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RESEARCH ARTICLE

ISOLATION, MOLECULAR CHARACTERIZATION OF DIAZOTROPIC BACTERIA FROM VIRGIN SOIL FROM ARAKU VALLEY AND ASSESSMENT OF OF AMMONIA ACCUMULATION AT SALINE CONDITIONS

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Abstract

Nitrogen-fixing bacteria are widely distributed in nature where they reduce atmospheric nitrogen in soil or in association with plant. They have been found in a wide variety of terrestrial and aquatic habitats in both temperate and tropical regions of the world. Nitrogen-fixing bacteria are found in symbiotic associations with plants free living in soil. The objective of the present research was to isolate free living Nitrogen fixing bacteria from virgin soil samples in araku valley and assessment of their ammonia accumulation at saline conditions. 10 soil samples were collected in different place from virgin areas. For isolation of the free living nitrogen fixing bacteria, Nitrogen free media like Jensen's Medium and Azotobacter Agar were used. Serially diluted soil samples were spread on the agar media and incubated for 48 hours. Eleven morphologically different bacteria were separated on made pure colonies on nutrient agar media. All bacteria were under go biochemical characterization which reveals that all these bacteria related to Azospirillum, Azotobacter and Clostridium. High ammonia liberating isolate MGN-10 was molecular characterizes as Azotobacter chroococcum and this soil application increase the plant growth in terms of growth parameters.

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Introduction:-

Nitrogen is one of the essential element in maximum biological processes occurs in plants (Ogura et al., 2006). Though Dinitrogen is abundantly available element in the environment, it is biologically unavailable for plants and some microbes oxidize or reduce forms of nitrogen. Though But the two nitrogen atoms were bonded with triple bond, it requires high energy to reduce to ammonia (Figg et al., 2012). Plant associated nitrogen fixing bacteria were considered as the best possible alternative for inorganic nitrogen fertilizer to promote plant growth and yield (Ladha and Reddy, 2000). Various species of nitrogen fixing bacteria were exists in nature like Arthrobacter, Acetobacter, Azotobacter, Azospirillum, Azoarcus, Beijerinckia, Bacillus, Derrxia, Enterobacter, Herbaspirillum, Klebsiella, Pseudomonas and Zoogloea have been isolated from the rhizosphere of various crops (Barraquio et al., 2000; James et al., 2000).

Interestingly in recent days potential nitrogen fixing growth promoting rhizobacterial biofertilizers usage were gradually increased (Vessey, 2003). The other beneficial effects like production of phytohormones (Tien et al., 1979; Haahtela et al., 1990) and competitive suppression of pathogens (Glick, 1995) were also revealed. In the

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coastal area nearly 20% of the cultivated and irrigated lands were affected by soil salinization and in some areas it salinization reached to 50 also (Khan et al., 2015). Every year salt affected cultivated land was increasing by 1-2% because of this it was becoming like a global leading issue in coming decades (Kasim et al., 2016). Because of the saline environments the diazotrophic bacteria growth in cultivated soil is a challenging task, saline tolerant, plant growth promoting diazotrophic bacteria is needed for today's agriculture. The objectives of the present work was to isolate diazotrophic bacteria from virgin soils and assessment of their other plant promoting traits and ammonia liberation at different saline conditions.

Materials And Methods:-

Sample collection

A total ten soil samples were collected from virgin areas (Zero chemical pesticides and fertilizer using area) of Araku valley, Visakhapatnam, Andhrapradesh. Rhizosphere soil samples were collected carefully by uprooting the root system and placed in a cool box for transport and stored at 4°C.

Isolation of free living Nitrogen fixing bacteria

Ten grams of rhizosphere soil was transferred to a 250 ml Erlenmeyer flask containing 90 ml sterile distilled water and shaken (120 rpm) for 30 min. Serial dilutions were made and 0.1 ml aliquots (10³–10⁵) were spread on plates containing Burk's N-free medium. The plates were incubated for 7 days at 30 °C and morphologically different colonies appearing on the medium were isolated and subcultured for further analysis.

Phenotypic characterization of the bacterial isolates

Physiological and biochemical characters of the bacterial isolates were examined according to methods described in Bergey's Manual of Systematic Bacteriology (Holt et al., 1994). The isolates were characterized for the following traits: color/pigment, form, elevation, margin, diameter, surface, opacity and texture.

