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Isolation and characterization of native *Rhizobium* from root nodules of raikia french bean growing area of Odisha

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Injudicious application of nitrogenous fertilizers leads to soil quality deterioration which results into yield loss. The application of biofertilizer containing native efficient rhizobia enhances the nodular properties, N-fixation and soil quality. Therefore, fifty strains of *Rhizobium* were isolated from root nodule of Raikia french bean and among them only two isolates *viz*., RBHR-15 and RBHR-21 were confirmed as *Rhizobium*. The isolates were unable to grow under anaerobic conditions and failed to produce ketolactase enzyme, showed a negative response for gelatin liquefaction and Simmon's citrate agar test, responded positively towards the indole test, MR-VP, TSI test and could produce NH_4^+ from peptone in the growth medium. The isolate RBHR-15, could reduce H_2S and nitrate whereas, RBHR-21 could not. The growth of both isolates was luxuriant in the nutrient broth containing 1% NaCl and decreased with an increase in the concentration of NaCl and grew profusely in the pH range of 6-8. The generation time of RBHR-15 and RBHR-21 were 16.4and 10.6 h, respectively. The 16s rRNA of both isolates was sequenced and submitted to the National Center for Biotechnology Information (NCBI). The isolates RBHR-15, and RBHR-21 were assigned accession numbers MN480514 and MN480516.

K**eywords**: Antibiotics, French bean, Generation time, Rhizosphere, Stress tolerance

French bean (*Phaseolus vulgaris* L) is an important legume vegetable belonging to family *Fabaceae*. It has many synonyms like the snap bean, kidney bean, haricot bean and also called 'raj mash' in Hindi. Beans are essentially used for their tender green pods with high protein. The dried pods are used as pulse and provide valuable protein to the human diet. Immature pods are marketed fresh, canned or frozen[1].The performance of different french bean varieties varies under different agroclimatic conditions due to their specific climatic requirement. In the Kandhamal district of Odisha tribal people have been cultivating one pole type of French bean known as "Raikia bean" in the rainy season in rainfed hill regions without much cultural practices. Due to the low temperature in the high hills of G. Udayagiri, and other parts, "Raikia bean" performs well and fetches an excellence price in the market. It has been cultivated in this region for decades. The "Raikia bean" is fleshy, good yielding and prized for its taste for which it has got very good market demand. It is a nutritious vegetable containing higher amount of protein, vitamin A and vitamin C, potassium, magnesium, calcium, and phosphorus. However, it is low in fat content[2].

The application of excess N fertilizer inhibits the nodule formation and N_2-fixation[3]. Many agricultural soils contain a high level of residual nitrogen[4]which limits legume nodulation and N_2-fixation. Furthermore, farmers frequently apply N-fertilizer to the seed bed of legumes to help crop establishment. This practice is likely to inhibit legume nodulation until the soil N supply has been depleted. Therefore, understanding how legumes sense and signal their N supply status to regulate nodulation is of fundamental importance for developing more sustainable agriculture using lower inputs of chemical fertilizer.

In leguminous plants, bioinoculation with *Rhizobium* as a substitute for costly N- fertilizer contributed for crop growth stimulation[5].Thus, emphasis should be given to the establishment of efficient symbiotic N_2-fixation in legume.

The utilization of native *Rhizobium* as inoculants promotes ecologically sustainable management of the agricultural ecosystem and enhance legume production due to their growth promoting traits and adaptability to soil and environmental stress[6]. Furthermore, crop production using inocultants could be cheaper and

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Suppl. Data available on respective page of NOPR

more affordable to the resource-poor smallholder farmer[7]. The ability of native strains to interact positively with resident soil micro biota and their adaptability to local agro-ecological climatic conditions often elucidates their superior performance over exotic commercial strains[8]. In the present investigation, rhizospheric soil and root nodules were collected from thirty locations in nineteen villages of Raikia bean growing areas of Kandhamal, Odisha to isolate native rhizobia.

Materials and Methods

Collection of rhizospheric soil and root nodule

The healthy Raikia bean plants of 45-50 DAS (days after sowing) were uprooted and adhering soil and healthy nodules were collected on a GPS basis. The collected soil was processed and the soil reaction and organic carbon status were estimated. The detached roots nodules were washed in sterile water and stored at 4°C in the refrigerator for further use.

