# Morphological and Biochemical characterization of bacteria isolated from root nodules and rhizosphere of *Glycine max* L.

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***Abstract*: *Glycine max* L. is an annual legume and cultivated worldwide for human consumption. In this study 21 isolates were obtained from the root nodules and rhizosphere of *Glycine max* L. The isolates were studied on the basis of their morphology and biochemical characterization. Isolates identified in the present investigation was made on the basis of biochemical tests performed. Further study is required for the best use of the isolated strains obtained in the production of biofertilizers.**

**Keywords: *Glycine max* L., Legume, Root nodules, Rhizosphere.**

## 1. INTRODUCTION

*Glycine max* L. (Soybean) is an important leguminous food crops belongs to family fabaceae. It was introduced in India from China in 10th century AD[1,2]. It contributes about 25% of the global edible oil and it is the world's most important seed legumes[2,3]. It is used in various ways like meat, milk, cheese, bread and oil. It is also good for heart disease and diabetes[2,4]. In grain legumes about one-third of human dietary protein is found[5]. Soybean can be grown under minimum agricultural inputs, management practices and climatic adversities so it helped in the improvement of socio-economic conditions of its grower[2]. Through symbiotic biological nitrogen fixation legumes produce sustainable amounts of organic nitrogen and also maintain the fertility of soil[5]. For the formation of nodule and its activity specific *Rhizobium* species require specific leguminous crops[6]. In symbiosis with the bacteria *Bradyrhizobium japonicum* soybean has the ability of fixing atmospheric nitrogen[7]. The area of the soil where plant roots secreates various metabolites is known as rhizosphere[8,9]. For sustainable soybean production rhizospheric microbes are needed[10]. *Rhizobium* spp., *Pseudomonas* spp., *Azospirillum* spp., *Bacillus* spp., are the plant growth stimulating bacteria[11].

## 2. MATERIALS AND METHODS

A. Collection of seeds

The seeds of *Glycine max* L. (Variety-BS-1 and BS-2) were collected from Birsa Agricultural University, Kanke, Ranchi, Jharkhand and were grown in pots.

B. Collection and Surface sterilization of root nodules

After flowering root nodules were taken from freshly uprooted plants. The roots of the plants were thoroughly washed under tap water to remove all the impurities. For the isolation of bacteria healthy root nodules were selected. After properly washing the root nodules were put in 70% ethanol for 30 seconds. Then they were treated with 3-5% $H_2O_2$ for 2 minutes. Then they were successively washed 3-4 times with sterile distilled water[12-15].

- Isolation of bacterial strains from root nodules

The sterilized root nodules were crushed with the help of pestle and mortar and the contents were spread on Yeast Extract Mannitol Agar (YEMA) plates. Then all the plates were incubated at 28± 2˚C for 24 hours. After 24 h colonies were picked for sub-culturing to obtain the pure culture.

C. Collection of rhizospheric soil

Soil samples were collected from rhizospheric area after flowering.

- Isolation of bacterial strains from rhizospheric soil

Isolation of the desired microorganisms was done by the serial dilution method (Dilutions- $10^{-1}$-$10^{-9}$)[16]. The dilutions were inoculated on YEMA plates and incubated at 28± 2˚C for 24 hours. Then the spreading technique was used for the separation of microbial colonies from each other present into the diluted sample in order to get single isolated colony in a mixed culture plate. This was followed by the streaking method which is used to get the only single type of culture on to the Yeast Extract Mannitol Agar plate. This technique is used to get the reduced number of the microbes from the rhizospheric soil samples.

D. Culture characterization

The isolates were studied on the basis of their size, form, elevation, margin, pigmentation, Gram staining, and appearance in YEMA media. Biochemical test like starch hydrolysis, casein hydrolysis, gelatin hydrolysis, indole test, methyl red test, voges-proskauer test, triple sugar-iron agar test, citrate utilization test, $H_2S$ production test, nitrate reduction test and catalase test were also done.

