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Characterization of *Rhizobium* sp (SAR-5) isolated from root nodule of *Acacia mangium* L.

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The use of efficient strain of *Rhizobium* is of prime importance for optimum N_2 harvest through legumes. The present investigation on microsymbiont associated with root nodulation of *Acacia mangium* L., an important forest species having wider adaptability. Certain biochemical and molecular techniques were used to characterize the microsymbiont. The 16S rRNA sequence was submitted to NCBI (National Center for Biotechnology Information) with an assigned accession number as MH 661260 (SAR-5). The dendrogram revealed that the strain MH 661260 (SAR-5) was *Rhizobium alamii,* exhibiting maximum similarity with *Rhizobium mesosinicum* strain: NR 043548. The maximum indole acetic acid (89.00 µg/mL) was produced by metabolizing glucose followed by fructose (70.4 µg/mL), mannitol (55.8 µg/mL), lactose (51.4 µg/mL), sucrose (46.2 µg/mL), starch (39.6 µg/mL), galactose (30.6 µg/mL) and maltose (26.4 µg/mL) and the least (16.5 µg/mL) was recorded in control. The plateau stage of growth was attained after 36 h of inoculation, but the exopolysaccharides (EPS) production was the highest (112 µg/mL) at 48 h after inoculation, thereafter reduced in yeast extract mineral medium. The most preferable carbon, nitrogen, and vitamin for EPS production were maltose, L-asparagine, and L-ascorbic acid, respectively and the least preferable were sucrose, KNO_3, and riboflavin, respectively. The isolate (SAR-5) could survive in the pH range of 6 to 8 and the salinity level up to 3% NaCl in laboratory conditions.

Keywords: Acidity tolerant, Exopolysaccharides, Indole-3-acetic acid (IAA), Salinity tolerant, Stress tolerance

Legumes are regarded as the third largest family of angiosperm plants, including 17000–19000 species distributed worldwide with nearly 3000 species identified as potential N_2 fixers[1]. The rhizobia can fix atmospheric nitrogen (N_2) in leguminous plants. These belong to thirty species and seven different genera – *Allorhizobium, Sinorhizobium*, *Rhizobium, Mesorhizobium*, *Bradyrhizobium*, *Methylobacterium*, and *Azorhizobium* – distributed in four distinct 16S rRNA branches within the alpha sub class of the Proteobacteria[2]. Several evidences indicate the nitrogen-fixing ability of tree legumes from natural forests[3] and agroforestry systems[4]. The *Bradyrhizobium* sp is reported to nodulate *Acacia mangium*[5] and *Acacia spirorbis*[6].

The symbiosis between legume and rhizobia starts with the exchange of signals between the host plant and its microsymbiont[7]. In nitrogen limiting conditions, the legume roots secrete flavonoid compounds into the rhizosphere and they serve to activate nod genes leading to the synthesis of a lipochitooligosaccharidic signal (Nod factor) which is essential for initiating symbiotic development in most legumes[8]. Compatible nod factors are recognized by legume nod factor receptors[9], initiating the symbiotic signalling cascades, which promote infection thread formation and nodule organogenesis[10]. A part of flavonoid compounds may also function as phytoalexins, acting to reinforce symbiosis specificity[11]. Other than Nod factors, exopolysaccharides, lipopolysaccharides, and capsular polysaccharides are also important for establishing legume-*Rhizobium* symbiotic relationships[12]. In a non-symbiotic context, the polysaccharides have an active role in plant development[13].

The abiotic factors like temperature, pH, and salinity may limit nodulation and N_2 fixation by rhizobia-acacia associations. There is substantial variability among strains in their ability to fix nitrogen under stress conditions[14]. The failure of N_2 fixing *Rhizobium*-legume symbioses in acid soils is a significant problem affecting crop productivity in nutrient- poor and adverse agro-climatic conditions across the world[15].