Isolation and screening of *Rhizobium*

Isolation of root nodulating rhizobia were carried out by following the method of Vincent[11]. Nodules were dipped in 0.1% sodium hypochlorite (NaOCl) solution for 30 seconds and then washed successively ten times with sterilized distilled water to remove traces of toxic NaOCl. Surface sterilized nodules were transferred to a test tube containing 5 mL sterilized distilled water. These nodules were crushed with the help of a sterilized glass rod to obtain a milky suspension of bacteroids. These were streaked on YEMA plates containing congo red. The plates were sealed by parafilm to avoid contamination and incubated at 28-30°C for 24-72 h. *Rhizobium* colonies appeared white, translucent, elevated and mucilaginous after 24-72 h, whereas, contaminants turned red. The distinct colonies were picked up and transferred to the YEMA plate for purification and further characterization. Further streaking, spreading and visual characterization of colony morphology helped in isolation of pure culture. Pure isolates were streaked on YEMA slants for preservation and further analysis. All tests were performed in triplicates.

Biochemical characterization of isolates

The biochemical characterization was performed by following standard protocols *viz*. growth on YEMA with congo red, growth on Glucose Peptone Agar (GPA) medium, Keto-Lactose test, growth on Hoffer's alkaline media, Gram's staining, mannitol mobility test, indole test, MR-VP test, citrate utilization test, triple sugar iron (TSI) test, anaerobic growth, sugar fermentation test, starch hydrolysis, liquefaction of gelatin, reduction of nitrate to nitrite, production of hydrogen sulphide as followed by Subudhi *et al*[9].

Enzymatic activity of bacterial isolates

The isolates were screened on selective media for the production of various industrially important extracellular enzymes like urease, amylase, cellulase, chitinase, caesinase, DNase and lipase following standard microbiological methods of Collins and Lyne[10].

Antibiotic sensitivity test

Pure rhizobia isolates were transferred on YEMA plates. The susceptibility test was performed by using a paper disc diffusion assay. Ten antibiotics were used *viz*. erythromycin, amphotericin, bacitracin, ciprofloxacin, polymyxin-B, tetracycline, penicilin-G, neomycin, amikacin, streptomycin. Each Petri plate containing 25 mL solidified medium was inoculated with one loop full of culture spread over whole Petri plates. Biodiscs were placed upon the surface of solidified agar medium. The plates were incubated for 24 h at 28±1°C and the zone formation was measured.

Molecular identification

The bacterial DNA was isolated by following the procedure given by Quick-DNA™ Fungal/Bacterial Miniprep Kit Catalog No. D6005 from Zymo Research. The concentration of each DNA sample was measured by Nanodrop (Biotech instruments, USA) and the DNA was stored at −80C for further use. The universal primers (Forward primer 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer5' CTTGTGCGGGCCCCCGTCAATTC-3') were used for the amplification of the 16S rDNA gene fragment[11]. The amplified DNA was separated by electrophoresis. The gel was photographed on gel documentation system. The sequencing was done by using instrument ABI 3730XL. After obtaining the 16 S rRNA sequence, each isolate was subjected to Basic Local Alignment Search Tool and the phylogenetic tree was prepared by using MEGA-X software[12,13].

Salinity and pH tolerance test

The identified isolates were inoculated to nutrient broth maintained with a salinity level of 1, 2,3,4,5 and 6% NaCl and maintained at pH 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 for studying salinity and pH tolerance. The broths were incubated at 30°C for 72 h. The turbidity

of the medium was measured at an interval of 24 h for 3 days at 660 nm wavelength using a visible spectrophotometer.

Generation time of isolates

The generation time of confirmed isolates was calculated on the basis of the growth rate of isolates at the active growth stage under optimum pH of 7.0 in nutrient broth media using the formula.

$g=\frac{0.301t}{logb-loga}$ where, g=generation time in hours.

t=growth duration (hours) at active stage
a=OD of growth medium at initiation of active growth
b=OD of growth medium at final stage of active growth

Results and Discussion

Characterization of soils

The pH of collected soils varied from 4.75 to 7.33. More than 30% of the soil was neutral (6.5-7.5) in reaction whereas, the rest 70% of the soil was slightly acidic (<6.50). The electrical conductivity (EC) was low in status. The organic carbon status of soil was medium to high and it varied from 5.3 to 19.9 g kg^{-1}, whereas, average was 10.3 g kg^{-1} (Suppl. Table 1).

Isolation of *Rhizobium* from root nodule:

The preserved nodules were surface sterilized, crushed and spread over the YEMAC medium. After 4 to 5 days of incubation of plates the colonies formed were selected according to their morphology. A total of fifty translucent, convex and circular colonies were selected for biochemical characterization (Suppl. Table 2). The isolates from nodules were coded as RBHR.

