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Isolation and characterization of saline tolerant *Azospirillum* strains from paddy field of Thanjavur district

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ABSTRACT

In the present investigation deals with the isolation and characterization of Azospirillum strains from paddy field rhizosphere soil of Thanjavur district .The Azospirillum strains were identified by morphological, physiological and biochemical characters.All the 10 Azospirillum strains were identified based on morphological character such as shape , temperature, pH and biochemical character such as IMViC,catalase,citrate,starch hydrolysis and urease test were studied. Out of 10 strains, selected the four strains (Azo 3,Azo 6,Azo8,and Azo 10) were highly tolerant upto 700mM .Based on this study saline tolerant strains of Azospirillum could be highly used to cultivation of paddy plant in coastal environment .

Key words :*Azospirillum*, NaCl, Rhizosphere soil.

INTRODUCTION

Azospirillum was first isolated from poor sandy soil in the Netherlands (Beijerinick 1925).*Azospirillum* is a free living plant growth promoting bacterium (PGPB),capable of affecting growth and yield of numerous plant species,many of agronomic and ecological significance.The leading theory concerning its growth promotion lies in its ability to produce various phytohormones that improve root growth adsorption of water &minerals that eventually yield larger, and in many cases,more productive plants (Dobbelaerae et al 2001).

Bacteria of the genus *Azospirillum* are widely distributed in soil and associated with the roots of forage grasses ,cereals and non gramineous plants (Bashan et al 1997) .*Azospirillum sp* are widely distributed soil nitrogen fixing bacteria that play an important role in the promotion of plant growth (Steenhoudt and Vandeleyden 2000) .

Azospirillum sp are among the most important bacteria involved in Nitrogen fixing in grasses .It is normal for *Azospirillum sp* to be on nitrogen free culture media with soil or roots ,However Dobereiner et al (1976)have reported that isolation of pure cultures from samples collected in temperature regions is difficult .Vlassak and Reynders (1978) have found that in many cases nitrogen fixing bacteria are closely associated with other microorganisms and that obtaining pure isolates is not easy .

The genus *Azospirillum* comprises free-living nitrogen fixing rhizosphere bacteria to a group that exerts beneficial effects on plant growth,namely the plant growth –promoting rhizobacteria (PGPR).Because of those properties,numerous studies on *Azospirillum* ecology,physiology,and biochemistry have been carried out during the past 15 years (Vande Broek and Vandeleyden 1995).*Azospirillum* are widespread in soils and comprise diverse diazotrophic rhizobacteria stimulating plant growth and development of polysaccharide components of the surface *Azospirillum* play an important role in the formation of associations with other rhizobacteria (Yogorenenkova et al 2001).

Salt stress has become an ever – increasing threat to food production,irrigation being a major problem of agricultural fields due to gradual salinization.Salt stress has three fold effects it reduces water potential,causes ion imbalance or disturbance in ion homeostasis and toxicity.This altered water status leads to initial growth reduction and limitation of plant productivity.Since salt stress involves both osmotic and ionic stress (Hayashi and murata 1998 ;Munns 2002).

Keeping the above mentioned points in the mind the present study has been justifiably planned to study the isolation,characterization and optimization to find out the saline tolerant *Azospirillum* strains.

MATERIALS AND METHODS

Collection of soil sample

The soil samples were collected from 10 different paddy fields.The samples were randomly collected, mixed thoroughly packed in polythene bag and labelled the location and depth of the soil,brought to the laboratory and stored for further studies

Isolation

Isolation of *Azospirillum* strains by using Nfb medium.The medium contains malic acid - 0.5g,$MgSO_4.7H_2O$-0.2g ,Nacl - 0.1g , $CaCl_2$ -0.02g ,Na_2 MoO_4 -0.002g ,$MnSO_4.H_2O$ -0.01g ,EDTA 1.64% -4 ml ,Bromothymol blue 0.5% (W/W in ethanol) -3 ml , KOH -4.5g,Biotin - 0.1mg ,distilled water -1000ml ,pH-6.8,One gram soil sample was taken and serialy diluted upto10-8.Adding 0.1ml of various dilutions to Nfb semisolid tubes and incubated for 24 hours .Identification of *Azospirillum* by morphological characteristics (cell shape,size,swarming experiment),physiological characteristics by effect of pH and effect of different temperature (27,32,40,45 and 50)

Tolerant to Nacl

Nfb medium was prepared with various concentration of Nacl (100, 200, 300, 400, 500, 600, 700mM). The isolated 10 strains (Azo 1 to Azo10)were inoculated separately and incubated for 48 hours .The growth level was measured by spectrometrically at 620nm.

