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Research Article

ISOLATION AND CHARACTERIZATION OF NITROGEN FIXING BACTERIA FROM SALINE HABITAT (GIR-SOMNATH DISTRICT): AS BIOFERTILIZER

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ABSTRACT

Human population in the world is increase day by day but land for agriculture purpose is limited and it will reduce with time. So food security is most important problem for world. Increase agriculture production farmers are applying excessive chemical pesticides and fertilizer, it may upset the soil health and ecosystems, biofertilizer contains plant growth promoting rhizobacteria (PGPRs), endo-and ectomycorrhizal fungi, cyanobacteria and many other useful microscopic organisms led to improve nutrient uptake and maintain soil health.

In present study, free living nitrogen fixing bacteria isolated from 10 different villages of the Gir-Somnath district, Gujarat (near to sea costal area and salinity affected), 27 Nitrogen fixer isolates have been selected and performed ARA (Acetylene reduction assay) to study their nitrogen fixation efficiency, potent isolates were checked (nitrogen fixing efficiency) with different pH, Salt and temperature range, Indole acetic acid activity (IIA), pesticides tolerance activity, ammonia production activity and siderophore activity were performed on isolates, prepared biofertilizer (individual isolates, mixture of isolates) and carried out plant growth promotion activities on wheat plants(Pot study experiments). Isolates and their mixture culture supported best plant germination (wheat plants) compare to standard biofertilizer (available from market), CHN01 isolates belongs to *myroides spp*, SUN13 isolates may belongs to *Beijerinckia fluminensis*, DHN14 isolates belongs to *Agrobacterium tumefaciens*.,PRN18 isolates belongs to *Delftia Spp*.,SAN26 isolates belongs to *Paenibacillus spp*. Phylogenitic tree was constructed of isolates by using neighbouring method from distance matrices by MEGA-X software Isolates were indigenous flora from saline habitat and gave best plant germination activities so, they can apply as biofertilizer in salinity affected area of the district.

Keywords: Nitrogen fixing bacteria, Indole acetic acid activity (IIA), Pesticides tolerance activity, Ammonia production activity, Siderophore activity, Pot study experiment.

1. INTRODUCTION

Biofertilizer contains bioinoculant it can helpful for plants growth and developments. It is a very cheaper and effective for increasing the crop productivity. Bacteria, fungi and blue green algae are use for biofertilizer preparation. It can apply at the rhizosphere of plants for improving their activity in the soil ecosystem. Healthy soil can produce higher amount of yields because of soil have enough micro and macro elements for plant growth development. Excessive use of chemical fertilizers and pesticides cause negative impact on soil and human health. Nitrogen fixing free living microorganisms has frequently been reported as plant growth promoters [1] and mostly active in plant root zone (rhizospheric area) ,they have nitrogenase (multimeric enzyme complex) enzyme which can play a crucial role in biological nitrogen fixation activity (biologically reduction of nitrogen to ammonia compound) [2], enzyme consist of two conserved protein complex: an iron (Fe) containing dinitrogenase reductase (Fe protein) coded by nifH gene and a molybdenum iron Dinitrogenase (MoFeprotein) coded by nifDk Genes [3]. Nitrogenase is oxygen sensitive enzyme and nitrogen fixer has adaptive mechanism which protects the enzyme from molecular oxygen concentration. It may be diverge in Physico-ecological patterns of the microbial morphology, physiology and community structure along a gradient from anaerobic to fully aerobic environments [4].

In present study, isolation of 27 nitrogen fixing bacteria from saline habitat soil of Gir-Somnath district. Nitrogen fixing efficiency of was done by acetylene

reduction assay, indole acetic acid production, ammonia production, pesticides tolerance activity and plant growth promotion activities (biofertilizer formulation) were performed on wheat plants.

2. MATERIAL AND METHODS

2.1. Isolation of Nitrogen fixing micro-organisms from saline soil samples

Enrichment culture method was used for isolation of nitrogen fixing microorganism from saline soil. Samples (1-2 g) were inoculated in 50 ml of ashby's mannitol broth, incubated for 2- 4 days at 37°C under shaking flask condition. Cell density was visually observed than transferred 0.1 ml of ASM broth culture to ASM Agar media and incubated plates for 3-4 day. Well isolated colonies was transferred and Preserved in ASM slant for further study [5].

2.2. Determination nitrogen fixing efficiency of Isolates by acetylene reduction assay (ARA Method)

Nitrogen fixation efficiency of isolates was quantified by Acetylene reduction assay (measuring the reduction of acetylene to ethylene). Isolates were transferred in vials containing 20 ml Jensen's Media medium and inoculated with 100µl of bacterial culture and incubate all vials at 28°C for 24h, 10% of the air in the vial was replaced with equivalent volume of acetylene gas (C2H2) and shacked it's for 30 minutes at appropriate temperature. Shimadzu 2010 gas chromatography equipment with a flame ionization detector and porapack N (Internal diameter 2.2 mm, length 2 mm) was used for ethylene concentration analysis. The reduction of acetylene to ethylene gas was measured for isolates [6, 7]. Physiological optimization (best performed) of isolates were selected and performed their ARA analysis with different temperature (25°C, 40°C), pH (06 and 09 pH) and salt concentration (2.5% and 5% NaCl).

2.3. Indole acetic acid (IAA) and ammonia production activities

Gordon and Weber method [8] was applied for quantitative measurement of Indole acetic acid from isolates, Ammonia production (qualitative analysis) was done by Bakker and Schippers method [9].

