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Phenotypic and Genotypic Characterization of *Rhizobium* species isolated from the root nodules of *Sesbania sesban* found in Mumbai and its suburban areas

KEYWORDS: *Rhizobium* species, *Sesbania sesban*, Biochemical and Molecular characterization

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ABSTRACT *Root nodulating bacteria were isolated and characterized from the root nodules of Sesbania sesban leguminous plants growing in regions of Mumbai and its suburban areas.*

A total of seventeen isolates isolated on Yeast Extract Mannitol Agar medium. These isolates were further studied for their morphological characters and biochemical characterization along with one reference culture (NCBI-TUR1). Out of seventeen, morphologically six Rhizobium strains were rod shaped, Gram negative and mucous producing. These Isolates were unable to grow in the presence of 0.1% Methylene blue and Lactose. With the help of biochemical characterization, it was confirmed that those all six isolates were Rhizobium species.

After the phenotypic characterization, Polymerase chain reaction and 16S rRna gene sequencing were used for the genotypic analysis of Rhizobium species. Out of seventeen isolates only six strains were selected as Rhizobium after nif gene amplification. Further six isolates were selected as fast growing Rhizobium species based on Bromothymol Blue test.

These Isolates were studied for different salt concentration range from 5- 9.0%, and culture shows less growth as the concentration of salt increased .All isolates grow at pH 6.0 to 11.0 but none of the isolates grow at ph 12.0. The optimum physical parameters for growth of fast growing rhizobia were found in pH between 7.0-8.0 and 28°C temperature. BIOLOG test was performed in order to understand the nutrient requirement and utilization pattern of Rhizobium species,

The goal of isolation of Rhizobium species associated with Sesbania sesban sample would be the assessment of Rhizobial genetic diversity .The tolerance to high sainity , pH and their survival in such harsh environmental conditions make these rhizobial isolates valuable to improve the productivity of the leguminous plants cultivated under extreme environments.

INTRODUCTION

Nitrogen fixation in leguminous plants by gram negative bacteria plays a key role in crop production or agricultural sustainability. Nitrogen was known to be essential nutrient supplement for plant growth. The extensive rise in Indian population increases the demands of crops production in last few years, resulting extensive use of chemical fertilizers which may lead to soil pollution. These gram negative bacteria collectively known as Rhizobia. Rhizobia have been widely used in agricultural systems for enhancing the ability of legumes to fix nitrogen (Teaumroong et al., 1998). From last few years soil pollution have drawn attention of ecologist due to fear for human health, Soil barreness and ultimately environmental menace. A study of microbial flora is important to understand their promising role in the ecological system .Physical environmental factors like pH, temperature, salinity etc. plays an important role in *Rhizobium*-legume symbiosis, a process involved in nitrogen fixation. Nodulated legumes have potential for nitrogen fixation, reforestation and to control soil erosion. (Lorite,et. al.,2010;Elsoni and Osman, 2011). Plant diversity significantly enhances rate of microbial process that mediate C and N cycling. (Mukherjee et al.2006) *Rhizobium* with their ability to fix nitrogen in symbiosis with legumes, play central role in the Nitrogen supply to ecosystem. Nitrogen fixation is a result of intimate Symbiotic relationship between the root nodule bacteria and the host plants.

Increasing the extensive role of biofertilizers including *Rhizobium* species in agricultural practices, it result higher yield and simultaneously helps in environmental restoration. Symbiotic nitrogen fixation plays a central role in production of million tones of total biological nitrogen. The importance of these microbes is evident from the fact that although a total of 100 million metric tones of synthetic nitrogen Produced per year, while nitrogen fixing microbes' yearly converts about 200 million tons of nitrogen to ammonia, and the major portion of this biological nitrogen fixation are carried out by the symbiotic nitrogen fixers such as *Rhizobium* (Glazer et al., 2007).

Nearly 40% of the world land surface can be categorized as suffering from potential salinity problem (Waraporn Payakapong , 2006). Mumbai is one of the region with soils, being characterized by low pH, temperature fluctuation and variability in the soil salt concentration, which might be limit Nitrogen fixation. Thus an increase of tolerance to salinity of Rhizobial bacteria might constitute another approach to improve plant productivity under symbiosis.

Several environmental conditions are the limiting factors to the growth and activity of nitrogen fixing plants. The characterization of *Rhizobium* isolates for their resistance to adverse or harsh environmental factors will be important before their selection for agricultural practices. Typical environmental stresses faced by legume nodules and microbes may include water stress, salinity, soil pH,be temperature, heavy metals and so on (Kucuk C.et al,2008). Nitrogen fixing Legumes might tolerant to high salinity represent an important alternative to improve the soil fertility (Keneni A, et al., 2010).

MATERIALS AND METHODS

1) Procurement of culture-

Recommended control culture of *Rhizobium* species TUR-1 Were procured from NCBI Pune.

2) Sample collection

Sesbania sesban plants were collected from different sites of Mumbai and its suburban areas in the spring season in year 2010.(Table no. 1).