In the present investigation, it has been attempted to isolate the efficient strain of *Rhizobium* from the

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root nodule of the *Acacia mangium* its natural association with subsequent characterization.

Materials and Methods

Root nodule collection

The 90 days old *Acacia mangium* plants were uprooted without damaging the root nodules. The roots were washed thoroughly with tap water and then the healthy nodules were collected and washed with sterile distilled water and surface sterilized in 95% ethanol followed by repeated washing in sterile distilled water for few sec at each time. The sterilized nodules were preserved at 4°C for further study.

Serial Dilutions of the collected root nodules

Healthy nodules were crushed with the help of a sterile glass rod in the petri plate to obtain the milky white bacteroids. After the extraction of bacteroids from nodules serial dilution was performed. In order to get the growth of the *Rhizobium*, about 1 mL of bacteroid solution were diluted to 10 mL of water in a test-tube which served as a stock solution. The stock solution was serially diluted up to 10^{-9}.

Biochemical characterization

Various biochemical tests were performed by following the standard procedures such as growth on YEMA with congo red, growth on glucose peptone agar (GPA) medium[16], growth on Hoffer's alkaline media[17]. ability to produce 3-ketolactase[18], growth in presence of 8% KNO_3[19], hydrolysis of urea[20], gelatinase activity[19], catalase activity[21], motility test[22], sugar fermentation tests[23]. Antibiogram was carried out using disc diffusion assay[24].

Isolation of Exo-polysaccharide

The cell- free culture filtrate after centrifugation at 10000 g [$g = (1.118 \times 10^{-5})$ R S^2, R = radius of the rotor in centimeters, and S = speed of the centrifuge in revolutions per min] for 20 min was used for extracting EPS. For precipitating the polysaccharides, three- volumes of acetone were added to a unit volume of cell- free culture filtrate. After centrifugation at 6000 g for 10 min, the precipitated polysaccharides were collected and suspended in 1 mL distilled water. Then three volume of acetone was added to the dissolved polysaccharide for reprecipitation, thereafter centrifuged. The process was repeated for three times. After that EPS solution in distilled water was used for taking observations.

Estimation of exopolysaccharide by spectrophotometry

The dissolved polysaccharide solution was used for the estimation of EPS by phenol sulphuric acid method[25]. The reaction mixture (1:1) in a test tube contained 1 mL of EPS solution and 1 mL of aqueous phenol. Then 5 mL of concentrated H_2SO_4 was added to it. The tubes were allowed to stand for 20 min after vigorous shaking. The absorbance was measured at 490 nm. The EPS solution in distilled water was used as control and determined against glucose standard. Specific productivity was calculated as EPS production/growth.

Optimization of Culture

To check maximum growth and EPS production by strains, different carbon, nitrogen, and vitamin sources were used. Individual sources were added separately to the tryptophan supplemented basal medium and the effect on growth and EPS production was recorded.

Effect of pH and salt concentration on the growth of isolates

The isolates were inoculated to nutrient broth medium maintained at pH 4.0, 5.0, 6.0, 7.0 and 8.0 for salinity tolerance, NaCl was added @ 1%, 2%, 3%, 4% and 5% to nutrient broth and incubated at 30^0 C for 96 h. The turbidity of the medium in each flask was measured at 3, 9, 15, 21, 30, 48, 72, and 96 h after inoculation, at 660 nm using a visible spectrophotometer.

Indole-3-acetic acid (IAA) production

The IAA was produced in 6 days at 30°C and 150 rpm cultures of SAR-5 in YEM broth with 0.1 g/L tryptophan. After centrifugation (6000 rpm for 30 min), an aliquot of broth was used to determine the production of indole-3-acetic acid by the method described by Glickman and Dessaux[26].

Molecular characterization

Quick-DNA™ Fungal/Bacterial Miniprep Kit Catalog No. D6005 from Zymo Research was used for the isolation of 16S rRNA. The amplified DNA was separated by electrophoresis in 0.8% agarose gel run in 1 × TAE buffer at 50 V for 30-45 min, till DNA fragments were migrated and documented using a gel documentation system.

