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ISOLATION AND CHARACTERISATION OF NON SYMBIOTIC NITROGEN FIXING BACTERIA (*Azotobacter* sp.) FROM TEA FIELD SOIL OF TERAI REGION OF NORTH BENGAL, INDIA

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Abstract: *Total 126 distinct colony has been isolated,31 colony from TS-1, 30 colony from TS-2, 25 colony from TS-3 and 40 colony from TS-4 from different location Terai Tea garden. Most of the colonies are 1.0-4.5 mm in diameter in size, circular even with rarely undulated colonies(shape), white or creamy translucent with central black dot. The selected 23 purified colonies so far tested for salinity test, TS-1-13, 19, 26, 27; TS-3-10, 24; TS-4-16 showed highest salinity tolerance upto 4.5% and TS-1-13, 26, TS-3-24, TS-4-16 showed highest salinity tolerance upto5.0%. Among 19 pure isolates from the Terai soil sample (TS-1- TS-4), maximum isolates (TS-1-1,18,19 TS-2-14,15,16,TS-3-27,28,33 and TS-4-32) exhibited highest resistance against Rifampicin (35mg/l). Among 19 pure isolates from the Terai soil sample TS-2-14, TS-3-27,TS-4-32 showed highest level(30mg/l of resistance against chloramphenicol. Among 19 isolated pure culture so far tested against tetracyclin TS-1-13,19,TS-2-7, TS-3-33 showed highest level (25mg/l) of resistance . Isolates number TS-1-3,19,TS-2-7,14,28 showed medium level of resistance at 30mg/l against tetracyclin. Among six selected isolated strains of Azotobacter, the strain no. TS-1--13,TS-1-26,TS-3-3 and TS-4-16 showed maximum level*

(0.006%) of nitrogen uptake capacity, which indicates that these strains would be used as biofertiliser in Tea field subject to their validation in field study.

Key Words: *Nitrogen fixing soil bacteria, Non symbiont, Azotobacter sp, salt tolerant, antibiotic assay, N estmationm biofertilizer.*

INTRODUCTION:

With the advent of chemical fertilizers and because of the prompt and high response of yield to it, chemical fertilizers have become the way of life in tea nutrition of N. E. India for not less than last seven- eight decades. Inorganic fertilizers like N, P and K are being applied at present in high quantities like 165 to 200 kg of N/ha/yr in most of the tea gardens (Barooah, 2006).

Application of inorganic fertilizers even at balanced amount does not sustain the soil fertility and productivity under continuous cropping (Kumpawat, 2004). The impact of such high dose of fertilizers on growth and yield of tea was well documented by many authors (Dev Chaudhury *et al.,* 1983; Sen and Paul, 1984, Paul and Sen, 1984a, b). Environmental pollution and residue left over by chemicals applied on soil are gaining due importance and looked into seriously in tea (Barooah, 2005) as tea is also a foreign exchange earner and has to face intrinsic network of tests of different certifying agencies. Nitrogen is the key element required for the crop and available in abundant quantity in nature. But it is not available directly to the plant. There are certain microorganisms which can convert this unavailable nitrogen (molecular nitrogen) into available form by fixing into the soil. Thus the biological nitrogen fixation is extremely important for every organism in the earth. The demand of agricultural products is ever rising with the rising population.

Biological nitrogen fixation by free-living bacteria in rice soils has been reported from alluvial, laterite, acid saline and acid sulphate saline soils (Sethunathan *et al.,* 1983). The presence of *Azotobacter* sp. and their rate of multiplication and nitrogen fixation are governed by many factors including soil pH (Jensen, 1961; Roy *et al.,* 1962).

Tea (*Camellia sinensis* (L), Kuntze*)* is a Dicotyledonous; (Family-Theaceac). Moreover 80 species have been identified. *Camellia sinensis* (China Variety of Tea) and *Camellia sinensis varassamics* (Assam Variety) are most important for commercial cultivation. Tea containing polyphenol like catechins, catechinol which have great antioxidant value and also caffeine

and some micro nutrient. Tea requires a soil of low pH (4.5-5.5) rich in humus. Tea is also heavy accumulator of aluminiums.

