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Halotolerent *Azospirillum lipoferum* N-29 As a Biofertilizer for Saline Soils

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In the present study halotolerent indigenous strain of *Azospirillum lipoferum* N-29 was isolated on modified Congored Nitrogen free agar with 6% NaCl concentration from the hyper saline soils of Kolhapur district of Maharashtra, India. It was identified as by using Bergey's manual of systematic bacteriology Williams *et, al.*,(1989). To study its nitrogen fixing ability acetylene reduction assay was performed as per Dobereiner (1997), as well as a pot experiment was carried out on sugarcane 8071 variety, which is cultivated on large scale in western Maharashtra region of India. Nine pots divided in to three sets. Set-1 labelled as control set seeded with sugarcane setts without any treatment. Set-2 seeded with sugar cane setts soaked in *Azospirillum lipoferum N-29* in sterile soil. Set-3 seeded with sugar cane setts soaked in *Azospirillum lipoferum N-29* one set each pot in normal hyper saline soil. For production of biofertilizers a biomass production experiment was carried using fully automatic bioreactor, using a modified Nitrogen free broth medium at 30°C temperature,pH7.0, 140rpm agitation. Results indicated that *Azospirillum lipoferum N-29* strain grows up to 8% NaCl concentration with optimum 4 % NaCl concentration, pH 7.0, temperature 30^{O}C and at 140 rpm. It tolerates about 12% NaCl salt concentration for 1 hour. Nitrogen fixing ability Results indicated that there was maximum nitrogen fixation (22%) observed at 30^{O}C temperature , 4% NaCl salt concentration, at 140 rpm agitation and at pH 7.0. Pot experiment showed that there was 58% increase in height of plant, diameter of stem, number of setts, and number of leaves as compared to control set and 20 % increase in sterile soil with *Azospirillum lipoferum N-29*, this indicated the combine effect of other microorganisms with that of *Azospirillum lipoferum N-29*.Biofertilizer production experiment indicated that maximum biomass (4*10^{9} cells ml^{-1}) can be produced within 48 hours at 30^{O}C and at 140 rpm using modified Nitrogen free broth medium with 20% sucrose.My study indicated the suitability of *Azospirillum lipoferum N-29* strain as a candidate for biofertilizer production for saline soils as well as for reclamation of saline soils.

Key words: *Azospirillum lipoferum* N-29, Hypersaline soils, Biofertilizers.Halotolerent,Saline soils.

Saline soils refers to soil that contains sufficient soluble salts with a conductivity of more than 4 mmhos/cm at 25° C. Hyper saline soils are soils that contains significant salts with a conductivity of saturation extract more than 18 m mhos/cm at 25° C Richards(1954).Saline soils have taken a serious mode in India. About 9 % of the total cultivated area in the country is affected by salinity and day by day the problem is becoming serious. Fertile soils are becoming nonfertile due excess use of chemical fertilizers or use of excess irrigation water, weak leaching due to table land and due to high temperature and evaporation of water.

On the global basis salt affected soils occupy an estimated 952.2 million hectares of land, constituting 7 % of total land affected by salinity Bresler et al (1983).The problem of soil salinity is wide spread in the world, amongst the affected country, Holland, Swedan, Hungary, Russia, South

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western USA, India , Pakistan and the Middle east are worstly affected Dahiya and Dahiya (1977). About 40,000 hectares of land annually becoming unfit for agricultural production in the world due to salinity.

In India the the problem has taken a serious mode about 9 % of the total cultivated area is affected by salinity Dahiya and Dahiya (1977). The problem is acute in the state of Maharashtra, Punjab, Hariyana and Uttar Pradesh states of India.

In Maharashtra about 34 million hectare has become salt affected. Such soils are predominant in Kolhapur, Sangli, Solapur, Ahmednagar, Dhule districts of Maharashtra state of India.