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Table 1. Different sites of sample collection -*Sesbania sesban* plant

Culture ID	Locations	Collection sites
CHA	Charkop goun Kandivali	Near charkop goun
VIR	Virar	Agashi
PAL	Palghar	Agricultural dept. Palghar
GHO	N. B. Mehta College, Nursery	Near Railway station
BEL	Belapur	Near River
TUR	Turbhe	Near Railway station

3) Isolation of *Rhizobium* species from Root nodules of *Sesbania sesban*
To isolate Rhizobia from the collected root nodules sample, pink and healthy root nodules of *Sesbania sesban* plant were selected. Isolation of *Rhizobium* sp. was done by the method described by (P.Somasegaran et al.,1994). Nodules were surface sterilized by immersing in Collected samples were washed several times with sterile distilled water. After washing 70% ethanol followed by 3% sodium hypochlorite solution for 3-4 minutes.Then surface sterilized nodules were again immediately washed with 5-6 times with sterile distilled water to remove the traces of Sodium hypochlorite solution. Surface sterilized nodules were crushed with a sterile glass rod in a sterile test tube containing 100µl. Loopful of nodule containing culture suspension was streaked on Yeast Extract Mannitol Agar(YEMA) plates containing 0.0025% congo red.(Vincent ,1970)and plates were incubated at 28 ± 1 °c temperature in dark condition .Single isolated colonies were selected and streaked on CREYMA medium for purity. (JORDAN 1984).Isolated pure Rhizobial cultures were preserved in -80°C with 15-20% glycerol until further use. (Elbanna et al.2009;EI-Akhal et al.,2009).

4) Phenotypic Characterization of *Rhizobium* isolates
a) Morphological characteristics
The colony morphology of the isolates was examined on Yeast extract Mannitol agar plate and CREYMA. After incubation of 48-72 hrs at 28°C isolated colonies were characterized on the basis of colony morphology. *Rhizobium* colonies appeared white glistening, gummy, mucous producing, small circular, elevated and transparent. (Aneja.,2003).

Gram stain reaction was carried out to confirm that culture is gram negative and does not contain any gram positive bacteria. Gram's procedure was done as per the method described by Somasegaran and Hoben 1994.

b) Biochemical Characterization
Glucose peptone agar assay (GPA)
GPA assay was performed to determine the capability of micro-organisms to utilize glucose as a sole carbon source for its growth. The Glucose peptone agar (40g/L glucose, 5g/L peptone, 15g/L agar, pH 7.0) medium was inoculated with *Rhizobium* isolates and growth was observed (Singh B. et al, 2008).

Bromothymol Blue Test (BTB)
To differentiate between fast and slow growers of *Rhizobium* species this assay was performed. YMA plates containing Bromothymol blue agar(10g/lMannitol,0.5gm/l,Potassium thiophosphate, 0.2gm/l Magnesium sulphate, 0.1gm/l Sodium chloride, 0.5gm/l yeast extract, 3gm/l Calcium carbonate, 20gm/l agar, 5 % bromothymol blue, and pH 6.8) Initially media was green in color.

After incubation of 72 hrs at 28°C.Slow growing *Rhizobium* are show an alkaline reaction in the medium, while fast growing bacteria are acidic in nature which changes color from green to yellow. (Vincent,1970).

Lactose assay (LA assay)
Lactose assay was performed to determine the capability of the micro-organisms to utilize lactose present in medium ((10gms/l lactose, 5gms/l peptone, 3gms/l beef extract, 20gms/l agar) as the sole carbon source for its growth.

Fluorescence assay
Fluorescence assay was performed (King et al, 1954) to determine the ability of isolates to fluoresce. King's medium (2g/l Peptone, 1.5g/l $MgSO_4$,1.5g/l K_2HPO_4 1.5G/l Glycerol, 15g/L agar, pH7) was prepared and inoculated aseptically with *Rhizobium* cultures. It was incubated at 28°c for 48hrs and observed under U.V. Light source. This test is a confirmatory test for the presence of Pseudomonas bacteria (Singh B.et al, 2008).

Methylene blue and Gentian violet Assay
Methylene blue and Gentian violet assay was performed to check the growth of the isolates .(SinghB. et al.,2008).Dye methylene blue (0.1%) was added in the medium and inoculated with isolated culture. Incubation was given at 28°c for 72hrs and observed the results. Similarly, Gentian violet treatment was done with the concentration of 0.1% (Gao et al, 1994).

Detection of Keto-lactose Production
Keto-lactose Production assay was specifically done for detection of *Agrobacterium* species. Test is based on the ability of *Agrobacterium*, common *Rhizobium* contaminant to produce Ketolactose enzyme which converts Lactose to 3-ketolactose .It is detected by the Benedicts reagent. *Rhizobium* culture were streaked on Lactose agar plates (10gms/l lactose, 5gms/l peptone, 3gms/l beef extract, 20gms/l agar) after incubation of 3-4 days at 28°C, plates were flooded with Benedict's reagent and kept at ambient temperature for 1-2 hrs.

If *Agrobacterium* species were present, then *Agrobacterium* growth was surrounded with yellowish zone of Copper di oxide. Where as yellow zone was absent on *rhizobium* growth. (Stowers and Elkans, 1980).