PCR amplification

The PCR was performed in a total volume of 25 µL master mix containing 10 pmol each of forward and reverse primers, 2.5 mM of $MgCl_2$, 200 µM each of the four deoxyribonucleotide triphosphates (dNTPs), 0.5 U of Taq DNA polymerase, 1x concentration of PCR buffer (Invitrogen, Life Technologies, Brazil) and 50 to 100 ng of isolated bacterial genomic DNA.

The template was denatured by heating at 95 °C for 5 min. This was followed by 39 cycles of denaturation of 30 sec at 95°C, 45 sec annealing, and 1 min elongation at 72°C, with a final extension of 7 min at 72°C. The amplicons were resolved in 1.5% agarose gel using 0.5x tris-acetate-EDTA (TAE) buffer by using forward (16SF- AGAGTTTGATCCTGGCTC AG) and reverse (16SR- TACGGTTACCTTGTTACG ACTT) primers.

Dendrogram and Data Interpretation

The consensus sequence of 16S rDNA gene was generated from forward and reverse sequence data using aligner software. The 16S rDNA gene sequence was used to carry out BLAST with the nr database of NCBI gene bank. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using the RDP database and the phylogenetic tree was constructed using MEGA X.

Statistical analysis

The data was analyzed statistically as per the procedure prescribed for a complete randomized design by using the software SPSS (*Statistical Package for the Social Sciences)*.

Results and discussions

Morphological and Biochemical Characterization

The colony of the isolate SAR 5 was circular in shape, convex in elevation, regular in the margin,and translucent. The isolate was able to grow in YEMA congo red medium but failed to grow on BTB added YEMA medium and glucose peptone agar medium. Likewise, Gram's reaction, absorption of congo red, reduction of ketolactase enzyme, and growth on 8% KNO_3 medium was observed to be negative. The strain was unable to grow in Hoffers alkaline medium. Similar findings were reported previously in *Rhizobium* sp isolated from chick pea[27], *Vigna mungo*, *Cicer arietinum*, and *Vigna radiata*[28].

Sugar utilization pattern of the isolate

Microorganisms generally use various sugars to meet their energy requirements. The utilization pattern of various sugars by the isolate SAR 5 is presented in (Table 1). It was observed that the isolate SAR 5 was able to degrade dextrose, fructose, galactose, and sucrose in both oxidative and fermentative modes, while the sugar mannitol was degraded only through the oxidative pathway. Further, it was revealed that the isolate was unable to utilize rhamnose, adonitol, inulin, trehalose, and inositol in any mode. Similar findings were reported by Niste *et al*.[29] in *Rhizobium Leguminosarum* bv. *Trifolii* and *Sinorhizobium Meliloti*.

Table 1 — Sugar utilization pattern of the isolated strain

Sugars	Conditions	
	Oxidation	Fermentation
Rhamnose	-ve	-ve
Dextrose	+ve	+ve
Cellobiose	-ve	-ve
Fructose	+ve	+ve
Galactose	+ve	+ve
Raffinose	-ve	+ve
Inositol	-ve	-ve
Mannitol	+ve	-ve
Inulin	-ve	-ve
Trehalose	-ve	-ve
Adonitol	-ve	-ve
Sucrose	+ve	+ve

Table 2 — Antibiogram profile and enzyme production of the isolated SAR 5

Enzymes	Response	Antibiotic*	Response
Urease	+Ve	E15	S
Oxidase	+Ve	B10	R
Catalase	+Ve	AP50	R
Amylase	-Ve	CIP5	S
DNase	-Ve	PB100	MS
Lipase	-Ve	P10	R
Nitrate reductase	+Ve	T	MS
Chitinase	-Ve	N30	MS
Gelatinase	+Ve	AK30	MS
Caseinase	+Ve	S10	MS

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

It was observed that the isolate SAR 5 could produce enzymes like urease, oxidase, catalase, nitrate reductase, gelatinase, and caseinase, whereas it couldn't produce amylase, lipase, DNase, and chitinase enzymes (Table 2). The isolate was resistant to Amphotericin, Bacitracin and Penicilin and moderately susceptible to Polymyxin, Tetracycline, Amikacin, Neomycin, and Streptomycin whereas susceptible to Erythromycin and Ciprofloxacin. The result corroborated with the findings of Deshwal and Chaubey[30].