Biochemical characterization of isolates

Among the 50 isolates RBHR-4, RBHR-5, RBHR-6, RBHR-11, RBHR-18 and RBHR-19 absorbed colour from YEMAC medium disqualifying them to be characterized as *Rhizobium* in consistent with the results of Trinick[14] who reported that rhizobia did not absorb congo red dye or absorbed very weakly compared with other bacteria. The isolates RBHR-1, RBHR-11, RBHR-22, RBHR-23, RBHR-24, RBHR-31, RBHR-35, RBHR-39, RBHR-44, RBHR-45, RBHR-47, RBHR-48, RBHR-49 and RBHR-50 were able to grow in Hoffer's alkaline broth medium. Thus, the above isolates might be inferred as *Agrobacterium* and not *Rhizobium* corroborating with the results of Hofer that *Agrobacteria* could grow at higher pH levels (pH 11.0) than rhizobia. Therefore, their growth in YEM broth with elevated pH of 11.0 is considered as a useful means to distinguish between two allied genera. The isolates RBHR-1, RBHR-6, RBHR-14, RBHR-19, RBHR-24, RBHR-40, RBHR-45 and RBHR-46 obtained from nodule were able to grow profusely on glucose peptone agar medium disqualifying them to be included under rhizobia. The isolates RBHR-1, RBHR-4, RBHR-5, RBHR-6, RBHR-8, RBHR-11, RBHR-14, RBHR-15, RBHR-20, RBHR-21, RBHR-22, RBHR-23, RBHR-24, RBHR-25, RBHR-26, RBHR-29, RBHR-30, RBHR-31, RBHR-33, RBHR-35, RBHR-36, RBHR-45, RBHR-46, RBHR-47, RBHR-48 were motile in nature indicating their competitive advantage for nodulating legumes. Our results show close conformity with the findings of Shahzad *et al.*[15] and Subudhi *et al*[9]. Positive test for motility proved that bacteria have flagella (peritrichous flagella) and the negative results for capsule presence. From above mentioned confirmatory tests, two isolates (RBHR-15 and RBHR-21) were confirmed to have the characteristics of *Rhizobium* and were considered for further biochemical characterization (Suppl. Table 3).

The isolates RBHR-15 and RBHR-21 were unable to grow under anaerobic condition. Earlier work of Daniel *et al.*[16] suggests that the simple turbidometric method of assessing anaerobic growth might not give a positive result with rhizobia which displays poor anaerobic growth. The authors also reported that anaerobic growth of *Rhizobium japonicum* appeared to be completely dependent upon the presence of nitrate and some rhizobia are capable of utilizing nitrate respiration to support anaerobic growth. The present finding also validates the above facts confirming rhizobial characteristics of the isolates RBHR-15 and RBHR-21which were able to reduce nitrate suggesting their ability to remove fixed nitrogen from soil by denitrification in free-living conditions. The isolatesRBHR-15 and RBHR-21 were able to produce H_2S.The production of H_2S and the utilization of citrate were restricted to some isolates of *Rhizobium meliloti*. Corroborating the present finding H_2S was also reported to stimulate soybean nodulation and enhance the N fixing potential of the soybean-rhizobia symbiotic system[17]. The isolates were positive towards triple sugar iron test in conformity with similar findings reported by Kucuk *et al*[18]. The *Rhizobium* strains were able to utilize glucose and sucrose more efficiently than YEMA.

All isolates were positive towards the indole test and MR-VP test and negative towards the citrate agar test.

These findings are in close agreement with Elsheikh and Wood[19], Javed and Asghari[20] who also characterized the *Rhizobium* from soil and sunflower root nodules with the same positive biochemical tests. Similarly, Oblisami[21] also studied the nodulation pattern in forage legume bacteria, by screening through the same tests. Singh *et al*.[22] characterized rhizobial strains from the roots of *Trigonella foenumgraecum*. IMViC test reactions(a set of four useful reactions viz. Indole test, Methyl red test, Voges Proskauer test and Citrate utilization test) is commonly employed for the identification of members of family *Enterobacteriaceae* which is a common contaminant and frequently isolated while isolating *Rhizobium* sp. Isolates which gave a negative reaction in the IMViC test were suspected as *Rhizobium* sp.[23], whereas, remaining isolates which were positive in the IMViC test may be the member of *Enterobacteriaceae* which is abundantly present in the soil and so firmly associated with the root and nodules of the legumes[23]. Surface sterilization sometimes cannot completely remove such bacteria. In the present investigation, all the isolates were positive towards indole and MR-VP test, negative towards Simmon's citrate agar test requiring further verification for presence of the members of *Enterobacteriaceace*.