Characterization of culture on the basis of nutrient utilization
The screened isolates were inoculated into biochemical media of the BIOLOG MicroPlate™ to test the ability of these *Rhizobium* strains to utilize different carbon, nitrogen, phosphorus, sulfur, nutrient supplements, peptide, nitrogen sources, osmolytes, pH and chemical sensitivity.

Biolog microplates were used for the characterization of microbial cells. A microbial cell can be characterized for nearly 1000 cellular phenotypes which are Preconfigured into 10 micro plates with 96 well formats. Each well represents an independent test centre. Cells were cultured and added to the wells (independent test centre) in different plates. Cells metabolize a substrate present in a well, respire, and release energy, which reduces colorless tetrazolium redox dye. The reduced dye which is purple in color can be detected visually.

Molecular Characterization
DNA Isolation
Isolation and purification of DNA was carried out according to the method described in (Sambrook and Rusell, 2001). The total genomic DNA was extracted from *Rhizobium* species and purified with the help of Agarose Gel Electrophoresis.

Identification of the isolated bacteria by 16s r-DNA gene sequencing
The most powerful tool to identify the unknown bacteria is to sequence the gene (DNA) coding for 16S-rdna, which is present in the chromosome of the bacteria. The prokaryotic specific primers used for 16S r-DNA gene amplification were as follows- FD1 (5' AGA GTT TGA TCC TGG CTC AG 3') and RP2 (5' ACG GCT ACC TTG TTA CGA CTT 3').These forward

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and reverse primers were used to amplify 16s r-DNA genes from the bacterial colony under standard PCR conditions

Bacterial Identification by nif H gene Amplification
Nirogenase enzyme plays an essential role in the reduction of N_2 to NH_3.

It is composed of Fe (dinitrogenase) and Mo-Fe protein (dinitrogenase reductase) which is encoded by Nif gene present in *Rhizobium* species (Raymond et al., 2004).

For the gene amplification, nifHF (5'TAC GGN AAR GGS GGN ATC GGC AA 3') and nifHR (5' AGC ATG TCY TCS AGY TCN TEC CA 3') were used to amplify nif genes from the bacterial colony under standard PCR conditions such as and initial denaturation 95°C for 3 min., followed 30 cycles of denaturation at 94°C at 45 sec, annealing 62°Cat 45min., elongation at 72°C for 1 min with final extension at 72°Cfor 7 min (Sara Nour et al,2001).which amplifies 601- bp fragement (Mullis.1990).

The amplified product has been subjected to sequencing with closely related sequences retrieved from EMBL by using CLUSTALW (Felsenstein,1993). Phylogenetic tree was constructed by using Neighbor joining method.

Impact of physical stress (pH, salt and temperature) on Isolated *Rhizobium* species
Test for High Acidity and alkalinity
The ability of the *Rhizobium* isolates strains to grow in acidic or basic media was tested by streaking them on Yeast Extract Mannitol Agar plates .Yeast mannitol agar media pH was adjusted to 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0,11.0,12.0,13.0 with Nacl or Hcl before autoclaving. After incubation of 2-3 days at 28°C isolates were survived at a particular pH in the soil. (Aurag and Sasson, 1992).

Salt tolerance
The ability of the isolates to grow in different concentrations of salt was tested by streaking isolates on YEM medium containing 0.1%, 0.5%, 1.0%,1.5%, 2.0%, 2.5%, 5.0%,10%, 20%, 30%,70% and 90% (w/v) NaCl. (El Sheikh et al. (1989), Hashem F.D.,et al.(1998)).

Temperature tolerance
Tolerance to high temperature had been tested by incubating the inoculated yeast mannitol agar plates of isolated *Rhizobium* species at 25°C, 28°C, 30°C, 37°C, 45°C and 60°C respectively (Hung et al.2005).

RESULTS AND DISCUSSION
Isolation and morphological characteristics
In the present study, seventeen *Rhizobium* sp. were isolated from root nodules of *Sesbania sesban* collected from different locations in Mumbai and its suburban areas. All the retrieved isolates were thrived on YEM agar media after incubation for 2-3 days.

Phenotypically all isolated Rhizobial colonies had sticky appearance showing the production of mucous at lower levels.

CRYMA agar YMA agar
Figure 1. Morphological and microscopical view of *Rhizobium* species

The *Rhizobium* isolates in the current study were further tested on YEMA Plates Containing BTB (Bromothymol blue) ,as an indicator in the media. On BTB agar plate both slow and fast growers formed convex, and circular colonies.All the Slow growing *Rhizobium* species are alkali producers which turn the dye from green to blue while, fast growing Rhizobum species are acidic in nature turns the media yellow (Somasegaran and Hoben,1985).All the 06 isolates grew faster on YMA plate containing bromothymol blue and showed an acidic reaction turning the medium from green to yellow in color. Results indicated that all the isolated colonies were fast growers and shows circular,white colored colonies till 1-3 days of growth and Changed from white to yellowish in color after 4 days due to pH change.(Vincent.,1982; Saha and Haque, 2005). It is a characteristic feature of *Rhizobium* speciesvthat they producesd acid during growth (DeVries et.al, 1980; Baoling et al, 2007). General microscopic view of the isolates showed them as rod cells and gram negative.