2.4. Pesticides tolerance activity on Isolates

Agar diffusion method was applied for pesticides tolerance activities on isolates. Different concentration of pesticides prepared in acetone solution (0.01%, 0.1% and 1%) was used for agar diffusion method. BILDOR (lmidacloprid 17.8% SL), Kush (Dichlorvos 76% E.C.) SIMBAA (Propargite 57% Ec.) and PROFEN Insecticides (Dichlorvos 76% E.C.) used respectively [10].

2.5. Molecular characterization of Nitrogen fixing bacteria (16S Rrna gene)

Potent isolates (Nitrogen fixer and Phosphate solubilizers) were used for molecular identification by 16S rna gene method. Total genomic DNA was extracted and purified by pospiech and Neumann method. Nitrogen fixer and PSB isolates were identified by using Microseq 500 16S rDNA bacterial sequencing Kit and DNA sequence (AbI 310: model, perkin-Elmer Applied Bisystem, CA, USA) (All sequences data were aligned and compared with available standard sequences of bacterial lineage in the national center for Biotechnology information Genbank (http:// www.ncbi.nlm.nih.gov/) using blast programme. Phylogenitic tree was constructed by using neighboring method from distance matrices by MEGA-X software.

2.6. Inoculum preparation (Biofertilizer) and plant growth germination activities of isolates (pot experiment)

Potent isolates of nitrogen fixer were separately transferred to 500 ml flasks containing 50 ml nutrient broth; isolates colonies were grown aerobically in a flask on a rotating shaker (150 rpm) for 48 h at 30°C. Prepared bacterial suspension of 10^8 CFU mL-1 and each isolates 50 ml of culture was used and mix with sterile charcoal (120gm) for preparation of solid biofertilizer. For dual inoculation, an equal volume (10^8 CFU mL-1 of each inoculant) of isolates cultures were mixed and then employed (Mixer) to treat on Wheat plant (*Triticum aestivum L.*) seeds for plant germinations activities.

3. RESULTS AND DISCUSSION

Free living nitrogen fixing bacteria were isolated from saline habitat of the gir-somnath district; ten different villages of the district were selected (based on their salinity data available from KVK-krushi Vigyan Kendra-Kodinar.) for isolation of nitrogen fixing bacteria from soil samples. Colonies morphology and growth characteristics of isolates on ASM agar, 27 isolates have been selected, given their name (identification code) according to their village code and performed gram staining (Table 1), CHN01, CHN02, VDN04, ADN07,

KDN10, DHN16, LDN20, LDN21, KAN23 and SUN27 isolates were gram positive and CHN03, VDN05, ADN08, KDN10, KDN11, SUN12, SUN13, DHN14, DHN15, PRN17, PRN18, LDN19, KAN22, SAN25 and SAN25 were gram negative free living nitrogen fixing bacteria (Table 2).

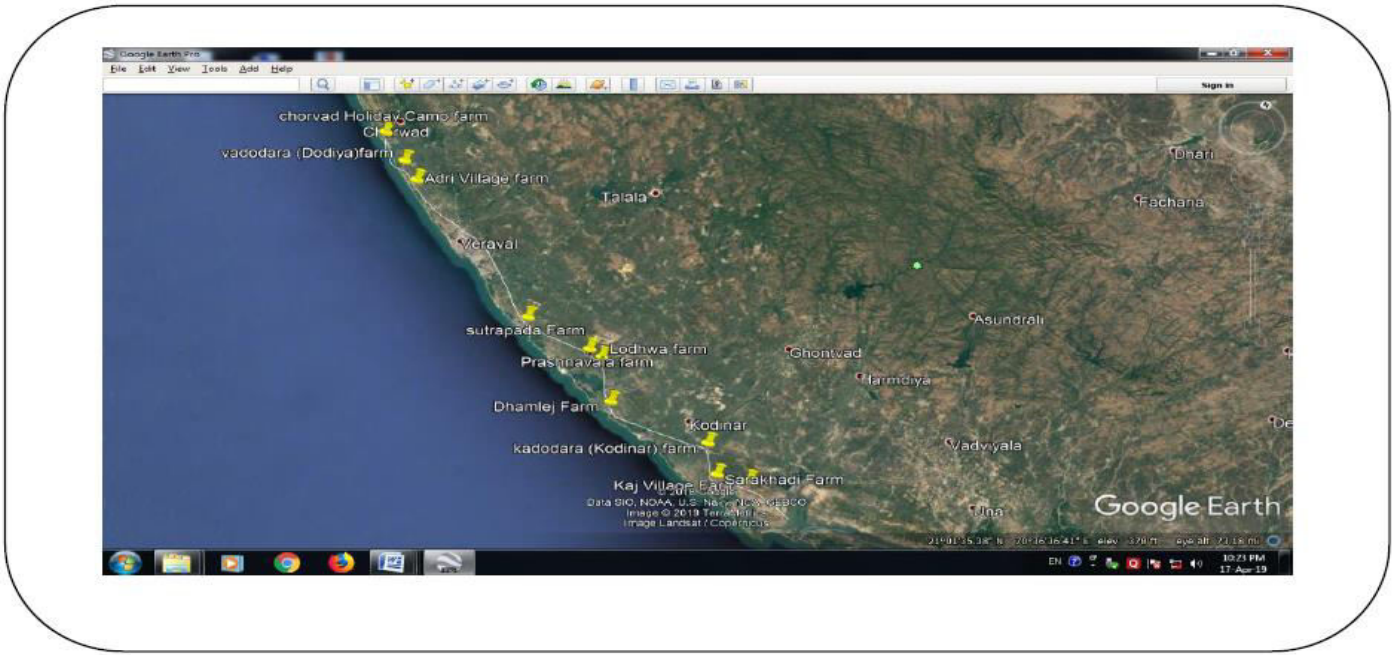


Fig. 1: Soil sampling site of Gir-somnath district

Table 1: Nitrogen fixing microorganisms and their code according to soil samples

Isolates	ASM Agar	Isolates	ASM Agar	Isolates	ASM Agar
CHN 1	+	KDN11	++	LDN21	++
CHN2	+	SUN12	+	KAN22	+
CHN3	++	SUN13	+	KAN23	+
VDN4	+	DHN14	+	KAN24	+
VDN5	+	DHN15	++	SAN25	+
ADN6	++	DHN16	+	SAN26	+
ADN7	++	PRN17	+	SAN27	+
ADN8	+	PRN18	+		
KDN9	+	LDN19	+		
KDN10	+	LDN20	+		

More than 35 Microorganism isolated from soil sample by Ashby Manitol Agar medium. Selected 27 microorganisms base on their colony morphology and growth characteristics.