Production of Indole Acitic Acid (IAA)

The indole acetic acid (IAA) production pattern by utilizing different carbon sources was presented in (Fig. 1). The isolate was able to metabolize the carbon for producing IAA. The highest (89 µg/mL) IAA was produced when medium was supplemented with glucose followed by fructose (70.4 µg/mL), mannitol (55.8 µg/mL), lactose (51.4 µg/mL), sucrose (46.2 µg/mL), starch (39.6 µg/mL), galactose (30.6 µg/mL), maltose (26.4 µg/mL), respectively. Similar findings were reported in *Rhizobium* sp from arhar[31].

Production of exopolysaccharides by utilizing carbon, nitrogen and vitamins sources

All the strains could reach their stationary phase of growth at 36 h of incubation, while maximum (112 µg/mL) EPS production by symbiont was recorded at 48hr of incubation and thereafter EPS production decreased (Fig. 2). A similar result was reported by Ghosh *et al*.[32] in enterobacter isolated from root nodule of *Abrus precatorius*.

The growth, EPS production, and specific productivity by the isolated strain were studied with mannitol (1%) and with the replacement of mannitol by eight different carbon sources (1%) from the yeast extract mineral medium (3) (Fig. 3). Evidently, maximum growth (4.25 OD) was recorded in maltose followed by manitol (4.21 OD), glucose (3.90 OD), starch (3.89 OD), lactose (3.52 OD), galactose (3.47 OD), fructose (2.35 OD), sucrose (2.27 OD) and control (1.94 OD), respectively. The highest EPS (122 µg/mL) and specific productivity (29) was recorded in maltose followed by manitol (108 µg/mL & 26), starch (91 µg/mL & 23), glucose (85 µg/mL & 22), lactose (72 µg/mL & 21), galactose (67 µg/mL & 19), fructose (43 µg/mL & 18), sucrose (39 µg/mL & 17) and control (25 µg/mL & 13), respectively. The utilization of different carbon sources for growth and EPS production by *Rhizobium* sp[33].

Different nitrogen sources (0.1%) were used to record the effect on the production of EPS, growth, and specific productivity (Fig. 4). The highest growth (OD 4.10), EPS ((102 µg/mL) and specific productivity