16S rRNA gene sequence analysis

DNA isolation was carried by the phenol chloroform extraction method described by Xia et al., (1995) with minor modifications. Two universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 907R (5'-CCGTCAATTCMTTTRAGTTT3') were used to amplify 16S rRNA genes. PCR reaction mixture of 25 µl total volume, containing 1/10 volume 10× Taqbuffer, 2 mM MgCl₂, 1 unit TaqDNA polymerase, 0.2 mM dNTP, 20 pmol forward primer, 20 pmol reverse primer and 100 ng DNA. DNA amplification was carried out in a Biorad Mini thermocycler with the following procedure: an initial denaturing step at 94°C for 5 min; 40 cycles for 1 min at 94°C (denature), 1 min at 48°C (annealing), 2 min at 72°C (extension) and a final elongation step at 72°C for 5 min. PCR products were separated by electrophoresis on 1.5 % agarose gel containing 0.5 µg/ml ethidium bromide, and photographed. The standard DNA samples (100 bp DNA ladder marker) were used as molecular size marker. The purified PCR products were subjected to Sanger's di-deoxy sequencing, in both forward and reverse directions, using BigDye terminator v3.1 cycle sequencing kit on ABI Prism3700 DNA Analyzer (Applied Biosystems Inc., USA) as per manufacturer's instructions. Related sequences were obtained from GenBank database National Center for Biotechnology Information (NCBI), National Library of Medicine (NLM) using BLAST, version 2 (Altschul et al., 1990). The sequences were aligned and the consensus sequence was computed using Mega-7 software. An evolutionary distance matrix was generated following the Kimura 2 parameter distance model (Kimura, 1980). Evolutionary trees for the data sets were inferred by the neighbor-joining method of Saitou and Nei (1987) by using the neighbor-joining program, MEGA version 2 (Kumar et al., 1993).

Estimation of ammonia liberation

100 ml Jensen's broth was inoculated individually selected eleven isolated pure bacterial cultures and incubated in rotatory shaker at 30°C. 1ml broth culture of each tube was aseptically transferred to the separate microcentrifuge tubes and centrifuged for 5min at 10,000rpm. 100 µl of each test supernatant was transferred to clean microcentrifuge tubes. 100 µl of Nessler's reagent was added to the supernatant including blank. 800 µl distilled water was added to the sample and incubated for 30min at room temperature. Absorbance was measured spectrophotometrically at 450nm.

Estimation of IAA production

The ability of the bacterial isolates to produce IAA was determined qualitatively and on Yeast Extract Mannitol broth medium. All pure isolates were inoculated onto 250ml conical flasks containing 50ml YEM broth media along with 0.1% L-tryptophan in triplicates. The cultures were incubated in orbital shaker at 28 ± 2°C and 120 rpm for 9

days. Cultures were centrifuged at 12000rpm for 5min and 500µl of supernatant from liquid cultures are taken into 1.5ml tube, 1ml of salkowaski reagent was added. Salkowski reagent was prepared by dissolving 2% of 0.5M FeCl₃ in 35% perchloric acid. Reaction tubes were incubated for 30 min in dark at room temperature. And the development of pink colour indicates the IAA production. Optical absorbance was measured at 530 nm. IAA concentrations were determined by using standard graph of known concentrations of IAA ranged between 1.25-100µg/ml. By extrapolating the obtained OD value onto standard graph, the quantity of IAA concentration produced by the bacterial isolates was obtained. (Dawwamet al., 2013).

Estimation of gibberellic acid

The bacterial strains were isolated for the presence of either of three activities i.e., IAA production, phosphate solubility and chitinolytic activity were also screened for their ability to produce GA by colourimetric analysis. 25 ml nutrient broth was inoculated in triplicate with pure strains of bacteria, and the culture flasks were incubated 28 ± 2°C on orbital shaker at 120 rpm for 48hrs. Culture was centrifuged at 12,000 rpm for 5min. The pH value of culture supernatants were adjusted to 2.5 using stock 3.75 N HCl. The culture supernatants were extracted using liquid liquid (ethyl acetate/NaHCO₃) extraction method. The amount of gibberellic acid in the ethyl acetate phase was measured by the UV spectrophotometer at 254 nm. The original concentration of GA present in medium was estimated with standard graph plotted with known concentrations of GA (Pandya and Desai 2014).

Estimating the effect of Salinity on ammonia accumulation in nitrogen free media from all bacteria

Total eleven NFB isolates were tested for their ammonia liberation at different salinity conditions. 100 ml Jensen's broth with different concentrations (1%, 2%, 3%, 4% & 5%) of NaCl was inoculated individually selected eleven isolated pure bacterial cultures and incubated in rotatory shaker at 30°C. 1ml broth culture of each the tube was aseptically transferred to the separate microcentrifuge tubes and centrifuged for 5min at 10,000rpm. The supernatants were tested for their ammonia liberation using Nessler's reagent.

Effect of NFB bacteria on plant growth.