All isolates are negative for the gelatine liquefaction test. This finding is in close agreement with Elsheikh and Wood[1927], Javed and Asghari[2028] who also characterized the *Rhizobium* from soil and sunflower root nodules with the same positive biochemical tests. The test was performed to determine the capability of microorganisms to produce gelatinase enzyme and use gelatin as a media source. Degradation of gelatin indicates the presence of gelatinase enzyme[24]. The cultures which produce gelatinase remain liquefied while others become solid due to presence of gelatin. It was observed that rhizobial cells do not produce gelatinase enzymes as a medium containing gelatin solidified when kept at 4°C for 30 as well as 60 min. Negative gelatinase activity is also a feature of *Rhizobium*[25]. All the isolates were able to produce ammonia from peptone. The present results are in conformity with those of Menna *et al*.[26] who also characterized strains based on morphophysiological characteristics. Kumar *et al*.[27] also observed similar characteristics for the genus *Rhizobium*. Results of ketolactose test revealed that all the isolates showed a negative result for the production of 3-ketolactose from lactose. Similar results were reported by Bernaerts *et al*.[28]. No yellow zone was observed around the colonies and such negative ketolactase activity confirmed the isolates to be free from any contamination of *Agrobacterium*[29]. All isolates except RBHR-21 and RBHR-26 responded positively to the nitrate reductase test. Nitrate reduction test is used for the differentiation of members of *Enterobacteriaceae* on the basis of their ability to produce nitrate reductase enzyme that hydrolyze nitrate (NO_3^-) to nitrite (NO_2^-) which may then be degraded to various nitrogen products like nitrogen oxide, nitrous oxide and ammonia (NH_3) depending on the enzyme system of the organism and the atmosphere in which it is growing.

Sugar utilization pattern of confirmed isolates

The data related to the sugar utilization pattern of confirmed isolates have been presented in (Table 1). The isolate RBHR-15 could utilize rhamnose, cellobiose, raffinose, inositol, mannitol and trehalose in both oxidation and fermentation condition. Dextrose and fructose were not utilized in both oxidation and fermentation condition. Galactose and inulin could be utilized only in oxidation conditions and glucose and sucrose could beutilized in fermentation condition. The isolate RBHR 21 could utilize dextrose and trehalose in both oxidation and fermentation condition. Galactose and inulin were utilized in oxidation conditions and glucose, mannitol, raffinose and sucrose were utilized in fermentation conditions whereas, rhamnose, cellobiose, fructose and inositol were not metabolized in both oxidation and fermentation conditions.

The confirmed isolates were tested for utilization of various sugars including mono, di, and tri-saccharides, aldose, ketose sugar alcohol *etc*. both under oxidation and fermentation condition lot of variation is observed in the sugar utilization pattern by the isolates. These findings corroborate the results of Oblisami[21] and Singh *et al*.[22] who reported these sugar tests during isolation and characterization of *Rhizobium meliloti*. These investigations show that nodule bacteria actually utilize a great variety of carbohydrates and related compounds, although different strains differ considerably in this respect. The utilization of a wide range of carbohydrates may be the key to surviving and competing for nodule occupancy[30]. Moënne-Loccoz and Weaver[31] found that the presence of some plasmids governing the assimilation of many carbohydrates such as glycerol, sorbitol, arabinose and raffinose is necessary for the

survival of rhizobia in soil under water and salt stress conditions. The disaccharides cellobiose, lactose, maltose, trehalose, and sucrose and the trisaccharide raffinose were metabolized by fast-growing strains but not by slow-growing rhizobia. Only the fast-growing soybean rhizobia have appreciable P-galactosidase activities similar to the activities of the other fast growers. The Carbohydrate utilization pattern divides these organisms based on the presence and absence of enzymes in the pentose phosphate pathway.

Production of enzymes and antibiotic sensitivity of isolates

The data related to the enzymatic test of isolates have been presented in (Table 2). All the isolates were able to produce urease, caseinase and cellulase whereas, amylase, DNase, lipase, chiitinase and gelatinase were not produced by any of the isolates. *Rhizobium*, being a micro-organism produces various enzymes like nitrogenase[3241]. Carbohydrate utilization assays indicated that *Rhizobium* isolates obtained from fenugreek roots were able to utilize different carbohydrate sources, thus it was assumed that they may produce important enzymes like amylase, cellulases, *etc*. Indeed, *Rhizobium* strains are reported to produce amylase[33] and cellulase[34]. These results with increased biomass yield, high activity of amylase and cellulase indicated that immobilized *Rhizobium* cells could be used at an industrial scale for production of these enzymes. Singh *et al.*[35] also observed positive results for catalase and oxidase activities. Wani and Khan[36] also reported that *Mesorhizobium* isolates were positive for catalase, oxidase and citrate utilization and were negative for lipase which corroborated the present observation.