Phenotypic Characterization of *Rhizobium* isolates
In Phenotypic characterization after Isolation and morphological characteristics Futher, affirmation of *Rhizobium* species was performed with Biochemical tests.

Six cultures were confirmed as *Rhizobium* species after biochemical characterization which is explained as follows-

Agrobacterium grows well on the Peptone Glucose Agar-Bromocresol blue plates with the significant change of pH. *Rhizobium* species shows poor growth or they are unable to grow on peptone Glucose agar without any change in pH. As there was no prominent change in pH, they failed to change the color of Bromocresol purple indicating that strains tested were not contaminated with *Agrobacterium*. (Vincent,982; Saha and Haque, 2005).

The *Rhizobium* isolates in the current study were further tested on YEMA Plates Containing BTB (Bromothymol blue) ,as an indicator in the media. On BTB agar plate both slow and fast growers formed convex, and circular colonies. All the Slow growing *Rhizobium* species are alkali producers which turn the dye from green to blue while, fast growing Rhizobum species are acidic in nature turns the media yellow (Somasegaran and Hoben,1985).All the 06 isolates grew faster on YMA plate containing bromothymol blue and showed an acidic reaction turning the Detection of Ketolactose Production assay was specifically done for the detection of *Agrobacterium* common contaminants of *Rhizobium* species. This reaction is mainly occurs in the *Agrobacterium* species, as they produce Ketolactase enzyme which converts lactose to 3-Ketolactose. Since, it utilizes the lactose as a sole source of carbon. On the oxidation reaction there is lactose get converted into 3- Ketolactose form. A yellow color was seen after overlaying YMA plates with Benedict's reagent. If yellow color appears then Contaminants was present. (Strowers and Elkan,1980). All the 06 isolates tested by us did not give yellow color hence; it is free of contaminant *Agrobacterium*. GPA and LA assay was performed to determine the capability of the microbes to utilize the glucose or lactose as the sole source of carbon for its growth.utilize the glucose or lactose as the sole source of carbon for its growth.Plates inoculated with *Rhizobium* species, after incubation showed the prominent growth.

Rhizobial cells are able to grow on GPA but they are growing poorly on LA.

Fluorescence assay was performed to determine the ability of the isolates to fluoresce (King, 1954). Mainly, it is done to confirm the presence of Pseudomonas species as they have the capacity to fluoresce under UV light. All the six isolates did not fluoresce,as *Rhizobium* is lacking of fluorescence ability.

Methylene blue and Gentian violet Assay was performed to

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confirm the isolated culture as *Rhizobium* species. Rhizobial cells were unable to grow either on medium containing 0.1% methylene blue and 0.1% Gentian violet (Wei et al, 2003).All the 06 isolates were not able to grow on media containing 0.1% methylene blue as well as 0.1% Gentian violet.

Table 2.Morphological and Biochemical Characterization

CULTURE ID	Morphological characters				Biochemical Characterization						
	Gram stain reaction	Micro scopy	Color	Mucosity	Peptide Glucose agar	Keto-lactose	Bromo-thymol Blue	Fluro-scence	Methylene blue	Gentian violet	Lactose Assay (LA)
CHA	Negative	Rod	white	mucous	-	-	+	-	-	-	-
VIR	Negative	Rod	white	mucous	-	-	+	-	-	-	-
PAL	Negative	Rod	white	mucous	-	-	+	-	-	-	-
GHO	Negative	Rod	white	mucous	-	-	+	-	-	-	-
BEL	Negative	Rod	white	mucous	-	-	+	-	-	-	-
TUR	Negative	rod	white	mucous	-	-	+	-	-	-	-

