Int.J.Curr.Microbiol.App.Sci (2018) *7*(8): 329-338

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 7 Number 08 (2018)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2018.708.037

Isolation, Morphological and Cultural Characterization of *Azospirillum* Isolated from Rhizospheric Soils of Various Non-Leguminous Crops of Ranchi Having Acidic pH

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Keywords

Azospirillum, Rhizosphere, Diazotrophic bacteria, Isolation, Characterisation, Microcyst, Capsule

Article Info

Accepted:
04 July 2018
Available Online:
10 August 2018

A B S T R A C T

Azospirillum is one of the versatile non-symbiotic, free living diazotrophic bacteria which appears to have a world-wide distribution and occurs in large number in the rhizosphere soil of a variety of grasses and cereals. The present study was carried out during *Rabi* and *Kharif* 2016-17 in the Department of Soil Science and Agricultural Chemistry, Birsa Agricultural University, Ranchi, Jharkhand. Efforts were made to screen out the presence of *Azospirillum* in rhizosphere of various non-leguminous crops and to characterize the isolates on the basis of morphological and cultural behaviours. On the basis of pH range (4.0-5.5), 54 rhizospheric soil samples were tentatively selected out of 100 samples for investigation. From the study conducted, presence of *Azospirillum* in rhizosphere of acidic pH was confirmed. Morphological characterization revealed that *Azospirillum* isolated from rhizosphere of various crops were gram negative and vibroid in shape. Cells were encapsulated i.e., were having capsules around them and formed microcyst in aged culture. Cultural characterisation revealed that colonies developed on agar slants were smooth, some of them were having raised while others were having flat elevation. Amount of growth of colonies observed were dense in 43 and thin in 11 colonies while they developed white sub-surface pellicle when grown in semi-solid Okon's media. Out of 54 colonies, 41 were white, 5 were red and rest colonies were found yellow in colour.

Introduction

Rhizosphere soil is a "hot-spot" for microbial growth and major microbial activities (Sachdev *et al.,* 2009). It is the narrow zone of soil specifically influenced by the root system (Dobbelaere *et al.,* 2003). This zone is rich in nutrients when compared with the bulk soil due to the accumulation of a variety of plant exudates such as amino acids and sugars providing a rich source of energy and nutrients for bacteria (Gray and Smith 2005). Root exudates are the substrate or fuel for the intense microbial (bacteria, fungi, algae, protozoa, nematodes and arthropods) activity within the rhizosphere. Thus it is the quantity and quality of the exudates and condition of the soil habitat that will determine the colonization potential of the rhizosphere (Lugtenberg *et al.,* 2002). *Azospirillum* spp. isolated from various geographical regions of the world is one of the best-characterized

genus of plant growth-promoting rhizobacteria (PGPR). They are known to associate with the roots of wheat, tropical grasses, maize, and other cereals (Oh *et al.,* 1999). The soil bacterium *Azospirillum* was first isolated from the Netherlands and originally named as *Spirillum lipoferum* by Beijerinck *et al.,* (1925). Later Schroder (1932) isolated from the soils in Germany and Austria. Till now, they have been isolated from the rhizosphere of many grasses and cereals all over the world, in a wide variety of terrestrial and aquatic habitats of tropical as well as in temperate climates (Yooshinan, 2001). Its occurrence in the rhizosphere varied from 1 to 10 per cent to the total rhizosphere population (Okon, 1985). *Azospirilla* are gram-negative, free-living, nitrogen-fixing rhizospheric bacteria.

