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OPTIMIZATION AND STANDARDIZATION OF CYST FORMATION IN AZOSPIRILLUM

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ABSTRACT

The objective of the study was to determine the most suitable cyst forming media in liquid conditions. Encystation in Azospirillum brasilense Cd was observed by using routine laboratory staining and microscopy. Best encystment occurred in liquid or semisolid media containing fructose (8 mM) and KNO3(0.5 mM). The encysted forms consisted of a central body filled with poly-β -hydroxybutyric acid granules, an electron-transparent intine like region,and a thick outer layer. The polymer content of the cells increased sharply and reached maximum on the 2nd day of growth followed by a gradual decline as the culture aged. Further they were subjected to heat treatment and stained to verify that these are cysts.

Keywords - Azospirillum, Liquid bioinoculant, Cyst formation, Cyst staining, Poly-β-hydroxybutyric acid (PHB)

I. INTRODUCTION

The free-living nitrogen-fixing rhizobacteria of genus *Azospirillum* lives in close association with plant roots and may have many beneficial effects on plant growth and yield of agroeconomic crops [1,2]. The cells of *Azospirillum brasilense* possess the capacity to aggregate and flocculate, and this makes it's dispersal and survival in soil easier.[3,4]. There is a direct correlation between the secretion of exopolysaccharides and flocculation in *Azospirillum brasilense* and *Azospirillum lipoferum* [4] . *Azospirilla* are particularly responsive to variations in the carbon:nitrogen (C:N) ratio. Rich media with a low C:N ratio tend to promote the growth mode, whereas high C:N ratio tend to aggregate and flocculant [4-8].

The medium for consistent induction of aggregation of *Azospirillum brasilense* cells was developed and the physical and chemical effects in various growth media was studied in detail. Growth of *Azospirillum brasilense* strain Cd in a medium with a high C:N ratio using fructose and ammonium chloride as C and N sources, respectively, respectively resulted in flocculation visible to the naked eye after 24 hours whereas no cell aggregates were formed after 72 hours of growth in a low C:N medium. The concentration of EPS produced by different strains of *Azospirillum brasilense* [9-12].

Hence the present study was based to determine the best liquid medium to produce cysts of *Azospirillum* in liquid conditions which can withstand high temperatures. The production of cysts was done by staining and measuring the polybutyrate content of the cysts [13-14].

II. MATERIAL AND METHODS Bacterial strain and growth conditions

For the present research work, *Azospirillum brasilence* standard Cd culture procured from Banaras Hindu University, Varanasi were maintained on malate medium slants with ammonium chloride (1g/lit.).

Screening of the most suitable cyst forming media for *Azospirillum* in liquid conditions. Cyst Induction in a suitable liquid culture medium.

For this experiment, *Azospirillum brasilence* standard Cd culture in the late log phase was harvested by centrifugation. The cell pellets were washed twice with sterile saline water and finally dissolved in the same. This was used as initial inoculum for all the experiments.

Cyst induction experiments such as reduced carbon source, incorporation of calcium carbonate in the growth medium and addition of organic compounds like butanol in the liquid medium failed to induce cyst induction suggesting that these compounds are not suitable for cyst induction in Cd culture. However in modified Dobereiner's medium containing 8 mM fructose and 0.5 mM potassium nitrate, the PHB formation was found to be highest (48.68 µg/ml) and cyst formation also occurred in large number as compared to control flask containing malate medium with and without ammonium chloride (Table 8). Similar reports were given by Aruna etal., 1992; Sadasivam and Neyra, 1985, 1987). They reported that by growing *Azospirillum* in the presence of fructose and potassium nitrate as the carbon and nitrogen source respectively, the synthesis of exocellular polysaccharides is promoted to induce the formation of cell aggregates and cysts. Cystic cells rich in PHB survive better than those without PHB (Assmus et al., 1995; Oliveira and de Souza; 1991; Zaady et al., 1993). Direct relationship between the cyst formation and the accumulation of PHB in *Azotobacter vinelandii* was also investigated by Stevenson and Socolofsky, (1966). They reported that in the presence of exogenous carbon source in the medium and absence of nitrogen, N-fixing bacteria such as *Azotobacter vinelandii* use the exogenous carbon faster than they can fix atmospheric nitrogen. Consequently cells accumulate larger amounts of PHB, a non-nitrogenous material however when ammonium chloride is added to the medium, the organisms convert the carbon source to nitrogenous and accumulate less PHB. They also reported that the PHB content of the cells is higher when the medium is not supplemented with nitrogen source whereas when combined nitrogen (NH_4Cl) is available in the medium, the PHB content is decreased. It is also found that bacteria grown in batch culture accumulates about 75% of PHB by their dry weight which functions as a carbon and energy source and is degraded under conditions of stress and starvation.

