measured by taking OD at 620 nm. These colonies which were resistant to neomycin 400.0 µg/ml and nalidixic 50.0 µg/ml acids were designated as mutants.
(ii) Random selection of mutants for nodulation experiments

Out of all the transconjugants (300 colonies) ten mutants were selected for nodulation experiments. Each of these ten mutants showed resistance to nalidixic acid and neomycin. Out of these ten mutants, five mutants were randomly selected based on their mucoid morphology for nodulation experiments. These mutants were designated as SB2M1, SB2M2, SB2M3, SB2M4 and SB2M5. These five mutants were also verified for Tn5 insertion by PCR amplification.

2.1.13 Nodulation Experiments

The capacity of roots of leguminous plants to develop nodules has been considered to be an important phenomenon to demonstrate the level of plant-rhizobial interaction and finally the atmospheric nitrogen fixation. The nodulation has been found to be increased with increasing infection by rhizobia. Therefore, the nodulation experiments were designed using SB2 and its mutants.

2.1.13.1 Growth and Nodulation of S. aculeata

The seeds of S. aculeata were surface sterilized with 70% ethyl alcohol, thoroughly rinsed with DW and then kept in water for 24 h. The seeds were then sprinkled with fungicide and kept overnight on sterile moist filter paper in a petri plate for germination. The germinated seeds were sown in autoclaved garden soil in sterile plastic pots. The seeds were then inoculated with 0.1 ml of SB2 culture or its mutants. The optical density of the culture in each case was observed to be 1.5 at 620 nm. The pots were then kept in the natural conditions for their growth for 4 wk.

2.1.13.2 Nitrogenase Activity

The plants of four wk of age were taken out of the pots. The roots along with nodules were removed with sterile blades and washed with tap water. The roots were gently pressed between two filter paper to remove excess of water and then placed in a 20.0 ml culture tube. Each tubes was sealed using subaseal stoppers and injected with 2.0 ml (10% of gas phase) of acetylene after removing equivalent amount of air. The tubes were then incubated at 28°C for 2.0 h. After incubation, 2.0 ml of gas samples were removed and analyzed for acetylene and ethylene content using a Hewlett-Packard 5890 –A Gas Chromatograph (GC) fitted with a flame ionization
detector and “Poropak N” in a 1.5 mm x 1.32 m column. This method was called acetylene reduction assay (ARA) technique.

The reduction of N2 and C2H2 by nitrogenase may be designated as follows:

\[ \text{N}_2 + 6\text{H}^+ + 6\text{e}^- \rightarrow 2\text{NH}_3 \]
\[ \text{C}_2\text{H}_2 + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{C}_2\text{H}_4 \]

Thus, the theoretical ratio of substrates reduced was 3:1, which was included in the equation used to quantify the rate of N2 fixation based on C2H2 reduction

\[ \text{mg N} = \frac{(\text{mM C}_2\text{H}_4 \times 28)}{3} \]
\[ \text{mM C}_2\text{H}_2 \text{ produced} = \mu \text{ moles of C}_2\text{H}_2 \text{ produced} / 1000 \]
\[ \mu \text{ moles of C}_2\text{H}_4 \text{ produced} / \text{h} / \text{g of fresh wt} = C \times P_s \times A_s \times V / P_{\text{std}} \times A_{\text{std}} \times T \times Wt \]

Where
C = concentration of C2H2 in standard in µ moles
Ps = Peak height or peak area of sample
As = Attenuation used for sample
Pstd = Peak area of standard
T = Time of incubation in h
Wt = Fresh weight of nodule in roots
V = Volume of air space in the assay tube

The ARA values were expressed as mg nitrogen produced per unit dry weight of nodules.

2.1.14 Characterization of Mutants of Rhizobium Specific to S. aculeata

2.1.14.1 Growth of Rhizobium Cultures

The SB2 and its mutant were grown in a flask containing 100.0 ml YEM medium. The flasks were kept in an orbital shaker (New Brunswick Scientific, USA) at 200 rpm and 28°C. The culture was grown till their stationary phase arrived (wild strain till 72 h and mutant strain till 120 h). The cell density was measured after every 4 h of interval by taking OD at 620 nm in Perkin Elmer spectrometer.