CHN1: Chorvad1, CHN2: Chorvad2, CHN 3 Chorvad3,VDN4: Vadodara (Dodiya) 4, VDN5: Vadodara (Dodiya)5ADN6:Adri6,ADN7:Adri7,ADN8:Adri8,KDN9:Kadodara9,KDN10:Kadodara10,KDN11:Kadodara11SUN12:Sutrapada12,SUN13:Sutrapada13,DHN14:Dhamlej14DHN15:Dhamlej15,DHN16:Dhamlej16PRN17:Prashnavala17,PRN18:Prashnavala18LDN19:Lodhva19,LDN20:Lodhva20,LDN21:Lodhva21KAN22:Kaj22, KAN23:Kaj23, KAN24:Kaj24SAN25: Sarkhadi 25, SAN26: Sarkhadi 26,SAN27: Sarkhadi 27

Table 2: Morphological characteristics of Isolates

No.	Characteristics	CHN02	DHN14	PRN18	SAN26	SUN13
1	Gram Staining	+ve	-ve	-ve	-ve	+Ve
2	Shape	Rod	Short rod	Short rod	Short rod	rod
3	Margin	Irregular	Irregular	Circular	Circular	Irregular
4	Elevation	Flat	Raised	Convex	Convex	Flat
5	Texture	Smooth	Smooth	Gummy	Wavy	Smooth
6	Consistency	Moist	Moist	Moist	Moist	Moist
7	Pigmentation	White	White	White	yellowish	White

Nitrogen fixing efficiency was done by acetylene reduction assay and results were recorded in table 3, reduction of acetylene gas (ethylene gas production) is directly proportional to the nitrogen fixing ability of the isolates. Acetylene reduction assay were performed for all the 27 isolates and results was recorded in 3.84 to 6.87 nmoles/24 hrs in range selected only five isolates (highest acetylene reduction value, nmoles of C2H4/hr) and performed the assay with different pH (6, 7 and 8 pH), Temperature (25, 35 and 40°C) and salt concentration (1%, 2.5% and 5% NacL Concentration), isolates were reducing the acetylene gas in 2.83 to 4.51 nmoles/24 hrs range and isolate PRN 18 formed highest acetylene gas reduction (4.51 nmoles/24 hrs) was recorded at 6 pH medium (Table 04). Where 8 pH of the medium the nitrogen fixing efficiency of the isolates was recorded in 3.20 to 4.53 nmoles/24 hrs ranges, isolates CHN02 offered maximum acetylene reduction efficiency (nitrogen fixing efficiency) at this medium (Table 4) two different salt concentrations (2.5 % and 5.0%) medium used for acetylene gas reduction assay (Nitrogen fixing efficiency) and at 2.5% salt concentration of the medium results were recorded 1.81 to 4.96 nmoles/24 hrs in range (Table 5) where at the 5% salt concentration results were recorded 2.53 to 3.53 nmoles/24 hrs in range (Table 5) nitrogen fixing efficiency studied with two different temperature range. isolates were grew in nitrogen free medium with ethylene gas and incubated for 24 hrs. Nitrogen fixing efficiency was recorded 3.36 to 4.91 nmoles/24 hrs at 20°C (Table 06) at 40°C, isolates nitrogen fixing efficiency (ethylene gas) were recorded in 3.23 to 3.98 nmoles/24 hrs Table 06 [7]. VDN4, VDN5, VDN6, KDN9, DHN14, DHN15, DHN16, LDN21, SAN25, SAN26 and SAN27 isolates served as potent IAA producer compare to other isolates. (Datta and Basu, 2000) (Fig. 2) ammonia production activities of 27 isolates was done by Bakker and Schippers (1987) methods method, development of deep yellow color indicated positive results.CHN3, KDN9 and SAN27 produced deep yellow colors.(as potent ammonia producers), (Table 7) KDN10, DHN14, SUN13, PRN18, LDN20, KAN24 and SAN26 were developed little yellow color indicated moderate ammonia producers than other isolates (Table 7) isolates were sensitive against 1% concentration of each pesticide (1% concentration). Isolates PRN18 was tolerance and other isolates were sensitive at 0.1% pesticides concentration.