Thermal death resistance and thermal death time of *Azospirillum*.

Thermal death resistance temperature.

Culture subjected to different temperatures for 60 minutes were plated and incubated. Colony growth was seen when the cultures exposed to 50^0C, 55^0C and 60^0C were plated.

III. THERMAL DEATH TIME

The colonies were observed on LB plates which were subjected to heat treatment in water bath at 50^0C for 10, 20, 30, 40, 50, 60, 70, 80 and 90 minutes. Colonies were observed only in flasks subjected to heat treat ment at 50^0Cfor 50 minutes. However, no growth was observed on plates when the aliquots from flasks subjected to 60-90 minutes heat treatment were plated.

IV. RESULTS

Thermal death temperature and thermal death time of *Azospirillum* brasilense, Cd strain.

To differentiate between the vegatative and the cysts cells of *Azospirillum* the thermal death temperature and death time were determined. It was found that the log phase cultures could not withstand 50^0C for the period of 60 minutes suggesting that the thresshold temperature of vegetative cells of *Azospirillum brasilence*, Cd culture is 50^0C beyond which only cysts cells will survive in the medium.

4.1 Cyst induction in *Azospirillum*

The experiments conducted using reduced carbon source, by incorporation of organic compound such as butanol in the medium or by adding calcium carbonate in the growth medium failed to induce cyst formation as no colonies were observed when the treatments were subjected to heat at 50^0C for 60 minutes in the water bath. However, colonies were observed on the plates both before and after heat treatment at 50^0C for 60 minutes from the flasks containing DB medium with 8 mM fructose and 0.5 mM potassium nitrate as carbon and nitrogen source respectively. Similar observations were made in malate medium with and without ammonium chloride but with lower log cfu values. The log cfu values before heat treatment in DB, malate medium without ammonium chloride and malate medium with ammonium chloride were observed to be 7.19, 5.95 and 6.00 and cfu count of 6.18, 4.87 and 3.95 after heat treatment respectively (Table 8).

PHB was found to be 48.68 µg/ml in the Modified DB medium. However in the malate medium with and without ammonium chloride it was found to be 22.18 and 22.85 µg/ml respectively (Table 8).

Cyst staining with Neutral red and Light green also confirmed the above results. Under the microscope, the outer layer of vegetative cell stained greenish in colour whereas the cysts took a brownish hue (Plate 4 a and b).

Modified DB medium containing 8 mM fructose and 0.5 mM potassium nitrate and N-free malate medium (Nfb) broth amended with trehalose at 5mM and 10 mM concentration, glycerol at 5 mM and 10 mM, PVP at 1% and 2%, either separately or in combinations i.e., 5C (5mM trehalose+5 mM glycerol+1% PVP) and 10C (10 mM trehalose+10 mM glycerol+2% PVP) were used to develop a liquid formulation.

The log cfu count (4.44) and PHB content (24.61 µg/ml) of *Azospirillum brasilense*, Cd culture after six months were recorded to be maximum in DB medium containing 8 mM fructose and 0.5 mM potassium nitrate as compared to other treatments and control (N-free broth without any chemical amendment). However the results were not found to be significant when compared with the best media supporting the shelf life of *Azospirillum,* i.e., 5 mM concentration of trehalose in malate medium without ammonium chloride in which the log cfu count (3.08) and PHB content (14.55 µg/ml) were recorded after six months period. his experiment, glycerol, EDTA, PVP and trehalose were used at different concentrations in sterile water.

Table 1. Log cfu values and PHB content in DB medium, malate medium without ammonium chloride and malate medium with ammonium chloride after 48 hours of growth.

Culture Medium	Log cfu value before heat treatment	Log cfu value after heat treatment	PHB content (µg/ml) expressed as crotonic acid
DB Medium	7.19	6.18	48.68
Malate Medium without ammonium chloride	5.95	4.87	28.85
Malate Medium with ammonium chloride	6.00	3.95	22.18
CD at 5%	**0.012**	**0.028**	**1.865**

Table 2. Log cfu values and PHB content of *Azospirillum* in different liquid culture media at monthly intervals.