There are different kinds of microorganism which could assimilate atmospheric nitrogen. They are symbionts like *Rhizobium* or Mycorrhiza free living non symbiont like *Azotobacter, Azospirillum*, *Azolla*, Cyanobacteria etc.

The most dominant non-symbiotic nitrogen-fixing heterotrophic bacterium in Indian soils is *Azotobacter chroococcum*. Later on several other species such as *A. vinelandii*, *A. beijerinckii*, *A. insigns,* ,*A. macrocytogenes* and *A. paspali* were recorded.

The first representative of the genus, *Azotobacter chromococcum*¸ was discovered and described by the Dutch microbiologist and botanist Martinus Beijerinck i 1901 . They are found in neutral and alkaline soils. *Azotobacter* is Gram-negative, motile, pleomorphic aerobic, free-living, nitrogen-fixing bacterium.

These bacteria have one of the most highly active cytochrome oxidases known, as well as notably active superoxide dismutase and catalase systems. *Azotobacter*s are the most intensively investigated heterotrophic group possessing the highest respiratory rates. Members of these genera are mesophilic, which require optimum temperature of about 30ºc.There are some microorganism which establish symbiotic relationships with different parts of plants and may develop special structures as the site of nitrogen fixation.

The beneficial effects of *Azotobacter* are not only due to its ability to fix atmospheric nitrogen, but also to secrete growth substances and antifungal antibiotics, which improve plant stand in inoculated field by inhibiting root pathogens. Apart from its nitrogen fixing ability, *Azotobacter* usually produces considerable amount of biologically active substances such as vitamins of B group like nicotinic acid, pantothenic acid, biotin, cytokinins, auxins and gibberellins. Recently, it has been also shown that strains of *Azotobacter* could be usefully employed in biofertilizers production, due to their ability of fixing nitrogen and solubilizing phosphates.

They are nonsymbiotic heterotrophic bacteria capable of fixing an average 20kg N/ha/year. Besides, it also produces growth promoting substances and are shown to be antagonistic to pathogens. *Azotobacter sp*. are found in the soil and rhizosphere of many plants and their population ranges from negligible to 104 g/l of soil depending upon the physico-chemical and microbiological (microbial interactions) properties (Ridvan, 2009). In soils, *Azotobacter*

sp. populations are affected by soil physico-chemical (e.g. organic matter, ph, temperature, soil depth, soil moisture) and microbiological (e.g. microbial interactions) properties (Ridvan, 2009). The genus *Azotobacter* includes 6 species, with *A. chroococcum* most commonly inhabiting various soils all over the world. The occurrence of other *Azotobacter* species is much more restricted in nature, e.g. *A. paspali* can be found only in the rhizosphere of a grass. Soil populations of *Azotobacter sp*. rarely exceed several thousand cells per gram of neutral or alkaline soils, and in acid (pH < 6.0) soils these bacteria are generally absent or occur in very low numbers (Martyniuk and Martyniuk, 2002).

The **Terai** is a plain region of Nepal and the plain land region in Bangladesh, Bhutan and India that lies in south of the outer foothills of the Himalaya, the Siwalik Hills, Terai region have an acidic soil profile. Tea grows best in pH ranging from 4.5-5.5. The carbon status, phosphorus status generally found low in most of the areas. In some areas also it is reported that there are some deficiency of potash. Total rainfalls markedly diminish from East to West. The monsoon arrives later, is much less intense and ends sooner.

No substantial data is available on occurrence, isolation and identification of *Azotobacter* in tea growing soil of North Bengal region. In the study an attempt has been made to isolate, characterize *Azotobacter* sp. from different soil samples collected from different Tea gardens of Terai region. The present study has been undertaken also aiming to identification and standardization of an efficient biofertilizers to produce organic tea crops as the use of chemical fertilizers and pesticides decreases the export demand of Tea.

MATERIALS AND METHODS:

Materials:

Soil samples were collected from four different location of Terai region of North Bengal in sterilized polythene bags (from Gangaram & Mohorgaon tea garden). The soil samples were taken below30 cm from the surface. Then samples were used for isolation and characterization of Nitrogen Fixing Bacteria.