Due to increased quantity of salts, the microbial flora is worstly affected, this also interfered with nitrogen fixing and phosphate solublizing ability of bacteria. As the prizes of nitrogenous fertilizers have nearly doubled during last 4 to 5 years, repeated use of use of chemical fertilizers also affect soil quality, this has necessitated to search for a cheaper source of nitrogen. To meet the needs of crops one of the important way is to go for biofertilizers using microorganisms.

The ***Azospirillum lipoferum N-29*** is a Gram negative obligate aerobic rods, actively motile . It fixes atmospheric nitrogen nonsymbiotically. It does not produce nodules but makes association by living in the rhizosphere region of the plant. ***Azospirillum*** species establish an association with many plants particularly with C4 plants such as maize, sorghum, sugarcane etc.It is the most common organism and can form associative symbiosis on a large variety of plants Dobereiner et al. (1976).

Biofertilizers are substances that contain living organisms, that includes nitrogen fixing, phosphate solublizing microorganisms etc. When they are applied to seeds, plant surfaces or soil, these microorganisms colonize in rhizosphere region and promote growth by increasing the supply or availability of primary nutrients to host plant. ***Azospirillum lipoferum N-29*** is one of the nonsymbiotic bacteria that have a great potential for use in production of biofertilizers due to its ability to fix atmospheric nitrogen (N2).

Excess accumulation of salts hampered the growth and activity of soil microflora. It affected the growth of N2 fixing and phosphate solublizing bacteria which has led to non fertility of soils.

By considereing this in the present study a halotolerent ***Azospirillum lipoferum N-29*** isolated from hypersaline soils of Kolhapur district of Maharashtra,India. Isolate was studied for its N2 fixing ability and also investigated for effective biofertilizers production for saline soils with the following objectives.

Objectives

· To study the effect of ***Azospirillum lipoferum N-29***on sugar cane 8071 variety.

· To optimize the process for maximum biomass production.

· To prepare a effective biofertilizer from ***Azospirillum lipoferum N-29*** for saline soils.

MATERIAL AND METHODS

Isolation of *Azospirillum lipoferum N-29*

Azospirillum lipoferum N-29 was isolated from hyper saline soil(fig.2), on modified Nitrogen free broth as per Dobereiner and Day (1976), by enrichment for 6 days and then on agar medium containing (DL-Malic acid 5 g^{-1},KH_2PO_4 0.5 g^{-1},KOH 4 g^{-1} ,$MgSO_4 7H_2O$ 0.2 g^{-1}, $FeSO_4.7H_2O$ 0.5 g^{-1}, $CaCl_2$ 0.02 g^{-1}, $NaMoO_4 2H_2O$ 2mg^{-1} ,$MnSO_4$ H_2O 10 mg^{-1} ,0.5 % Alcoholic solution of Bromothymol blue-2 ml^{-1} ,Agar 0.5%,NaCl 2%. ,4%,6%,8%,10%,respectively,pH-7.0,Distilled water 1000ml.) Plates were incubated at RT for 48 h.The growth of *Azospirillum lipoferum N-29* was monitored by observing change in colour of medium from yellowish green to blue colour with the formation of pellicle.

Finally *Azospirillum lipoferum N-29* was isolated on modified Congo-red Nitrogen free agar medium containing above medium inoculated with 15 ml/L of 1:400 aqueous solution of Congo-red,Rodriguez Caceres (1982). Plates were incubated at RT for 48 hours.

Plates with highest NaCl concentration was observed for prominent red colour colonies of *Azospirillum lipoferum N-29.* Colony showing red colour were transferred on modified Congo-red Nitrogen free agar medium slant and further used for identification using Bergey's manual of systematic bacteriology Williams *et, al.*,(1989), and for other experimental studies.

Effect of environmental factors on growth

Preparation of inoculum: Inoculum was prepared by growing *Azospirillum lipoferum N-29* Modified Nitrogen free broth, it was incubated on rotary shaker at 30^{O}C temperature, 140 rpm agitation for 24 hours.