Table 3: Acetylene reduction assays of Nitrogen Fixing bacteria:

NO.	Culture ID	nmoles of C2H4/hr	NO.	Culture ID	nmoles of C2H4/hr
1	AD6	4.72	16	LD20	5.45
2	AD7	4.72	17	LD21	4.72
3	AD8	5.23	18	PR17	4.38
4	CH01	4.94	19	PR18	7.21
5	CHO2	5.63	20	SA25	4.74
6	CHO3	4.09	21	SA26	6.1
7	DH14	6.87	22	SA27	5.73
8	DH15	4.72	23	SU12	4.49
9	DH16	4.38	24	SU13	5.73
10	KA22	6.4	25	VD05	4.1
11	KA23	4.6	26	VD22	4.86
12	KA24	4.86	27	VD04	4.87
13	KD10	4.1			
14	KD11	4.6			
15	KD09	3.84			

Table 4: Nitrogen fixing efficiency of potent isolates at 6 & 8 pH, 37°C with 1% NaCl

No.	Name of Isolates	nmoles of C2H4/hr (6 pH)	No.	Name of Isolates	nmoles of C2H4/hr (8 pH)
1	CHN02	3.6	1	CHN02	4.55
2	DHN14	3.1	2	DHN14	3.21
3	PRN18	4.51	3	PRN18	3.2
4	SAN26	2.83	4	SAN26	4.2
5	SUN13	3.21	5	SUN13	4.13

Table 5: Nitrogen fixing efficiency of potent isolates at 2.5 % & 5 % NaCl 7 pH, 37°C

No.	Name of Isolates	nmoles of C2H4/hr (2.5 %)	No.	Name of Isolates	nmoles of C2H4/hr (5.0%)
1	CHN02	3.21	1	CHN02	3.13
2	DHN14	4.43	2	DHN14	2.85
3	PRN18	4.1	3	PRN18	3.2
4	SAN26	1.81	4	SAN26	2.53
5	SUN13	4.96	5	SUN13	3.53

Table 6: Nitrogen fixing efficiency of potent isolates at 20°C & 40°C with 1% NaCl concentration

No.	Name of Isolates	nmoles of C2H4/hr (2.5 %)	No.	Name of Isolates	nmoles of C2H4/hr (5.0%)
1	CHN02	3.21	1	CHN02	3.13
2	DHN14	4.43	2	DHN14	2.85
3	PRN18	4.1	3	PRN18	3.2
4	SAN26	1.81	4	SAN26	2.53
5	SUN13	4.96	5	SUN13	3.53

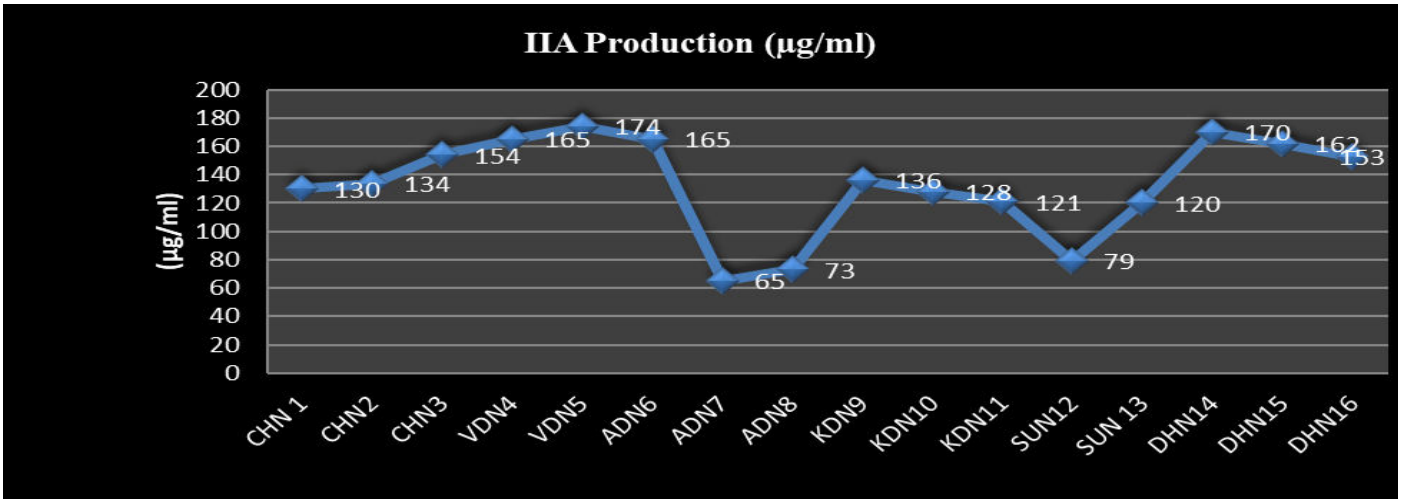


Fig. 2: IAA producing Isolates (µg/ml)

Table 7: Ammonia production activities of Isolates

No.	Name of Isolates	Ammonia Production
1	CHN02	++
2	DHN14	++
3	PRN18	++
4	SAN26	++
5	SUN13	++

Except SUN13 isolates other isolates were tolerance against 0.01 % (Bildor) pesticides concentration Kush and Profen insecticides have same chemical composition (Dichlorvos 76 % E.C) insecticide. All isolates were sensitive against 0.1% concentration of pesticides where CHN02, PRN18 and SAN26 were reported as tolerance at 0.01% pesticides concentration. Simbaa (Propargite 57% Ec) was the third insecticides used for pesticide tolerance activity. Three different pesticides concentration (0.01%, 0.1% and 1% prepared in acetone) were used against isolates, isolates were sensitive at 0.1% concentration except DHN14 isolates other isolates recorded sensitive at 0.01% of pesticides concentration. Siderophore production activity was done by chrome azurol sulfonate assay, CAS agar medium used for siderophore production activities. (Schwyn and Neilands isolates SAN26, PRN18, DHN14 and SUN13 gave siderophore production activity but CHN02 isolates not given siderophore activity results were recorded in table: 08 potent nitrogen fixer isolates were used for biofertilizer preparation. Charcoal used as carrier for isolates and performed plant germination activity (growth promotion activity) on