Media preparation:

One liter of *Azotobacter* specific Mannitol agar medium(MA), was prepared by weighing the components(K_2PO_4 1g, $MgSO_4$ 0.200g, Nacl 0.200g, $FeSO_4$ 0.05g,Mannitol 20.0g, Agar 15.0g) dissolved in water following the volume make up to 1000ml with double distilled water,

then pH adjusted to 5.0 with 1N NaOH /HCl.. After mixing the agar, the medium was autoclaved. Finally, the medium was poured in sterile Petri Plates @ 20-25ml.

Isolation of Bacteria:

Serial dilution and plating of samples:

Soil sample(1.0g) of TS-1, TS-2, TS-3 and TS-4 was added to 10 ml of water in a test tube which served as stock solution. Remaining four test tubes were filled with 9 ml of water. Transferring 1ml of solution from the previous test tube with the help of pipette to make the solution dilute. Series continued up to 10^{-5} dilution. Sterility is the hallmark of any bacteriological isolation so entire process was carried out in the laminar air flow cabinet.

Bacterial colony identification and morphology:

Using the spread plate technique, the bacterial colony identification and external morphology were studied for which Mannitol agar media was prepared. Therefore 250 ml of MA medium was prepared for 4 petri plates. From this a portion of 100 ml was taken for identification of colonies in 4 different plates and the remaining portion was used for 0.5%NaCl assay. The MA media was autoclaved and then poured in 4 different petri plates which were also sterilized by autoclave. Then the serial dilution of $10^{-1}$to 10^{-5} were chosen and from that 0.5 ml of culture was transferred from each serially diluted test tubes and spreaded on the petri plates by means of spreader. Then petri plates were kept in incubation for 28°C for 2 days for the incubation and growth of bacteria.

After 48 hours of incubation the petri plates were taken out from the incubator and the bacterial colony morphology were studied.

Pure culture preparation and Maintenance:

Well developed and separated colonies which were identified on Mannitol Agar media were marked and then these separated colonies were chosen. By the help of sterile toothpicks the colonies were inoculated separately on 4 plates(TS-1,TS-2,TS-3 and TS-4) by streaking method containing Mannitol Agar media. These culture (plates) were considered as Master Plate.

Assays:

Two types of assay have been conducted for the characterization of soil bacterium (*Azotobacter* sp) Nacl assay and Antibiotic assay.

Nacl assay:

Nacl assay usually done for determining the optimum condition of salinity at which the soil bacteria can grow. Five concentration have been made ranging from 0.5% to 5 % Nacl in Mannitol Agar Media then were poured on petri plates. Two plates were prepared for each of the concentration. In one plate TS-1, TS-2 and in another plate TS-3, TS-4 bacterial colonies were streaked by toothpicks followed by incubation for 2 days at 28°C.

Antibiotics Sensitivity Assay:

Antibiotic sensitivity test usually done for determining the level of sensitivity of bacterial strain against a particular antibiotic. So mainly here 3 kinds of antibiotics were used- Chloramphenicol, Rifampicin and Tetracyclin. Seven different concentration were made (10,15,20,25,30,35,40mg/l) in Mannitol Agar Media. Two plates were made for each antibiotic concentration. The plates were divided into 2 halves. In one plate TS-1, TS-2 and in another plate TS-3, TS-4 bacterial colonies were streaked by toothpicks followed by incubation for 48 hours at 28^0C. This was done for each pair of antibiotic plate.

Kjeldahl Process for N estimation:

It is a method for the quantitative determination of organic nitrogen in chemical substances like ammonia developed by Johan Kjeldahl in 1883.On the basis of salt tolerant and antibiotics resistivity, the samples TS1-13, TS1-26, TS2-14, TS3-28, TS3-33, TS4-16 has been selected for nitrogen estimation. The nitrogen in Bacterial cultures (three days) were estimated by kjeldhal method (Williams and Wasington,1996). The acid digested sample was distilled and subjected to quantification of nitrogen through titrimetric method using 0.0N NaOH.