RESULTS AND DISCUSSION

Ten strains were isolated from different paddy rhizophere soil of Thanjavur District (Table 1).All the 10 strains of *Azospirillum* were observed its colony colour ,shape and size(Plate I;Fig.2). Out of 10 strains,6 strains were rod and another 4 strains were vibroid.All the isolated strains were gram-negative bacteria(Table 2).

In the present study ten different isolated strains showed white dense and undulating fine pellicle.The pellicle formation is characteristic of *Azospirillum* . Dobereiner and Day (1976) reported microaerophilic growth in semisolid agar stagnant conditions were helpful for the inoculation of the organism .Since *Spirillum lipoferum* grows in a typical pellicle 1 to 4mm below the surface ,this method was particularly useful for studying the substrates and growth conditions for nitrogen fixation .The isolated strains were rod and vibroid in shape.This result was confirmed by Doberenier and Baldani (1979) and Krieg et al.(1984) and also reported that microscopic examination revealed polymorphism ,but the dominant forms on a solid malate medium are characteristic curved rods of various sizes with predominant refractive fat droplets .

Table 1.Orgin of the *Azospirillum* strains

S.No	Strain No.	Place for isolation
1	Azo1	Ammapettai
2	Azo2	Saliyamangalam
3	Azo3	Poondi
4	Azo4	Punnainallur
5	Azo5	Vallam
6	Azo6	Thirubuvanam
7	Azo7	Thanjavur
8	Azo8	Udaiyarkovil
9	Azo9	Ukkadai
10	Azo10	Soorakottai

Table 2.Morphological characteristics of *Azospirillum* strains

S.No.	Strain No.	Cell shape	Cell size (µm)	Colony character (BMS medium)	Swarming experiment(cm)
1	Azo1	Rod	0.5	Pale yellow	6
2	Azo2	Vibroid	0.9	White	6
3	Azo3	Rod	0.6	Pale yellow	4
4	Azo4	Rod	0.6	Pale yellow	5
5	Azo5	Vibroid	0.6	White	10
6	Azo6	Rod	0.9	White	9
7	Azo7	Vibroid	0.6	White	8
8	Azo8	Rod	0.9	White	4
9	Azo9	Rod	0.6	White	5
10	Azo10	Vibroid	0.9	Pale yellow	5

Table 3.Physiological characteristics of *Azospirillum* strains

(a) Effect of different temperature

S.No	T˚	Azo1	Azo2	Azo3	Azo4	Azo5	Azo6	Azo7	Azo8	Azo9	Azo10
1	27˚	+	+	+	+	+	+	+	+	+	+
2	32˚	+	+	+	+	+	+	+	+	+	+
3	40˚	+	+	+	+	+	+	+	+	+	+
4	45˚	+	+	+	+	+	+	+	+	+	+
5	50˚	+	+	+	+	+	+	+	+	+	+

(b) Effect of different pH

S.No	pH	Azo1	Azo2	Azo3	Azo4	Azo5	Azo6	Azo7	Azo8	Azo9	Azo10
1	5.5	-	+	+	+	+	-	-	-	-	-
2	6.0	-	+	+	+	+	-	+	+	+	+
3	6.8	+	+	+	+	+	+	+	+	+	+
4	7.0	+	+	+	+	+	+	+	+	+	+
5	7.5	+	+	+	+	+	+	+	+	+	+

