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Isolation and screening of *Azotobacter Spp.* for plant growth promoting properties and its survival under different environmental stress conditions

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ABSTRACT

Because of detrimental changes in the soil condition, the substitution of chemical fertilizer is essential. Quite a lot of alternatives are available to improve the soil productivity now a day such as biofertilizers. Plant growth promoting rhizobacterias (PGPR) is being used as efficient biofertilizers known to influence plant growth by direct or indirect methods. Seeking competent PGPR strains with diverse activities, a total of six *Azotobacter* were isolated from Orissa University of Agriculture and Technology (OUAT) rice field of Odisha. All these test isolates were screened on the basis of morphological and biochemical characteristics. The result reveals that all isolates are gram negative small rods in shape; all were positive for catalase and oxidase but negative for starch and gelatine hydrolysis. Isolates were then screened for their plant growth promoting properties such as production of indoleacetic acid (IAA), nitrate reduction, ammonia (NH_3), phosphate solubilization and antifungal activity. All isolates show IAA, nitrate and phosphate solubilization positive. One of the major constraints on agricultural yield is drought and this circumstance is likely to be intensified in the future due to water shortage worldwide. A number of mitigation approaches and alterations are involved to survive drought stress. Plant growth-promoting rhizobacteria (PGPR) could play a major role in decreasing different stress conditions in plants. From an overall study of six isolates A_4 isolate shows significant PGPR and drought tolerant properties.

Keywords: *Azotobacter; Plant growth promoting rhizobacteria traits(PGPR);Biofertilizer; EPS production.*

1. INTRODUCTION

Soil is a dynamic natural resource not only for agriculture but also it plays a vital role towards maintenance of life processes. It is a critical component and foundation of ecosystem as nation's entire agricultural system directly depends on it. At present situation foremost difficulties faced in agricultural sector are the pollution and contamination of soil caused by profound use of chemical fertilizers. Application of these are currently under debate due to environmental disturbance, fear for consumer's health and food security concern. Because of current social concern about the side effect of agrochemicals, several lines of research focuses on interaction of plant and microbial population of rhizospheric region. Plant rhizosphere is a complex niche where soil, plant and microbes interaction occur. Nitrogen deficiency is frequently a major limiting factor for crops production now-a day. In agriculturally important crops, N-fertilizers widely applied for better yield purposes. To reduce the use of N-fertilizer, exploitation of plant growth promoting bacteria (PGPB) in the crop field is an interesting replacement because microbial products are considered target precise, harmless and self replicating. They also maintained soil health by increasing soil fertility and enhance the production to a greater level.

Azotobacter is a genus of free-living diazotrophic bacteria found in neutral to alkaline soils, some of the plants and in aquatic surroundings. It is an aerobic, gram-negative, pleomorphic, live in singly, chains, or clumps, may or may not provided by flagella, nitrogen-fixing bacteria that are found throughout the world [1,2]. Their resting stage is spent as a thick walled cyst, which protect the organism from harsh climate. *Azotobacter* sp. play a key role in maintaining soil fertility through several beneficial effects on plants.

The main objective of this research study is to isolate *Azotobacter* species from paddy field soil sample having ability to produce growth hormones, inhibit pathogenic fungal species and solubilize phosphate which will help in the development of potent biofertilizer to sustain in harsh climatic conditions.

2. MATERIALS AND METHODS

2.1. Sample collection and bacterial isolation.

Rhizospheric soil sampling were done from the rice field of different locations of Orrisa University of Agriculture and Technology, Khordha , Odisha(Latitude $20^0.2650'$N, Longitude $85^0.811'$E). Under aseptic conditions, the rhizospheric soil samples were collected carefully and put in to the labelled plastic bags. Soil samples were air dried along with that physico-chemical properties like pH, moisture content of the samples were measured. Then soils were prepared to isolate the bacteria by spread plate technique in Burk's culture media ($MgSo_4$ = 0.2gm,K_2HPo_4 =0.8gm,$KHPo_4$ =0.200gm,$CaSo_4$ =0.130gm,$FeCl_3$=0.00145gm,$NaMoO_4$ =0.000254gm per litre) which is a most suitable medium for growth and cultivation of nitrogen fixing bacteria as well as diazotrophs such as *Azotobacter* species. The plates were incubated for 2–5 days at 28^0C. Well-isolated and differentiated

colonies were transferred to Burk's slants and maintained at 4^0C for further study. Isolated bacterial strains from the rhizospheric soil sample were further considered for biochemical and enzymatic activities.

