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ISOLATION OF *AZOTOBACTER* SPP. FROM MARINE THONDI REGION AND TO CHECK ITS STABILITY BY EARTHERN POT CULTURE METHOD

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ABSTRACT

In this study, the sample collected from different Tondi marine region showed positive results (80 samples). The *Azotobacter* spp. was isolated from the samples by the use of selective medium containing 3.5% sodium chloride. Marine and sediments samples were tested for the presence of chemical ingredients at TNAU, Coimbatore. The primary screening was done for the collected samples using nutritional parameters such as salt and sugar concentrations, and physical parameters such as temperature and pH, in which 10 strains have been selected based on their highest OD values .The nitrogen fixing ability of the isolated *Azotobacter* spp. was determined by pot cultivation method by assessing the growth of green gram. Of the 80 *Azotobacter* spp. isolated, ten efficient strains (soil and marine culture) and three reference strains were used for the experiment. The various characteristics of growth such as seed germination, shoot length, root length and vigour index were measured and analyzed. The plant leaf was analyzed to check the difference in chlorophyll content. Significant difference was observed. Hence, the use as bioinoculant has economic relevance which will increase further with the intensification of research and development.

KEYWORDS: *Azotobacter* spp., biofertilizers, pot culture method, seed germination, plant growth

Azotobacter is used as a biofertilizer in the cultivation of most crops. *Azotobacter* is an obligate aerobic diazotrophic soil-dwelling organism with a wide variety of metabolic capabilities, which include the ability to fix atmospheric nitrogen by converting it to ammonia. There are different strains of *Azotobacter* each has varied chemical, biological and other characters. However, some strains have higher nitrogen fixing ability than others (Burgmann et al., 2003).

Biofertilizers play a main key role for selective adsorption of immobile (P, Zn, Cu) and mobile (C, S, Ca, K, Mn, Cl, Br, and N) elements to plants. The rhizosphere bacteria secrete growth substances and secondary metabolic, which contribute to seed germination and plant growth (Dwivedi et al., 1989). There is firm evidence that indole -3-acetic acid ,gibberellins (Brown and Burlingham, 1976) and cytokinins all produced by plants and essential to their growth and development, are produced also by various bacteria which live in association with plants. There is also evidence that the growth hormones produced by the bacteria can in some instances increase growth rates and improve yields of the host plants (Brown and Burlingham, 1976). When *Azotobacter* is applied to seeds, seed germination is improved to a considerable extent, so also it controls plant diseases due to above substances produced by *Azotobacter.*

Mishustin and Skilnikova (1969) studied that the effect of *Azotobacter* inoculants on cotton, Barley, Oat, Potato, Sorghum and Sugar Beat plants which showed an increase in yield over the inoculated control. The beneficial effects of *Azotobacter* are that ability to produce ammonia, vitamins and growth substances that enhance seed germination (Narula and Tauro, 1986). Production of Indole acetic acid and other auxins, such as gibberellins and cytokinins (Martinez-Toledo et al., 1988) which enhance root growth and aid in nutrient absorption. *Azotobacter* seed-inoculation was tested in a field trial in North-India with ten Indian wheat cultivars (Manske et al., 2000).

MATERIALS AND METHODS

Sample Collection

Samples were collected in different locations of Tondi marine region at the depth of 15 m. The randomly collected samples in the sterile plastic bags (soil sample) and water sampling bottles (water sample) bottles were kept in an ice-cold box and transported safely to the lab for further analysis with in 12 hrs. The sample with media tubes were packed and transported safely to the laboratory.

Isolation of *Azotobacter* From Water and Sediment Samples

Different selective media were used for the

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isolation of *Azotobacter* sp from marine source. Media used for the isolation of nitrogen fixing organism (*Azotobacter*) from marine sources were Jensen's agar medium, *Azotobacter* agar medium, Burk's Medium and marine agar medium. *Azotobacter* strains used for this study were maintained and cultured in Burk medium. The collected soil samples were serially diluted, plated in *Azotobacter* agar media (3.5% Nacl) and incubated at 28°C for 4 days. As the isolates are of marine origin, the media were prepared by the 3.5% sodium chloride (Nacl). Followed by Gram staining, catalase test, starch hydrolysis test and motility tests were also carried out.