Test isolates were assessed against ten antibiotics namely erythromycin (E15), amphotericin (AP50), bacitracin (B10), ciprofloxacin (CIP15), polymixin B(PB100), tetracycline (T), penicilin-G (P10), neomycin (N30), amikacin (AK30) and streptomycin (S10) and the data related to antibiotic sensitivity have been presented in (Table 3). The isolate RBHR-15 was sensitive to erythromycin, amphotericin, ciprofloxacin and neomycin, resistant to bacitracin and penicillin whereas, moderately sensitive to polymixin-B, tetracycline, ambikacin and streptomycin. The isolate RBHR-21 was sensitive to erythromycin and ciprofloxacin, resistant to bacitracin, amphotericin, and penicillin whereas, moderately sensitive to polymixin-B, tetracycline, neomycin, ambikacin and septromycin.

The evaluation of intrinsic resistance to antibiotics showed that all the test isolates exhibited high resistance to Penicillin and Bacitracin. All isolates were sensitive towards Erythromycin and Ciprofloxacin. All isolates were moderately susceptible to Tetracycline. The resistant isolates have a higher survival value in natural habitats.

The bacteria possess a differential response towards the antibiotics as some show resistance and some susceptibility to a particular antibiotic**.** The majority of the fast-growing species of *Rhizobium* tested was found to be resistant to > 32 μg/mL

Table 1— Sugar utilization pattern of isolates

Sl. No.	Sugars	O/F	RBHR-15	RBHR-21
1	Rhamnose	Oxidation	+ve	−ve
		Fermentation	+ve	−ve
2	Dextrose	Oxidation	−ve	+ve
		Fermentation	−ve	+ve
3	Cellobiose	Oxidation	+ve	−ve
		Fermentation	+ve	−ve
4	Fructose	Oxidation	−ve	−ve
		Fermentation	−ve	−ve
5	Galactose	Oxidation	+ve	+ve
		Fermentation	−ve	−ve
6	Glucose	Oxidation	−ve	−ve
		Fermentation	+ve	+ve
7	Raffinose	Oxidation	+ve	−ve
		Fermentation	+ve	+ve
8	Inositol	Oxidation	+ve	−ve
		Fermentation	+ve	−ve
9	Mannitol	Oxidation	+ve	−ve
		Fermentation	+ve	+ve
10	Trehalose	Oxidation	+ve	+ve
		Fermentation	+ve	+ve
11	Sucrose	Oxidation	−ve	−ve
		Fermentation	+ve	+ve
12	Inulin	Oxidation	+ve	+ve
		Fermentation	−ve	−ve

Table 2 — Enzyme production by the confirmed isolates

	Enzyme									
Isolates	Urease	Oxidase	Catalase	Amylase	DNase	Lipase	Chitinase	Gelatinase	Caseinase	Cellulase
RBHR-15	+ve	+ve	+ve	−ve	−ve	−ve	−ve	−ve	+ve	+ve
RBHR-21	+ve	+ve	+ve	−ve	−ve	−ve	−ve	−ve	+ve	+ve

Table 3 — Antibiotic sensitivity tests

	Antibiotics									
Isolates	E15	B10	AP50	CIP5	PB100	P10	T	N30	AK30	S10
RBHR-15	S	R	S	S	MS	R	MS	S	MS	MS
RBHR-21	S	R	R	S	MS	R	MS	MS	MS	MS

**R-Resistant (<5mm), S-Susceptible (>20mm), MS-Moderately susceptible (>10 to 20mm), E15-Erythromycin, AP50-Amphotericin, B10-Bacitracin, CIP15-Ciprofloxacin, PB100-Polymyxin-B, T-Tetracycline, P10-Penicilin-G, N30-Neomycin, AK30-Amikacin, S10-Streptomycin*

penicillin and to < 1 µg/mL tetracycline. On the other hand, most of the slow-growing species of *Rhizobium* were susceptible to penicillin concentrations < 16 µg/mL while they were resistant to tetracyclin concentrations > 1 µg/mL. There are three known determinants of bacterial permeability to an antibiotic: hydrophobicity, electrical charge, and amount of the antibiotic and the *Rhizobium* that showed a high level of resistance did not take up the antibiotics.