I.Morphological Characterization

Size, form, elevation, margin, pigmentation and appearance in YEMA media were observed.

Gram Staining:

The bacterial smear was prepared with a loop full of isolated strains of bacteria via spreading over a clean slide in a drop of distilled water and is allowed to dry in air. The smear is heat fixed by passing over the flame and then stained with crystal violet solution for 1 minute followed by rinsing with distilled water and is allowed to dry. The slide is then poured with Gram's Iodine solution (Mordant) for 1 minute followed by rinsing with distilled water and decolorized with alcohol. Then again rinsed with distilled water and is allowed to dry in air. The smear thus obtained was stained with counter stain for 1 minute followed by rinsing with distilled water and is allowed to dry in air then the slide was observed under the microscope. Gram negative cell appears pink-red in colour and Gram positive as violet[17].

II. Biochemical Characterization

Biochemical test like starch hydrolysis, casein hydrolysis, gelatin hydrolysis, indole test, methyl red test, voges-proskauer test, triple sugar-iron agar test, citrate utilization test, $H_2S$ production test, nitrate reduction test and catalase test were also done[18-22].

a) Starch hydrolysis

Starch agar media was prepared and inoculated with isolated strains then incubated at 28± 2˚C for 24 hours. After 24 hours drops of Iodine solution were spread on the culture grown on petri-plates. Then observed a clear zone of hydrolysis surrounding the growth of the culture shows positive results.

b) Casein hydrolysis

Milk agar media was prepared and inoculated with isolated strains then incubated at 28± 2˚C for 24 hours. After 24 hours observed a clear zone of hydrolysis surrounding the growth of the culture shows positive results.

c) Gelatin hydrolysis

Gelatin media was prepared and poured in test tubes then inoculated with isolated strains and incubated at 28± 2˚C for 24 hours. After 24 hours, the cultures were placed in refrigerator at 4˚C for 30 minutes. After 30 minutes observed the cultures. The cultures that remain liquefied show positive results.

d) Catalase test

With the help of a sterile loop, took a small amount of cultures on the clean slide then place 1 drop of 3% hydrogen peroxide on the culture. The evolution of air bubbles indicates positive results.

e) Indole test

Nutrient broth medium was prepared and poured into the test tubes. The isolated strains were inoculated to the broth and incubated at 28± 2˚C for 2 days. Then 1 ml of Kovac's reagent was added to each test tube. The tubes were gently shaken and allowed to stand until the reagent reaches the top. The formation of red color ring indicates positive results.

f) Methyl red test

Nutrient broth medium was prepared and poured into the test tubes. The isolated strains were inoculated to the broth and incubated at 28± 2˚C for 2 days. Then 5 ml of methyl red indicator was added to each test tube. The red coloration of the broth indicates positive results.

g) Voges-Proskauer test

Nutrient broth medium was prepared and poured into the test tubes. The isolated strains were inoculated to the broth and incubated at 28± 2˚C for 2 days. Then 5 ml of Baritt's reagent A and B was added to each test tube. The formation of red color indicates positive results.

h) Citrate utilization test

Simmons citrate agar medium was prepared and poured in the test tubes. The isolated strains were inoculated and incubated at 28± 2˚C for 2 days. After incubation, the green color of the medium turned to blue color indicates the positive results.

i) Triple sugar iron (TSI) agar test

Triple sugar iron agar medium was prepared and poured in the test tube and allowed to solidify. The isolated strains were inoculated and incubated at 28± 2˚C for 24 hours. The color of both the butt and slant were observed.

j) Hydrogen sulfide production test

Triple sugar iron agar medium was prepared and poured in the test tube and allowed to solidify. The isolated strains were inoculated and incubated at 28± 2˚C for 24 hours. The black color of the medium shows positive results.

k) Nitrate reduction test

For nitrate reduction test, nutrient broth with 1% potassium nitrate was prepared and poured into the test tubes and then each test tube was inoculated with the isolated strains and incubated at 28± 2˚C for 3 days. Then add 0.5ml solution of sulphuric acid and alpha naphthylamine in the culture broth. Development of red color indicates the positive results.