Wheat plants for thirty days incubation treatment (Table 9). Fifteen seeds were seeded in different plates (Pot) for plant growth promotion activity for 30 day and Sterile water was applied to each plate (tree day intervals), Standard biofertilizer used (available from Veraval agro market) for evaluation of growth promotion activity with Potent Isolates (Nitrogen fixer) (Fig. 10) Shoot length, root length, dry weigh and wet weight of plants was recorded after 30 days (Fig. 9). Plant germination activity of mixter isolates (plate) recorded highest (91.80 %) compare to standard biofertilizer (68.20%) and controls (No isolates) (57.50 %) Plates, (Fig. 8) Isolates PRN18, DHN14, SUN13, SAN26 and CH02 gave potential plant germination activities compare to standard and Control plates. (Fig. 08-09), plant were harvested from each individual plates ,mixer isolates gave highest weight(wet and dry weight) compare to other isolates of the plates, 6.57 gm/plate wet weight of plants recorded of mixer (biofertilizer) plate which was higher compare to standard (5.38 gm/plate) biofertilizer and control (4.22 gm/plate) plate (Fig. 9) Other isolates (as biofertilizer) wet weight were represented in graphical data which may point out that isolate were support the plant germination activities in saline habitat. It may apply as nitrogen biofertilizer propose in saline habitat soil. (Fig. 10) carbohydrate utilization test, nitrate reduction test, ammonia production test, starch hydrolyzing test, casein hydrolysis test, Catalase test and Gelatin liquefaction test were performed on five isolates (biochemical analysis) (Table 09) and after the molecular characterization of isolates (Bisystem, CA, USA) (12) were aligned and compared with available standard sequences of bacterial lineage in the national center for Biotechnology information Genbank (http://www.ncbi.nlm.nih.gov/) using blast programme. CHN01 isolates belongs to *myroides spp*, (Fig. 3) SUN13 isolates may belongs to *Beijerinckia fluminensis*, (Fig. 4) DHN14 isolates belongs to *Agrobacterium tumefaciens* (Fig. 5), PRN18 isolates belongs to *Delftia Spp*., (Fig. 6), SAN26 isolates belongs to *Paenibacillus spp*. (Fig. 7), Phylogenitic tree was constructed of isolates by using neighbouring method from distance matrices by MEGA-X software (Thompson *et.al.,* 1997).

Table 8: Siderophore production activity of Nitrogen fixing Isolates

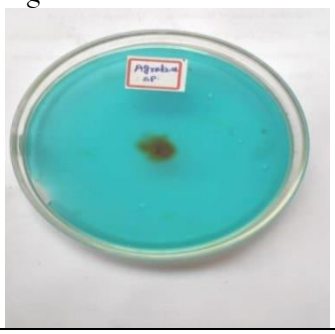
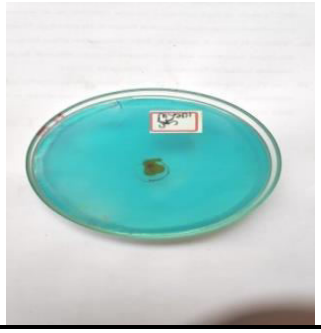
Isolates	Siderophore activity Results	Plates
Isolates 01 CHN02 Myroids ororatus	Isolates02 SAN26 Beijerinckia fluminensis	Isolates 03 SUN13 Agrobacterium tumefaciens
		
Isolates04 DHN14 Delftia spp.	Isolates04 DHN14 Delftia spp.	
		

Table 9: Biochemical characterization of Nitrogen fixing isolates

		Biochemical characteristics Isolates				
No.	sources of carbon	CHN01	SUN13	DHN14	PRN18	SAN26
1	Sucrose	-	+	+	+	+
2	Sorbitol	-	-	+	-	-
3	Fructose	-	-	-	-	-
4	Galactose	-	+	+	-	-
5	Glucose	+	+	+	+	+
6	Xylose	-	-	-	-	-
7	Lactose	+	+	+	+	-
8	Raffinose	-	-	-	-	-
9	Maltose	-	-	-	-	-
10	Mannitol	+	-	+	+	+
11	Reduction of nitrate	+	+	+	-	+
12	Hydrolysis of Urea	+	-	+	+	+
13	Production of Ammonia	+	+	+	+	+
14	H2s Production	-	-	+	+	
15	Oxidase	+	+	+	+	+
16	Gelatin liquefaction	+	-	+	-	-
17	Casein Hydrolysis	+	-	-	-	+
18	Starch Hydrolysis	+	-	+	-	+
19	IAA production	+	+	+	+	+