Isolate MGN-10 was used for the plant growth experiments on paddy plant. Every treatments 100 gm soil was taken in cup. Bacterial isolate was added in gradient increasing concentrations (1×10^5 , 1×10^6 & 1×10^7). In each treatment 20 paddy seeds were taken and keep in the cups and sufficient water was added. All treatments were grown in glass chamber with sufficient light. After 10 days of seeding percentage of germination, root length, shoot length and dry weight were estimated.

Results and Discussion:-

Our complete study was focused on virgin forest land microbes because of novel microbial diversity. Although soil characteristics (particularly pH) have been frequently reported as strong drivers of microbial diversity in forest ecosystems (Lauber et al., 2008; Kaiser et al., 2016), tree species have been shown to exhibit a stronger impact on community structure than the soil environment (Bonito et al., 2014). Among the 10 rhizospheric soil samples collected near Araku valley Visakhapatnam, 11 different bacterial colonies were isolated by using Nitrogen free Burks medium. Plant root morphology, the stage of plant growth, root exudates, and the physical and chemical properties of the soil are reported to influence the occurrence and distribution of microbial communities in the soil and rhizosphere (Vessey, 2003).

The isolates were named as MGN-01 to MGN-11. According to their physical characters they were isolated individually. MGN-01 and MGN-10 isolates were transparent in nature, MGN-02, MGN-03, MGN-06 isolates were cream in color, MGN-04 and MGN-09 isolates were white in color and remaining isolates were showed yellow color. In case of staining results all isolates bacteria were rod shaped bacteria only, MGN-01, MGN-02, MGN-04, MGN-07, MGN-09, MGN-10 and MGN-11 were gram negative bacteria and the remaining bacteria were gram positive bacteria.

All these eleven isolates were characterized using biochemical techniques. MGN-05 isolate showed negative result for nitrate reduction remaining isolates were found positive. MGN-04 and MGN-05 isolate showed negative result for urea hydrolysis and remaining isolates were found positive. MGN-01, MGN-02, MGN-03, MGN-04, MGN-06, MGN-07 & MGN-09 isolates showed positive result for H₂S production and the remaining isolated showed negative result. Isolate MGN-01 and MGN-10 showed negative result for oxidase activity and the remaining isolates showed positive result. Isolates MGN-06, MGN-07 and MGN-09 showed negative result for citrate and catalase production where as remaining isolated showed positive result. Isolates MGN-02 and MGN-06 showed positive result for MR

rest and the remaining showed negative result. MGN-01 and MGN-06 showed positive result for VP test and the remaining were showed negative results. MGN-01, MGN-05 MGN-11 isolated showed positive results for catalase hydrolysis and the remaining isolates showed negative result. MGN-02 MGN-05 and MGN-09 isolates showed positive results for starch hydrolysis and the remaining isolates showed negative results. MGN-01, MGN-04, MGN-06, MGN-09, MGN-10 and MGN-11 isolates showed positive result for lipid hydrolysis and the remaining isolates showed negative result. All isolates showed positive result for IAA production. Only MGN-07 isolate able to produce spore. Except MGN-04, MGN-08 and MGN-09 remaining isolated showed motility.

Estimation of ammonia liberation

Every plant in the earth can absorb Nitrogen in the form of Ammonia/ Nitrate, in our study we studied the ammonia liberation of the all bacterial isolated in Nitrogen free media, this study was performed for 15 days with 3 days interval. The major postulate observed was accumulated ammonia was increasing with every interval in all isolates. Among the isolated bacteria MGN-07 and MGN-10 accumulated high ammonia i.e. 306 and 349 µg/ml at 3rd day interval, the remaining isolates liberated Ammonia in between 90 to 300 µg/ml. On 15th day of incubation isolate-10 liberate maximum 580 µg/ml of ammonia.

IAA production

Diversified microbes groups, including soil, epiphytic and tissue colonizing bacteria have been found to synthesize IAA (Patten and Glick, 1996). In the present study all 10 isolates were able to produce some or more IAA which is comparable with previous reports on different bacteria like Azotobacter, Azospirillum, Pseudomonas and Stenotrophomonas (Malik et al., 1997; Suckstorff and Berg, 2003). We have observed the IAA production for 15 days with 3 days interval and the values were tabulated. The range of the IAA production was 17.5 µg/ml to 385.1 µg/ml at 3rd day of incubation, highest IAA was produced by the MGN-02 next MGN-10 produced 348.2 µg/ml IAA at 3 day of incubation. In every isolate gradually the IAA production was increased in every interval. On 15 day of incubation MGN-10 was produced 750.4 µg/ml of IAA and this was best among the remaining isolates on 15th day of incubation. IAA production in study was higher than the earlier reports (Malik et al., 1997; Suckstorff and Berg, 2003)

Gibberellic acid production

Among them only 9 isolates able to produce gibberellic acid, MGN-06 and MGN-09 were not produced GA. Remaining 9 isolates produced GA in between 12.5 to 56.4 µg/ml. MGN-04 was produced maximum GA production at 56.4 µg/ml. GA production was increased gradually in every interval for every isolate studied. On the final day of incubation also MGN-04 was produced maximum GA i.e. 75.6 µg/ml, on the other hand MGN-10 produced second best GA production i.e. 72.3 µg/ml.