2.2 Biochemical characterization and enzymatic activities study of bacterial isolates.

The probable isolates were categorized based on their staining characteristics and total of six isolates were further considered in terms of biochemical properties like indole, methyl red, voges-proskauer ,citrate ,Mannitol motility ,oxidase, catalase, urease and enzymatic activities like starch and gelatine hydrolysis for confirmation of genus according to Bergey's manual of systematic bacteriology[3]. The five carbons sources viz. glucose, maltose, inositol, mannose, rhamnose, were investigated to observe their utilization by the six isolates.

2.2.1. Cyst Formation.

Cysts are the means of asexual reproduction of *Azotobacter* species under favourable conditions[4]. The *Azotobacter* isolates were grown on Burk's medium and incubated for 7 days. These isolates were stained by crystal violet for detection of cyst and observed under oil immersion.

2.3. *In vitro* screening of bacterial isolates for their plant growth promoting (PGP) activities.

All six bacterial isolates obtained by the confirmatory morphological and biochemical test were subsequently screened for diverse plant growth promoting properties. To check the phosphate solubilisation by the isolates , the cultures were placed on modified Pikovskaya agar containing insoluble tricalcium phosphate (TCP) and incubated at 28±0.1ºC for 2-4 days [5] . Indole acidic acid production was analysed by means of qualitative method reported by Bric *et al*, 1991[6]. For the production of ammonia bacterial isolates were inoculated in peptone water and incubated for 48 h at 28±2°C. Nessler's reagent (0.5 ml) was added to it. Transformation of brown to yellow colour was positive result for ammonia [7]. To check nitrate to nitrite reduction by isolates, they were inoculated into nitrate broth and incubated at 30 ±1ºC for 96 h. With the addition of sulphanillic acid and α-naphthylamine mixture (1:1) detection of red colour indicate positive result. Conformation of N_2-fixation capability of the isolates were examined in N-free agar based Jensen agar media and incubated for 72 h at 28±1ºC[8].

2.4. Seed germination by roll towel method.

Bacterial isolates were grown in respective broth at 28±2°C for 24 h. Rice seeds (Naveen) were surface sterilized and inoculated in culture medium for 30 min [9]. To determine the effect on seed germination of the isolates in germination paper this test was performed using the roll towel method [9]. A control was taken with sterile Burk's medium for comparison. Initially, the brown germination papers were soaked in distilled water then seeds were put on the paper. Another pre-soaked paper towel was wrapped on the first one so that the seeds were supposed to be in stable position. The towels were then rolled and covered with polythene to prevent drying, After 14 days, the towels were removed, the number of germinated seeds were counted and determined the length of root and shoot each plant separately comparing with the control seeds. The germination percentage was calculated by the formula:

Germination percentage (%) =

$$\frac{\text{Seeds germinated}}{\text{Total seeds germinated}} \times 100$$

Root and shoot length of germinated plants were measured.

2.5. Effect of stress tolerance Parameter on growth of isolates.

Different stress parameters like pH(5,6,7,8, and 9) temperature (50^0c ,15^0c ,37^0c ,25^0c) and salt tolerance capability (0% 2% 5% 7%and 10%) were investigated for the growth of isolated strains .After 48 hour of growth ,optical density was taken by the spectrophotometer(620nm) .Various pH of Burk's media was maintained by using diluted Hcl and NaOH. A range of temperature was retained in hot air oven, freeze, bacterial incubator and room temperature. Screening for different salt tolerance properties of the *Azotobacter* isolates was tested by taking 5ml Burk's broth containing different salt concentration.