The variation in antibiotic resistance pattern of different isolates might also be due to variation in edaphic – climate factors of the locations from where they were isolated. An increase in antibiotic resistant rhizobia population was associated with an increase in soil phosphorus and Aluminum contents.

Molecular characterization

Biochemical tests for the isolates (RBHR-15 and RBHR-21) were found to be in agreement with molecular (16S rRNA) analysis. The 16S rRNA was PCR amplified with the universal primers (5'-AGAGTTTGATCCTGGCTCAG -3' and 5' CTTG TGCGGGCCCCCGTCAATTC-3'). The band image was taken by gel documentation system (Fig. 1). The sequence data were aligned with other 16S rRNA sequences for multiple alignments using MEGA X software from the NCBI gene bank (Fig. 2). The dendrogram was built with a homology pattern which revealed that 16S rRNA sequence of the isolated strains (RBHR-15 and RBHR-21) from root nodule were *Rhizobium etli* and *Rhizobium pisi*. The sequence was submitted to NCBI gene bank and the strains were assigned the accession number – MN480514 and MN480516 for RBHR-15 and RBHR-21, respectively.

Stress tolerance behavior of confirmed isolates

Acid tolerance behavior of isolates

The growth expressed as a change in optical density (OD) of nutrient broth inoculated with test isolate at different pH values was measured till 96 h after inoculation. The data have been presented in (Figs 3A & 4A) for isolate RBHR-15 and RBHR-21, respectively.

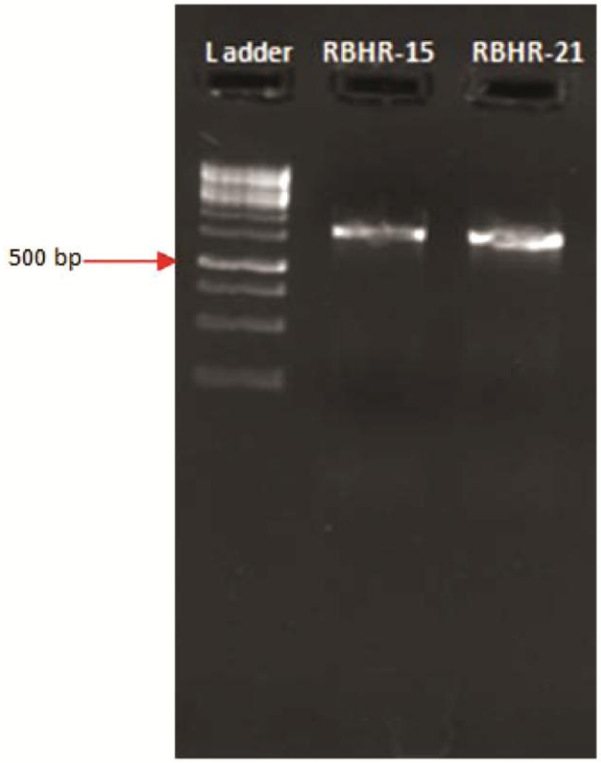

Fig. 1 — PCR amplified product of 16S rRNA of bacterial isolates. (Lad: molecular size marker 100 bp; lane 1: isolate RBHR-15 and lane 2: isolate RBHR-21)

The stationary phase in the growth curve of isolate RBHR-15 was attained after 48h of incubation with maximum OD of 1.89 in pH-7.0 followed by 1.79 and 1.39 in pH-6.0 and pH 8.0, respectively, whereas, the stationary phase was attained after 24 h in pH-4 and pH-5 with maximum OD of 0.40 and 0.42, respectively. The maximum OD (0.22) in pH-9 was recorded at 25 to 30 h after incubation (Fig. 3A).

The stationary phase in the growth curve of isolate RBHR-21 was attained after 42 h of incubation with maximum OD 1.82 in pH-7.0 followed by 1.65 and 1.18 in pH-6.0 and pH 8.0, respectively, whereas, the stationary phase was attained after 30 h in pH-4 and pH-5 with maximum OD of 0.40 and 0.41. The maximum OD (0.40) in pH-9 was recorded at 30h

after incubation (Fig. 3B). All two isolates tested could grow profusely at pH within the range of 6 to 8. At when pH 4 and 9 all isolates showed a drastic decrease in growth. The present finding is corroborated by Sethi *et al*.[37] and Subudhi *et al*.[9]. The decrease in growth under prevailing conditions might be attributed to the deficiency of glutathione in the different strains.