Effect of salinity on ammonia accumulation

Azotobacter is a free-living nitrogen-fixing microbial genus widely distributed in soil and rhizosphere (Martinez et al., 1985; Kennedy & Tchan, 1992). There are also several previous reports, Zahran (1999) found that the nitrogen-fixing bacteria was able to grow in media containing NaCl. The main theme of the present research is to evaluate the effect of salinity on the ammonia accumulation and evaluating the high ammonia accumulating isolate at high saline environment. At 1% NaCl also ammonia accumulation was decreased in every isolate. In case of MGN-10, 8.44 % ammonia production was decreased at 1% NaCl concentration. At 5% NaCl concentration only MGN-05, MGN-07, MGN-10 & MGN-11 were able to accumulate ammonia. Among them MGN-10 produced highest ammonia production i.e. 254.9 µg/ml, this was 56% decrease compared to control. Similarly Ravikumar et al., 2004 reported that some types of Azotobacter spp. (*A. chroococcum*, *A. berijerinkii*, *A. vivelandii*) isolated from saline environments can tolerant to high salinity concentrations up to 3.5 g / 100ml. But in our study Isolate MGN-10 can tolerate NaCl at 5 g/100ml.

Molecular characterization

High yielding bacteria in all accepts in the present study was MGN-10. 16s rRNA gene was amplified, sequenced and phylogenetic tree was constructed using MEGA software and the results revealed that the organism was *Azotobacter chroococcum*. Our sequence was showed 98.5% homology with the existing sequence and in the phylogenetic tree it was found in the same branch. *Azotobacter*, *Pseudomonas* and *Bacillus* also has been reported earlier from rhizosphere of various crop plants (Chan et al., 1994; Vessey, 2003). *Azotobacter* is a free-living nitrogen-fixing microbial genus widely distributed in soil and rhizosphere (Martinez et al., 1985; Kennedy & Tchan, 1992).

Plant studies

Basic level plant growth were carried out with rice plants. Among the four treatments one treatment was control and other three were MGN-10 at different concentrations (10^5 , 10^6 and 10^7 cells). Gradient bacterial concentration seems to increase all the plant growth promoting factors. Plant germination was found 95.3% in control treatment and 98.9% in bacterial treatment-4 (10^7 cells). The effect was found major in root length, 113% root length was increased compared to the control. As the Azotobacter is a non-symbiotic microbe, its maximum potential to enhance plant productivity can be exhausted by co-inoculating it with some other biofertilizers as compared to its single application. In addition to directly benefitting the plants through enhanced mineral uptake, Azotobacter also accelerate beneficial activities of other biofertilizers, if used in consortium. Moreover, reports of other microorganisms enhancing the plant growth activity of Azotobacter are also available. Currently, several reports of Azotobacter being utilized along with other microbes are found to be highly preferable among researchers as well as farmers (Aisha et al., 2020).

Conclusion:-

Among the 10 soil samples studied a total 11 different nitrogen fixing bacteria were isolated. All bacteria were biochemically characterized. Eleven bacteria were evaluated for the Ammonia accumulation, IAA production and GA production, among them MGN-10 was found to be the high yielding isolate. It produced 580 µg/ml Ammonia, 750 µg/ml IAA and 72.3 µg/ml GA at 15 days of incubation. And interestingly at 5% NaCl concentration also MGN-10 was able to produce 254.9 µg/ml Ammonia. Finally MGN-10 was molecularly characterized as Azotobacter chroococcum and this bacteria application increases the rice plant germination, root length, shoot length and dry weight also.

Tables

Table 1:- Biochemical characterization of free living nitrogen fixing bacteria.