2.1.14.2 Effect of Salt and pH Stress on Growth

Growth rate of SB2 and its mutant were monitored in the presence of the stress conditions namely salt and pH. The SB2 and its mutant strains were grown
in a flask containing YEM broth with 0.2 M, 0.6 M, 1.0 M, 1.5 M, 2.0 M NaCl. The cultures were also grown in a different flasks containing YEM medium at various pH ranging from pH 3.0 to pH 11.0. The flasks were kept in an orbital shaker (New Brunswick Scientific, USA) at 200 rpm and 28°C. The culture was grown till their stationary phase arrived (wild strain till 72 h and mutant strain till 120 h). The growth rate was monitored at regular intervals of 4 h. Cell density measurements were done at an OD of 620 nm with Perkin Elmer (Lambda Bio 20) spectrophotometer.

2.1.14.3 Hydrophobicity Assay

The bacterial cell surface hydrophobicity was tested by the two-phase partition method described by Rosenberg et al. (1980) using n-hexadecane as the hydrocarbon phase. Briefly, bacteria grown in YEM broth were harvested by centrifugation at 5,000 x g for 10 min and resuspended in phosphate buffered saline (pH 7.4) to give OD$_{400}$ = 1.0. For qualitative monitoring of hydrophobicity, equal volume of cell suspension (5 ml) and hexadecane were mixed for each type of cells and were monitored for cell partitioning.

The OD at 400 nm was recorded for the aqueous phase after allowing it to stand at RT for 15 min for the phase separation. E. coli, a hydrophilic strain, was taken as a control. The hydrophobicity was expressed as the percentage of the initial absorbance of the aqueous suspension.

2.1.14.4 Sudan Black B Staining

The mutant strains of SB2 were examined for Sudan Black B staining for their screening as EPS deficient mutants (Liu et al. 1998). Briefly, the bacteria were grown as single colonies on plates containing TY agar. Approximately, 8.0 ml of the 0.02% Sudan Black B solution (0.02% solution of Sudan Black B dissolved in 96% ethanol) was applied to the plate and allowed to remain undisturbed for about 10 min. The dye was then decanted, and the plates were gently rinsed by adding 10.0 ml of 100% ethanol and swirling for a few min. Colonies unable to incorporate the Sudan Black B stain appeared white, while colonies able to incorporate the dye appeared bluish black.
2.1.14.5 Protein Profile of Mutants

(a) Sample preparation

The SB2 and SB2M3 were analyzed for their protein profile by performing two dimensional gel electrophoresis. The strains were grown in a flasks containing 500.0 ml YEM medium at 28°C until their OD reached to 1.5 at 620 nm. The sample preparation was carried out by pelleting the cell from culture medium by centrifuging at 6000 x g for 1 h at 4°C. The cells were washed with PBS (pH 7.2) and then suspended in 10.0 ml of lysis buffer. The cells were sonicated for 10 min and centrifuged again for 1 h at 6000 x g at 4°C. The supernatant thus obtained was treated as protein sample. The protein estimation of these samples was carried out by Bradford method.

(b) 2-D Gel Electrophoresis

First dimension gel electrophoresis was carried out in PROTEIN IEF cell, BIO-RAD. The protein sample (200.0 μg/strip) were applied to the 7.0 cm ready made IPG strip (4/7 pH) along with 125.0 μl rehydration buffer. The strips were rehydrated for 12 h at 20°C. The IEF was performed after the rehydration for 4 to 6 h at 10,000 Vh.

Second dimension gel electrophoresis was carried out in Mini-PROTEAN II slab cell, BIO-RAD. The first dimension IEF strip were equilibrated for 30 min in equilibrium buffer and then run in a 12% SDS-PAGE. The gels were then silver stained and analyzed for differential protein pattern.