Key : Growth = + No growth = -

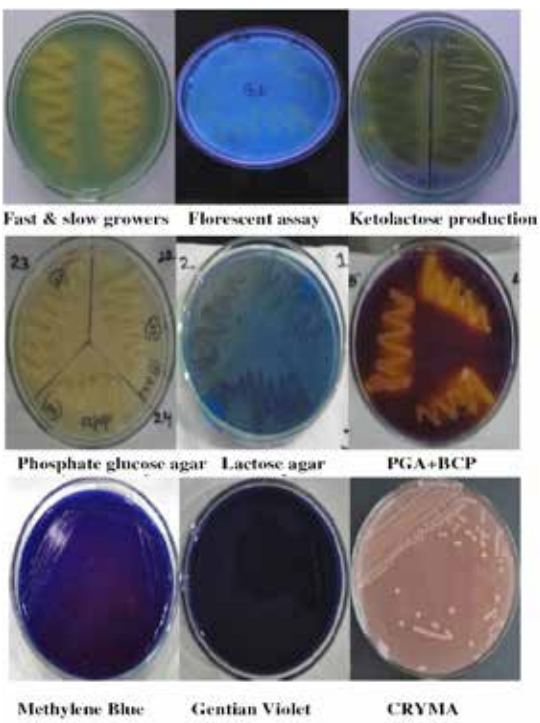
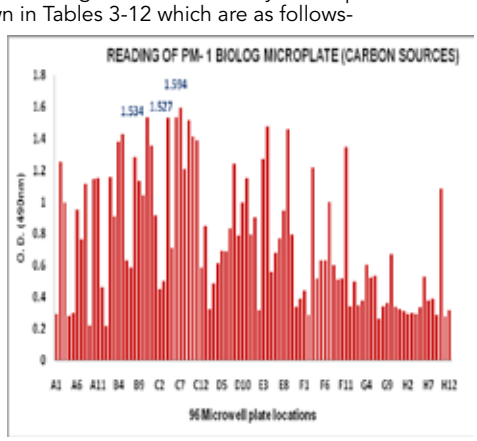
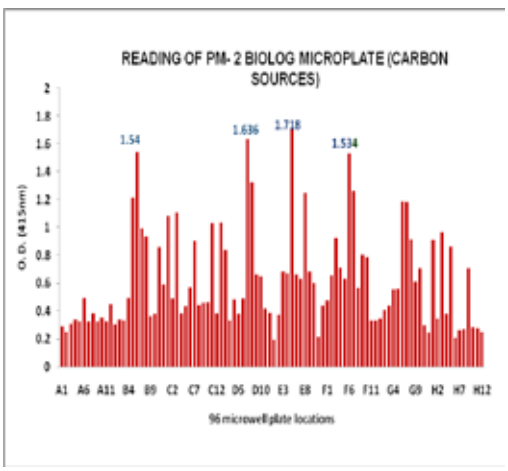


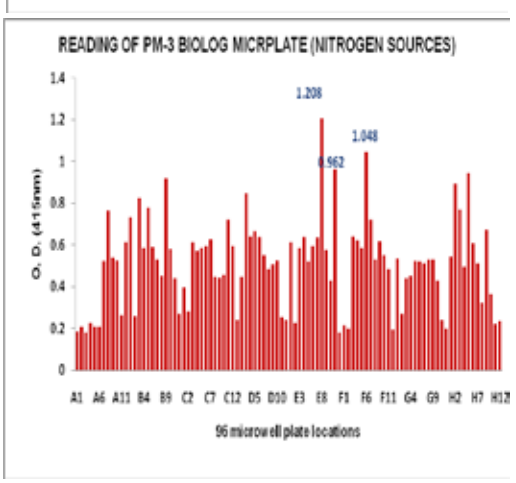
Figure 2. Biochemical characterization of isolates

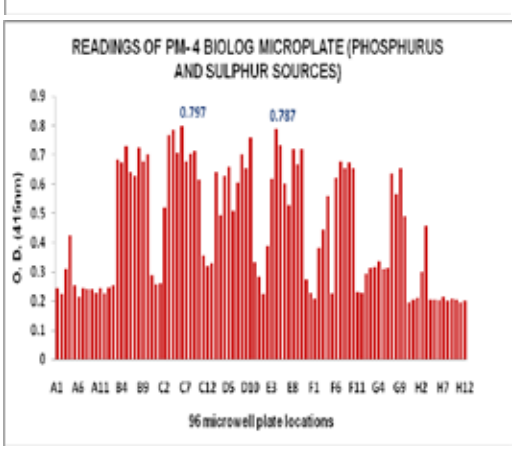
Characterization of culture on the basis of nutrient utilization

Rhizobium culture was tested for their ability to utilize various carbon, nitrogen, phosphorus, sulfur, nutrient supplement, peptide-nitrogen sources, osmolyte and pH. The results are shown in Tables 3-12 which are as follows-









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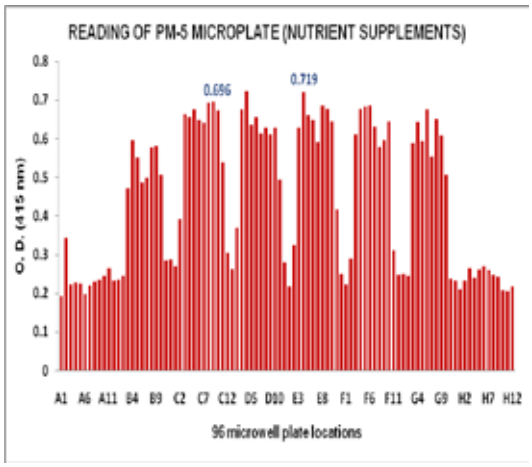