Test Name	Bacterial isolate names										
	MGN-01	MGN-02	MGN-03	MGN-04	MGN-05	MGN-06	MGN-07	MGN-08	MGN-09	MGN-10	MGN-11
Reduction of nitrate	+	+	+	+	-	+	+	+	+	+	+
Urea Hydrolysis	+	+	+	-	-	+	+	+	+	+	+
H ₂ S Production	+	+	+	+	-	+	+	-	+	-	-
Oxidase	+	-	+	+	+	+	+	+	+	-	+
Gelatinase production	-	+	-	-	-	-	-	-	-	-	+
Citrate	+	+	+	+	+	-	-	+	-	+	+
Catalase	+	+	+	+	+	-	-	+	-	+	+
MR	-	+	-	-	-	+	-	-	-	-	-
VP	+	-	-	-	-	+	-	-	-	-	-
Casein Hydrolysis	+	-	-	-	+	-	-	-	-	-	+
Starch Hydrolysis	-	+	-	-	+	-	-	-	+	-	-
Lipid Hydrolysis	+	-	-	+	-	+	-	-	+	+	+
IAA production	+	+	+	+	+	+	+	+	+	+	+
Spore formation	-	-	-	-	-	-	+	-	+	-	-
Motility	+	+	+	-	+	+	+	+	-	-	+

‘-’ means negative result; ‘+’ positive for result.

Table:- Physical characters of isolated bacteria.

	Colony Colour	Colony shape	Bacteria shape	Grams staining
MGN-01	Transparent	Uneven	Rod	Negative
MGN-02	Cream	Uneven	Rod	Negative
MGN-03	Cream	round	Rod	Positive
MGN-04	White	Uneven	Rod	Negative
MGN-05	Light yellow	round	Rod	Positive
MGN-06	Cream	Uneven	Rod	Positive
MGN-07	Yellow	Round	Rod	Negative
MGN-08	Yellow	Uneven	Rod	Positive
MGN-09	White	Uneven	Rod	Negative
MGN-10	Transparent	Round	Rod	Negative
MGN-11	Yellow	round	Rod	Negative

Table 2:- Ammonia accumulation in nitrogen free media from all bacteria.

S.No	Isolate	Ammonia Accumulation ($\mu\text{g/ml}$)				
		3 rd day	6 th day	9 th day	12 th day	15 th day
1	MGN-01	181 $\pm$ 0.33	238 $\pm$ 1.33	261 $\pm$ 0.22	289 $\pm$ 1.09	301 $\pm$ 0.77
2	MGN-02	090 $\pm$ 0.45	098 $\pm$ 0.56	117 $\pm$ 0.38	142 $\pm$ 0.39	184 $\pm$ 0.45
3	MGN-03	241 $\pm$ 0.23	266 $\pm$ 0.45	284 $\pm$ 0.98	289 $\pm$ 0.45	294 $\pm$ 0.32
4	MGN-04	095 $\pm$ 1.67	128 $\pm$ 0.49	137 $\pm$ 0.45	159 $\pm$ 0.94	166 $\pm$ 0.11
5	MGN-05	218 $\pm$ 0.35	226 $\pm$ 0.26	278 $\pm$ 0.35	290 $\pm$ 0.23	317 $\pm$ 0.90
6	MGN-06	220 $\pm$ 0.33	228 $\pm$ 0.89	234 $\pm$ 0.92	278 $\pm$ 0.09	290 $\pm$ 0.04
7	MGN-07	306 $\pm$ 1.09	389 $\pm$ 0.38	394 $\pm$ 0.21	413 $\pm$ 0.11	419 $\pm$ 0.11
8	MGN-08	198 $\pm$ 1.33	228 $\pm$ 0.67	287 $\pm$ 0.11	294 $\pm$ 0.56	298 $\pm$ 0.24
9	MGN-09	112 $\pm$ 0.45	143 $\pm$ 0.90	160 $\pm$ 0.94	190 $\pm$ 0.23	216 $\pm$ 0.33
10	MGN-10	349 $\pm$ 0.33	389 $\pm$ 1.03	449 $\pm$ 0.98	516 $\pm$ 0.33	580 $\pm$ 0.45
11	MGN-11	288 $\pm$ 0.39	315 $\pm$ 0.98	338 $\pm$ 1.09	360 $\pm$ 0.85	368 $\pm$ 0.98

Table 3:- IAA Production from isolated bacteria.