Total Nitrogen was calculated and expressed as % (percentage) using the formula:

$$\text{Total N \%} = \frac{(\text{Blank value} - \text{Titrated value}) \times \text{Normality of NaOH} \times 0.014}{\text{Sample weight taken}} \times 100$$

RESULTS AND DISCUSSION:

Bacterial colony identification and morphology:

After incubation the diluted soil sample in *Azotobacter* specific media, total 126 distinct colony has been isolated,31 colony from TS-1, 30 colony from TS-2, 25 colony from TS-3 and 40 colony from TS-4. Among them morphological features of 23 isolated pure colonies has been enumerated in this study. These 23 isolated pure cultures are maintained by sub

culturing in fresh media for every month and treated as master plate, the representative of which is shown in figure -1.

Total 31 distinct colony (data not shown) of TS-1 has been morphologically characterized, among them six most promising colony namely TS-1-3,TS-1-6,TS-1-13,TS-1-19,TS-1-26 and TS-1-27 are considered for NaCl, antibiotic and N content assay. Most of the colonies are 1.0-4.5 mm in diameter in size, circular even with rarely undulated colonies(shape), white or creamy translucent with central black dot (opacity, Table-1).

In TS-2,total 30 distinct colony (data not shown) has been morphologically characterized, among them six most promising colony namely, TS-2-5,TS-2-7,TS-2-8,TS-2-9,TS-2-15 and TS-2-22 has been considered for salt tolerance, antibiotic and N content assay. Most of the colonies are of 1.0-4.0 mm in diameter (size); circular even with rarely undulated colonies (shape) ; white or creamy translucent.(opacity, Table-1)

In TS-3, total 25 distinct colony (data not shown) has been morphologically characterized, among them six most promising colonies namely,TS-3-3, TS-3-4,TS-3-6,TS-3-10,TS-3-15 and TS-3-24 are considered for salt tolerance, antibiotic and N content assay. Most of the colonies are of 1.5-5.0 mm in diameter (size); circular elevated even with rarely undulated colonies (shape); white or creamy translucent (opacity, Table-1)

In TS-4, total 40 distinct colony (data not shown) has been morphologically characterized, among the five most promising colony namely, TS-4-12,TS-4-14,TS-4-16,TS-4-23 and TS-4-33 are considered for salt tolerance, antibiotic and N content assay..Most of the colonies are of 0.5- 5.0 mm in diameter (size); circular even with rarely undulated colonies (shape); creamy translucent with some bluish translucent (opacity, Table-1)

Salinity(Nacl) tolerance assay: The selected 23 purified colonies so far tested for salinity test, TS-1-13, 19, 26, 27; TS-3-10, 24; TS-4-16 showed highest salinity tolerance up to 4.5% andTS-1-13, 26, TS-3-24, TS-4-16 showed highest salinity tolerance upto5.0% .Others colonies like TS-2-5, 7, 8 showed minimum and TS-4-14 showed no salinity tolerance. (Table-2). Similar work has done by Akhter et al (2012) and observed that some strains of *Azotobacter* showed medium level (6%) and some showed highest level (10%) of salinity. A strain of Azotobacter which showed maximal N2 fixation at 30% of NaCl, with good fixation still observed at 10 to 40% was isolated by Blinkov (1963).

Antibiotics Sensitivity Assay:

Rifampicin: Among 19 pure isolates from the Terai soil sample (TS-1- TS-4), maximum isolates (TS-1-1,18,19 TS-2-14,15,16,TS-3-27,28,33 and TS-4-32) exhibited highest resistance against Rifampicin (35mg/l). Isolates number TS-1-18, TS-2-14,TS-3-27,28,31,33 showed reistance against 30mg/l of rifampicin. Isolates number TS-1-6, 12, 14 showed no resistance properties against rifampicin. Some isolates namely TS-1-18,19, TS-2-14,15,16,TS-3-27,28,33 and TS-4-32 showed certain level of resistance against antibiotic rifampicin at 40mg/l concentration.(Table-3).

Chloramphenicol: Among 19 pure isolates from the Terai soil sample TS-2-14, TS-3-27,TS-4-32 showed highest level(30mg/l) of resistance against chloramphenicol. Isolates number TS-2-14,15,16,TS-3-27,28,33 and TS-4-32 showed medium level (35mg/l) of resistance against chloramphenicol. The isolates number TS-2-14,15,16,TS-3-27,28 and TS-4-32 showed lower level of chloramphenicol resistance at a concentration of 40mg/l. whereas TS-3-31 and TS-4-16 showed no resistance against chloramphenicol.(Table-4). The resistance property of Azotobacter sp against chloramphenicol (30µg/ml) has been reported by Bhattacharjee et al 2016.