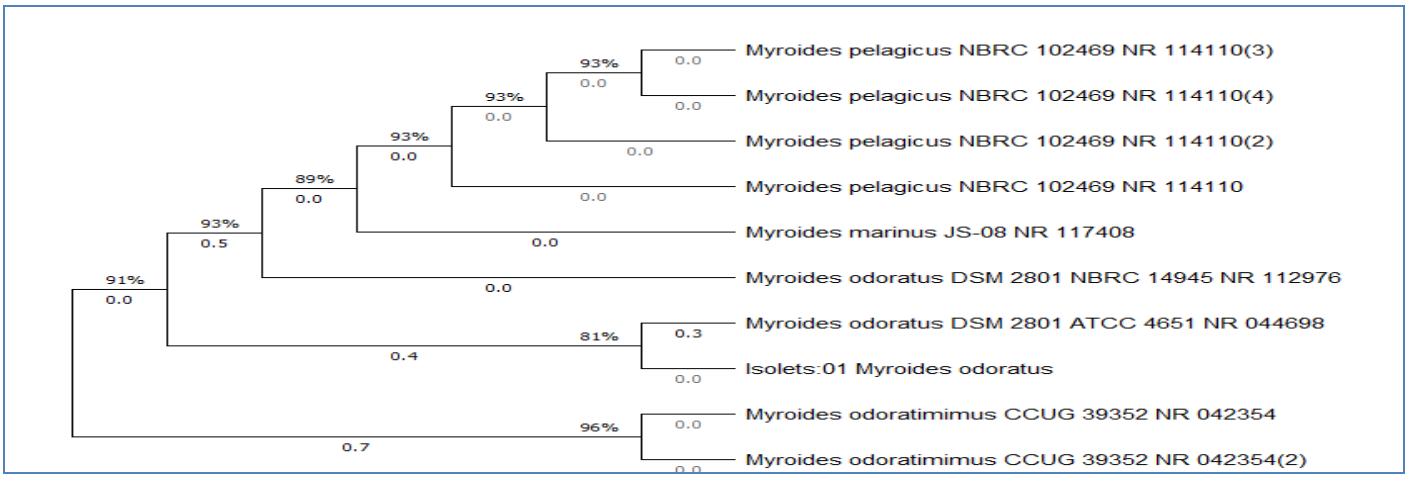


Fig. 3: Phylogenitic tree of Isolates: CHN02 (Chorwad Village) Nitrogen Fixing Bacteria

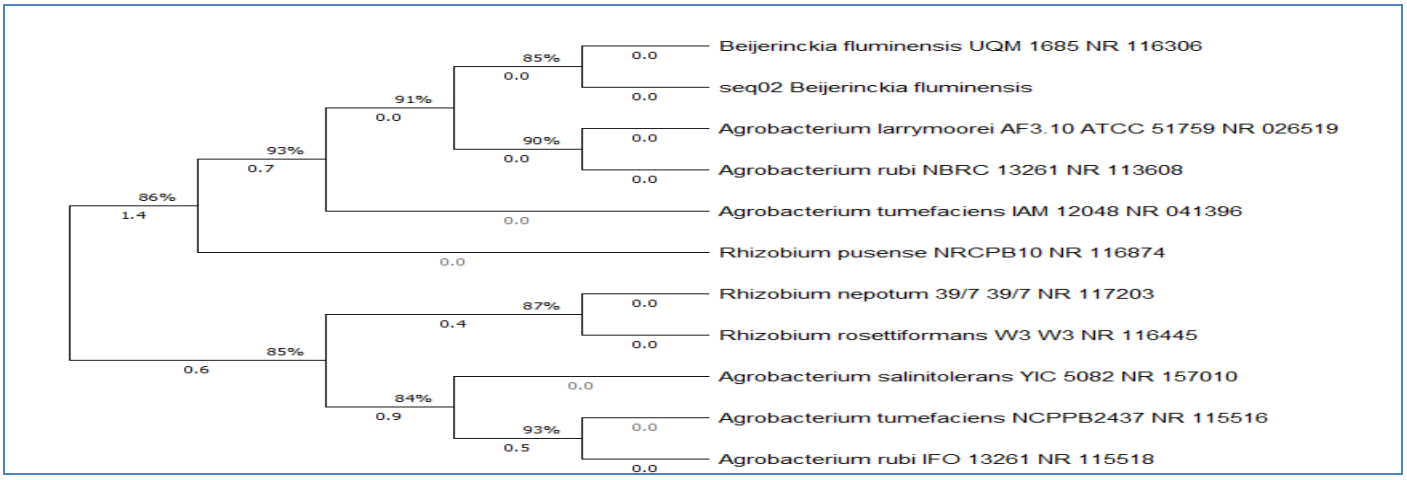


Fig. 4: Phylogenitic tree of Isolates: SUN 13 (Sutrapada Village) Nitrogen Fixing Bacteria

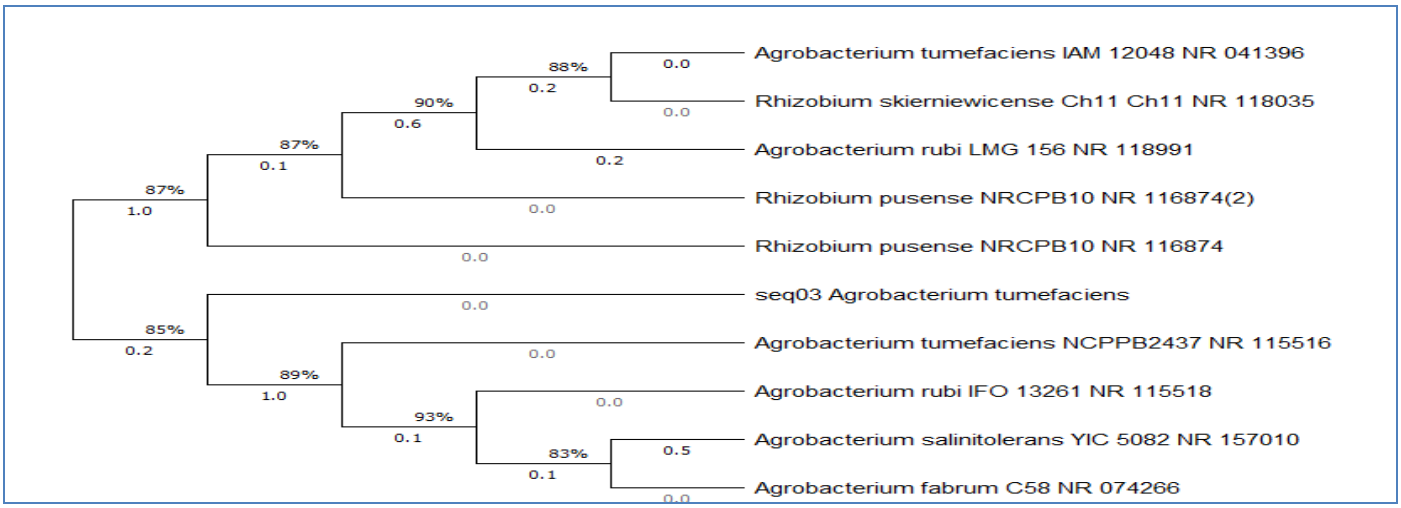


Fig. 5: Phylogenic tree of Isolates: DHN 14 (Dhamlej Village) Nitrogen Fixing Bacteria