Chemical and Nutrient Analysis of Marine Sediments and Water Samples

The collected samples were tested for its chemical and nutrient contents at department of soil science, Tamil Nadu Agricultural University, Coimbatore.

Effect Of Various Physico-chemical Parameters

Effect Of Salt And Sugar Concentrations

The growth of isolates was checked for different salt and sugar concentrations (0.5%, 1.0%, 1.5% and 2%) by incorporating the nutrients into Burk's Media and incubated at 28° for 2 days. The growth of isolates was checked by OD at 670 nm.

Effect of Temperature

Different temperatures were maintained viz., 28°C, 37°C, and 45°C and incubated and incubated at 28° for 2 days. The growth of isolates was checked by OD at 670 nm.

Effect of pH

Different pH of media were maintained viz., 6, 7,8, 9 and incubated at 28° for 2 days. The growth of isolates was checked by OD at 670 nm.

Pot Culture Experiment

The nitrogen fixing ability of the isolated *Azotobacter* spp was determined in garden soil by pot cultivation method by accessing the growth of green gram. The broth containing active culture of *Azotobacter* sp was selected. Five marine strains, five soil strains, three standard *Azotobacter* sp procured from MTCC and NCIM were used. Forty healthy seeds of green gram were mixed with 3ml of *Azotobacter* inoculum and 3ml of cooled rice porridge. The seeds were dried and sown in each pot. The control is devoid of the inoculum. The pots were watered regularly and the effect of bacterial inoculum on seed germination on 5th day was checked and then the growth of plant root and shoot length was measured at interval of 5 days.

Growth Characters of The Plant

1. Percentage of Germination

The germination rate was calculated for all the treated pots. It was calculated using the formula.

Percentage of germination =

$$\frac{\text{Number of seeds germinated}}{\text{Number of seeds sown}} \text{ X } 10$$

2. Shoot Length

The shoot length between collar and top of the primary shoot was measured in cms scale at interval of 5 days. The shoot length was measured for maximum number of plants.

3. Root Length

The plants were uprooted gently without disturbing the root system and then roots were washed with distilled water. The root length between collar and tip of primary root was measured in cms scale at interval of 5 days. The root length was measured for maximum number of plants.

4. Vigour Index

Vigour index value was computed using the formula and was expressed as whole number.

Vigour index = Germination percent

x (Root length + Shoot length)

The seed germination percentage was recorded on 5th day after the sowing of seed. The root length between collar and tip of the primary root was measured in cms and mean value was recorded as root length. The shoot length between collar and top of the primary shoot was measured in cms and mean value was recorded as shoot length.

Biometric Analysis

Chlorophyll estimation was performed for the plant materials using appropriate standard procedure. After 15 days interval leaves were collected for estimating the chlorophyll content by spectrophotometric method. Spectrophotometric analysis of chlorophyll pigments were developed in the 1930's and 1940's. Chlorophyll is extracted

in 80% acetone and the absorption at 663nm and 645nm are read in spectrophotometer.

Calculation

The amount of chlorophyll in the extract was calculated using the formula:

The amount of chlorophyll in the extract was calculated using the formula:

$$\text{mg chlorophyll a /g tissue} = 12.7\,(A_{663}) - 2.69\,(A_{645}) \times \frac{V}{1000 \times W}$$

$$\text{mg chlorophyll b/g tissue} = 22.9\,(A_{645}) - 4.68\,(A_{663}) \times \frac{V}{1000 \times W}$$

$$\text{mg total chlorophyll/g tissue} = 20.2\,(A_{645}) + 8.02\,(A_{663}) \times \frac{V}{1000 \times W}$$