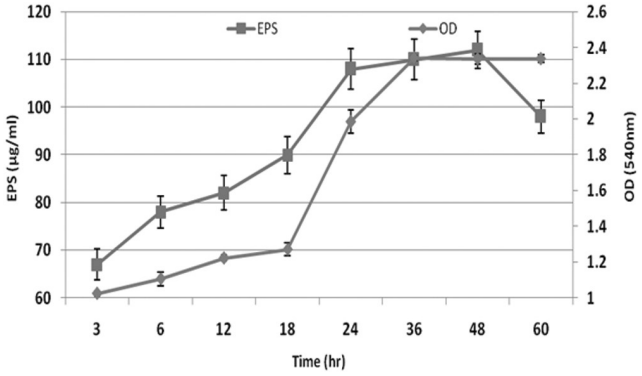

Fig. 2 — Growth and EPS production in YEMM

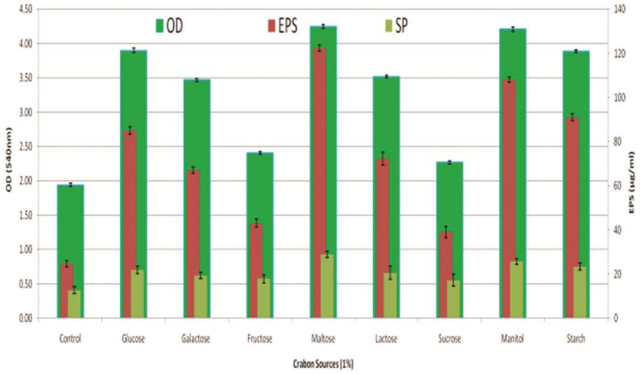

Fig. 3 — Growth and EPS production media by utilizing different carbon sources

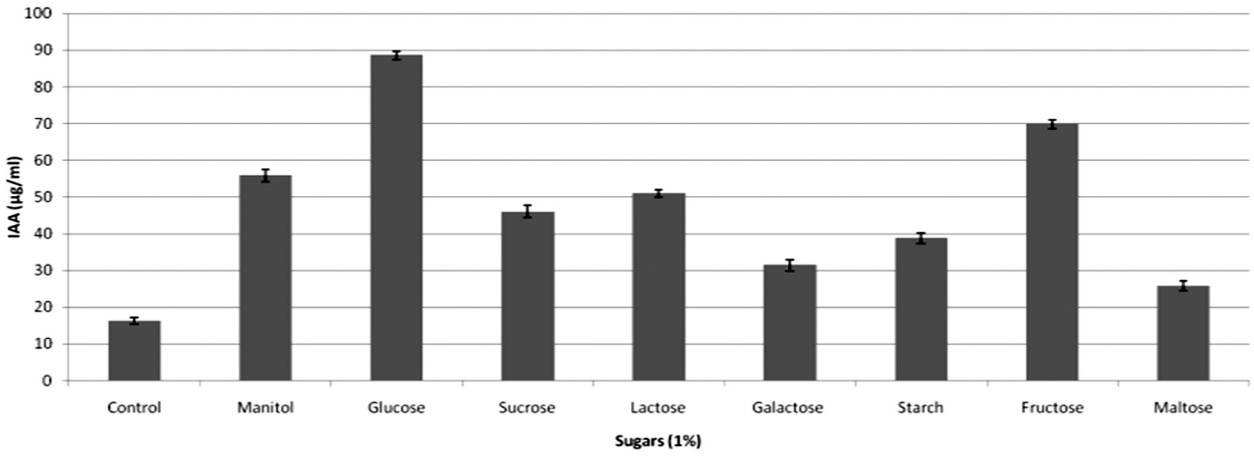

Fig. 1 — Production of IAA by utilizing different carbon sources and the head bar represents the standard error

(25) was recorded with L-aspergine (0.1%) followed by other nitrogen sources: glycine> $(NH4)_2SO_4$ > NH_4Cl > $NaNO_3$ > KNO_3, and the least growth (OD 1.24), EPS (20 µg/mL) and specific productivity (16) was recorded in control. Different nitrogenous compounds were reported to utilized by *Rhizobium tropici*[34] for their growth and EPS production.

The effect of five different vitamins (1 µg/mL) on growth, EPS production, and specific productivity by the symbiont were monitored. The maximum growth (OD 2.49), EPS (79 µg/mL) and specific productivity (32) was obtained when the medium was supplemented with L-ascorbic acid followed by biotin, thimine, nicotinic acid, and riboflavin, respectively (Fig. 4). The least growth (OD 1.6), EPS (40 µg/mL), and specific productivity (24) were obtained in control. Similar findings were reported in EPS production by *Rhizobium* sp[33].

