INNOVARE JOURNAL OF AGRICULTURAL SCIENCE

Vol 6, Issue 1, 2018

ISSN- 2321-6832

Research Article

BIOCHEMICAL CHARACTERIZATION AND PROTEIN PROFILE BY SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESISOF FRENCH BEAN (*PHASEOLUS VULGARIS* L.) ASSOCIATED RHIZOBIA

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Received: 13 February 2018, Revised and Accepted: 04 April 2018

ABSTRACT

Objectives: Plant growth-promoting rhizobacteria are beneficial soil bacteria that colonize plant roots and increase plant growth promotion activity by various mechanisms in numerous ways. Nitrogen is essential element for all forms of life. Legume symbioses have a significant role in agriculture; the plants tolerate to grow on nitrogen deprived soils and reduce the essential for nitrogen inputs for leguminous crops. French bean (*Phaseolus vulgaris L.*) is nodulated with numerous *Rhizobium* species. In the present study, biochemical characterization of six *Rhizobium* strains isolated from French bean nodules collected from different regions of Andhra Pradesh, India.

Methods: The French bean plants were uprooted and loosely adhering soil was detached by gentle shaking. The roots along with mature nodules.

Results: All the isolates of rhizobia were positive to the indole test, nitrate reduction test, urease, catalase test, oxidase, and MacConkey agar test. Starch hydrolysis and gelatin hydrolysis test were positive for all isolates except Rh01 and Rh3 isolates. The maximum amount of protein content 92.22 mg/ml was recorded from Rh6 *Rhizobium* strain and minimum was found, 75.38 mg/ml in Rh3 *Rhizobium* strain.

Conclusion: The conclusion of the present study, the French bean rhizobial isolates toenhance the plant growth and nodulation of bean plant under the different environmental conditions.

Keywords: Rhizobacteria, Adhering, Indole test, Mature nodules, Protein.

INTRODUCTION

Rhizobium is Gram-negative bacteria; they have the capability to produce nodules on the roots of leguminous plants. Rhizobia exist as a free-living saprophyte and in a symbiotic relationship through leguminous plants [4] (Rai *et al.*, 2014). The latter interaction begins with a precise molecular signal exchange among the legume and the free-living *Rhizobium*. One of the most significant sites of biological N2 fixation is inside nodules that form on legume species as a result of a symbiosis among the bacteria and host plant. The *Rhizobium*-legume relationship is greater to other nitrogen-fixing organisms due to its high potential [2] (Deshwal and Chaubey, 2014). The nitrogen fixation is attained by bacteria inside the cells, the nodules, which typically develop on roots and on stems. This mutualistic association is beneficial for both partners, the plant providing dicarboxylic acids as a carbon source to bacteria and getting in return, ammonium. Legume symbioses have a significant role in agriculture. The plants tolerate to grow on nitrogen deprived soils and reduce the essential for nitrogen inputs for leguminous crops. Nitrogen-fixing legumes also give to nitrogen enrichment of the soil and have been used from antiquity as crop rotation species to increase soil fertility. They produce high protein-containing leaves and seeds, and legumes such as soybeans, groundnuts, peas, beans, lentils, and alfalfa are a chief source of protein for human and animal consumption [7]. The French bean (*Phaseolus vulgaris* L.) is nodulated with different fast-rising *Rhizobium* sp., such as *Rhizobium leguminosarum* bv. *phaseoli* [5]. A greenhouse experiment was conducted by Srivastava *et al.* (2006) to examined the effect of N and Zn and their communication in French bean inoculated with *R. leguminosarum* bv. *phaseoli* on nodulation at 40 days after sowing and shoot and root, seed, dry weights, and yields at nutrient concentration, maturity, and uptake at both 40 DAS and maturity. The aims of the present study were to identify the *Rhizobium* isolates and biochemical characters, the carbon source utilization of rhizobial isolates, and protein profiles by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

METHODS

Sample collection

Plants were collected from local agricultural field sites in different districts of Andhra Pradesh. For sample collection, selected 15 sites for French bean sample collections.