They display a versatile C and N metabolism which makes them well adapted to establish in the competitive environment of the rhizosphere (Hartmann and Zimmer 1994). *Azospirillum* flocs comprise a mixture of vegetative and encysted cells surrounded by a polysaccharide-rich network (capsule), conferring advantages such as stress tolerance, extended shelf life and enhanced survivability (Sadasivan and Neyra, 1985). *Azospirillum* cells appear in two distinct forms: the slightly vibroid form (V-form) occurring in young laboratory cultures and on plant roots (Tarrand *et al.,* 1978), and the cyst form (C-form), occurring under stress or in old laboratory cultures (Sadasivan and Neyra, 1985). The C-form may be a survival structure. Occurrence of *Azospirillum* in soil is strongly pH-dependent with a pH around 7, being optimal. However, sporadic occurrence was observed even in soils with pH 4.8 (Magalhaes *et al.,* 1983). Hence the present work was undertaken with a view to screen out the presence, isolate *Azospirillum* spp. from the rhizospheres of acidic soils of Ranchi (Jharkhand) and characterise them on the basis of their morphological and cultural behaviour.

Materials and Methods

Material

Azospirillum species studied in the present investigation were isolated from soil of rhizosphere having pH range of 4.0 to 5.5 of different non-leguminous crops grown in various blocks viz., Kanke, Aangara, Nagri, Bero, Itki of Ranchi district. Details of the location, soil pH and crop grown selected for isolation of *Azospirillum* are mentioned in Table 1.

Collection of rhizosphere soil

Rhizosphere soils were collected from the rhizospheric region of the plant at the depth of 5-6 cm near the periphery of roots of different crops from different blocks of Ranchi district in plastic bags. The soil samples were preserved in refrigerator.

pH of soil samples

Soil samples were collected from 100 different locations from Ranchi districts for pH analysis. The soil samples were air dried, grounded, sieved for estimation of pH by adopting standard methods**.** Soil pH was determined in a soil water suspension of 1:2.5 w/v, stirred at regular intervals for 30 minutes using pH meter (Jackson 1973). Details of selected 54 soil samples selected for isolation of *Azospirillum* has been presented in Table 1.

Isolation of *Azospirillum* spp.

Isolation of *Azospirillum* species from rhizospheric soils was done following the methods of serial dilution. From the soil samples selected on the basis of pH range (4.0-5.5), 1 g of soil was taken and serially diluted using sterile distilled water upto 10^{-6} dilutions. One ml of diluted sample from 10^{-4} to 10^{-6} dilutions were taken and 1ml of aliquot

was inoculated in tubes containing Okon's Nfb (Nitrogen free bromothymol) semi-solid media. All the tubes were incubated at 35°C for 48 h and observed the growth by the formation of pellicles. Pellicles formation is considered as positive for *Azospirillum.*

Pellicles were streaked on petriplates containing Nfb Okon's solid media and incubated at 35°C for 48 hours. Morphologically divergent *Azospirillum* colonies were picked from the plates of dilution 10^{-5} and streaked on basal minimal salt agar medium and incubated at 35°C for 24-48 hrs.

After attaining sufficient growth, all the isolates were preserved in a refrigerator for further investigation. The colonies developed on Okon's agar medium (pH adjusted to 6.8) were transferred to slants of same medium and stored at 4^0C.

Okon's Media

Malic acid 5.00 g, KOH 4.00 g, K_2HPO_4 0.50 g, FeSO4.7H2O 0.05 g, $MnSO_4.7H_2O$ 0.01g, $MgSO_4.7H_2O$ 0.10g, NaCl 0.02 g, $CaCl_2$ 0.01g, Na_2MoO_4 0.002g, Bromothymol blue (0.5% in 95% methanol) 2.00 ml, Agar 1.8 g (semi-solid)/18 g(solid), NH_4Cl 1 g, Water 1 litre.