## 3. Results and Discussion

The results of the present study were shown in Tables and figures.

Table 1. Morphological Characterization of the bacteria isolated from *Glycine max* L. (Variety-BS-1)

(RNBS-1:- Bacteria isolated from root nodule of *Glycine max* L. of variety BS-1;

| S.No. | Strain | Morphological Characterization of the bacteria isolated from *Glycine max* L. (Variety-BS-1) in YEMA media | | | | |
|---|---|---|---|---|---|---|
| | | Size | Form | Elevation | Margin | Pigmentation |
| 1 | RNBS-1 | Small, Moderate | Circular | Raised | Entire | White |
| 2 | BS-1-(I) | Large | Circular | Convex | Entire | White |
| 3 | BS-1-(II) | Pinpoint, Small | Circular | Raised | Entire | Off Yellow |
| 4 | BS-1-(III) | Pinpoint, Small | Circular | Raised | Entire | Off Yellow |
| 5 | BS-1-(IV) | Large | Irregular | Convex | Entire | White |
| 6 | BS-1-(V) | Pinpoint, Small, Moderate | Circular | Raised | Entire | Off White |
| 7 | BS-1-(VI) | Pinpoint, Small | Circular | Raised | Entire | Off Yellow |
| 8 | BS-1-(VII) | Pinpoint, Small, Moderate | Circular | Raised | Entire | White |
| 9 | BS-1-(VIII) | Pinpoint, Small | Circular | Raised | Entire | Off Yellow |
| 10 | BS-1-(IX) | Pinpoint, Small | Circular | Convex | Entire | Off White |
| 11 | BS-1-(X) | Pinpoint, Small | Circular | Raised | Entire | Off Yellow |

BS-1-(I-X):- Bacteria isolated from rhizosphere of *Glycine max* L. of variety BS-1)

Table 2. Morphological Characterization of the bacteria isolated from *Glycine max* L. (Variety-BS-2)

| S.No. | Strain | Morphological Characterization of the bacteria isolated from *Glycine max* L. (Variety-BS-2) in YEMA media | | | | |
|---|---|---|---|---|---|---|
| | | Size | Form | Elevation | Margin | Pigmentation |
| 1 | RNBS-2 | Pinpoint,Small, Moderate | Circular | Convex | Entire | Off White |
| 2 | BS-2-(I) | Pinpoint, Small | Circular | Raised | Entire | White |
| 3 | BS-2-(II) | Pinpoint, Small | Circular | Raised | Entire | Off Yellow |

| | | | | | | |
|---|---|---|---|---|---|---|
| 4 | BS-2-(III) | Pinpoint, Small, Moderate | Irregular | Convex | Entire | Off White |
| 5 | BS-2-(IV) | Pinpoint, Small | Circular | Raised | Entire | Off Yellow |
| 6 | BS-2-(V) | Pinpoint,Small | Circular | Raised | Entire | Off White |
| 7 | BS-2-(VI) | Pinpoint, Small, Moderate | Circular | Raised | Entire | White |
| 8 | BS-2-(VII) | Pinpoint, Small | Circular | Raised | Entire | Off White |
| 9 | BS-2-(VIII) | Pinpoint,Small | Circular | Raised | Entire | Off White |
| 10 | BS-2-(IX) | Pinpoint, Small | Circular | Raised | Entire | Yellow |

(RNBS-2:- Bacteria isolated from root nodule of *Glycine max* L. of variety BS-2;
BS-2-(I-IX):- Bacteria isolated from rhizosphere of *Glycine max* L. of variety BS-2)