Tetracyclin: Among 19 isolated pure culture so far tested against tetracyclin TS-1-13,19,TS-2-7, TS-3-33 showed highest level (25mg/l) of resistance . Isolates number TS-1-3,19,TS-2-7,14,28 showed medium level of resistance at 30mg/l against tetracyclin. Few isolates showed lower level of resistance against tatracyclin at 35mg/l and 40mg/l concentration (Table-5). It has revealed from our observation that tetracyclin is the strongest antibiotic against *Azotobacter* sp so far tested in our study. The similar work has carried out by Bhattacharjee et al 2016 and showed that *Azotobacter* sp has a resistance property against tetracyclin at a concentration of 30µg/ml.

Estimation of N by Kjeldahl process: The result of the Kjeldahl process of the selected bacterial colonies (TS-1-13, 26; TS-2-15; TS-3-3, 24 and TS-4-16) revealed the level of nitrogen uptake percentage by bacteria.(Table-6, Fig:5). Among six selected isolated strains of *Azotobacter*, the strain no. TS-1--13,TS-1-26,TS-3-3 and TS-4-16 showed maximum level (0.006%) of nitrogen uptake capacity, which indicates that these strains would be used as biofertiliser in Tea field subject to their validation in field study. One more thing that has to be discussed is that, it has been recognized to all that how finely does the "*Azotobacter*"

species had the ability to acquire “Nitrogen” as strains of “non symbiotic” “bacteria” isolated during Nikul *et al.*

CONCLUSION:

The use of chemical fertilizer in crop plants becoming decreased day by day as the demand of “organic crops”, gaining momentum. To combat this problem search and the use of suitable alternatives is most important. The biofertilizer is a right candidate for such alternatives. The present study has been focused for the isolation, identification and characterization of one of the alternatives of most important “cash crops” like Tea. The isolation and characterization and estimation of N uptake capacity of *Azotobacter* sp from soil sample in North Bengal Tea estates mainly in Terai region will give an insight among researcher and tea growers to use the *Azotobacter* as potential right candidate as biofertiliser. The further study in this field will require for molecular characterization of this potent organism in details.

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Table: 1- Colony identification and morphology analysis of TS-1 , TS-2, TS-3 and TS-4 soil sample

Sample code	Colony number	Dilution	Colour(opacity)	Shape(margin)	Size(mm)
TS-1	3	10^{-5}	Translucent white with central black dot	Circular(even)	2.0
TS-1	6	10^{-5}	Translucent white with central black dot	Circular(even)	4.5
TS-1	13	10^{-4}	Creamy(translucent)	Circular(even)	2.5
TS-1	19	10^{-4}	Bluish(iridescent)	Circular(even)	4.0
TS-1	26	10^{-4}	White(translucent)	Circular(even)	2.5
TS-1	27	10^{-4}	White(translucent)	Circular(even)	2.0
TS-2	5	10^{-5}	Reddish(translucent)	Circular(even)	1.5
TS-2	7	10^{-4}	Translucent white with central black dot	Circular(even)	4.0
TS-2	8	10^{-4}	Creamy(translucent)	Circular(undulated)	2.0
TS-2	9	10^{-4}	Creamy(translucent)	Circular(undulated)	1.5
TS-2	15	10^{-4}	Creamy(translucent)	Circular(undulated)	1.5
TS-2	22	10^{-3}	Translucent white with central black dot	Circular(even)	2.0
TS-3	3	10^{-5}	White(translucent)	Circular elevated(even)	4.0
TS-3	4	10^{-5}	White(translucent)	Circular elevated(even)	3.5
TS-3	6	10^{-5}	Creamy(translucent)	Circular(even)	1.5
TS-3	10	10^{-5}	White(translucent)	Circular elevated(even)	5.0
TS-3	15	10^{-4}	White(translucent)	Circular elevated(even)	2.5
TS-3	24	10^{-4}	White(translucent)	Circular elevated(even)	2.5
TS-4	12	10^{-5}	Creamy(translucent)	Circular(even)	2.5
TS-4	14	10^{-5}	White(translucent)	Circular(even)	0.5
TS-4	16	10^{-5}	Creamy(translucent)	Circular(even)	4.5
TS-4	23	10^{-4}	Creamy(translucent)	Circular(even)	5.0
TS-4	33	10^{-3}	Bluish(iridescent)	Circular(even)	4.0