Purification of the culture

Purification of the culture was carried out by frequent transfer of colony of *Azospirillum* developed on Okon's agar media to seal solid nitrogen free malate medium on petriplates (Okon *et al.*, 1977) having the following constituents: K_2HPO_4 6.0 g, KH_2PO_4 4.0 g, $MgSO_4.7H_2O$ 0.2 g, NaCl 0.1 g, $CaCl_2$ 0.2 g, NH_4Cl 0.1 g, NaOH 3.0 g, Yeast extract 0.1 g, $FeCl_3$ 10.0 mg, Na_2MoO_4 20.00 mg, $MnSO_4$ 2.10 mg, H_3BO_3 2.80 mg, $Cu(NO_3)_2$ 0.04 mg, Agar 18 g, Water 1 litre

Morphological characterization

Gram reaction

Smears prepared from 48 hours old cultures were gram stained as per Huker modification (Rangaswami and Bagyaraj, 1996).

The slides were observed under compound microscope (oil immersion).

Capsule staining

Presence of capsules around the cells was observed on acetic crystal violet stained smears under oil immersion.

Microcyst formation

Stained smears of two weeks old cultures were observed under oil immersion.

Observations were recorded regarding presence of round thick walled cells as the preparation of microcysts.

Shape

Smears prepared from 48 hours old cultures were obtained and examined under oil immersion.

Cultural characterization

Different isolates of *Azospirillum* species were grown on respective standard media and their characteristic growth patterns were observed.

Serially diluted isolates of *Azospirillum* species were grown on Okon's agar medium (Okon *et al.*, 1977) in petriplates and in tubes (for agar strokes) at 35^0C for 72 hours then purification of colonies were done.

Observations were made with regard to nature of colonial growth.

Results and Discussion

In the present study, selectivity to grow on specific Nfb (Nitrogen free bromothymol) media and subsequently confirming their morphological, cultural and physiological identity with the type cultures as described in Bergey's Manual (Buchanan and Gibbons, 1974) and *Aquaspirillum* taxonomy for *Spirillum* (Kreig and Hylemon, 1976) were taken as reference for investigation and characterization of *Azospirillum* isolates. A total of 54 isolates were studied under various morphological and cultural behaviours.

Morphological characteristics

All the isolates were studied for their morphological characteristics and results are presented in Table 2. Isolates were microscopically observed for their gram reaction, cell shape, presence of capsule and microcyst formation. Results revealed that the 54 isolates were gram negative in reaction and cell shape of all the isolates was vibroid when observed under microscope. These findings were confirmed by Rosemary *et al.*, 2013 and Rasool *et al.*, 2015. All the isolates were having capability of forming microcysts. Transition into cyst-like cells were observed in older cultures of *Azospirillum* was reported by Berlman (2004). Extracellular capsule was present in all 54 isolates which is in confirmity with reports of Madi *et al.*, (1988).

Cultural characteristics

Data related to cultural characterisation has been presented in Table 2.

Colony morphology

Study revealed that colonies developed on agar slants were smooth, some of them were having raised while others were having flat elevation. Amount of growth ranged from large to slight. 43 colonies were dense and 11 were thin in amount of growth.

Azospirillum displays high degree of pleomorphism with cellular and colony variations among the species as well as within each species depending on the strain, medium composition and culture conditions as reported by Becking, 1985. The same was investigated by Rasool *et al.*, (2015).