2.5.1. Extraction of EPS.

EPS (Exo PolySaccharide) facilitate the isolates to develop in stress conditions by improving the regulation of carbon source diffusion and water holding capacity. EPS protects bacteria from desiccation under drought stress[10,11,12].Isolated *Azotobacter* strain was cultured in Burk's broth medium with 50ppm of Cr (50mg $K_2Cr_2O_7$ in 1000ml of distilled water) and incubated at 28±1ºC in shaking for 72 hours. A control was also maintained without $K_2Cr_2O_7$ to compare the growth of *Azotobacter.* Bacterial cell biomass was separated out by centrifugation for 20 min at 10,000 rpm .After centrifugation, cell pellet was collected in an eppendorf tube and supernatant was further used for exo polysaccharide extraction. Two volumes of ice cold Isopropanol were added into it, and stored overnight at 4^0C. The supernatant was centrifuged at 8000rpm for 10 min. The pellets were collected for bacterial exo polysaccharide. After drying weigh the pellet to know which organism was showed higher production of exo-polysaccharide.

3. RESULTS

An attempt was made to isolate the native *Azotobacter* spp. and study their potential plant growth-promoting properties. *Azotobacter* species abundance depends upon several factors viz. microbiological and soil physicochemical properties (pH, soil moisture) [13]. The large quantity of *Azotobacter* in rice field has also been confirmed by Sariv and Ragoviv (1963)[14] who reported that the *Azotobacter* population is more in the rhizosphereic region and also depends according to the plant species.

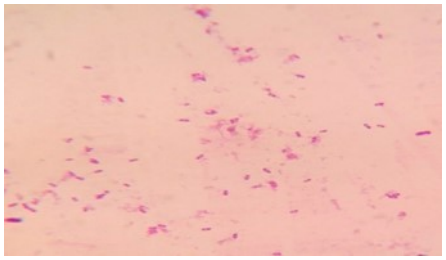

Figure 1. Gram staining of the bacteria under compound microscope.

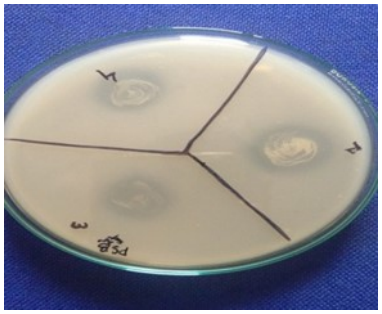

Figure 2. clear zone around the colonies indicating phosphate solubilisation activity by *Azotobacter* isolates.

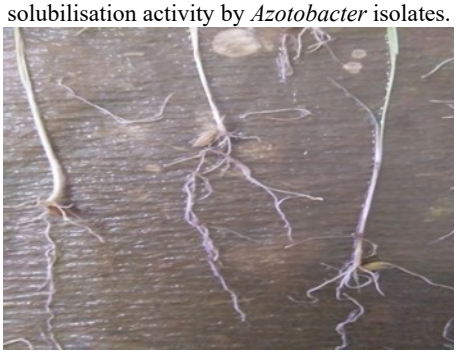

Figure 3. Effect of azotobacter formulation on the growth of roots of rice seeds.

The Physico-chemical properties of soil viz. pH and moisture content percentage were measured along with the bacterial load and the result is presented in table 1. A total of 4 soil samples were collected from rhizosphere region of different rice fields of OUAT Khordha, Odisha. The pH values of soil samples were found to be acidic and varied from 5.93 to 6.14. Soil moisture content differed with the sampling sites ranged from 19.38 to 30.57. The bacterial load was detected and differ from 3.2 to 5.9×10^4CFU/g in Burk´s medium which indicated abundant number of nitrogen fixing bacteria in rice field(Table:1). Six isolates were selected, the morphological characters were studied and all the isolates were found circular, entire, flat, transparent, slimy, mucoid, gummy, white and motile gram –ve rods (fig:1).

All the isolates from cyst after 7 days of incubation. Isolates were then maintained as pure culture. From the biochemical and enzymatic study, it was revealed that all the isolates were found catalase and oxidase positive(Table:2). The isolates were studied for their carbon source utilization. These results confirm that these isolates belong to the genus *Azotobacter* [15].