(+) Presence of growth
(-) Absence of growth

Table 4.Biochemical characteristics of *Azospirillum* strains

S.No	Biochemical characters	Azo1	Azo2	Azo3	Azo4	Azo5	Azo6	Azo7	Azo8	Azo9	Azo10
1	Biotin	+	+	+	+	+	+	+	+	+	+
2	Starch	+	+	+	-	+	-	+	+	+	+
3	Citrate	-	+	+	-	+	+	+	+	+	+
4	Indole	+	+	+	-	+	+	+	+	+	+
5	Methyl red	+	+	+	-	+	+	+	+	+	+
6	Voges proskauer	+	+	-	+	-	-	+	-	-	+
7	Urease	+	+	+	-	+	+	+	+	+	+
8	Catalase	+	+	+	+	-	-	+	+	+	+

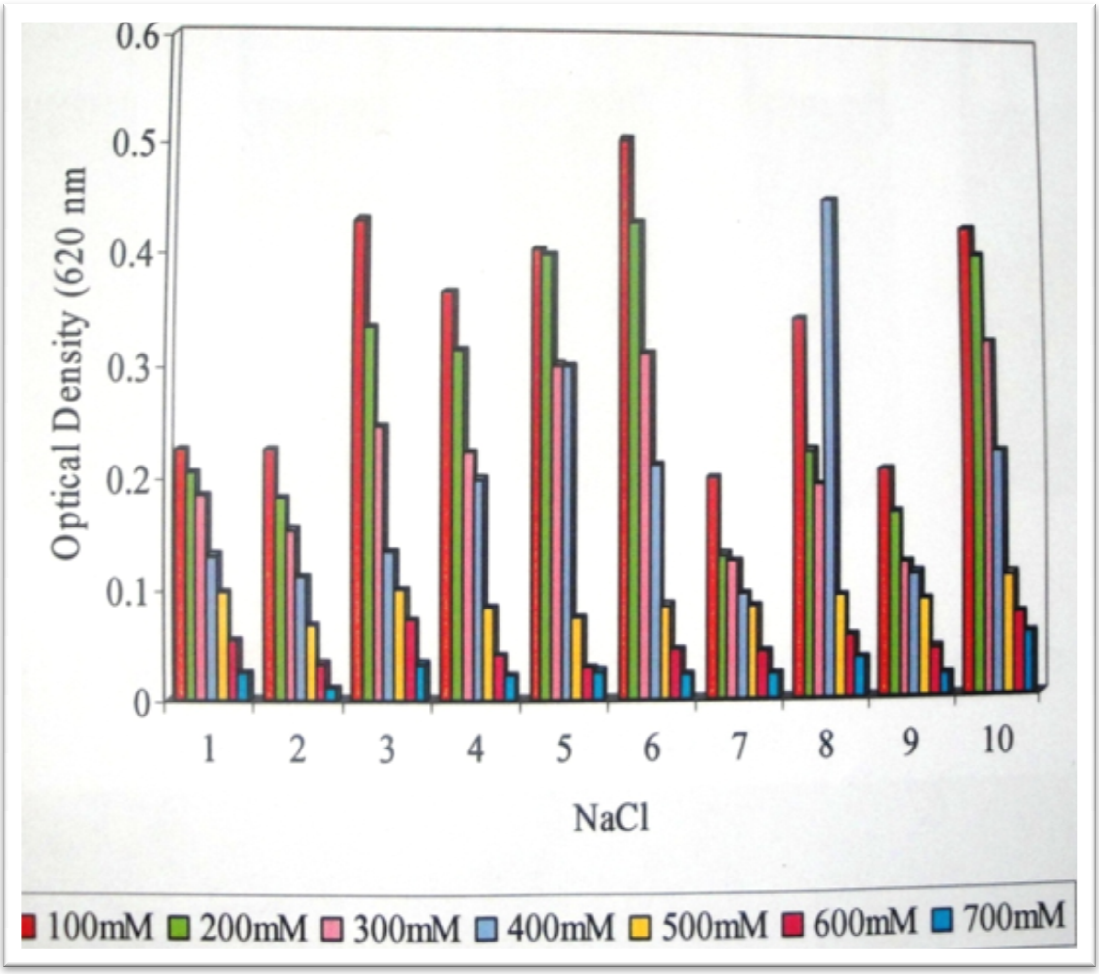
\+ *(Positive)*
\- *(Negative)*

Table-5 utilization of different carbon sources

S.No	Strain No	Manitol	Sucrose	Fructose	Lactose	Succinate
1	Azo1	+	+	+	+	+
2	Azo2	+	+	+	+	+
3	Azo3	+	+	+	+	+
4	Azo4	+	+	+	+	+
5	Azo5	+	+	+	+	+
6	Azo6	+	+	+	_	+
7	Azo7	+	+	+	+	+
8	Azo8	+	_	+	+	+
9	Azo9	+	+	+	+	+
10	Azo10	+	+	+	+	+

(+) presence of growth
(-) absence of growth

Fig I Effect of NaCl in different mM concentration



Among the 10 strains isolated, the cell size was varied from 0.5 to 0.9µm . The size range (0.7 to 1.5µm) is really reported by Bergey's manual 9th ed.(1994) for different azospirillum strains. In BMS medium, 6 strains showed white colour other's are pale yellow. There is no colour formation in Azospirillum strains reported by Lakshmi Sadasivam et al.(1985).

Eckert et al 2001 reported that all these features were very similar to other *Azospirillum sp* . The trait that differeniates ,the species from other based on its ability to use several sugars and some minute genetic detail optimum growth occurs at 30ºC and at pH values between 6.0 and 7.0 but not 37ºC. In my research found that all the isolated strains were grow well on 5 different temperatures (27,32,40,45 and 50ºC)(Table 3a). Among the isolates studied,Azo2,Azo3,Azo4,and Azo5 tolerate and well grown in different pH range (5.5,6.0,6.8,7.0, and 7.5). But Azo1,Azo6,to Azo10 could not tolerate pH 5.5. TheAzo6 and Azo1 strains were also not in a position to tolerate pH 6.0(Table 3b)

In biochemical character study, all the 10(Azo1 to Azo10) isolated strains grow well with biotin medium (Table 4). Bergey's manual 9th editon (1994) reported that *A. lipoferum* and saline