Fig.1 White colonies of *Azospirillum*

Fig.2 Yellow colonies of *Azospirillum*

Fig.3 Red colonies of *Azospirillum*

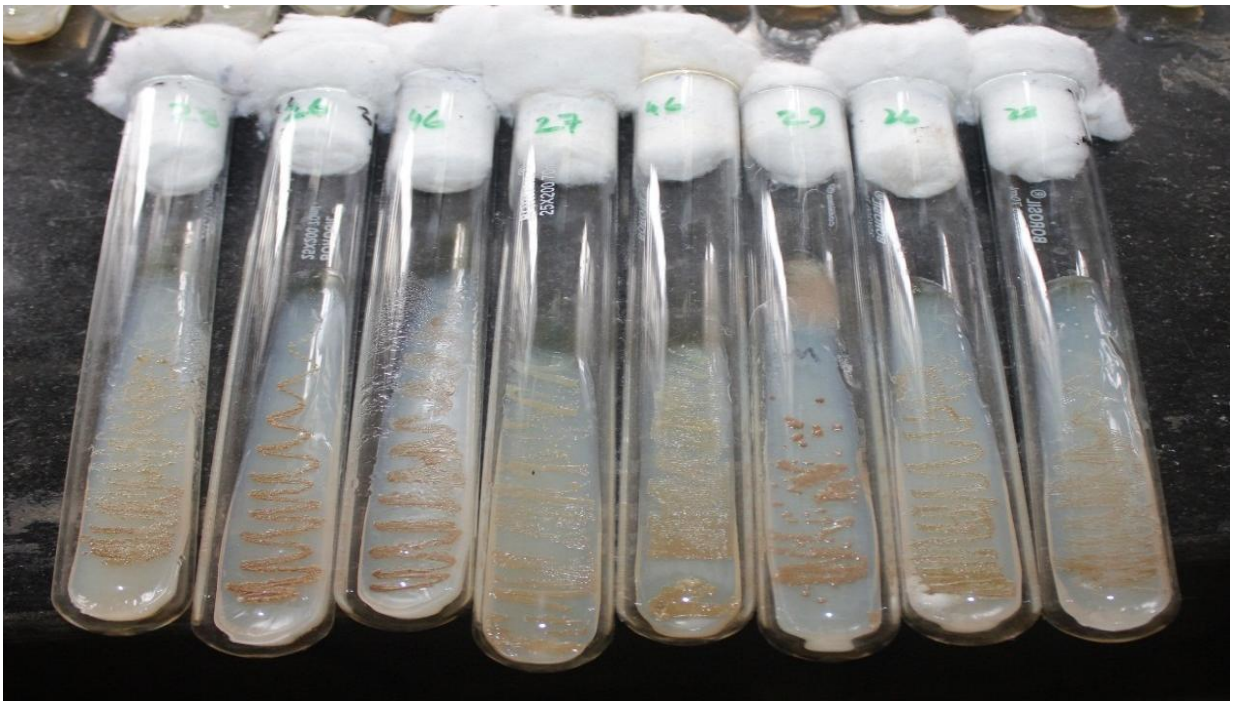

Table.1 Details of 54 rhizospheric soil samples selected for isolation of *Azospirillum*