Collection of nodules

The French bean plants were uprooted and loosely adhering soil was detached by gentle shaking. The roots along with mature nodules were thoroughly washed in running water until the removal of adhering soil and dust particles. The collected nodules were reserved in sterile polythene bags and transported to the laboratory for further studies. The collected nodules were washed 4–5 times with sterile distilled water. They were surface sterilized using 0.1% mercuric chloride solution for 1 min, 70% ethanol for 4–5 min and washed in distilled water; it was transferred to 70% ethanol for 2 min and finally washed in distilled water to remove all the traces of sterilants. The sterilized root nodules were crushed with the pestle and mortar by adding small aliquots of sterilized water, which was 10–1 dilution. This suspension was serially diluted up to 10-7. The diluted suspensions 10-5–10-7 were selected and 0.1 ml of suspension was inoculated in Petri dish containing sterile YEMA with Congo red. The inoculated plates were incubated at 28±2°C for 3 days. At the end of the incubation time, the rhizobial colonies appeared translucent white and elevated. They were picked out using a sterile inoculating loop and uniformly streaked on YEMA medium. The rhizobial isolates were purified, subculture, and stored for further investigation.

Biochemical characterization of *Rhizobium* (Vincent, 1970)

Indole test

Tryptone broth medium was prepared. The medium was poured into the test tubes. The rhizobial isolates were inoculated separately to the broth and incubated at 30±2°C for 2 days. The uninoculated broth was maintained as control. After the period of incubation, 1 ml of Kovac's reagent was added to each tube, including control. The tubes were gently shaken at an interval of 10–15 min and allowed to stand until the reagent reaches the top. The formation of red color ring is indicating the positive results; whereas yellow color ring indicates negative result.

Methyl red (MR) tests

The MR-Voges Proskauer (VP) broth was prepared. 5 ml of the broth was poured into sterile test tubes. The rhizobial isolates were inoculated separately into the tubes and incubated at 30±2°C for 2 days. After the incubation period, 5 ml of MR indicator was added to the each tube. Red coloration of the broth indicates the positive result while turning of MR to yellow is a negative result.

VP test

The MR-VP broth was prepared. 5 ml of the broth was poured into sterile test tubes. The rhizobial isolates were inoculated separately into the tubes and incubated at 30±2°C for 2 days. After the incubation period, 5 ml of Barritt's reagent A and B was added. Development of red color indicates the negative results.

Citrate utilization test

Simmons citrate agar medium was prepared. The medium was poured into the sterile test tubes. The rhizobial isolates were inoculated separately into the test tubes and incubated at 30±2°C for 4 days. After the incubation period, the green color turned to blue indicates the positive results.

Hydrogen sulfide production test

SIM agar medium was prepared. The medium was poured into the sterile test tubes. The rhizobial isolates were inoculated separately into the test tubes and incubated at 30±2°C for 4 days; the hydrogen sulfide production was observed.

Nitrate reduction test

Nitrate broth was prepared and 5 ml of medium dispensed into test tubes. The test tubes were sterilized and one loop full of culture of rhizobial isolates were inoculated separately and incubated at 30±2°C for 4 days. After the period of incubation, three drops of alpha naphthylamine and three drops of sulfanilic acid reagent were added. The cherry red color indicates the positive results.

Urease test

Christensen's urea agar medium was prepared. The medium was poured into the sterile test tubes and allowed to solidify. The rhizobial isolates were inoculated separately into the test tubes and incubated at 30±2°C for 4 days. After the incubation period, the appearance of deep pink color indicates positive results.

Catalase test

A clean glass slide was taken and a drop of rhizobial culture suspension was placed. Few drops of hydrogen peroxide were added to the culture. The evolution of air bubbles from the suspension is indicates the positive results.

Oxidase test

Trypticase Soy agar medium was prepared. The medium was poured into the Petri plates and allowed to solidify. The rhizobial isolates were streaked separately over the agar surface and incubated at 30±2°C for 4 days. After the incubation period, 2–3 drops of p-aminodimethylaniline oxalate were added to the surface of the plates and observed the color change. The formation purple color is indicates the positive results.

Starch hydrolysis

Starch agar medium was prepared. The medium was poured into the sterile Petri plates and allowed to solidify. The rhizobial cultures were incubated into the Petri plates separately and incubated at 30±2°C for 4 days. After the incubation period, 5 ml of iodine solution was added and observed a clear zone of hydrolysis surrounding the growth of the organisms and is indicates the positive results.