where,

A = absorbance at specific wavelength

V = final volume of chlorophyll extract in 80% acetone

W = fresh weight of tissue extracted

RESULTS

Totally 80 samples were collected in marine region of both water and sediments in the intervals of approximately 20 days. Out of 40 marine water and sediment samples collected, all the 40 samples were showing the presence of *Azotobacter*. These samples were processed through the commonly used procedures such as selective media, Gram staining, catalase test, starch hydrolysis test and motility tests for identification of free-living diazotrophic organism. The colony morphological features of the isolates were examined in different growth selective media. The colonies were very clear, large, mucoid, watery due drops like initially i.e. from the marine source. Other tests show that *Azotobacter* sp is gram negative, motile, catalases and starch hydrolysis positive. All the isolated *Azotobacter* strains were numbered for the easy identification and convenience.

Chemical and Nutrient Analysis of Marine Sedimentand Water Samples

He collected samples were tested for its chemical and nutrient contents at TNAU, Coimbatore (Table 1a and 1b)

Screening of Strains

The total collected strains were subjected to primary screening, using nutritional parameters such as salt and sugar concentrations, and physical parameters such as temperature and pH, in which 10 strains have been selected based on their highest OD values (Figure 1, 2, 3 and 4).

Pot Culture Experiment

The main objective of the pot culture study is to examine the influence of *Azotobacter* on green gram, 5 days interval after sowing various characteristics of growth such as percentage of germination, shoot and root length was measured results were noted. The pot culture experiment results showed that, inoculation with *Azotobacter* influence the growth of black gram by increasing their shoot and root length and chlorophyll content. Five marine strains (400, 401, 402, 404, 414), 5 soil strains, (415, 417, 432, 438, 440) and 3 standard strains MTCC (123) and NCIM (2452 and 2821) were used for the experiment. Approximately (10×10^7 CFU/pot) broth inoculums were introduced in all the 26 pot (original and duplicate) except the control pot. The various characteristics of growth such as percentage of germination, shoot and root length was measured and results were noted. The percentage of seed germination was found to be 80% to 90% (Figure 5). The growths of plant root length values ranges from 4 to 12 cms and shoot length values range from 6 to 25 cms. Marine strain 400 and soil strain 432 shows remarkable effect on the shoot length of plant and marine strain 404 and soil strain 440 shows remarkable effect on the root length of the plant than others on 15th day. The Vigour index values range from 1538.32 to 1841.72. Significant differences were observed. The plants inoculated with *Azotobacter* sp were taller than that of control pot.

Biometric Analysis

Chlorophyll content was estimated using leaf materials by spectrophotometric method. The leaves were collected from each pot and chlorophyll contents were estimated. The plants inoculated with efficient strains of Azotobacter spp. have more chlorophyll contents. The OD values at 645nm ranges 0.125 to 0.250 and OD value at 663nm ranges from 0.414 to 0.561.

DISCUSSION

Azotobacter is a strict aerobe and its metabolism consists primarily in the simple oxidation of substrate to

carbon-dioxide and water (Lewis et al., 1973).The estimated contribution of free-living N-fixing prokaryotes to the N input of soil ranges from 0-60 kg/ha /year (Burgmann et al., 2003). Effect of *Azotobacter chroococcum* inoculants on cotton Maize and sorghum was increased the crop yield in 0.7-26.2, 36.5-71.7 and 9.3-38 respectively (Shende and Apte, 1982).

Rhizosphere-colonizing bacteria, including *Azotobacter chroococcum*, that possess the ability to enhance plant growth when applied to seeds, roots or tubers are called plant growth-promoting rhizobacteria (PGPR) (Kukreja et al., 2004). PGPR was first defined by Kloepper and Schroth (1978) to describe soil bacteria which, when used as an inoculant, enhance plant growth. Preeti Vasudevan et al., (2002) studied that the increase in shoot length in rice plants treated with biological preparations (*Bacillus* sp.) when compared with control plants.