No. of days	30 day s		60 day s		90 day s		120 day s		150 day s		180 day s	
Treatments	**Log cfu**	**PHB (µg/ml**	**Log cfu**	**PHB (µg/ml**	**Log cfu**	**PHB (µg/ml**	**Log cfu**	**PHB (µg/ml**	**Log cfu**	**PHB (µg/ml**	**Log cfu**	**PHB (µg/ml**
D B Medium	7.50	58.95	6.55	47.40	6.29	41.55	4.91	26.20	4.59	25.30	4.44	24.61
5 mM Trehalose.	7.28	56.45	6.45	47.52	5.80	41.40	4.48	24.82	4.17	23.35	3.08	14.55
10 mM Trehalose	7.42	57.65	6.53	49.90	6.17	40.46	4.51	24.82	4.34	24.10	3.25	15.05
5 mM Glycerol	7.21	56.55	6.37	47.40	5.07	26.77	3.90	20.35	3.46	16.53	3.05	14.31
10 mM Glycerol	7.46	57.55	6.45	45.80	5.15	28.30	4.35	24.05	3.66	19.25	2.02	13.15
1 % PVP	7.39	56.75	6.27	41.96	4.99	26.17	3.93	19.60	2.74	13.30	0.00	0.00
2 % PVP	7.18	55.02	6.60	47.70	5.71	31.45	4.27	24.04	3.20	16.89	2.05	10.50
5C(5mMtre+ 5mMgly+1 PVP)	6.38	45.65	5.37	34.76	5.07	25.80	2.24	11.41	0.00	0.00	0.00	0.00
10C(10mMtre+ 10mMgly+2 % PVP)	6.41	49.50	5.44	35.55	4.88	28.25	2.60	13.80	0.00	0.00	0.00	0.00
Control	6.13	40.75	4.28	24.00	2.45	13.25	0.00	0.00	0.00	0.00	0.00	0.00
CD at 5 %	**0.278**	**3.632**	**0.759**	**5.249**	**0.413**	**7.306**	**0.457**	**2.743**	**0.538**	**3.519**	**0.407**	**1.695**

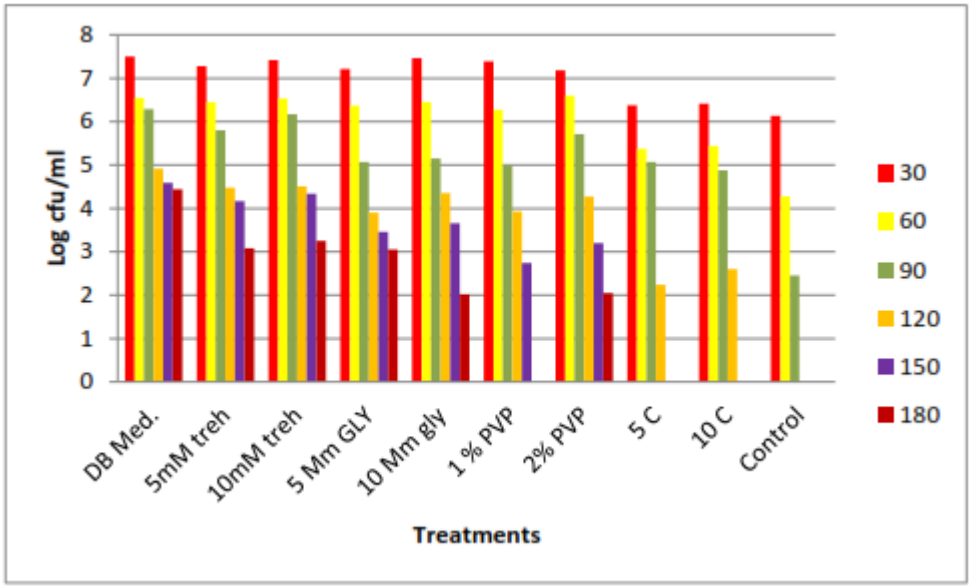

Figure 1(a) Log cfu values of DB medium and malate medium without ammonium chloride containing osmoprotectants at different concentrations.