Table: 2- Nacl tolerant study of *Azotobacter* sp

Sample code	Concentration of Nacl(%)									
	0.5%	1.0%	1.5%	2.0%	2.5%	3.0%	3.5%	4.0%	4.5%	5.0%
TS-1-3	+++	+++	+++	+	-	-	-	-	-	-
TS-1-6	+++	+++	+++	+++	++	-	-	-	-	-
TS-1-13	+++	+++	+++	+++	+++	+++	+++	+++	++	-
TS-1-19	+++	+++	+++	+++	+++	+++	+++	++	++	-
TS-1-26	+++	+++	+++	+++	+++	+++	+++	+++	++	-
TS-1-27	+++	+++	+++	+++	+++	+++	+++	++	++	-
TS-2-5	+++	++	-	-	-	-	-	-	-	-
TS-2-7	+++	++	-	-	-	-	-	-	-	-
TS-2-8	+++	++	-	-	-	-	-	-	-	-
TS-2-9	+++	+++	++	++	-	-	-	-	-	-
TS-2-15	+++	+++	++	++	-	-	-	-	-	-
TS-2-22	+++	+++	++	-	-	-	-	-	-	-
TS-3-3	+++	+++	+++	+++	+++	++	+++	+++	+	-
TS-3-4	+++	+++	+++	+++	+++	++	+	-	-	-
TS-3-6	+++	+++	+++	+++	++	+++	+++	+	+	-
TS-3-10	+++	+++	+++	+++	+++	+++	+++	++	++	+
TS-3-15	+++	+++	+++	+++	+++	++	-	-	-	-
TS-3-24	+++	+++	+++	+++	+++	+++	+++	+++	++	-
TS-4-16	+++	+++	+++	+++	+++	+++	+++	+++	++	+
TS-4-12	+++	+++	+++	-	-	-	-	-	-	-
TS-4-14	-	-	-	-	-	-	-	-	-	-
TS-4-23	+++	+++	+++	++	-	-	-	-	-	-
TS-4-33	+++	+++	+++	+++	-	-	-	-	-	-

Table: 3-The antibiotic (Rifampicin) resistance study of *Azotobacter* sp

Sample code	10mg/l	15mg/l	20mg/l	25mg/l	30mg/l	35mg/l	40mg/l
TS-1-3	+++	+	-	-	-	-	-
TS-1-6	-	-	-	-	-	-	-
TS-1-12	-	-	-	-	-	-	-
TS-1-14	-	-	-	-	-	-	-
TS-1-18	+++	+++	+++	+++	+++	++	++
TS-1-19	+++	+++	+++	+++	++	++	+
TS-2-7	+++	+++	+++	+	-	-	-
TS-2-14	+++	+++	+++	+++	+++	++	+
TS-2-15	+++	+++	+++	+++	++	++	+
TS-2-16	+++	+++	+++	+++	++	++	+
TS-2-17	+++	+++	+++	++	++	+	-
TS-3-27	+++	+++	+++	+++	+++	++	+
TS-3-28	+++	+++	+++	+++	+++	++	+
TS-3-31	+++	+++	+++	+++	+++	+	-
TS-3-33	+++	+++	+++	+++	+++	++	+
TS-4-16	++	++	++	+	-	-	-
TS-4-32	+++	+++	+++	++	++	++	+
TS-4-12	+++	++	++	+	+	+	-
TS-4-23	+++	++	++	+	+	+	-