Gelatin hydrolysis test

Gelatin agar medium was prepared. The medium was poured into the sterile Petri plates. The rhizobial isolates were inoculated separately into the plates and incubated at 30±2°C for 4 days. After the incubation period, HgCl2 solution was flooded over the medium. The formation of clear zone around the culture indicates the positive results.

Triple sugar iron (TSI) agar test

TSI agar medium was prepared. The medium was poured into the sterile test tubes and allowed to solidify. The rhizobial cultures were inoculated into the tubes and incubated at 30±2°C for 24 h; the results were noted.

MacConkey agar test

MacConkey agar medium was prepared. The medium was poured into the sterile Petri plates and allowed to solidify. The rhizobial isolates were inoculated into the plates separately and incubated at 30±2°C for 3 days. The growth rate of the rhizobial isolates was noted.

Estimation of total protein (Lowry *et al*., 1951)

The rhizobial culture was centrifuged at 10,000 rpm for 10 min. From these, 500 mg of pellet was taken in a tube and suspended the pellet in 1 ml of distilled water and centrifuge again for 3 min and the collect the pellet, add 200 µl of 2× sample buffer, leave it in a boiling water bath for 5 min and add 200 µl of distilled water and centrifuge again for 5 min collect the supernatant for estimation process. 0.2–1 ml of extracted protein sample was taken in a test tube and makeup to 4 ml with distilled water. 5.5 ml of alkaline copper sulfate reagent was added and allowed to stand for 10 min; finally, 0.5 ml of Folin phenol reagent was added and vortex the tubes immediately and let stand for room temperature at 60 min. The optical density was measured at 750 nm in spectrophotometer. The amount of protein was calculated with a standard curve prepared using bovine serum albumin.

Extraction

The rhizobial culture was centrifuged at 10,000 rpm for 10 min. From these, 500 mg of pellet taken in a tube, 1.5 ml of 1 M sodium chloride was added and mixed thoroughly. 250 µl of 10% SDS was added to the same tube and mixed well. The tube was placed in water bath for 15 min (a temperature higher than 60°C but lesser 70°C was needed) to destroy the enzyme that degrades diphenylamine (DNA). The tubes were cooled till to reaches the room temperature and add equal volume of 80% phenol mixed well and again centrifuged at 10,000 rpm for 10 min. Then, equal volumes of 95% ethanol were added and mixed the tube and allowed to stand for 10 min at room temperature. The DNA will precipitate. It will again centrifuge at 5000 rpm for 5 min and collect the pellet for estimation process.

Estimation of DNA method

The rhizobial culture was centrifuged at 10,000 rpm for 10 min. From these, 500 mg of pellet taken in a tube, 1.5 ml of 1 M sodium chloride was added and mixed thoroughly. 250 µl of 10% SDS was added to the same tube and mixed well. The tube was placed in water bath for 15 min (a temperature higher than 60°C but lesser 70°C was needed) to destroy the enzyme that degrades DNA. The tubes were cooled till to

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Estimation

About 0.2 ml of 1 ml of extracted DNA sample was taken in a test tube and makeup to 1 ml with distilled water added 5 ml of DNA reagent to each tube. Vortex the test tubes and covers the tubes with aluminum foil and keep in the boiling water bath for 10 min. Then, cool the test tubes at room temperature for 10 min; the optical density was measured at 595 nm in spectrophotometer. The amount of DNA was calculated with a standard curve prepared using calf thymus DNA.

Chemicals and reagents

All the chemicals used in these experiments were purchased from M/s Hi-Media, Mumbai, India, unless otherwise mentioned. All the experiments were conducted at least for 3 times in triplicates.

Analysis of data

The data were statistically analyzed using analysis of variance and continued with standard error from at least three replications.

RESULTS AND DISCUSSION

The results of the present study were shown in Tables 1-3 and Figs. 1-12. The results indicated that all the isolates of rhizobia were positive to the indole test, nitrate reduction test, urease, catalase test, oxidase, and MacConkey agar test. None of the isolates produced H2S and utilized citrate as sole source of carbon. Starch hydrolysis and gelatin hydrolysis test were positive for all isolates except Rh01 and Rh3 isolates. These isolates were slow growers and unable to utilize starch and gelatin components.