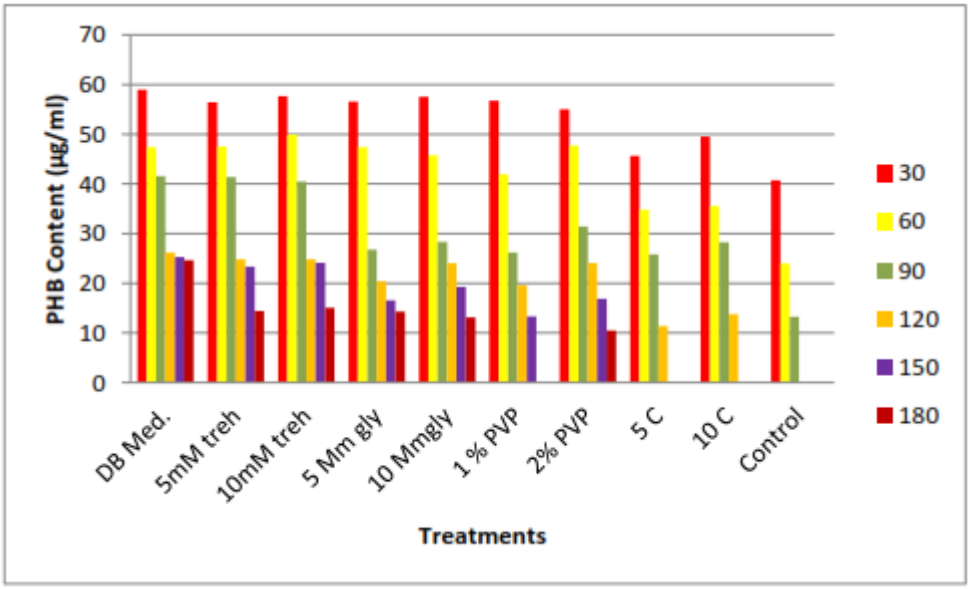

Figure 1(b). PHB content of cells in µg/ml in DB medium and malate medium without ammonium chloride containing osmoprotectants at different concentrations.

Note- 5C denotes (5mM treh. + 5mM gly. + 1% PVP) in N-free malate medium.
10 C denotes (10mM treh. + 10mM gly. + 2% PVP) in N-free malate medium.

V. DISCUSSION

In the study period of three months, among all the osmoprotectants used viz., glycerol (1M, 2M and 3M); EDTA (100 µM, 200 µM and 400 µM); trehalose (50 µM, 100 µM and 200 µM) and PVP (100 µM, 200 µM and 400 µM), the highest cfu values were obtained at 50 µM trehalose concentration in sterile water

(Table 6). The possible reason for the maintenance of higher population in DB medium may be attributed to the higher amount of PHB content in the medium which serves as an electron and carbon sink and also functions as an energy source by degrading under conditions of stress and starvation. Tal and Okon, (1985) also reported that the ability of *Azospirillum brasilense* to tolerate various stress was affected by the lack of PHB accumulation which resulted in lower stress endurance

The role of PHB as an intracellular energy and carbon storage compound, which can enhance the survival during storage of bioinoculants, under suboptimal conditions, such as lack of moisture, heat stress and limited available carbon source has been examined in various other bacteria. In field experiments carried out in Mexico with maize and wheat, better and more consistent results were obtained when peat inoculants prepared with PHB-rich *Azospirillum* cells were used by Fallik and Okon, 1996 and Dobbelaere et al., 2001 respectively. Hense it can be concluded that PHB rich formulations have better shelf life as compared to other formulations. Another reason that makes PHB rich formulations more suitable as bioinoculants is the presence of nitrogenase enzyme, a prerequsite for nitrogen fixation activity, which is much more in PHB-rich bacteria (Paper and Werner, 1980 and 1982; Tal and Okon (1985). Moreover the possible role of PHB in *Azospirillum* may be in the regulation of the oxygen environment for nitrogen fixation and also in permitting the cells to increase their oxidative activity (respiratory protection) in the absence of exogenous substrate (Tal and Okon 1965). All this accounts for the better suitability of DB medium for the liquid formulations in which cysts are formed in maximum number and accumulate maximum amount of PHB which serves as a source of energy under stress conditions.

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REFERENCES

1. S.P. Wani, and K.K. Lee, (1992). Role of biofertilizers in upland crop production. In: Fertilizers of Organic Manures, Recycle Wastes and Biofertilizers.(ed)
2. Role of Azospirillum in the improvement of crop production and plant nutrition. In: Nitrogen. Proc. Indian Natl. Sci. Acad. B 59: 315-324.
3. Umali-Garcia M, Hubbell DH, Gaskins MH, Dazzo FB. Association of Azospirillum with grass roots. Appl Environ Microbiol. 1980 Jan; 39(1) : 219–226.
4. Vela GR, Wyss O. Improved Stain For Visualization of Azotobacter encystment. J Bacteriol. 1964 Feb;87:476–477
5. Beaman BL, Jackson LE, Shankel DM. Formation of multiple central bodies in giant cysts of Azotobacter vinelandii. J Bacteriol. 1968 Jul;96(1):266–269.
6. Cagle GD, Vela GR. Giant cysts and cysts with multiple central bodies in Azotobacter vinelandii. J Bacteriol. 1971Jul;107(1):315–319.