Salinity tolerance behaviour of isolates

The growth expressed asa change in optical density (OD) of nutrient broth inoculated with test isolates at different salt concentration was measured till 96 h after inoculation. The data have been presented in (Fig. 4A & 4B) for isolate RBHR-15, and RBHR-21, respectively. In 1% NaCl medium, the isolate RBHR-15 grew luxuriantly up to 36 h with maximum OD 3.3 thereafter, the growth was stagnant (Fig. 4A). In 2% solution, the growth of the isolate was higher up to 21 h and stagnant thereafter. The trend recorded with 3% NaCl was that the growth rate was lower and growth becomes stagnant after 42 h. In 4% NaCl, luxuriant growth started after 18 h and reached maximum at 42 h after that the growth became stagnant. In 5% and 6% NaCl very less growth was observed and the maximum OD was 0.3.

In 1% NaCl medium, the isolate RBHR-21 grew luxuriantly up to 30 h with maximum OD 3.7 thereafter, the growth was stagnant (Fig. 4B). In 2% solution, the growth of isolate was increased up to 18 h with OD 2.4 became and stagnant thereafter. The OD of the isolatewith 3% NaCl increased slowly up to 36 h. In 4% NaCl, the growth started after 15 h and increased up to 42 h after which it becomes stagnant. In 5% and 6% NaCl very less growth was observed and the maximum OD was 0.5. The result corroborated with the finding of Sethi *et al*.[37] and Subudhi *et al*[9].

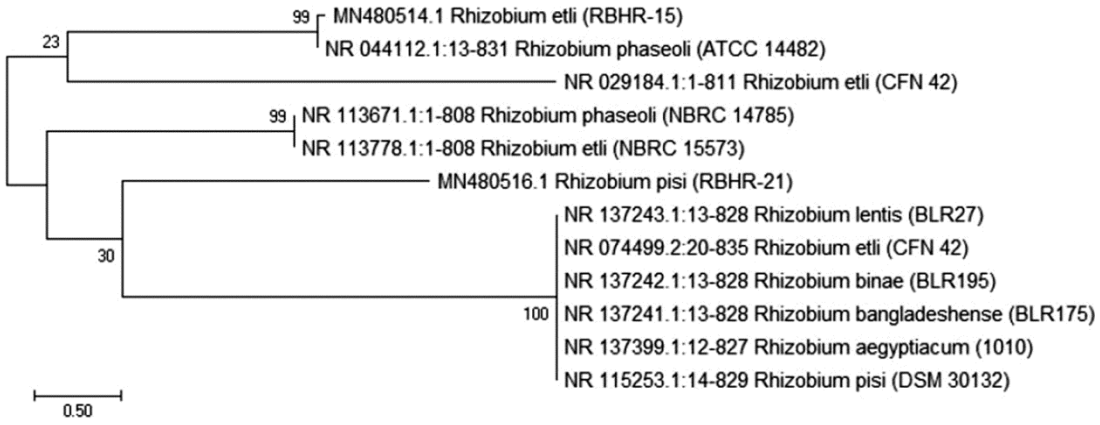

Fig. 2 — The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 11.05876416 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 12 nucleotide sequences. Allambiguous positions were removed for each sequence pair. There were total of 870 positions in the final dataset. Evolutionary analyses were conducted in MEGA X