Sl. No.	Sample. No.	Place of collection	pH of the soil	Crop (previous/ present)
1	AZM5	B.A.U Campus, SSAC, Kanke block	5.4	Maize
2	AZM6	B.A.U Campus, SSAC, Kanke block	5.3	Maize
3	AZM10	B.A.U Campus, SSAC, Kanke block	5.5	Rice
4	AZM15	B.A.U Campus, Tech park,Kanke block	5.1	Rice
5	AZM16	B.A.U Campus, Tech park, Kanke block	5.4	Ragi
6	AZM17	R.A.C Farm, W-section, Kanke block	5.3	Rice
7	AZM18	R.A.C Farm, W-section, Kanke block	5.1	Ragi
8	AZM19	R.A.C Farm, W-section, Kanke block	5.2	Rice
9.	AZM22	R.A.C Farm, W-section, Kanke block	5.5	Wheat
10.	AZM23	R.A.C Farm, W-section, Kanke block	5.4	Wheat
11.	AZM25	Chamghati, Aangara block	5.5	Rice
12.	AZM26	Chamghati, Aangara block	5.3	Rice
13.	AZM27	Chamghati, Aangara block	5.1	Rice
14.	AZM29	Chamghati, Aangara block	5.2	Rice
15.	AZM30	Chamghati, Aangara block	5.4	Rice
16.	AZM32	Chamghati, Aangara block	5.4	Rice
17.	AZM33	Chamghati, Aangara block	5.3	Rice
18.	AZM34	Chamghati, Aangara block	5.2	Rice
19.	AZM35	Chauli patra, Nagri block	4.9	Pea
20.	AZM36	Chauli patra, Nagri block	4.6	Ragi
21.	AZM 39	Itki mor, Itki block	4.7	Potato
22.	AZM 40	Itki mor, Itki block	4.6	Ragi
23.	AZM 42	Itki mor, Itki block	4.7	Mustard + Pea
24.	AZM 45	Garhgao, Itki block	4.6	Pea + Sugarcane
25.	AZM 46	Garhgao, Itki block	5.1	Wheat
26.	AZM 53	Devali, Itki block	5.4	Ragi
27.	AZM 55	Devali, Itki block	4.7	Potato
28.	AZM 56	Devali, Itki block	4.8	Maize
29.	AZM 60	Bhandra, Itki block	4.2	Maize
30.	AZM 61	Bhandra, Itki block	4.7	Onion
31	AZM 62	Karmatoli, Bero block	4.1	Pea + Potato
32.	AZM 63	Karmatoli, Bero block	4.0	Potato
33.	AZM 64	Karmatoli, Bero block	4.0	Potato
34.	AZM 65	Kalanji, Bero block	4.0	Ginger
35.	AZM 66	Didhiya, Bero block	4.1	Mustard + Pea
36.	AZM 70	Tuko, Bero block	5.1	Pea
37.	AZM 71	Tuko, Bero block	4.4	Potato
38.	AZM 75	Parepara, Bero block	4.6	Pea
39.	AZM 76	Parepara, Bero block	4.9	Potato
40.	AZM 77	Parepara, Bero block	4.7	Lentil
41.	AZM 80	Jainathpur, Bero block	4.8	Pea
42.	AZM 81	Jainathpur, Bero block	4.9	Mustard
43.	AZM 83	Bhaishmuro, Bero block	4.4	Ginger
44.	AZM 84	Bhaishmuro, Bero block	4.8	Mustard
45.	AZM 85	Bhaishmuro, Bero block	4.4	Pea
46.	AZM 87	Bhaishmuro, Bero block	4.1	Ragi
47.	AZM 88	Bhaishmuro, Bero block	4.3	Potato
48.	AZM 89	Bhaishmuro, Bero block	4.4	Potato
49.	AZM 90	Bhaishmuro, Bero block	4.1	Potato
50.	AZM 93	Kundo, Bero block	4.2	Potato
51.	AZM 94	Kundo, Bero block	4.8	Ragi
52.	AZM 95	Kundo, Bero block	4.6	Maize
53.	AZM 99	Bero, Bero block	4.3	Potato
54.	AZM 100	Bero, Bero block	4.7	Ragi

Table.2 Morphological and cultural characterization of the new isolates of *Azospirillum*