Unless otherwise stated 250ml Erlenmeyer flask containing 50 ml of Nitrogen free broth medium was inoculated with 1 % inoculum containing 4*10^{6} cell ml^{-1} and flasks were incubated on rotary shaker at 20^{O}C,30^{O}C,40^{O}C,50^{O}C for temperature and For pH at pH3, pH4, pH5, pH6, pH7, pH 8, pH9.

For Agitation at 40rpm, 60rpm,80rpm,100rpm,120rpm,140rpm,160rpm,180rpm,200rpm.

For NaCl concentration medium was inoculated with 1%,2%,3%,4%,5%,6%,7%,8%,9%,10% NaCl salt.

For NaCl tolerance 0.1 ml of inoculum containing 4*10^{6} cell ml^{-1} was inoculated in 10ml Nitrogen free broth medium containing 1%, 2% up to 18% NaCl salt and incubated at 30^{O}C for 24 hours. During incubation 0.1 ml of medium was removed from broth and analysed for viable population as CFU ml^{-1} as per Travors and Cook (1992).

Finally by keeping all parameters optimum 100 ml of Nitrogen free broth medium was inoculated with 1ml inoculum containing 4*10^{6} cells ml^{-1}. For optimum growth 1 ml of sample was analysed after every 6 hours for optical density at 540 nm.

Bioreactor study

For bioreactor study, fully automatic bioreactor (Biostat B. B .Brown International, Germany, Fig.1) was used the parameters which were found optimum during shake flask study were kept optimum and biomass production was carried out until a constant cell number was observed .

Nitrogen fixation measurement

Nitrogen fixation capacity of *Azospirillum lipoferum N-29* was determined by Acetylene reduction test using gas chromatography as per Dobereiner (1997) and Hardy et.al. (1975) Nitrogen free broth medium containing. NaCl 4%, Sucrose 20 g-l using glass bottles with rubber stoppers.

Azospirillum lipoferum N-29 was grown in 100 ml above medium. Flask was incubated on rotary shaker for 48 hours to obtain full growth of *Azospirillum lipoferum N-29*. From this 20 ml was transferred to a empty sterile glass bottle 30 ml capacity with rubber stopper. To this bottle 10 ml of acetylene gas was added and bottle was closed with rubber stopper and allowed to stand in shed for 1 hour for reaction time of enzyme nitrogenase on acetylene gas. From this bottle 1 ml of the gas was removed and ethylene percentage was determined using gas chromatography.

Biomass production

For biomass production Nitrogen free broth medium was inoculated with 40 g-l of glucose and 5g-l of Yeast extract. Biomass production was carried out until a constant biomass was obtained. During biomass production oxygen (pO_2) was maintained approximately 35% of air saturation and culture was feeded when the carbon source was depleted below 2 g-l. For growth pattern and culture density optical density was measured after every 6 hours at 540 nm wavelength. Biomass was measured by dry weight measurement and CFU ml^{-1}.

Pot experiment

In order to detect effect of *Azospirillum lipoferum N-29* on sugarcane variety 8071, a pot experiment was designed. 9 earthen pots with 45 cm height and 40 cm diameter were used. 3 sets each with 3 pots was prepared. Soil collected from hypersaline soils was used to conduct experiment. 25 Kg of saline soil was mixed with 10 Kg of manure and 15 Kg of garden soil in each pot and final concentration of salts were kept 4 %. Total 3 sets were prepared. Set-1. Kept as control set without treatment of sugar cane setts with *Azospirillum lipoferum N-29*. Set.2 sterile soil seeded with three sugar cane setts one in each pot soaked in *Azospirillum lipoferum N-29*. Set.3 Normal saline soil seeded with three sugar cane setts one in each pot soaked in *Azospirillum lipoferum N-29*suspension with a cell density of 4*10^{9} cells ml^{-1}.