Table 3. Biochemical Characterization of the bacteria isolated from *Glycine max* L. (Variety-BS-1)

| S.No. | Strain | Biochemical Characterization of the bacteria isolated from *Glycine max* L.(Variety-BS-1) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Gram Staining | Starch Hydrolysis | Casein Hydrolysis | Gelatin Hydrolysis | Catalase test | Indole test | Methyl Red test | Voges-Proskauer test | Citrate Utilization test | TSI test | $H_2S$ Production test | Nitrate Reduction test |
| 1 | RNBS-1 | -ve | +ve | +ve | +ve | +ve | -ve | -ve | -ve | -ve | +ve | -ve | +ve |
| 2 | BS-1-(I) | -ve | +ve | +ve | +ve | +ve | -ve | -ve | -ve | -ve | +ve | -ve | +ve |
| 3 | BS-1-(II) | -ve | +ve | +ve | +ve | +ve | -ve | -ve | -ve | +ve | +ve | -ve | +ve |
| 4 | BS-1-(III) | -ve | +ve | +ve | +ve | +ve | -ve | -ve | -ve | -ve | +ve | -ve | +ve |
| 5 | BS-1-(IV) | -ve | -ve | +ve | +ve | +ve | -ve | -ve | -ve | -ve | +ve | -ve | +ve |
| 6 | BS-1-(V) | -ve | +ve | +ve | +ve | +ve | +ve | -ve | -ve | -ve | +ve | -ve | +ve |
| 7 | BS-1-(VI) | -ve | +ve | +ve | +ve | +ve | +ve | -ve | -ve | +ve | +ve | -ve | +ve |
| 8 | BS-1(VII) | -ve | +ve | +ve | +ve | +ve | +ve | -ve | -ve | +ve | +ve | -ve | +ve |
| 9 | BS-1-(VIII) | -ve | +ve | +ve | +ve | +ve | +ve | -ve | -ve | +ve | +ve | -ve | +ve |
| 10 | BS-1-(IX) | -ve | +ve | +ve | -ve | +ve | +ve | +ve | -ve | -ve | +ve | -ve | +ve |
| 11 | BS-1-(X) | -ve | +ve | +ve | -ve | +ve | -ve | +ve | -ve | +ve | +ve | -ve | +ve |

(RNBS-1:- Bacteria isolated from root nodule of *Glycine max* L. of variety BS-1;
BS-1-(I-X):- Bacteria isolated from rhizosphere of *Glycine max* L. of variety BS-1)

Table 4. Biochemical Characterization of the bacteria isolated from *Glycine max* L. (Variety-BS-2)

| S.No. | Strain | Biochemical Characterization of the bacteria isolated from *Glycine max* L.(Variety-BS-2) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Gram Staining | Starch Hydrolysis | Casein Hydrolysis | Gelatin Hydrolysis | Catalase test | Indole test | Methyl Red test | Voges-Proskauer test | Citrate Utilization test | TSI test | $H_2S$ Production test | Nitrate Reduction test |
| 1 | RNBS-2 | -ve | +ve | +ve | +ve | +ve | -ve | -ve | +ve | -ve | +ve | -ve | +ve |
| 2 | BS-2-(I) | -ve | +ve | +ve | +ve | +ve | -ve | -ve | +ve | -ve | +ve | -ve | +ve |
| 3 | BS-2-(II) | -ve | +ve | +ve | +ve | +ve | -ve | +ve | -ve | -ve | +ve | -ve | +ve |
| 4 | BS-2-(III) | -ve | +ve | +ve | +ve | +ve | +ve | +ve | -ve | -ve | +ve | -ve | +ve |
| 5 | BS-2-(IV) | -ve | -ve | +ve | +ve | +ve | -ve | +ve | -ve | -ve | +ve | -ve | +ve |
| 6 | BS-2-(V) | -ve | +ve | +ve | +ve | +ve | -ve | -ve | -ve | +ve | -ve | -ve | +ve |
| 7 | BS-2-(VI) | -ve | +ve | +ve | +ve | +ve | +ve | +ve | -ve | -ve | +ve | -ve | +ve |
| 8 | BS-2-(VII) | -ve | -ve | +ve | +ve | +ve | +ve | +ve | -ve | -ve | +ve | -ve | +ve |
| 9 | BS-2-(VIII) | -ve | +ve | +ve | +ve | +ve | +ve | +ve | -ve | -ve | +ve | -ve | +ve |
| 10 | BS-2-(IX) | -ve | -ve | +ve | +ve | -ve | +ve | +ve | -ve | -ve | +ve | -ve | +ve |