Experiment on soil *Azotobacter* on the growth of maize was carried out by Hegazi et al., (1979) result showed significant increase in the count of *Azotobacter* in 6 week-old plant. Kavimandan (1986) was carried out a pot experiment with an *Azotobacter chroococcum* along with 50 Kg N /Ha. He found an adverse effect of bacterial inoculation on the yield of wheat. It is also found that inoculation with *Azotobacter* in mangrove soil increase seedlings, root biomass, shoot biomass, total chlorophyll of plant. Thus azotobacterisation is beneficial in raising vigorous seedlings of mangrove in coastal wetlands.

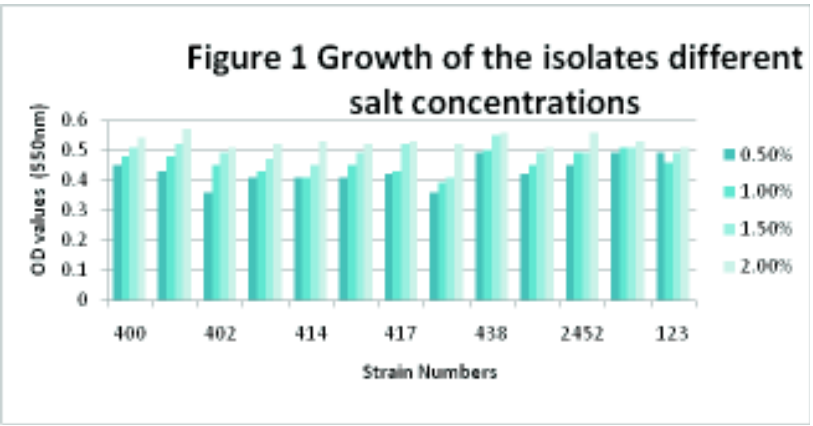
Experiments with *Azotobacter* cultures and crop

Table 1a: Details of soil analytical test

Soil Analytical Results		
Particulars	**Sample**	**Interpretation**
pH	8.29	Alkaline
Electrical Conductivity(dSm -1)	6.05	Very high salt
Texture	SL	Sandy loam
Lime	C	Calcareous
N(kg/ha)	115	Low
P(kg/ha)	20.9	Medium
K(kg/ha)	548	High
Copper(ppm)	1.1	Low
Manganese(ppm)	0.94	Low
Iron(ppm)	9.48	High
Zinc(ppm)	1.1	Low

Table 1b: Details of water analytical test

Water Analytical Results		
Particulars	**Levels**	**Interpretation**
pH	7.76	Normal
Electrical Conductivity(dSm -1)	10.13	Increasing salt problem
Carbonate(meL -1)	10.4	-
Bi-Carbonate(meL-1)	21.6	-
Calcium(meL -1)	18.4	-
Magnesium(meL-1)	84.65	-
Potassium(meL-1)	2.91	-
Sodium(meL-1)	97.8	Severe problem
Chloride(meL-1)	521.2	Severe problem
Residual sodium carbonate(RSC)	-71	Safe
Sodium adsorption ratio(SAR)	13.63	Moderately Safe
Adjusted SAR	54.5	Severe problem
Ca/Mg ratio	0.22	Safe



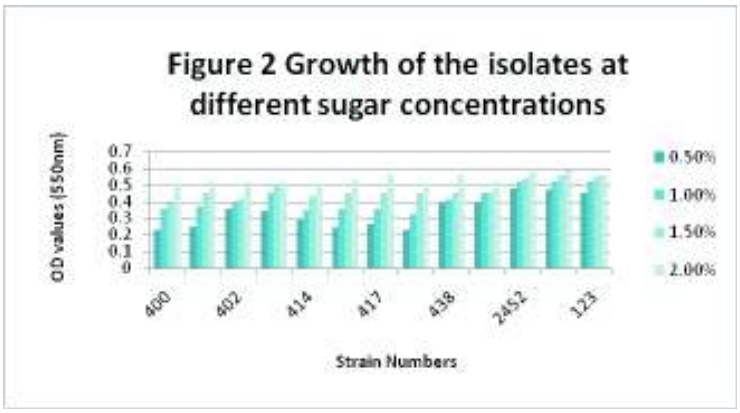


Figure 2 Growth of the isolates at different sugar concentrations
OD values (550nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
400
402
414
417
438
2452
123
Strain Numbers
0.50%
1.00%
1.50%
2.00%