All pots were then kept under natural environment for a period of 60,90,120 days and watered at an alternate day. At the end of 60,90,120 days incubation, plant were studied for biomass, shoot growth, diameter of stem, number of leaves and number of setts.

RESULT AND DISCUSSION

Azospirillum lipoferum N-29 was

isolated from hyper saline soil Fig.2. and identified as per bergeys manual. Fig.3 shows the growth of *Azospirillum lipoferum N-29* on Congo-red Nitrogen free agar .

Table 1. Growth pattern of *Azospirillum lipoferum N-29* in terms of OD at 540nm

Time in hours	OD_{540}nm
0	0.01
6	0.1
12	0.2
18	0.4
24	0.8
30	1.2
42	1.3
48	1.5
54	1.5
60	1.5

Table 2. Growth and nitrogenase activity of *Azospirillum lipoferum N-29* at different pH

pH	Growth (OD_{540})	Rate of nitrogen fixation(%)
3	0.02	0
4	0.02	0
5	0.02	0
6	0.04	7
7	0.16	16
8	0.14	12
9	0.14	0

Effect of environmental factors:Shake flask study

Temperature was found to be optimum 30^{O}C, pH-7.0, Agitation-140 rpm

Bioreactor study

Bioreactor study indicated that optimum growth was obtained at 30^{O}C temperature ,pH 7.0, Agitation 120rpm, maximum biomass was obtained at 48 hours. Table.1 and Fig.4 shows the growth pattern of *Azospirillum lipoferum N-29* in terms of OD_{540} nm.

Nitrogen fixation

Table-2.indicates the rate of nitrogen fixation, it was found to be maximum at pH-7.0 and temperature 30^{O}C. Table-3 indicates the effect of NaCl concentration on growth of *Azospirillum lipoferum N-29* .

The growth was maximum at 4 % NaCl and it was completely stopped at 8% NaCl

Table 3. Growth and nitrogenase activity of *Azospirillum lipoferum N-29* at different NaClnconcentrations

NaCl %	Growth (OD_{540})	Rate of nitrogen fixation(%)
1	0.1	12
2	0.1	15
3	0.2	16
4	0.5	17
5	0.4	15
6	0.4	10
7	0.4	08
8	0.3	0
9	0.3	0
10	0.3	0
11	0.2	0

Table 4. Effect of *Azospirillum lipoferum N-29* on sugarcane 8071 variety

Set No.	Treatment	Height (inch)			Diameter of stem			Number of Setts			Number of leaves		
Days		60	90	120	60	90	120	60	90	120	60	90	120
1	Control Setts without treatment	08	12	-	0.7	0.8	-	4	5	-	4	6	-
2	Setts with *Azospirillum lipoferum N-29* in sterile soil	10	15	18	1.0	1.2	2.3	6	7	10	6	9	13
3	Setts with *Azospirillum lipoferum N-29* in normal hypersaline soil	13	19	34	1.6	2.1	3.1	6	11	14	7	11	16

Fig. 1. Fully automatic bioreactor

Fig. 2. Hyper saline soil from Kolhapur district

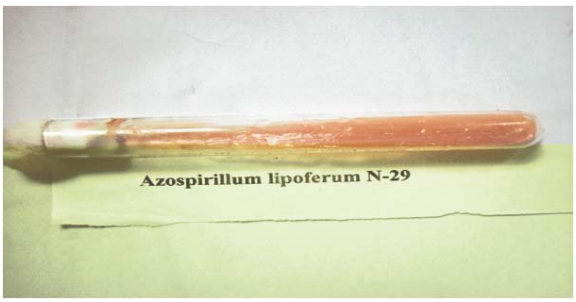

Fig. 3. Growth of *Azospirillum lipoferum N-29* on Congo-red Nitrgen free agar slant