In MR test, acid produced from the glucose, the isolates Rh01, Rh03, and Rh6 were positive, whereas remaining *Rhizobium* isolates were negative. In Voges Proskauer test, acetone produced from glucose, the isolates Rh02, Rh4, and Rh5 were positive, whereas other isolates were negative. In TSI agar test, all the rhizobial isolates were fermented the glucose and sucrose and also produced gas in the test tubes. However, the isolates were not fermented the lactose (Figs. 3-8).

The maximum amount of protein content 92.22 mg/ml was recorded from Rh6 *Rhizobium* strain and minimum was found, 75.38 mg/ml in Rh3 *Rhizobium* strain. Protein content of all rhizobial strains was shown in Table 2. Six *Rhizobium* isolates protein was analyzed by SDS-PAGE; electrophoresis was shown in Fig. 12. The maximum amount of DNA content 52.36 mg/ml was recorded from the Rh1 and 38.74mg/ml for Rh2 strain, followed by 45.14, 29.28, 44.39, and 21.69 mg/ml were estimated. This data are shown in Table 3.

All the rhizobial isolates were positive to the indole, nitrate reduction, urease, catalase, oxidase, and MacConkey agar test. Some of the isolates were not produced H2S and consumed citrate as a sole source of carbon. Positive results were found from the starch hydrolysis assay. On subjecting inoculated plates to iodine test, clear zones from place to place were observed and the colonies changed to yellow color; however, blue color appears on no growth areas. It designates that the isolates have the potential to hydrolyze starch present occur in the medium. Similar findings reported by De Oliveira *et al.,* [1] on the *Rhizobium* strains utilize the starch obtained from the various sources.

Rhizobial cells yield gelatinase enzyme and negative gelatinase activity also feature of Rhizobium [3]. Yellow slants and red butt were obtained showing the utilizing of glucose and sucrose in the TSI agar medium [8]. Naz *et al*. [9] also found the similar findings. Carbohydrate utilization by *Rhizobium* isolates, six isolates were selected on the basis of their growth behavior Rh1–Rh6 from French bean root nodules.

The present investigation, six isolates were able to grow vigorously in the presence of different carbohydrate sources such as glucose, galactose, maltose, xylose, and fructose. Rh1 *Rhizobium* strains were

Table 1: Carbohydrate utilization by *Rhizobium*

S. No	Carbon sources	Rh 1	Rh2	Rh 3	Rh4	Rh5	Rh6
1	Glucose	+	+	+	+	+	+
2	Galactose	+	+	+	+	+	+
3	Maltose	−	−	+	+	+	+
4	Lactose	+	+	−	−	+	+
5	Arabinose	+	+	−	+	−	−
6	Xylose	+	+	+	−	−	+
7	Fructose	++	++	++	++	++	++
8	Sucrose	+	+	−	−	−	+

Table 2: Estimation of DNA in rhizobial isolates

S. No	Rhizobium DNA sample (µl)	Standard OD value	Rhizobial strains at 595 nm					
			Rh1	Rh2	Rh3	Rh4	Rh5	Rh6
1	20	0.003	0.356	0.387	0.366	0.452	0.394	0.344
2	40	0.006	0.377	0.441	0.347	0.335	0.388	0.473
3	60	0.012	0.425	0.352	0.359	0.365	0.422	0.372
4	80	0.015	0.436	0.349	0.336	0.382	0.399	0.423
5	100	0.021	0.449	0.459	0.356	0.380	0.387	0.485
Amount of protein (mg/ml)			52.36	38.74	45.14	29.28	44.39	21.69

DNA: Diphenylamine

Table 3: Estimation of protein in rhizobial isolates

S. No	Rhizobium protein sample (ml)	Standard OD value	Rhizobial strains OD value at 660 nm					
			Rh1	Rh2	Rh3	Rh4	Rh5	Rh6
1	0.1	0.03	0.21	0.23	0.28	0.19	0.25	0.29
2	0.3	0.06	0.32	0.28	0.32	0.22	0.35	0.31
3	0.5	0.10	0.33	0.32	0.29	0.28	0.44	0.35
4	0.7	0.24	0.46	0.40	0.39	0.31	0.45	0.37
5	0.9	0.18	0.19	0.48	0.42	0.41	0.49	0.55
Amount of protein (mg/ml)			89.22	90.17	75.38	95.01	90.14	92.22