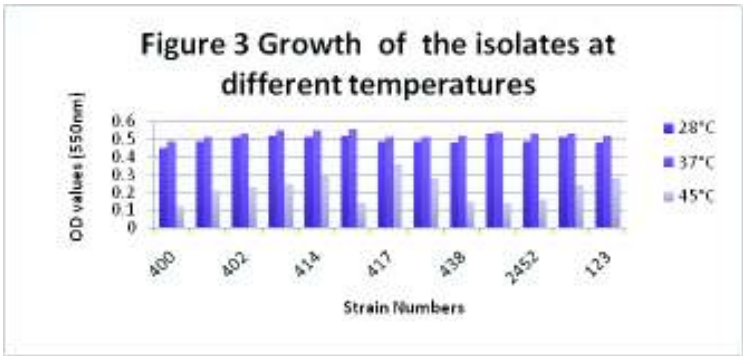


Figure 3 Growth of the isolates at different temperatures
OD values (550nm)
0.6
0.5
0.4
0.3
0.2
0.1
0
400
402
414
417
438
2452
123
Strain Numbers
28°C
37°C
45°C

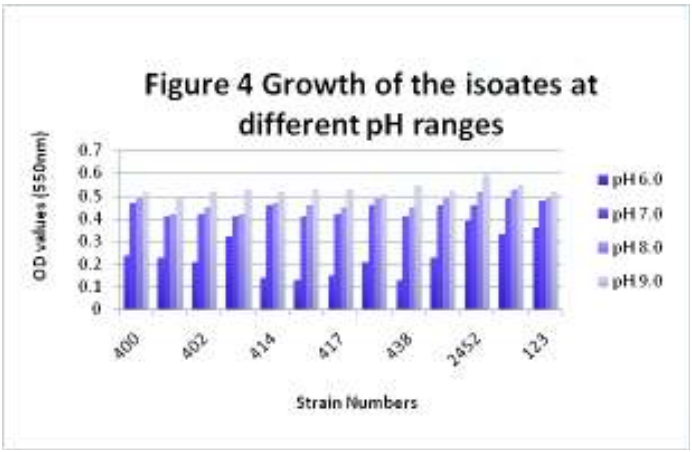


Figure 4 Growth of the isoates at different pH ranges
OD values (550nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
400
402
414
417
438
2452
123
Strain Numbers
pH 6.0
pH 7.0
pH 8.0
pH 9.0

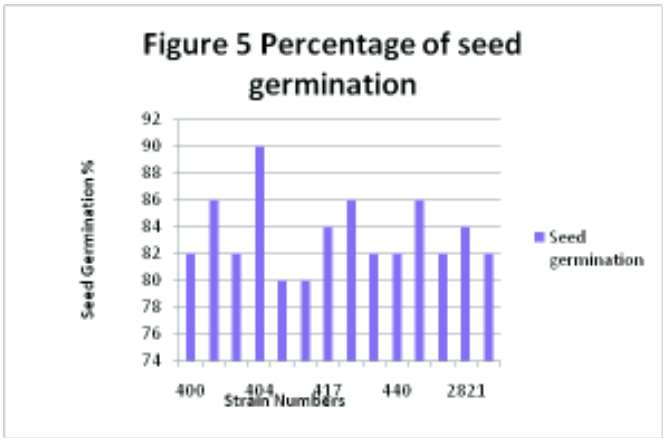


Figure 5 Percentage of seed germination
Seed Germination %
92
90
88
86
84
82
80
78
76
74
400
404
417
440
2821
Strain Numbers
Seed germination

plants at the Indian Agricultural Research Institute, New Delhi, lead us to believe that significant increases in growth and yield of wheat, rice and vegetable crops could be obtained in pot trials.)

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