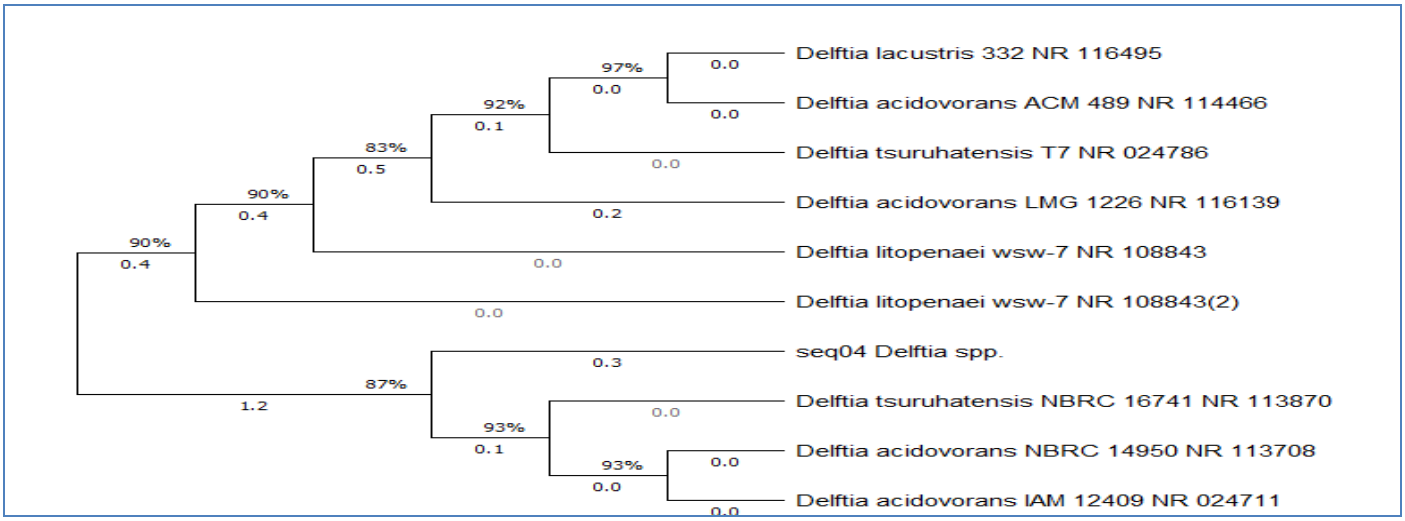


Fig. 6: Phylogenic tree of Isolates: PRN18 (Prashavala Village) Nitrogen Fixing Bacteria

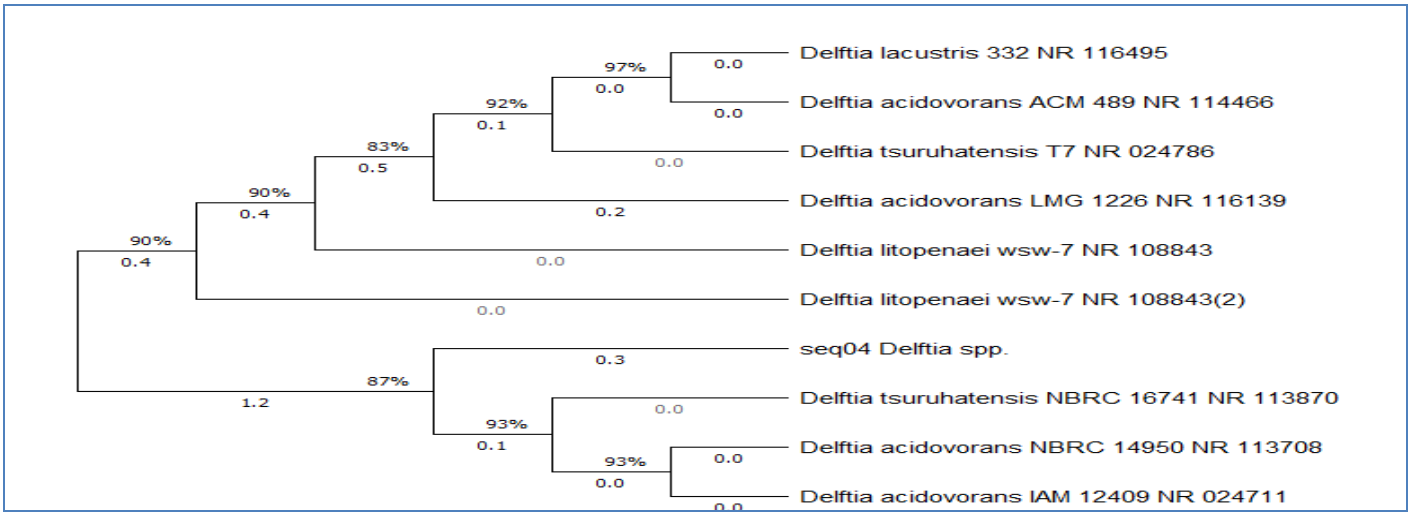
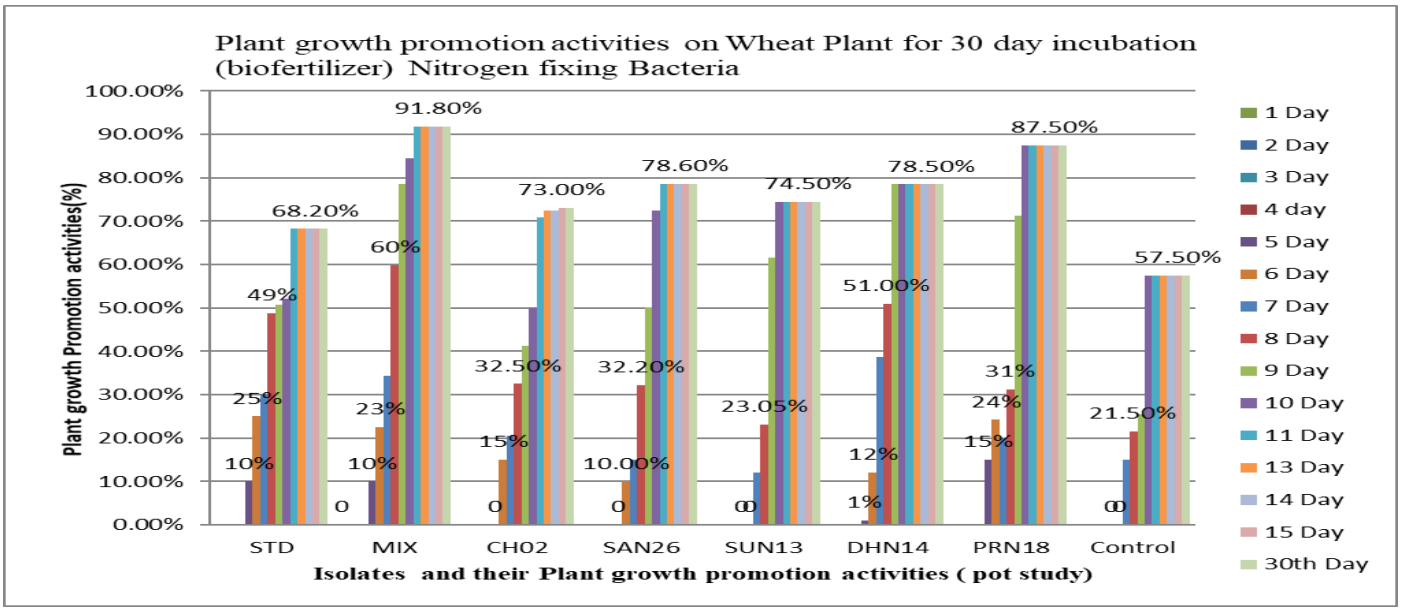