S.No	Isolate	IAA ($\mu\text{g/ml}$)				
		3 rd day	6 th day	9 th day	12 th day	15 th day
1	MGN-01	17.5 $\pm$ 0.03	19.5 $\pm$ 0.34	25.0 $\pm$ 0.21	29.4 $\pm$ 1.32	32.8 $\pm$ 0.90
2	MGN-02	385.1 $\pm$ 0.21	389.5 $\pm$ 0.90	434.8 $\pm$ 0.92	542.5 $\pm$ 1.89	674.1 $\pm$ 0.23
3	MGN-03	156.9 $\pm$ 0.90	190.4 $\pm$ 1.09	199.5 $\pm$ 1.90	231.8 $\pm$ 2.34	289.5 $\pm$ 0.45
4	MGN-04	19.3 $\pm$ 0.04	52.8 $\pm$ 0.33	127.9 $\pm$ 1.43	180.5 $\pm$ 0.91	234.8 $\pm$ 0.33
5	MGN-05	228.0 $\pm$ 0.33	245.0 $\pm$ 0.13	249.5 $\pm$ 0.31	252.0 $\pm$ 1.90	277.2 $\pm$ 0.33
6	MGN-06	184.6 $\pm$ 0.45	190.3 $\pm$ 0.88	227.9 $\pm$ 0.49	260.1 $\pm$ 3.42	279.4 $\pm$ 0.61
7	MGN-07	121.0 $\pm$ 0.95	176.3 $\pm$ 1.20	190.3 $\pm$ 0.90	254.8 $\pm$ 0.90	289.1 $\pm$ 1.87
8	MGN-08	118.0 $\pm$ 0.01	143.2 $\pm$ 0.85	149.0 $\pm$ 1.05	170.4 $\pm$ 0.93	189.9 $\pm$ 1.12
9	MGN-09	56.9 $\pm$ 0.51	85.3 $\pm$ 2.19	116.2 $\pm$ 0.42	189.3 $\pm$ 0.33	230.1 $\pm$ 0.11
10	MGN-10	348.2 $\pm$ 0.59	390.2 $\pm$ 2.05	482.8 $\pm$ 1.45	550.2 $\pm$ 1.09	750.4 $\pm$ 0.29
11	MGN-11	134.6 $\pm$ 0.29	139.2 $\pm$ 1.88	185.4 $\pm$ 1.08	198.6 $\pm$ 0.76	220.6 $\pm$ 0.32

Table 4:- GA Production from isolated bacteria.

S.No	Isolate	GA ($\mu\text{g/ml}$)				
		3 rd day	6 th day	9 th day	12 th day	15 th day
1	MGN-01	18.5 $\pm$ 0.11	18.9 $\pm$ 0.83	22.4 $\pm$ 0.21	24.1 $\pm$ 0.31	24.9 $\pm$ 0.05
2	MGN-02	27.3 $\pm$ 0.02	29.0 $\pm$ 0.33	29.5 $\pm$ 0.62	31.0 $\pm$ 0.90	34.6 $\pm$ 0.90
3	MGN-03	12.5 $\pm$ 0.09	13.9 $\pm$ 0.06	14.0 $\pm$ 0.96	14.5 $\pm$ 0.72	16.2 $\pm$ 0.39
4	MGN-04	56.4 $\pm$ 0.33	56.9 $\pm$ 0.09	61.4 $\pm$ 0.28	69.9 $\pm$ 0.49	75.6 $\pm$ 0.82
5	MGN-05	32.6 $\pm$ 0.29	33.5 $\pm$ 0.19	39.0 $\pm$ 0.02	39.5 $\pm$ 0.45	39.9 $\pm$ 0.67
6	MGN-06	0.0	0.0	0.0	0.0	0.0
7	MGN-07	34.9 $\pm$ 0.01	40.6 $\pm$ 0.31	41.9 $\pm$ 0.73	43.1 $\pm$ 0.42	44.5 $\pm$ 0.33
8	MGN-08	49.1 $\pm$ 0.72	52.0 $\pm$ 0.41	55.1 $\pm$ 0.09	55.6 $\pm$ 0.94	55.6 $\pm$ 0.92
9	MGN-09	0.0	0.0	0.0	0.0	0.0
10	MGN-10	53.4 $\pm$ 0.92	55.8 $\pm$ 0.90	60.9 $\pm$ 0.01	71.2 $\pm$ 0.05	72.3 $\pm$ 0.10
11	MGN-11	23.0 $\pm$ 0.05	24.0 $\pm$ 0.39	25.1 $\pm$ 0.69	26.4 $\pm$ 0.90	26.9 $\pm$ 0.45

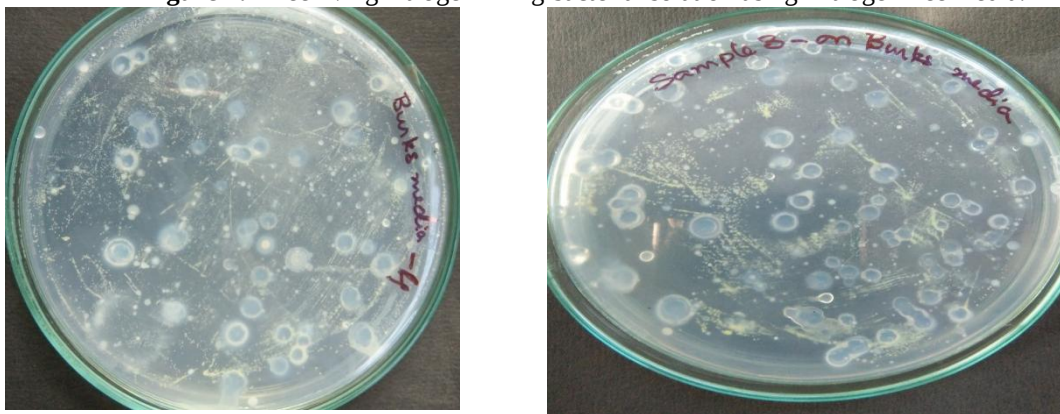
Table 5:- Effect of Salinity on ammonia accumulation in nitrogen free media from all bacteria.