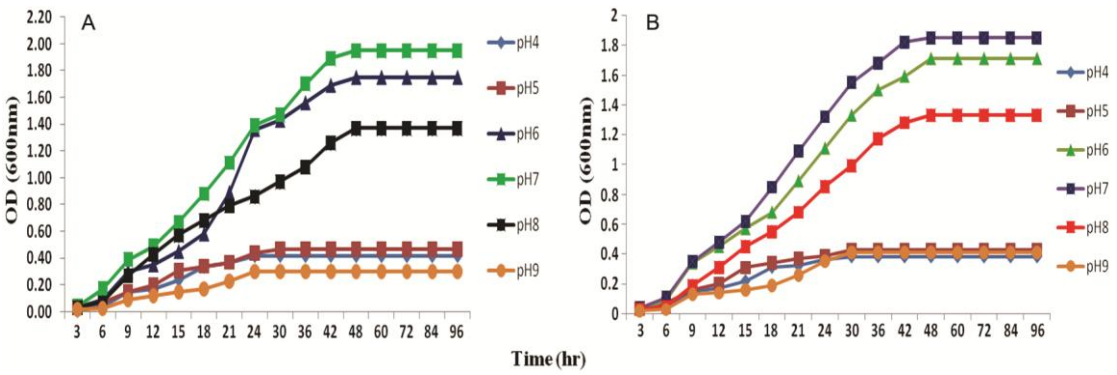

Fig. 3 — Growth of (A) RBHR-15; and (B) RBHR-21 in different pH levels

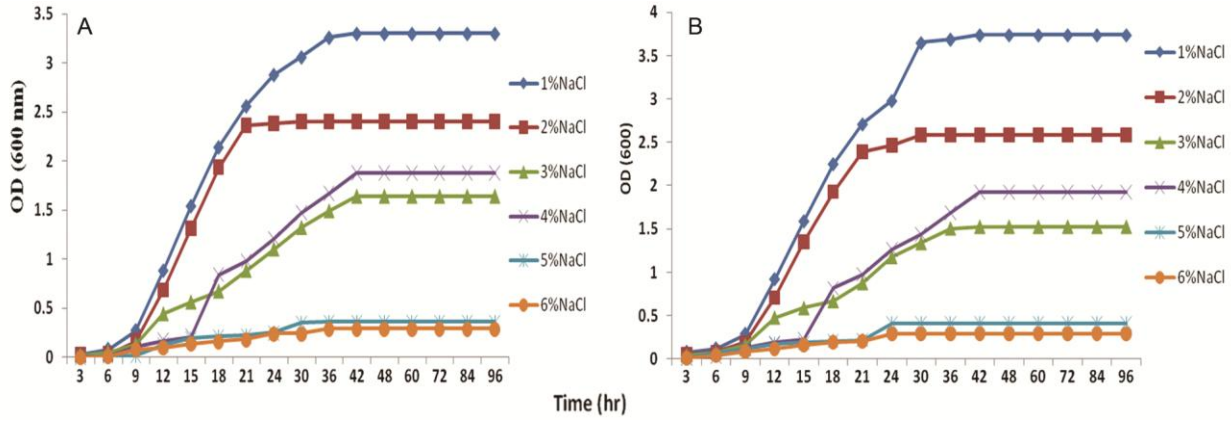

Fig. 4 — Growth of (A) RBHR-15; and (B) RBHR-21 in different salt

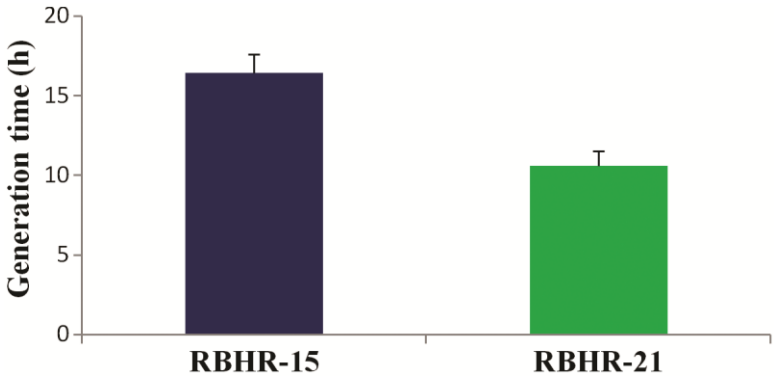

Fig. 5 — Generation time of RBHR-15 and RBHR-21

The salt stress decreases the efficiency of the *Rhizobium*-legume symbiosis by reducing plant growth and photosynthesis survival and proliferation of rhizobia in the soil and rhizosphere or by inhibiting very early symbiotic events, such as chemotaxis and root hair colonization, thus directly interfering with root nodule function.

Generation time of confirmed isolates

The generation time of two isolates was determined from their growth rate in nutrient broth medium under optimum pH of 7.0 at active growth stage (Fig. 5). The generation time of isolates RBHR-15 and RBHR-21 were calculated to be 16.4 h and 10.6 h, respectively. Thus, the isolates were characterized as slow growers as the generation time was more than 5 h[38].

Conclusion

The following conclusions were drawn from the present investigation. The two isolates were confirmed to have characteristics of *Rhizobium* on the basis of tests like growth on Hoffer's alkaline media (pH-11.0), Gram's reaction, Cell shape, absorption of Congo red medium, *etc*. The isolates exhibited a high degree of resistance to antibiotics like penicillin and bacitracin. The isolates had salt tolerance to 1% NaCl and the optimum pH for growth was found to be 6-8. The isolates RBHR-15 and RBHR-21 were tested for abiotic stress tolerance and were found to be slow-growing in nature with a generation time of 16.4 and 10.6 h, respectively.

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Conflict of interest

All authors declare no conflict of interest.

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