2.1.14.6 Electron Microscopy

Changes in the cell morphology and its internal structure were monitored by electron microscopy. For transmission electron microscopy (TEM), the cultures of SB2 and its mutant SB2M3 were inoculated at initial OD of 0.1 (660 nm) and then grown till the OD reached 0.6. After harvesting, cells were washed three times with phosphate buffer (pH 7.4) and then resuspended in fixative solution containing 2.5% glutaraldehyde prepared in 0.1 M phosphate buffer (pH 7.4) and incubated overnight. Samples were then mounted on microscope stubs and sputter coated on sputter coater and were examined for surface structure analysis on LEO 435 VP scanning Electron Microscope. TEM analysis for internal structure changes was examined on Philips CM-10 TEM.
2.1.15 Isolation of LPS

Isolation of LPS was carried out by growing SB2 and SB2M3 in 500.0 ml batches at 28°C to late log phase (OD_{620} = 1.5) in a flask containing YEM medium. Cells were harvested by centrifugation at 2000 x g for 30 min at 4°C. The yield was 2.0-5.0 g wet wt / L of culture. The pellet was suspended in phosphate buffered saline (PBS, pH 7.2) and centrifuged to remove any loosely bound polysaccharides. The LPS of the bacterial cell wall was isolated by the hot water-phenol extraction procedure (Westphal and Jann 1965). The washed rhizobial pellet (2.0 g) was thoroughly suspended in 30.0 ml hot water at 65°C. After a homogeneous suspension was obtained, 30.0 ml of phenol at 65°C was added to it. The suspension was stirred for 15 min at 65°C. The suspension was cooled immediately in an ice water bath for 15 min and then centrifuged at 6000 x g for 30 min at 4°C. The upper phase was separated and the phenol phase was reextracted twice with same volume of hot water at 65°C. The water layers were pooled and exhaustively dialyzed against distilled water. DNase I (1000.0 kunitz/mg) and of RNase A (100.0 kunitz/ mg) were added to the concentrated water phase and stirred overnight to remove ribose sugars. The sample was then treated with proteinase K (100.0 µg/ml) at 37°C for 1 h. The sample was redialyzed and lyophilized. The lyophilized powder was designated as crude LPS and stored at 4°C till further use.

2.1.16 Isolation of EPS

Isolation of EPS was carried out by centrifuging SB2 and SB2M3 culture at 3000 x g for 30 min at 4°C. The supernatant thus obtained was dialyzed against DW. The dialyzed was lyophilized to minimum volume. The concentrated volume thus obtained was precipitated with absolute alcohol. The amount of precipitate was dissolved in minimum volume of DW. It was designated as EPS and was stored at 4°C till further use.

2.1.17 Analysis of LPS by Colorimetric Assays

2.1.17.1 Hexoses

Neutral hexoses were determined by orcinol sulfuric acid method (White and Kennedy 1979).
2.1.17.2 Uronic Acids

Uronic acids were estimated by the carbozole method (Bitter and Muir 1962). Two reagents were prepared for this assay:

(a) Sodium tetraborate dihydrate (0.95 g) was dissolved in 2.0 ml hot water and 98.0 ml ice cold Conc. H$_2$SO$_4$.
(b) Dissolved 125.0 mg carbozole in 100.0 ml ethanol.

The samples and standards were made to a volume of 250.0 μl and kept in an ice bath. Reagent A (1.5 ml) was added while stirring. The mixture was heated at 100°C for 10 min. After cooling the tubes rapidly in an ice bath, 50.0 μl of reagent B was added and stirred. The tubes were reheated at 100°C for 15 min. The tubes were rapidly cooled and spectrophotometrically read at 525 nm. Commercially available glucuronic acid was used as a standard.