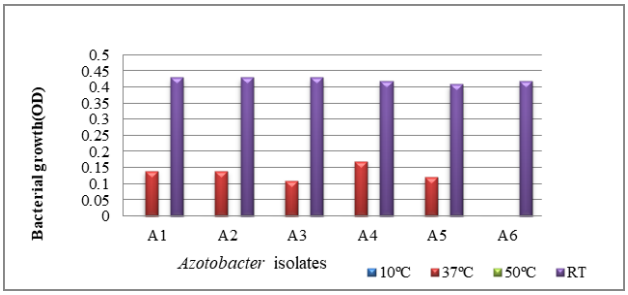

Figure 4. Effect of temperature on the growth of *Azotobacter* isolates.

3.1. The Plant growth promoting activities exhibited by bacterial isolates.

PGPR properties such as indole acetic acid production, phosphate solubilization, ammonia, nitrate production and antibiosis properties of the six isolates were studied and presented (Table :3). Brakel & Hilger, (1965) reported that *Azotobacter* generated Indol-3-Acetic Acid (IAA) while tryptophan was added to the culture media[16]. Althaf and Srinivas (2013) formulated that phosphate solubilisation (Fig: 5) and gibberillic acid production is higher in Azotobacter[17]. He also described that many strains of *Azotobactor* also exhibited fungicidal properties against certain species of fungus.

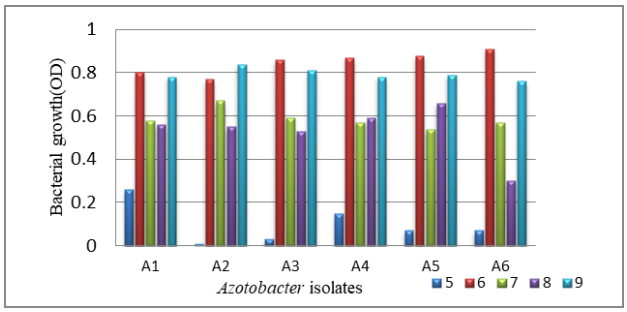

Figure 5. Effect of pH on the growth of *Azotobacter* isolates.

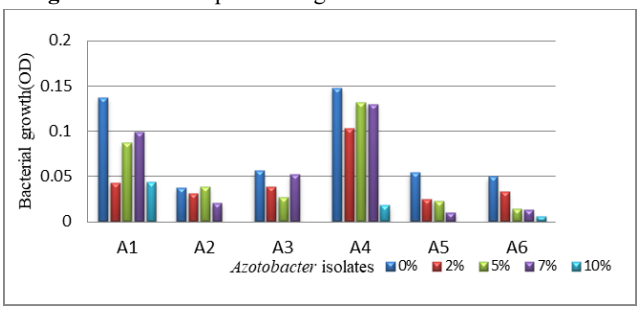

Figure 6. Effect of salt concentration on the growth of *Azotobacter* isolates.

Table 1. Physicochemical properties and bacterial load count of soil.

Soil sample name	pH	Moisture content (%)	Bacterial load (10^4 CFU/g)
S1	5.93	20.09	5.3
S2	5.92	23.10	3.2
S3	6.14	19.38	5.9
S4	5.86	30.57	4.5

These responses intended that by producing growth regulatory substances *Azotobacter* maybe influences the improvement of plants. Hence, *Azotobacter* species is considered as a member of plant growth promoting rhizobacteria[18]. *Azotobacter* produces phytohormones that are thiamine, riboflavin, nicotin, IAA and gibberallin which can stimulate root and shoot development [19]. Appling Azotobacter formulation to seeds improves seed germination to a considerable extent and control plant diseases (Fig: 6, Table: 4). Bacteria producing phytohormones enhance the plant stress tolerance by stimulating endogenous hormones [20,21].The six bacterial isolates are regarded as palnt growth promoting rhizobacteria as they enhanced the root and shoot growth by producing phytohormones in rice plant (Naveen) as well as inhibit pathogens. All isolates have the ability to solubilization phosphate(Fig:2); produce ammonia, nitrate, IAA but A1, A2, and A4 showed antifungal properties.