tolerant species *A.halopraeferens* require biotin for its growth and N_2 fixation.Isenberge et al. (1958) identify anaerobic gram-negative non-sporeformers, an important characteristics is the mability to produce indole relatively little information is available concerning the production of information available from studies with aerobes. In the present study 9 strains were producing indole,only one (Azo4) strain could not produce indole and also methyl red (Table 4).

In the present study, all the 10 strains utilized mannitol, fructose and succinate. But only one strain could not utilize sucrose (Azo4)and lactose (Azo6) (Table 5). Kreig (1984) reported that the organic acids support the vigorous growth and nitrogen fixation of all *Azospirillum spp*. But the organisms have very different capabilities for the utilization of sugars. Masthiz et al (1986) obsverd that *A.brasilense* cannot grow and fix nitrogen with most sugars. *A.lipoferum* effectively utilizes glucose and *A.amazonense* utilizes sucrose. It has been suggested that chemotaxis to organic acid, sugars and amino acids is important for the establishment of root-associated growth of Azospirilla in their ecological niche (Table 4).

Growth of the bacteria in the presence of different NaCl concentrations showed that the strain could tolerate upto 300mM NaCl.The higher concentration of NaCl total growth was seen by Villiana Rivorala (1998).In the present study,the strains(Azo3,Azo5,Azo8 andAzo10)were highly tolerant to the NaCl.But the growth rate is declined in other study (Fig I).The results obtained were similar to those observed in the presence of 700mM NaCl indicating that the decrease in growth rate was purely osmotic and not the result of specific ions present at high concentration.In the present study,the suitability of *Azospirillum* strains for the paddy field of thanjavur(Dt) were screened.Out of 10 isloates selected,Azo3,Azo5,Azo8 and Azo 10 were grown very well upto 200mM concentration. Hence the said strains could be effectively used as Biofertilizer for the paddy field of thanjavur, popularly called as granaries of South India.

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