Table: 4- Antibiotic (Chloramphenicol) resistance study of *Azotobacter* sp

Sample code	10mg/l	15mg/l	20mg/l	25mg/l	30mg/l	35mg/l	40mg/l
TS-1-3	+++	++	++	++	-	-	-
TS-1-6	+++	+++	+++	++	++	+	-
TS-1-12	+++	++	++	+	-	-	-
TS-1-14	+++	+	-	-	-	-	-
TS-1-18	+++	+++	+++	++	-	-	-
TS-1-19	+++	++	++	+	-	-	-
TS-2-7	+++	+++	+++	+	-	-	-
TS-2-14	+++	+++	+++	+++	+++	++	+
TS-2-15	+++	+++	+++	+++	++	++	+
TS-2-16	+++	+++	+++	+++	++	++	+
TS-2-17	+++	+++	+++	++	++	+	-
TS-3-27	+++	+++	+++	+++	+++	++	+
TS-3-28	+++	+++	+++	+++	++	++	+
TS-3-31	+	-	-	-	-	-	-
TS-3-33	+++	+++	+++	++	++	++	-
TS-4-12	+++	+++	+++	+++	++	+	-
TS-4-16	-	-	-	-	-	-	-
TS-4-23	+++	+++	+++	++	+	-	-
TS-4-32	+++	+++	+++	+++	+++	++	+

Table: 5- Antibiotic (Tetracyclin) resistance of *Azotobacter* sp

Sample code	10mg/l	15mg/l	20mg/l	25mg/l	30mg/l	35mg/l	40mg/l
TS-1-3	+++	+++	+++	+++	++	+	+
TS-1-6	+++	+++	++	++	+	-	-
TS-1-12	+++	++	++	+	+	-	-
TS-1-14	+++	++	++	+	-	-	-
TS-1-18	+++	+++	+++	++	+	-	-
TS-1-19	+++	+++	+++	+++	++	+	-
TS-2-7	+++	+++	+++	+++	++	+	-
TS-2-14	+++	+++	++	++	++	+	+
TS-2-15	+++	+++	+++	++	+	+	-
TS-2-16	+++	+++	++	+	+	-	-
TS-2-17	+++	+++	++	+	-	-	-
TS-3-27	+++	+++	+++	++	+	-	-
TS-3-28	+++	+++	+++	++	++	+	+
TS-3-31	+++	+++	+++	++	+	+	-
TS-3-33	+++	+++	+++	+++	++	++	++
TS-4-12	+++	+++	+	-	-	-	-
TS-4-16	++	+	+	-	-	-	-
TS-4-23	+++	+++	+	-	-	-	-
TS-4-32	+++	+++	+++	+	+	-	-

Table-6: The result of Nitrogen estimation (Kjeldahl process)

Sample No	%of N uptake	N uptake per 150ml pure culture
TS-1-13	0.006	0.009
TS-1-26	0.006	0.009
TS-2-15	0.005	0.0075
TS-3-3	0.006	0.009
TS-3-24	0.005	0.0075
TS-4-16	0.006	0.009

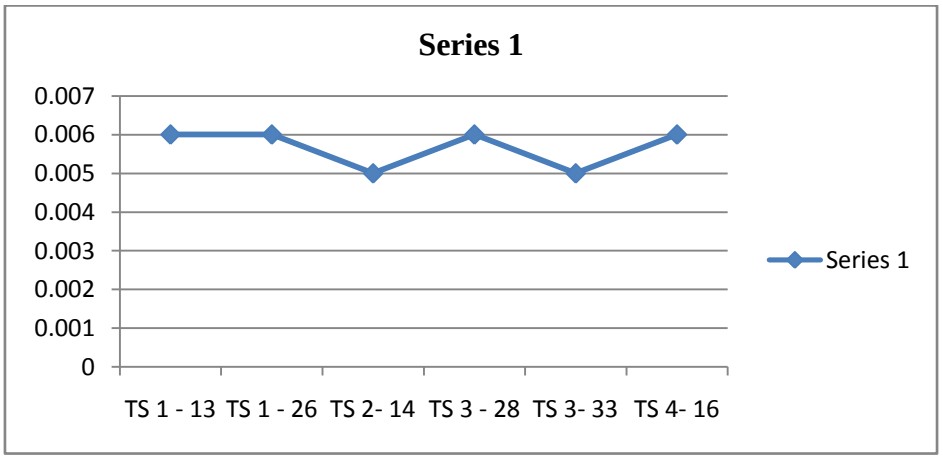

Figure-5: Graphical representation of N content of selected isolated *Azotobacter* sp