(RNBS-2:- Bacteria isolated from root nodule of *Glycine max* L. of variety BS-2;
BS-2-(I-IX):- Bacteria isolated from rhizosphere of *Glycine max* L. of variety BS-2)

A. B. C. D.

**Fig.1 Showing**: A. Plants of *Glycine max* L. Variety (BS-1)

B. Plants of *Glycine max* L. Variety (BS-2)

C. Root Nodules of *Glycine max* L. Variety (BS-1)

D. Root Nodules of *Glycine max* L. Variety (BS-2)

A. B. C. D.

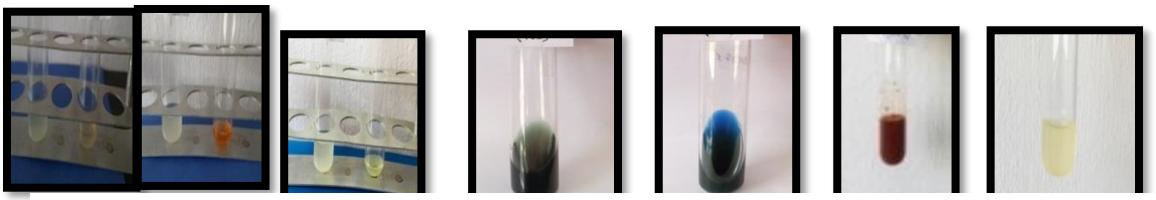
**Fig.2 showing:** A: Pure culture of bacteria isolated from root nodules of *Glycine max* L. (Var. BS-1)

B: Pure culture of bacteria isolated from root nodules of *Glycine max* L. (Var. BS-2)

C: Pure culture of bacteria isolated from rhizosphere of *Glycine max* L. (Var. BS-1)

D: Pure culture of bacteria isolated from rhizosphere of *Glycine max* L. (Var. BS-2)

A. B. C. (Negative) D.(Positive) E. F.



G. H. I. J.(Negative) K.(Positive) L.(Positive) M.(Negative)

**Fig.3 showing**: **A**- Starch Hydrolysis test **B-** Casein Hydrolysis test **C-D-** Gelatin Hydrolysis test
**E-** Catalase test **F-** TSI test **G-** Indole test
**H-** Methyl Red test **I-** VP test **J-K-** Citrate Utilization test
**L-M-** Nitrate Reduction test

A total of 21 isolates of bacteria were isolated from root nodules and rhizosphere of *Glycine max* L. All the 21 isolates were Gram negative, 18 isolates shows starch hydrolysis test positive, all isolates shows casein hydrolysis test positive, 18 isolates shows gelatin hydrolysis positive, 5 isolates shows indole test positive, 8 isolates shows methyl red test positive, no isolates shows positive for voges-proskauer,7 isolates shows citrate utilization test positive, 20 isolates shows triple sugar-iron agar test positive, no isolates shows $H_2S$ production test positive, 20 isolates shows nitrate reduction test positive and all the isolates shows catalase test positive.

Isolates identified in the present investigation was made on the basis of biochemical tests performed. According to biochemical test it was confirmed that isolates BS-1-(I), BS-1-(V),BS-1-(IX), BS-2-(III), BS-2-(VI), BS-2-(VII) and BS-2-(VIII) are *Rhizobium* species[13,23-26], isolates BS-1RN,BS-1-(VII), BS-2RN, BS-2-(V) are *Bradyrhizobium* species[18,27], isolates BS-1-(II),BS-1-(III),BS-1-(VI), BS-1-(VIII), BS-1-(X), BS-2-(II), BS-2-(IV) and BS-2-(IX) are *Azotobacter*[28] species, isolates BS-1-(IV) and BS-2-(I) are *Azospirillum* species[29] .

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