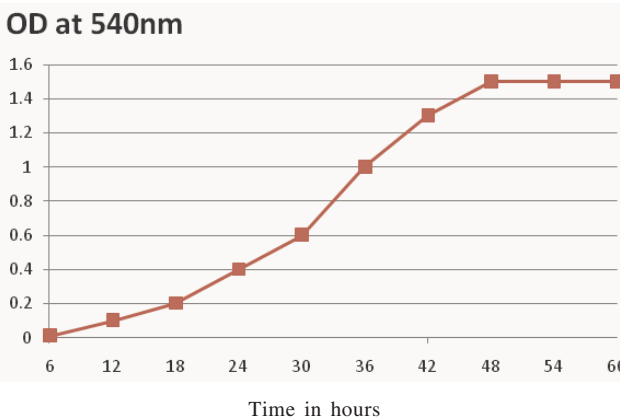

Fig. 4. Growth pattern of *Azospirillum lipoferum N-29* in terms of OD_{600}nm

concentration and there was no nitrogen fixation at 8 % NaCl concentration. Hardy et.al.(1975) reported that *Azotobacter sp.* from mangrove plants showed maximum growth at 3 % NaCl, and nitrogen fixation was maximum at 1%. My results showed maximum growth and nitrogen fixation at 4 % NaCl, it may be due to the Hyper saline origin of the isolate.

Dhanasekar et al.,(2003), Mishustin and Shilnikova (1969), Kisten et al.,(2006) studied the effect of pH and temperature on ***Azotobacter sp.*** ,their results indicated the maximum cell density at pH7.0, temperature 30°C, I also report similar findings. Brock et al.,(1994), Pena et al.,(2000) found that *Azotobacter sp.* showed maximum growth at 150rpm and at 36th hour of incubation, my results indicated the maximum growth at 140rpm and at 48th hour of incubation with a cell density of 10^9 cells ml^{-1}.

Effect on Sugarcane 8071 variety Table-4 indicates the effect of *Azospirillum lipoferum N-29* on growth of sugar cane 8071 variety.

Results indicated that there was 46% more increase in height of plant, number of setts, number of leaves, and biomass as that of control and 30% increase in height as compared to pot inoculated with sterile soil with *Azospirillum lipoferum N-29* .This indicated that there was a combine effect of organisms along with *Azospirillum lipoferum N-29* on growth.

Jayaraman and Ramiah,(1986) studied the effect of *Azospirillum*, on Rice grains they observed that there was significant increase in flowering, increased growth, dry matter, and grain, they also observed that single culture inoculation of *Azospirillum* as an biofertilizer is sufficient to fulfil the demand of nitrogen than mixed culture.Subbarao (1999;1979), Talukdar et al. (2001) also studied the effect of *Azospirillum* on growth of rice, they also found similar effect, however there are no reports in the literature on effect of *Azospirillum lipoferum N-29* on sugar cane. This might be first report on the use of Halophillic *Azospirillum lipoferum N-29* and its potential use as candidate for biofertilizer production.

CONCLUSION

Azospirillum lipoferum N-29 tolerates grows up to8%NaCl concentration, tolerates 12% NaCl up to one hour and grows optimally at 4% NaCl concentration at 30°C,at pH 7.0, and at 140rpm agitation.

It fixes maximum nitrogen at 4% NaCl salt concentration.

Process optimization is carried out in order to produce biofertilizer for saline soils.

There is a scope for use of nitrogen fixing ***Azospirillum lipoferum N-29*** as potential biofertilizer for reclamation of saline soils.

On presenting this work, i am impressed with the ability of halophillic ***Azospirillum lipoferum N-29*** to fix nitrogen at high salt concentration. Further there is lack of comparative results primarily due to difficulty in comparing results obtained, our work will encourage researcher to obtain comparative results. It may hope that my investigations may inspire others to carry out work on Halophillic nitrogen fixing ***Azospirillum lipoferum N-29*** on other aspects which have not yet studied.

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