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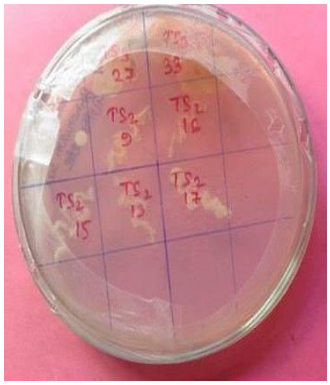
Figure-1: Maintainence of pure Culture as master plate



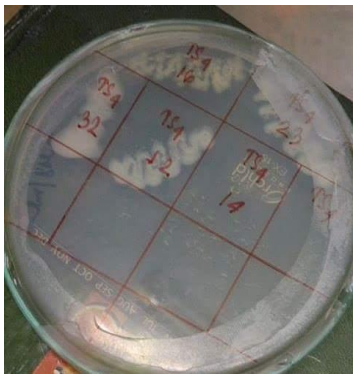
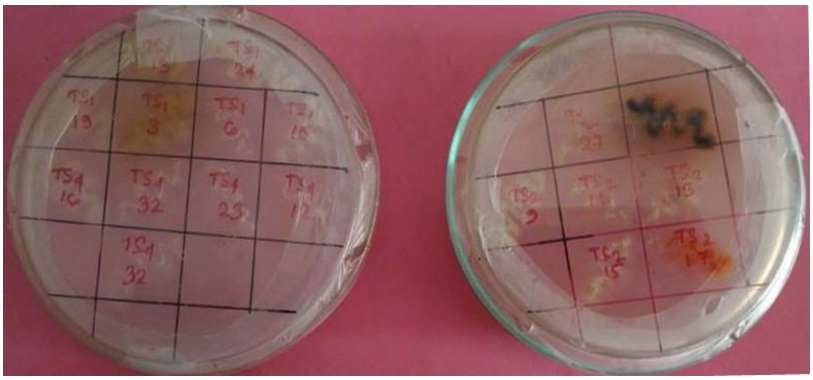


Figure-4: Antibiotic (Chloramphenicol and Tetracyclin) resistance assay of *Azotobacter* sp



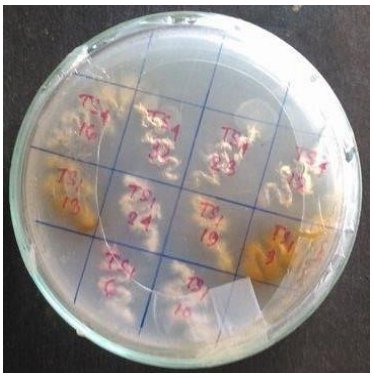


Figure-2: Nacl tolerant test of isolated pure culture of *Azotobacter* sp

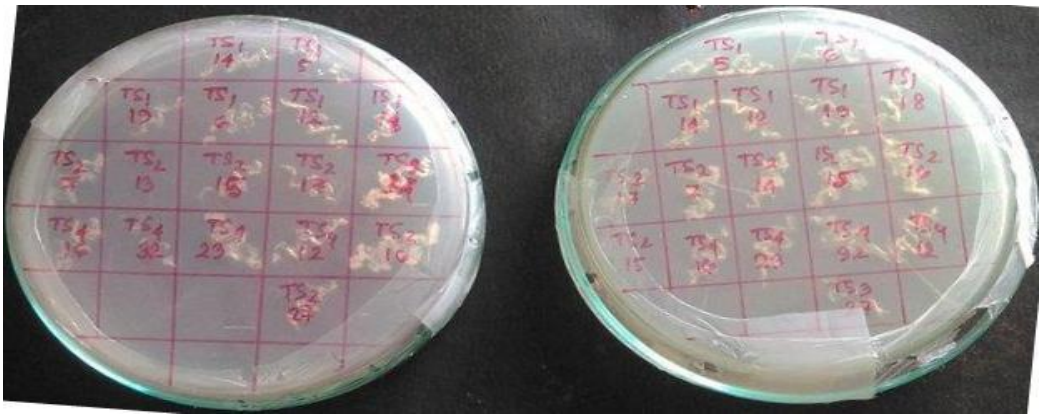


Figure-3: Antibiotic(Rifampicin)resistance assay of isolated pure culture of *Azotobacter* sp