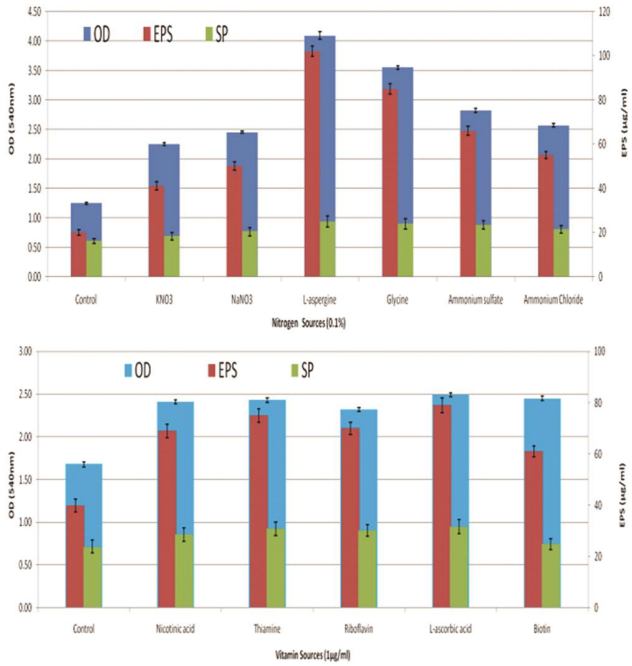

Fig. 4 — Growth and EPS production by utilizing different 'N' and different Vitamin sources

Acidity and salinity tolerant behaviour of microsymbiont

The isolate SAR-5 was grown in the nutrient broth medium and the growth in terms of optical density was measured in spectrophotometer. The growth pattern at different pH was represented in (Fig. 5A). The plateau stage of growth curve was attained after 48 h of inoculation in the medium of pH 6.0 and 8.0, whereas at pH 7.0 the plateau stage was attained after 84hr of inoculation. The highest (OD 3.52) growth was recorded in pH 7.0 after 84 h followed by pH 6.0 (OD 1.97), pH 8.0 (OD 1.89), pH 5.0(OD 0.29) & pH 4.0 (OD 0.18), respectively, after 60 h of inoculation. The isolate could grow well in neutral pH and growth was less in alkaline and acidic medium. A similar finding was reported in *Rhizobium* sp[31].

The growth of isolate SAR-5 was measured at different concentrations and presented in the graph (Fig. 5B). The stationary phase of growth came after 48 h of inoculation in all concentrations of NaCl. The highest growth (OD 1.79) was recorded in 1% NaCl followed by 2% (OD 1.68), 3% (OD 1.49), 4% (OD 0.27) and 5% NaCl (OD 0.05), respectively.

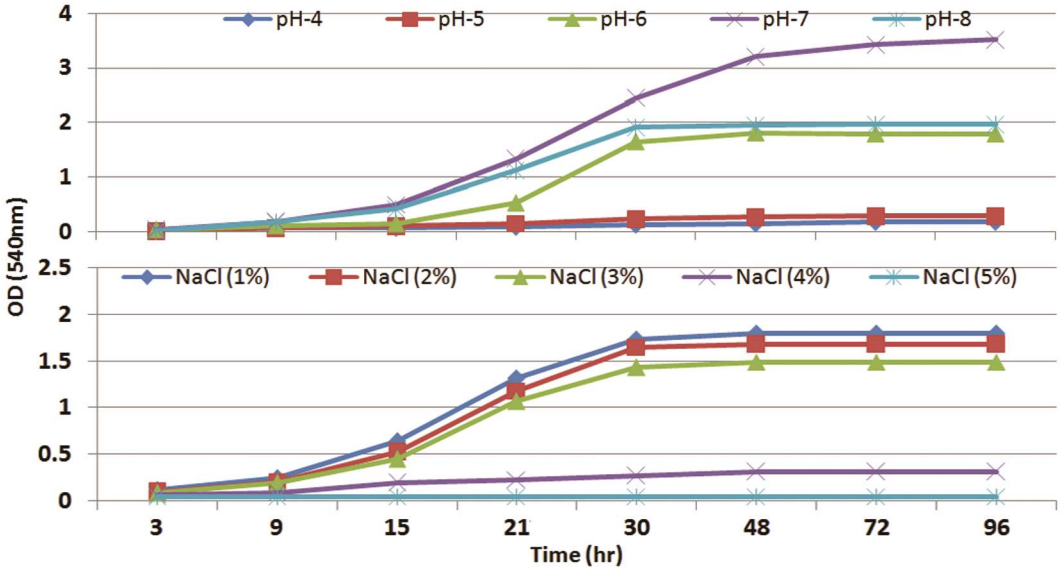

Fig. 5 — Growth curve of Strain SAR-5 at different (A) pH levels; and (B) salt concentration