Sl. No.	*Azospirillum* isolates	Gram reaction	Capsule	Microcyst formation	Shape of cell	Solid agar media	Semi-solid media	Color of colony
1.	AZM 5	Negative	Present	+	Vibroid	Smooth, Raised, Dense	White sub-surface pellicle	White
2.	AZM 6	Negative	Present	+	Vibroid	Smooth, Raised, Dense	White sub-surface pellicle	White
3.	AZM 10	Negative	Present	+	Vibroid	Smooth, Flat, Dense	White sub-surface pellicle	White
4.	AZM 15	Negative	Present	+	Vibroid	Smooth, Flat, Dense	White sub-surface pellicle	White
5.	AZM 16	Negative	Present	+	Vibroid	Smooth, Flat, Dense	White sub-surface pellicle	White
6.	AZM 17	Negative	Present	+	Vibroid	Smooth, Flat, Dense	White sub-surface pellicle	White
7.	AZM 18	Negative	Present	+	Vibroid	Smooth, Flat, Dense	White sub-surface pellicle	White
8.	AZM 19	Negative	Present	+	Vibroid	Smooth, Flat, Dense	White sub-surface pellicle	White
9.	AZM 22	Negative	Present	+	Vibroid	Smooth, Raised, Dense	White sub-surface pellicle	White
10.	AZM 23	Negative	Present	+	Vibroid	Smooth, Raised, Dense	White sub-surface pellicle	White
11.	AZM 25	Negative	Present	+	Vibroid	Smooth, Flat, Dense	White sub-surface pellicle	White
12.	AZM 26	Negative	Present	+	Vibroid	Smooth, Raised, Dense	White sub-surface pellicle	White
13.	AZM 27	Negative	Present	+	Vibroid	Smooth, Raised, Dense	White sub-surface pellicle	White
14.	AZM 29	Negative	Present	+	Vibroid	Smooth, Raised, Dense	White sub-surface pellicle	White
15.	AZM 30	Negative	Present	+	Vibroid	Smooth, Flat, Dense	White sub-surface pellicle	White
16.	AZM 32	Negative	Present	+	Vibroid	Smooth, Flat, Dense	White sub-surface pellicle	White
17.	AZM 33	Negative	Present	+	Vibroid	Smooth, Flat, Dense	White sub-surface pellicle	White
18.	AZM 34	Negative	Present	+	Vibroid	Smooth, Flat, Dense	White sub-surface pellicle	White
19.	AZM 35	Negative	Present	+	Vibroid	Smooth, Flat, Dense	White sub-surface pellicle	White
20.	AZM 36	Negative	Present	+	Vibroid	Smooth, Flat, Dense	White sub-surface pellicle	White
21.	AZM 39	Negative	Present	+	Vibroid	Smooth, Flat, Dense	White sub-surface pellicle	White
22.	AZM 40	Negative	Present	+	Vibroid	Smooth, Flat, Dense	White sub-surface pellicle	White
23.	AZM 42	Negative	Present	+	Vibroid	Smooth, Flat, Dense	White sub-surface pellicle	White
24.	AZM 45	Negative	Present	+	Vibroid	Smooth, Flat, Dense	White sub-surface pellicle	White
25.	AZM 46	Negative	Present	+	Vibroid	Smooth, Flat, Dense	White sub-surface pellicle	White