3.2. Stress tolerance capacity of bacterial isolates.

The EPS protect these bacteria from desiccation under drought stress by enhancing the water retention and any regulation of organic carbon source diffusion[10,11,12]. All the six *Azotobacter* isolates showed production of EPS, which enable the isolates to grow at stress condition (Table: 2).

With respect to temperature all strains showed maximum growth at 30^0C followed by 37ºC and no growth was observed

at10ºC and 50ºC(Fig:4). Regarding the effect of pH most of the isolates such as A1, A3, A4, and A6 show maximum growth at pH 6 followed by pH 9(Fig: 5) whereas the isolate A2 showed highest growth at pH 9 followed by pH 6. Increase in concentration of the salt from 2% to 10% the growth was decreased and there was negligible growth in the presence of 10% NaCl. (Fig: 6). *Azotobacter* species is sensitive to acidic pH, high salt and temperature [22].

Table 2. Biochemical, enzymatic and carbon source utilization, EPS production properties by the bacterial isolates.

Sl.no.	Biochemical test		A1	A2	A3	A4	A5	
1.	Indole test		+	+	+	+	+	+
2.	Methyl red test		+	+	+	+	+	+
3.	Vogues-proskaur test		-	-	-	-	-	-
4.	Citrate utilization test		-	-	+	-	-	+
5.	H_2S production		-	-	-	-	-	-
6.	Mannitol motility test	Mannitol	-	+	-	-	-	-
		Motility	+	+	+	+	+	+
7	Catalase test		+	+	+	+	+	+
8	Oxidase test		+	+	+	+	+	+
9	Urease test		+	-	+	+	+	+
10	Amylase		-	-	-	-	-	-
11	Gelatinase		-	-	-	-	-	-
12	Sugar utilization	Glucose	+	+	+	+	+	+
		Maltose	-	+	+	-	-	-
		Inositol	-	+	+	-	+	-
		Mannose	+	+	+	+	+	+
		Rhamnose	-	+	+	-	-	-
13	Cyst formation		+	+	+	+	+	+
14	EPS production		+	+	+	+	+	+

+:positive, -:negetive

Table 3. The Plant growth promoting activities exhibited by *Azotobacter* isolates.

Isolates	IAA	Phosphate solubilization	Ammonia	Nitrate	Antibiosis
A1	+	+	+	+	+
A2	+	+	+	+	-
A3	+	+	+	+	+
A4	+	+	+	+	+
A5	+	+	+	+	-
A6	+	+	+	+	-

+:positive, -:negetive

Table 4. Germination percentage, shoot and root length of the rice seed treated by *Azotobacter* isolates.

Germination Properties of rice(Naveen)	Control	A1	A2	A3	A4	A5	A6
Percentage of germination	88	92	88	88	96	96	92
Shoot length Mean(cm)	7.29	10.91 49.65[a]	9.96 36.62 [a]	9.61 31.82 [a]	10.98 50.61 [a]	10.15 40.60 [a]	10.06 37.99[a]
Root length Mean (cm)	10.42	11.18 7.2[b]	10.15 -2.59 [b]	11.06 6.1 [b]	12.61 21.01 [b]	12.60 20.92 [b]	9.99 -4.12 [b]

a: % increase in shoot length with respect to control, b: % increase in root length with respect to control,-: decrease of root length with respect to control

4. CONCLUSIONS

The successful isolation of local isolates of *Azotobacter* shows important role in soil fertility. They also considerably showed their capabilities of solubilizing tricalcium phosphates, producing Indole acetic acid and antifungal metabolites against indicator fungal species. In addition, they have also the potential for production of EPS on large scale. Isolate A4 showed improved seed germination and has a beneficiary response on the growth of shoot and root length. *Azotobacter* inoculants have a major promoting outcome on the growth of root; shoot length and germination of rice seeds. For PGPR mediated drought stress tolerance plants have phytohormonal activities and EPS production mechanisms. With advance research, the organism can be of immense agricultural importance with its application in agricultural field.

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