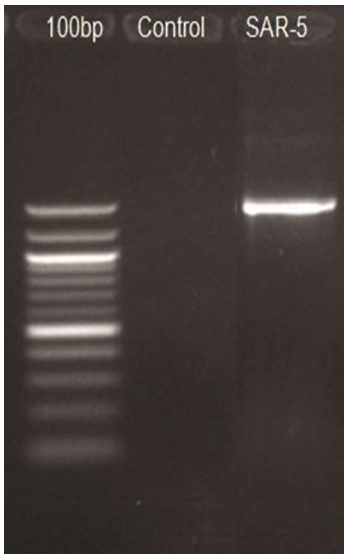

Fig. 6 — Gel documentation photograph of 16S rRNA genes of the isolates SAR 5 (MH 661260)

The isolate was able to grow with up to 3% NaCl. Similar findings were reported by Patil *et al*.[35] in *Rhizobium* sp.

16S rRNA gene sequencing and analysis of phylogeny

Biochemical tests for the isolate (SAR-5) were found to be in agreement with molecular (16S rRNA) analysis (Fig. 6). The sequence data were aligned with other 16S rRNA sequences for multiple alignments using Mega X software. The dendrogram was built with a homology pattern and revealed that isolate SAR 5 is identified as a strain of *Rhizobium alamii*. The 16S region was PCR amplified with 16sF (5′AG AGTTTGATCCTGGCTCAG3′) and 16sR (5′TACG GTTACCTTGTTACGACTT3′) primers.

The sequence data were aligned with other 16S rRNA sequences for multiple alignments using MEGA X software from the NCBI gene bank (Fig. 7). The dendrogram was built with a homology pattern which revealed that 16S rRNA sequence of the isolated strain (SAR 5) from root nodule of *Acacia mangium* was *Rhizobium alamii* exhibiting maximum similarity with *Rhizobium mesosinicum*: NR 043548. The sequence was submitted to NCBI gene bank with an accession number - MH 661260**.**

Conclusion

The study concluded that the microsymbiont (SAR-5) could produce enzymes like urease, oxidase, catalase, nitrate reductase, gelatinase and caseinase. It was resistant to *Bacitracin, Penicillin* and could metabolize different carbons for producing phytohormone indole

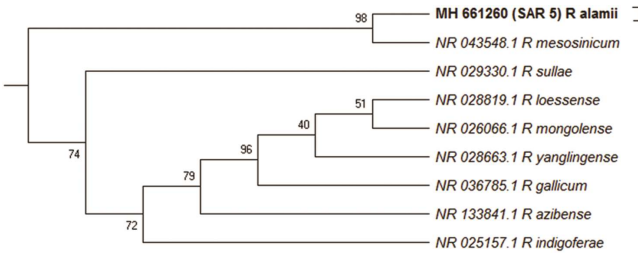

Fig. 7 — Dendrogram of symbiont, SAR 5 (MH 661260)

acetic acid as well as exopolysaccharides needed for better root growth and the survival during abiotic stress. It could tolerate the salinity up to 3% NaCl and could grow under slightly acidic (pH 6.0) to slightly alkaline (pH 8.0) condition which is also optimum for the establishment of forest species. Inoculation of *Acacia mangium* seeds with SAR-5 strain is expected to have a greater impact in raising robust seedlings during the nursery phase for better establishment in the future.

Conflict of interest

All authors declare no conflict of interest.

Acknowledgement

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