2.1.17.3 Electrophoretic Analysis of LPS

LPS samples were loaded on a 12% polyacrylamide gel with 0.1% SDS. The gels were run at a constant current of 20.0 mA / well. After the run, the LPS gels were fixed overnight in 200.0 ml of 25% isopropanol in 7% acetic acid and silver stained for carbohydrates (Hitchcock, 1983). The gels were treated with periodic acid (1.05 g periodic acid dissolved in 150.0 ml distilled water containing 4.0 ml of 25% isopropanol and 7% acetic acid) for oxidation. After washing in 200.0 ml triple DW each, the gels were silver stained in a solution consisting of 0.1 N NaOH (28.0 ml), ammonium hydroxide (1.0 ml), 20% (w/v) silver nitrate (5.0 ml) and DW (115.0 ml). After four washings with 10 min intervals, the gels were developed in 250.0 ml solution (citric acid- 12.5 mg and formaldehyde- 125.0 μl). The gels were finally kept in a stop solution (22.0 ml distilled water with 10.0 ml of 7% acetic acid) for 1.0 h and stored in DW.

2.1.18 Lectin-LPS Binding

2.1.18.1 Biotinylation of Lectins

Lectins were biotinylated in accordance with manufacture’s guidelines for labeling of proteins by carbodiimide coupling. The following steps were followed.
(i) 5.0 mg/ml of purified lectin was dissolved in 250.0 mM NaCl, maintained at 5.0-6.0 pH.

(ii) Dissolved biotin (long arm) hydrazide in dimethylsulfoxide at a concentration of (50.0 mg/ml)

(iii) Dissolved 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide–HCl (EDC) in 150.0 mM NaCl at a concentration of 100.0 mg/ml.

(iv) Dissolved N-hydroxysulfosuccinimide (NHS) in 150.0 mM NaCl at a concentration of 10.0 mg/ml.

(v) Added 20.0 μl of biotin, 100.0 μl of EDC and 100.0 μl NHS per ml of lectin solution.

(vi) Incubated for 3 h for overnight at RT.

(vii) Dialysed the sample to separate the unreacted material from the biotinylated lectin.

(viii) The biotinylated lectin was stored in 4°C till further use.

2.1.18.2 Dot blot

The biotinylation of the lectin was checked by dot blot. The biotinylated lectin (1.0 μg) was loaded on the nitrocellular (NC) membrane. The non-specific binding sites were blocked by 4% BSA (periodate treated) for 2 h at 37°C. After washing several times with PBS the NC was incubated with streptavidin–peroxidase at a concentration of 0.1 mg/ml in 1:700 times dilution. The washing was done in buffer and finally it was developed in 3,3’- diaminobenzidine.

2.1.18.3 Enzyme Linked Lectin Binding Assay (ELLBA) for LPS

Purified lectins SSA, SRA, SSTα and SNA were labeled with biotin as described above. The LPS suspension from strain SB2, SB2M3, GN17, M28, M19, SB16, P-14, IGR92, Rcd301 and LM were coated at a concentration of 10 μg/100.0 μl in triplicate, in 96 well microtitre plate (Nunc, Denmark) and was allowed to incubate overnight at 37°C. The wells were washed five times with washing buffer (10.0 mM PBS, pH 7.2 with 0.02% Tween 20) followed by blocking with 100.0 μl of 1% BSA in PBS and incubation for 2.0 h at 28°C. The blocking buffer was then removed from the wells and then washed by five consecutive washes with 100.0 μl washing buffer. The plates were then incubated for 3 h at 28°C after adding 100.0 μl (2.0 μg) biotynilated lectin. The wells were washed five times with washing buffer followed
by addition of 100.0 μl of streptavidin- HRP conjugate (1:700 dilution in DW) to each well and subsequent incubation at 28°C for 45 min. After incubation the plate was washed five times with washing buffer and the final colour was allowed to develop with OPD (1.0 mg/ml in 0.05 M citrate buffer and 5.0 μl/ml H₂O₂) for 30 min. The yellow colour was allowed to develop for 30 min at RT. The reaction was then stopped by adding 100.0 μl of 2 N H₂SO₄ to each well. The absorbance was then read at 492 nm in an ELISA reader (spectramax plus Molecular Devices, USA).