S.No	Isolate	Ammonia Accumulation at 15 days of incubation ($\mu\text{g/ml}$)				
		1% NaCl	2% NaCl	3% NaCl	4% NaCl	5% NaCl
1	MGN-01	281.5 $\pm$ 1.95	153.8 $\pm$ 1.95	39.2 $\pm$ 1.06	0.0	0.0
2	MGN-02	123.9 $\pm$ 2.74	52.1 $\pm$ 3.05	0.0	0.0	0.0
3	MGN-03	271.0 $\pm$ 2.31	153.8 $\pm$ 0.90	84.3 $\pm$ 2.07	0.0	0.0
4	MGN-04	137.9 $\pm$ 1.86	110.5 $\pm$ 0.56	85.9 $\pm$ 0.90	30.4 $\pm$ 1.01	0.0
5	MGN-05	262.0 $\pm$ 3.39	205.2 $\pm$ 2.64	110.7 $\pm$ 0.39	56.2 $\pm$ 0.63	21.6 $\pm$ 0.09
6	MGN-06	251.6 $\pm$ 3.02	186.2 $\pm$ 2.91	95.2 $\pm$ 0.33	0.0	0.0
7	MGN-07	398.5 $\pm$ 3.09	221.7 $\pm$ 3.09	185.2 $\pm$ 0.54	132.6 $\pm$ 0.90	18.90 $\pm$ 0.33
8	MGN-08	221.7 $\pm$ 2.09	104.8 $\pm$ 0.89	39.6 $\pm$ 0.90	0.0	0.0
9	MGN-09	176.4 $\pm$ 1.36	44.8 $\pm$ 3.51	0.0	0.0	0.0
10	MGN-10	531.5 $\pm$ 1.33	489.4 $\pm$ 2.53	338.5 $\pm$ 2.10	299.4 $\pm$ 2.01	254.9 $\pm$ 1.65
11	MGN-11	324.6 $\pm$ 1.45	206.2 $\pm$ 0.90	196.3 $\pm$ 3.21	143.8 $\pm$ 0.99	95.3 $\pm$ 0.09

Table 6:- Effect of MGN-10 isolate on growth parameters of rice plant.

S.No	Bacterial concentration / ml	Percent germination	Root length (cm)	Shoot length (cm)	Dry weight (mg)
1	Control	95.3	2.6	4.5	115.3
2	1 x 10 ⁵	95.5	3.2	4.8	115.9
3	1 x 10 ⁶	97.2	4.9	5.3	118.4
4	1 x 10 ⁷	98.9	5.8	6.1	129.8

Figures

Figure 1:- Free living nitrogen fixing bacteria isolation using Nitrogen free media.

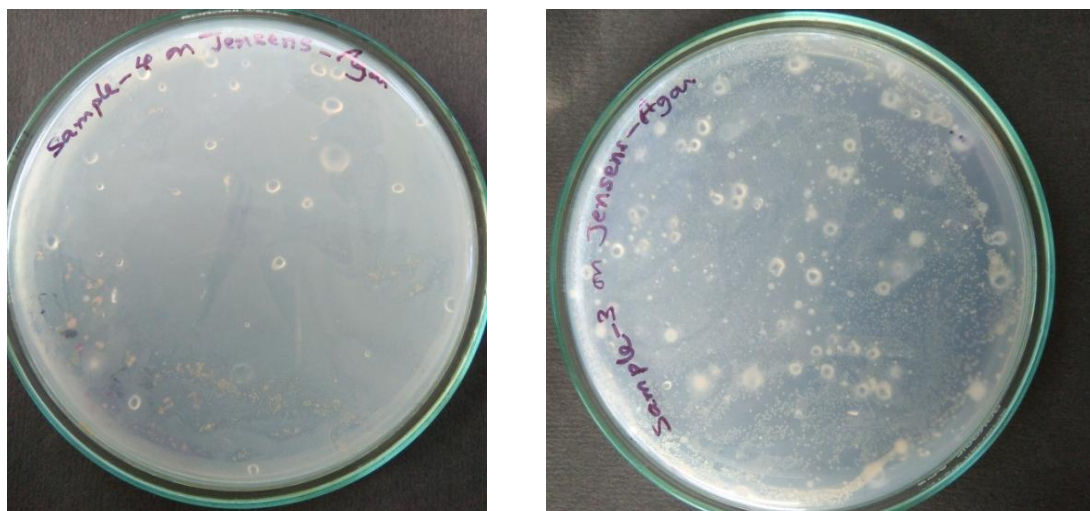


Figure 2:- Pure colonies of isolate bacteria.