26.	AZM 53	Negative	Present	+	Vibroid	Smooth, Flat, Dense	White sub-surface pellicle	White
27.	AZM 55	Negative	Present	+	Vibroid	Smooth, Flat, Dense	White sub-surface pellicle	White
28.	AZM 56	Negative	Present	+	Vibroid	Smooth, Flat, Dense	White sub-surface pellicle	White
29.	AZM 60	Negative	Present	+	Vibroid	Smooth, Flat, Dense	White sub-surface pellicle	White
30.	AZM 61	Negative	Present	+	Vibroid	Smooth, Flat, Dense	White sub-surface pellicle	White
31.	AZM 62	Negative	Present	+	Vibroid	Smooth, Flat, Thin	White sub-surface pellicle	Red
32.	AZM 63	Negative	Present	+	Vibroid	Smooth, Flat, Thin	White sub-surface pellicle	Red
33.	AZM 64	Negative	Present	+	Vibroid	Smooth, Flat, Thin	White sub-surface pellicle	Red
34.	AZM 65	Negative	Present	+	Vibroid	Smooth, Flat, Thin	White sub-surface pellicle	Red
35.	AZM 66	Negative	Present	+	Vibroid	Smooth, Flat, Thin	White sub-surface pellicle	Yellow
36.	AZM 70	Negative	Present	+	Vibroid	Smooth, Flat, Thin	White sub-surface pellicle	Yellow
37.	AZM 71	Negative	Present	+	Vibroid	Smooth, Flat, Thin	White sub-surface pellicle	Yellow
38.	AZM 75	Negative	Present	+	Vibroid	Smooth, Flat, Dense	White sub-surface pellicle	White
39.	AZM 76	Negative	Present	+	Vibroid	Smooth, Flat, Dense	White sub-surface pellicle	White
40.	AZM 77	Negative	Present	+	Vibroid	Smooth, Flat, Dense	White sub-surface pellicle	White
41.	AZM 80	Negative	Present	+	Vibroid	Smooth, Flat, Dense	White sub-surface pellicle	Yellow
42.	AZM 81	Negative	Present	+	Vibroid	Smooth, Flat, Dense	White sub-surface pellicle	Yellow
43.	AZM 83	Negative	Present	+	Vibroid	Smooth, Flat, Thin	White sub-surface pellicle	White
44.	AZM 84	Negative	Present	+	Vibroid	Smooth, Flat, Thin	White sub-surface pellicle	White
45.	AZM 85	Negative	Present	+	Vibroid	Smooth, Flat, Thin	White sub-surface pellicle	White
46.	AZM 87	Negative	Present	+	Vibroid	Smooth, Flat, Thin	White sub-surface pellicle	Red
47.	AZM 88	Negative	Present	+	Vibroid	Smooth, Flat, Dense	White sub-surface pellicle	White
48.	AZM 89	Negative	Present	+	Vibroid	Smooth, Flat, Dense	White sub-surface pellicle	White
49.	AZM 90	Negative	Present	+	Vibroid	Smooth, Flat, Dense	White sub-surface pellicle	White
50.	AZM 93	Negative	Present	+	Vibroid	Smooth, Flat, Dense	White sub-surface pellicle	White
51.	AZM 94	Negative	Present	+	Vibroid	Smooth, Flat, Dense	White sub-surface pellicle	White
52.	AZM 95	Negative	Present	+	Vibroid	Smooth, Flat, Dense	White sub-surface pellicle	Yellow
53.	AZM 99	Negative	Present	+	Vibroid	Smooth, Flat, Dense	White sub-surface pellicle	Yellow
54.	AZM 100	Negative	Present	+	Vibroid	Smooth, Flat, Dense	White sub-surface pellicle	Yellow

Colour production by colonies

Out of 54 colonies, colour of 41 was white, 5 were red and rest were yellow in colour (Fig. 1, 2 and 3). Tarrand *et al.*, (1978) have reported that colonies of different N_2 fixing *Azospirillum* strain showed pink, deep pink, red or yellow colour. This was due to presence of different carotenoid pigment in that isolates as reported by Baldani *et al.*, (1986) and Rasool *et al.*, (2015).

Growth in semi-solid media

Investigation revealed that all the 54 isolates of *Azospirillum* were developed as white sub-surface pellicle in semi-solid agar media. In this zone the concentration of dissolved oxygen permits optimal respiration rates without inhibiting nitrogen fixation (Day and Dobereiner, 1976).

As growth continues and more oxygen is consumed, the pellicle moves towards the surface where a dense pellicle forms. This growth pattern of *Azospirillum* in semi-solid media was reported by Hossain *et al.*, (2015). Free living diazotroph, *Azospirillum* are able to survive even at pH 4.0 i.e., under highly acidic conditions and they have wider availability in rhizospheric soils of different blocks of Ranchi district. They are negative to Gram's reaction.

They are vibroid shaped cells having capsule and are able to form thick walled microcysts during unfavourable conditions which is their adaptive mechanism to survive in adverse conditions. *Azospirillum* spp. show high degree of polymorphism in respect to their colonial patterns, elevation etc which may be attributed to their isolation from different rhizospheric and soil conditions where they were surviving. Colour development in few colonies is due to presence of carotenoid pigments.

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How to cite this article:

Narayan, R., N.C. Gupta and Shahi, D.K. 2018. Isolation, Morphological and Cultural Characterization of *Azospirillum* Isolated from Rhizospheric Soils of Various Non-Leguminous Crops of Ranchi Having Acidic pH. *Int.J.Curr.Microbiol.App.Sci.* 7(08): 329-338.
doi: https://doi.org/10.20546/ijcmas.2018.708.037