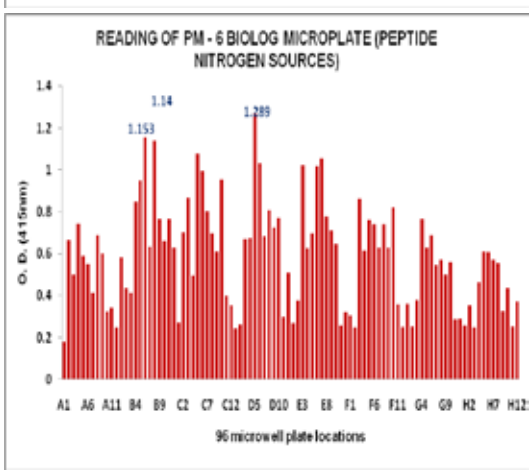

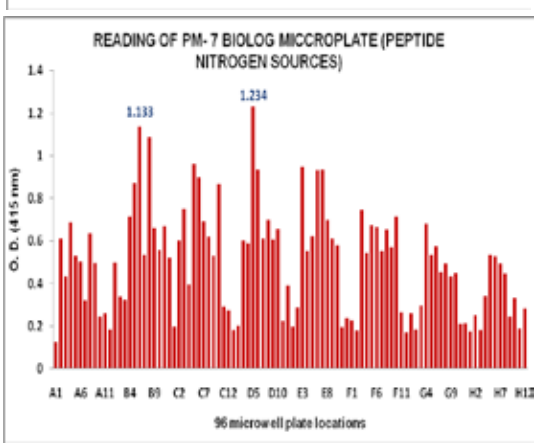

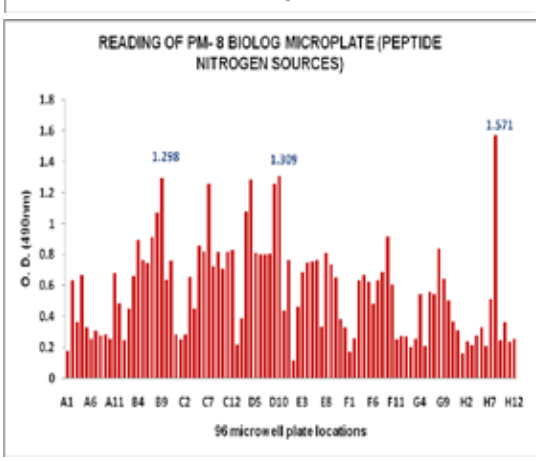

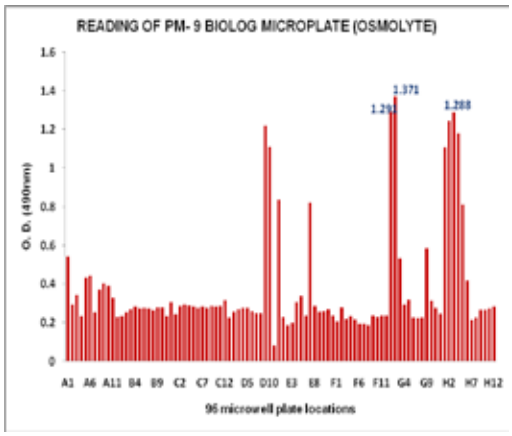

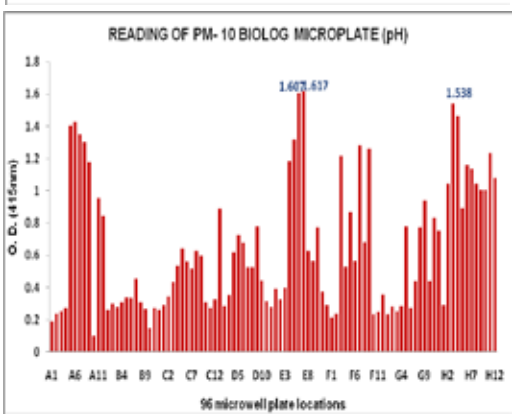

Graph 1- 10 Biolog Microplate result

Table 3. Carbon sources (PM-1)

Sr. No.	RESULT OF PM-1	O.D. reading	Nutrient source
1	C7	1.594	D- GLUCOSE
2	B11	1.534	D- MANNITOL
3	E4	1.479	D-FRUCTOSE-6-PHOSPHATE

Table 4. Carbon sources (PM-2)

Sr. No.	RESULT OF PM-2	O. D. Reading	Nutrient source
1	E5	1.718	D- GLUCOSAMINE
2	D7	1.636	TURANOSE
3	B6	1.544	D-ARABITOL

Table 5. Nitrogen sources (PM-3)

Sr. No.	RESULT OF PM-3	O. D. reading	Nutrient sources
1	E8	1.208	D- GLUCOSAMINE
2	F6	1.048	GUANINE
3	E11	0.962	N- ACETYL - D-GLUCOSAMINE

Table 6 Phosphorus and sulphur sources (PM- 4)

Sr. No.	RESULT OF PM -4	O. D. Reading	Nutrient sources
1	C6	0.797	D-GLUCOSAMINE -6-PHOSPHATE
2	E4	0.787	PHOSPHORYL CHOLINE

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Table 7 Nutrient supplements (PM- 5)

Sr. No.	RESULT OF PM- 5	O.D. Reading	Nutrient sources
1	D4	0.721	(-) SHIKIMIC ACID
2	E4	0.719	PYRIDOXINE
3	C9	0.696	CARBOXAMIDE

Table 8. Peptide Nitrogen sources (PM- 6)

Sr. No.	RESULT OF PM- 6	O. D. Reading	Nutrient sources
1	D5	1.269	Gln-Gln
2	B6	1.153	Arg-Arg
3	B8	1.14	Arg-Gln

Table 9 Peptide Nitrogen sources (PM- 7)