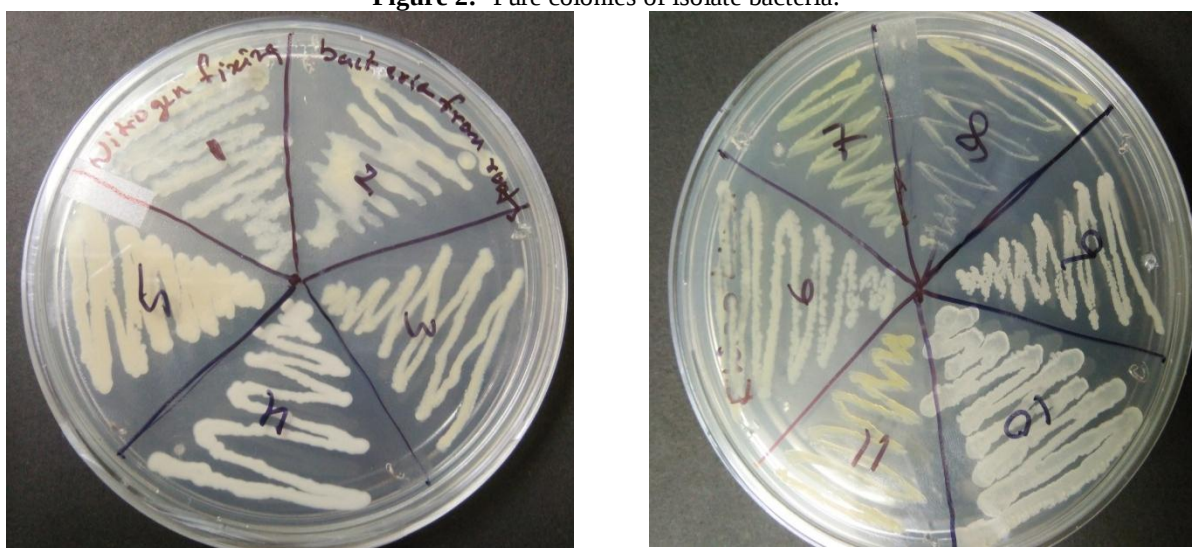
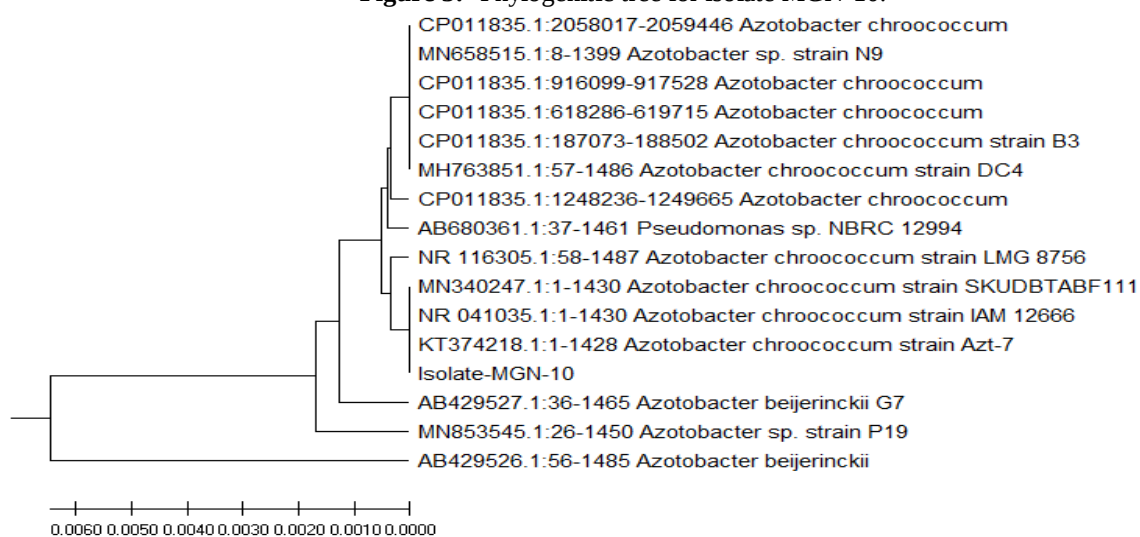


Figure 3:- Phylogenetic tree for isolate MGN-10.



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