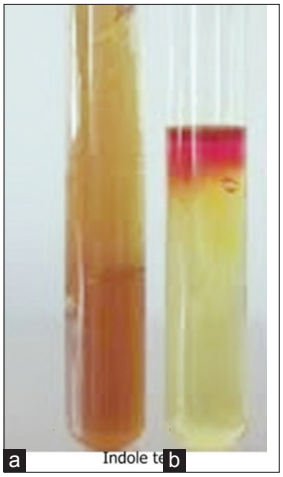

Fig. 1: (a and b) Indole test for Rhizobium

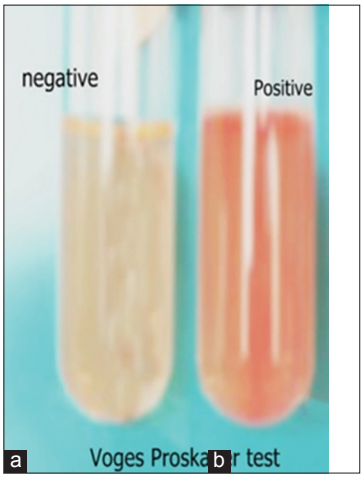

Fig. 2: (a and b) Voges Proskauer test for Rhizobium

Fig. 3: (a and b) Methyl red test for Rhizobium

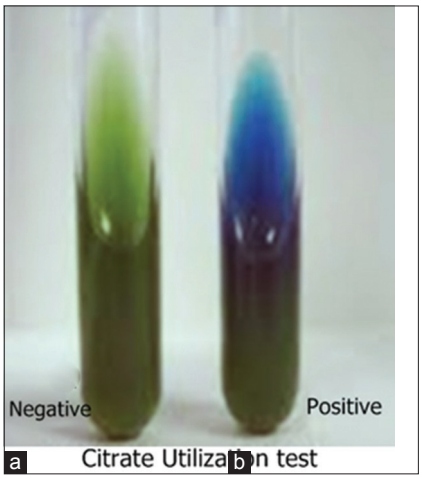

Fig. 4: (a and b) Citrate utilization test for Rhizobium

Fig. 5: Nitrate reduction test for Rhizobium

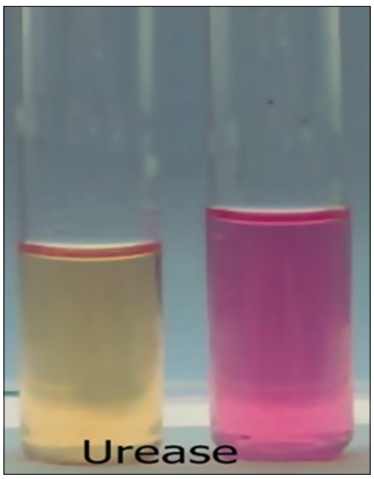

Fig. 6: Urease test for Rhizobium

utilized all carbohydrates as a carbon source. Arabinose and sucrose were not utilized by Rh3 and Rh5 isolates. Kucuk *et al*. [10] have proposed that the Eskisehir isolates were able utilize the several compounds as sole sources of carbon. All isolates were able to grow healthy in the occurrence of D (−) fructose, D (+) galactose, D (+) glucose, D (+) mannitol, sucrose, starch, succinate, and rhamnose. French bean rhizobial isolates utilized a wide range of carbohydrates and salts of organic acids as carbon sources [11]. In the present study, all the strains showed virtuous growth on carbohydrates. Similar results were also reported in *Rhizobium* isolates from *Sesbania sesban* root nodules [12]. From the current study, it is evident that all the strains efficiently consumed a wide range of carbohydrates; it is one of the significant criteria to be measured as plant growth promoting bacteria.