Sr. No.	RESULT OF PM- 7	O. D. Reading	Nutrient sources
1	D5	1.234	Pro-Asp
2	B6	1.133	Lys-Val

Table 10 Peptide Nitrogen sources (PM- 8)

Sr. No.	RESULT OF PM- 8	O. D. Reading	Nutrient sources
1	H8	1.571	Gly-Phe-Phe
2	D10	1.309	Ser-Asp
3	B9	1.298	Leu-His

Table 11 Osmolytes (PM- 9)

Sr. NO.	RESULT OF PM-9	O. D. Reading	Nutrient sources
1	G2	1.371	SODIUM PHOSPHATE Ph 7.50mM
2	G1	1.291	SODIUM PHOSPHATE Ph 7.20mM
3	H3	1.288	SODIUM NITRATE 40mM

Table 12 pH (PM-10)

Sr. NO	RESULT OF PM -10	O. D. Reading	Nutrient sources
1	E7	1.617	pH 9.5 +L-GLUTAMINE
2	E6	1.607	Ph 9.5 + L-GLUTAMIC ACID
3	H3	1.538	x-β-D-GLUCOSIDE

Genotypic Characterization
Molecular Characterisation

DNA isolation was carried out on 0.8% Agarose gel.The obtained bands were visualized under UV-transilluminator. In this study the isolated DNA was compared with the control bacterial DNA. All the 06 isolated samples showed the presence of DNA.

The almost complete 16S r-DNA gene sequences (1500bp) were aligned. Analysis of the 16s rDNA of the 06 isolates was performed using NCBI BLAST (National Centre for Biotechnology Information) with following gene sequences. primers (16S-F, 5'AGAGTTTGATCCTGGCTCAG 3' and 16S-R, 5'ACGGCTACCTTGTTACGACTT 3'). The complete sequences were aligned to the homologous sequences available for *Rhizobium* strains. The BLAST (NCBI) search using the sequences showed 99% homology of isolates to other GenBank as Rhizobia with 16s r-DNA .

The 16s r-DNA genes are directly linked to the phylogenic of micro-organism (Schwieger and Tebbe, 1998).

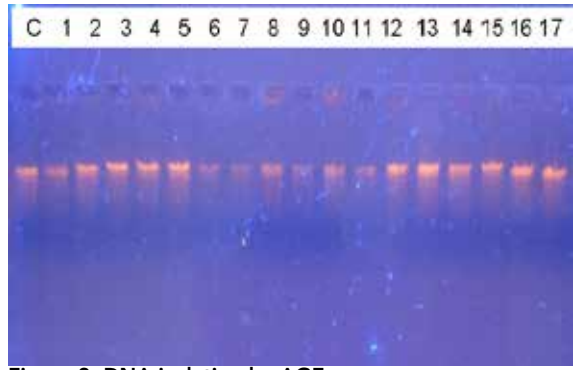

Figure 3. DNA isolation by AGE

Nif-H gene amplification

Nitorgenase enzyme is the enzyme which is highly essential for reducing nitrogen to ammonia and is encoded by nif gene. Since, the nifH gene only occurs in nitrogen fixing micro-organisms, it has been used to monitor the presence of the diazotrophs.

The nifH gene encodes for a subunit of nitrogenase reductase in nitrogen fixing organisms. The 06 strains gave PCR amplified product with an approximate length of 780bp. On the basis of this, it was confirmed that the 06 *Rhizobium* strains have nitrogen fixing capability (M. P Nuti,1979)]

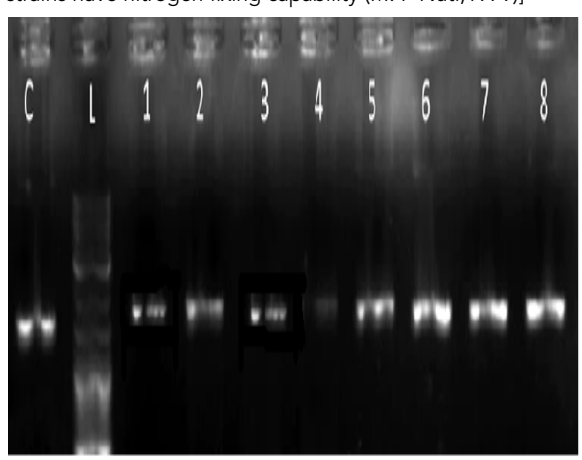

Figure 4. Nif gene amplification

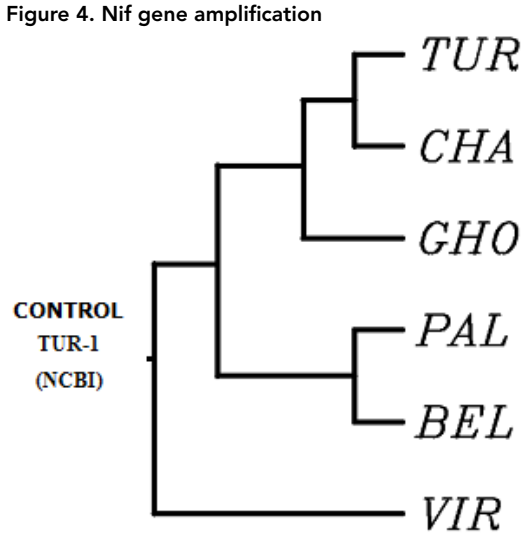

Figure 5.Phylogenetic Tree view of *Rhizobium* isolates by neighbor joining method