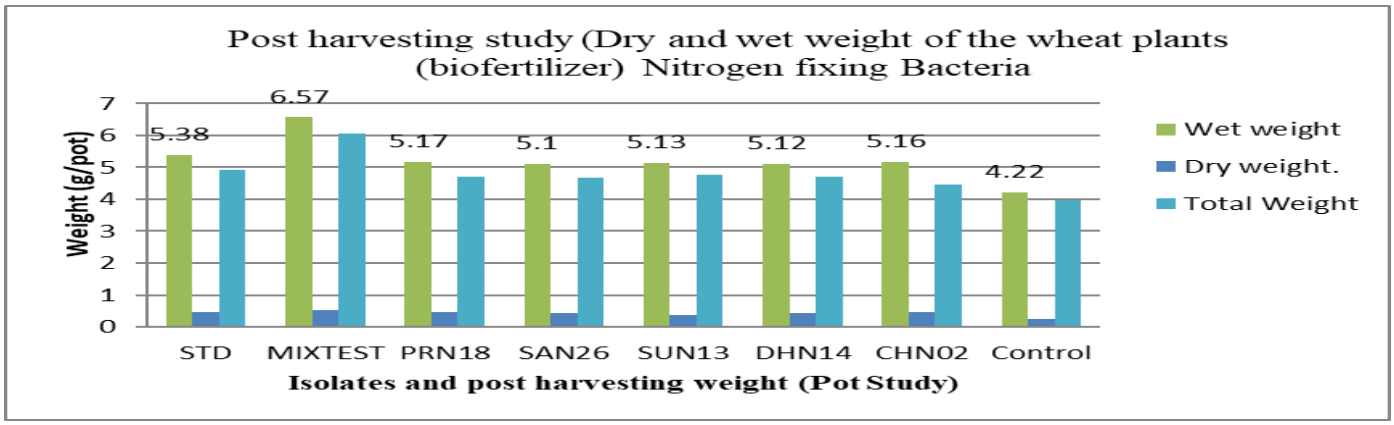
Fig. 7: Phylogenic tree of Isolates: SAN26 (Sarkhadi Village) Nitrogen Fixing Bacteria



(N.B: plants germination activity experiments performed in duplicate set)

(STD: commercial biofertilizer, Test Mix: PRN18, CHN02, SAN26, SUN13, DHN14, control: No Isolates)

Fig. 8: Plant growth promotion activities on Wheat Plant.



(N.B: plants germination activity experiments performed in duplicate set)

(STD: commercial biofertilizer, Test Mix: PRN18, DHN14, SUN13, SAN26, CHN02, control: No Isolates)

Fig. 9: Dry weight and wet weight study of wheat plants

Fig. 10: Wheat Plant growth obsered in control,Mixter and standar Biofertilizer in pot

4. CONCLUSION

Nitrogen is important element for plant growth and developments, it is not easily available in saline habitat to plants, in present study isolation and characterization of nitrogen fixing bacteria from saline habitats and performed their plant growth promotion activities (as biofertilizer) with standard biofertilizer (available from market) on wheat plants, recorded results indicated that indigenous flora (potent isolates) apply as biofertilizer and it will give optimum results in salinity affected soil, Prepared solid biofertilizer is an alternative option for reducing dependence on chemical fertilizer/their negative impacts on soil and it may support the plants rhizospheric activity, improve plant growth and yield.

5. ACKNOWLEDGMENT

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