Fig. 7: Triple sugar iron test for Rhizobium

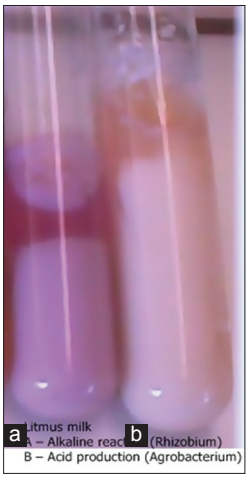

Fig. 8: (a and b) Litmus test for Rhizobium

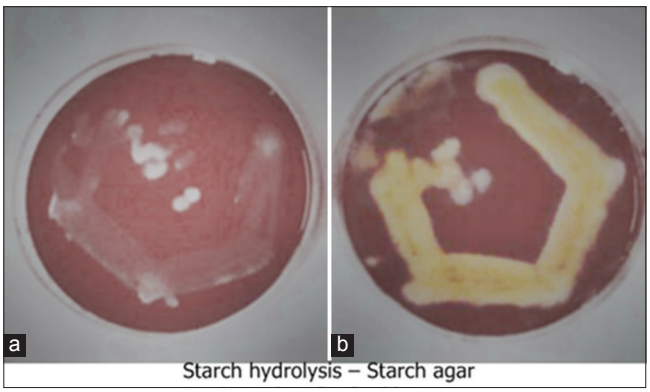

Fig. 9: (a and b) Starch hydrolysis for Rhizobium

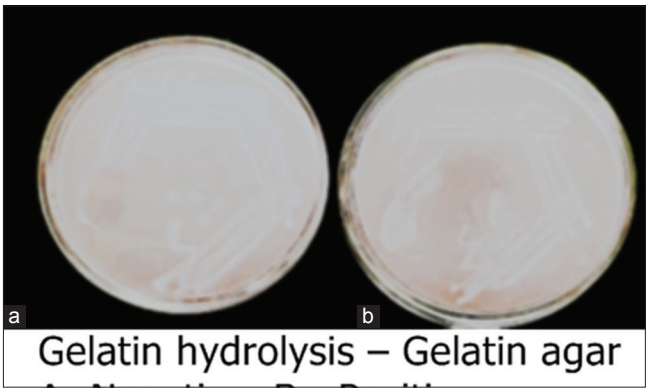

Fig. 10: (a and b) Gelatin hydrolysis for Rhizobium

Fig. 11: (a and b) Rhizobial colonies on MacConkey agar medium

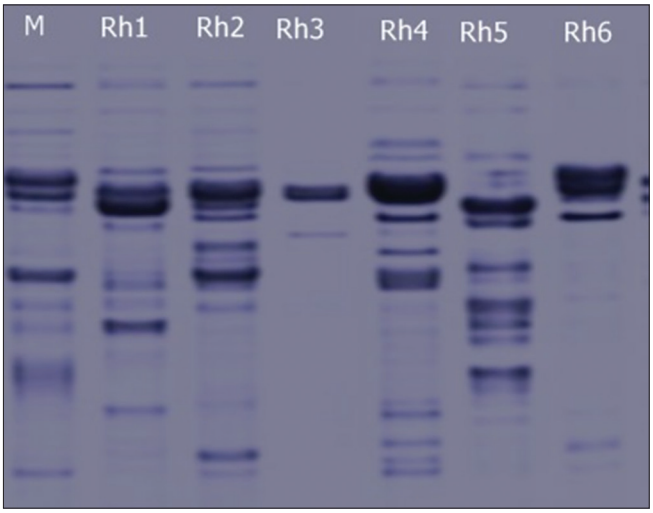

Fig. 12: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis gel electrophoresis of protein isolated from rhizobial strains of French bean

The electrophoretic protein banding pattern of cell of six *Rhizobium* isolates as performed by SDS-PAGE electrophoresis. SDS-PAGE of whole cell proteins of rhizobial strains from the wild legumes exhibited protein profiles with bands ranging after 5–19 bands per profile was observed by Zahran *et al.* [13]. Analysis of whole cell proteins helps in identifying of the rhizobial strains and useful in the differentiation among the isolates within the same group, Fabiano and Arias [14]. The total protein profiles, thus suggest that the patterns are isolate specific and can help as fingerprints, it can, hence, be concluded that chickpea-rhizobia isolated from same soil-climatic region is diverse [15]. The French bean rhizobial isolates were characterized based on the growth behavior in Hofer's alkaline broth, Congo red medium, lactose agar, polyhydroxybutyrate staining, and litmus milk reaction. All these tests confirmed that findings of the present study have been isolated *Rhizobium* species. All the isolates were identified on the basis of biochemical characters showed differences among the six rhizobial isolates and established as rhizobia.

CONCLUSION

The conclusion of the present study, the French bean rhizobial isolates to enhance the plant growth and nodulation of bean plant under the

different environmental conditions. The symbiosis among *Rhizobium* and legumes is an inexpensive and usually effective for ensuring tolerable supply of nitrogen for legume-based crop and pasture fabrication than the application of organic fertilizers.

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