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Impact of environmental stress on *Rhizobium* species
Test for High Acidity
pH is playing an important role in the growth of the organism. Slight variations in the pH of a medium might have enormous effects on the growth of an organism. *Rhizobium* has been reported to grow the best at neutral pH that is 7 (Baljinder Singh et. al, 2008). All the strains were able to survive well at pH 4.0 to 7.0 and 8.0 to 13.0.

Test for salt tolerance
All *Rhizobium* species which had been isolated from *Sesbania sesban* samples had grown in almost all ranges of the various Sodium Chloride concentrations tested except 90%. The ability to tolerate high salt concentration was far greater from 0.1% to 2.0%.Hence,it indicates that, all six strains were more adapted to highly saline conditions.

Temperature tolerance
The isolated *Rhizobium* strains had been restricted to thrive on YMA and incubated for 48hrs at different temperature degrees (25°C, 28°C, 30°C, 37°C, 45°C and 60°C) to test their tolerance to higher temperature. The results indicated that, all *Rhizobium* strains grew in between 25°C to 30°C after 48 hrs. At 25°C, 37°C, 45°C and 60°C, no strains had grown on YMA plates after 48 hrs.

Table 13. Effect of different pH on *Rhizobium* species

Culture ID	pH range									
	4	5	6	7	8	9	10	11	12	13
CHA	+	+	+	+	+	+	+	+	+	+
VIR	+	+	+	+	+	+	+	+	+	+
PAL	+	+	+	+	+	+	+	+	+	+
GHO	+	+	+	+++	+	+	+	+	+	+
BEL	+	+	+	+++	+	+	+	+	+	+
TUR	+	+	+	+++	+	+	+	+	+	+
CON	+	+	+	+++	+	+	+	+	+	+

Key :+=Growth++ =Moderate growth, +++ = Good growth

Table 14. Effect of different concentrations of Sodium chloride on *Rhizobium* species

Culture ID	Salt Concentrations (%)											
	0.1	0.5	1	1.5	2	2.5	5	10	20	30	70	90
CHA	+++	+++	+++	++	++	++	+	+	+	+	+	-
VIR	+++	+++	+++	++	++	++	+	+	+	+	+	-
PAL	+++	+++	+++	++	+	++	+	+	+	+	+	-
GHO	+++	+++	+++	++	++	++	+	+	+	+	+	-
BEL	+++	+++	+++	++	++	++	+	+	+	+	+	-
TUR	+++	+++	+++	++	++	++	+	+	+	+	+	-
CON	+++	+++	+++	++	++	++	+	+	+	+	+	-

Key : + = Growth , ++ =Moderate growth, +++ = Good growth,- = No growth

Table 15. Impact of temperature on *Rhizobium* species

Culture ID	25°C	28°C	30°C	37°C	45°C	60°C
CHA	-	+++	++	-	-	-
VIR	-	+++	++	-	-	-
PAL	-	+++	++	-	-	-
GHO	-	+++	++	-	-	-
BEL	-	+++	++	-	-	-
TUR	-	+++	++	-	-	-
CON	-	+++	++	-	-	-

Key : + = Growth , ++ =Moderate growth, +++ = Good growth,- = No growth

CONCLUSION:
In conclusion, it can be said that phenotypically and genotypically all the retrieved isolates were Fast growing *Rhizobium* species. The impact of environmental stress studies shows a high diversity among isolated rhizobial strains. The sequencing results revealed the existence of little genetic variability .The goal of isolation of *Rhizobium* species associated with *Sesbania sesban* would be for the assessment of Rhizobial genetic diversity which will contribute to both purposes such as to raise the worldwide knowledge of biodiversity of soil microbes and the usefulness of Rhizobial collections in the agricultural field.

In the present study, *Sesbania sesban* plants were collected from different locations of Mumbai as well as Mumbai suburban regions. The root nodules of *Sesbania sesban* plant was used as a source for the isolation of *Rhizobium* species. Out of seventeen isolates, six isolates belonging to *Rhizobium* species and characterized by using morphological, biochemical, biolog and molecular studies. These isolates showed the presence of Nif gene which is encoding for Nitrogenase enzyme, a typical characteristic of *Rhizobium* species.

These isolates might be useful to increase the symbiotic nitrogen fixation in leguminous trees. The study suggesting adaptability of isolates to different ecological environments with many factors at stress levels such as temperature, pH and high concentration of salt. Thus, culture can be used for bioremediation purpose like other microbes like Pseudomonas spps.

Continued efforts needs to understand the complex association between legumes and their symbiotic partners, with an emphasis on their ecological role, soil fertility, evolution and transfer of nitrogen fixation genes and ultimately to employ efficient strains in the sustainable agricultural practices in India.

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