7. Eskew DL, Focht DD, Ting IP. Nitrogen fixation, denitrification, and pleomorphic growth in a highly pigmentedSpirillum lipoferum. Appl Environ Microbiol. 1977 Nov;34(5):582–585.
8. Pope LM, Wyss O. Outer layers of the Azotobacter vinelandii cyst. J Bacteriol. 1970 Apr;102(1):234–239.
9. Sadoff HL. Encystment and germination in Azotobacter vinelandii. Bacteriol Rev. 1975 Dec;39(4):516–539.
10. Socolofsky MD, Wyss O. Cysts of Azotobacter. J Bacteriol. 1961 Jun;81(6):946–954.
11. Sadasivam, L. and Neyra, C.A. (1985). Flocculation in Azospirillum brasilense and Azospirillum lipoferum : exopolysaccharides and cyst formation. J. Bacteriol., 163 : 716-723.
12. Sadasivam, L. and Neyra, C.A. (1985). Cyst production and brown pigment formation in aging cultures of Azospirillum brasilense ATCC 29145. J. Bacteriol., 169 : 1670-1677.
13. Arunakumari, A., Lamm, R.B. and Neyra-Estens,C.A. (1992) Changes in cell surface properties of Azospirilla in relation to cell pleomorphism and aggregation. Symbiosis 13: 291-305.
14. Burdman, S., Jurkevitch, E., Schwartsburd, B., Hampel, M. and Okon, Y. (1998). Aggregation in Azospirillum brasilense; effects of chemical and physical factors and involvement of extracellular components. Microbiology 144: 1989-1999
15. Neyra, C.A., Atkinson, A. and Olubayi, O. (1995) Coaggregation of Azospirillum with other bacteria: basis for functional diversity. NATO ASI Ser.G 37: 429-439
16. Lamm, R.B. and C.A. Neyra. (1981). Characterisation and cyst production of Azospirilla isolated from selected grasses growing in New Jersey and New York. Can. J. Microbiol., 27 : 1320-1325.
17. Law J.H. and Slepecky, R.A. (1961). Assay of poly-beta-hydroxybutyric acid. J. Bacteriol., 82 : 33-36.
18. Layne, J.S. and Johnson, E.J. (1964). Natural factors involved in the induction of cyst formation in Azotobacter. J.Bacteriol., 87 : 684-689.
19. Narula, N., Suneja, S. and Vasudeva, M. (1998). Encystment in Azotobacter and Azospirillum. Indian J. Microbiol.,38 : 1-8.
20. Narula, N. and Kleiner, D. (1992). Encystation in strains of Azospirillum. Annals of Biology, 8 (2) : 188-193.
21. Stevenson, L.H. and Socolofsky, M.D. (1966). Cyst formation and polybetahydroxybutyric acid accumulation in Azotobacter. J. Bacteriol., 91 : 304-310.
22. Stevenson, L.H. and Socolofsky, M.D. (1972). Encystation of Azotobacter vinelandii in liquid cultures. Antonie van Leeuwenhoek J. Microbiol. Serol., 38 : 605-610.
23. Tal. S., and Y. Okon (1985). Production of the reserve material polybetahydroxybutyrate and its function in Azospirllum brasilense Cd. Can. J. Microbiol., 31 : 608-613.
24. Ward, A. C., Rowley, B.I. and E. A. Dawes. (1977). Effect of oxygen and nitrogen limitation on poly-β- hydroxybutyrate biosynthesis in ammonium-grown Azotobacter beijernckii. J. Gen Microbiol., 102 : 61-68.
25. Westby, C. A., Cutshall, D.S. and G. V. Vigil. (1983) Metabolism of various carbon sources by Azospirillum brasilense. J. Bacteriol., 156 : 1369-1372.

ISBN: 978-93-86171-38-2

26. Das, A. and A.K. Mishra. (1983). Utilization of fructose by Azospirillum brasilence. Can. J. Microbiol, 29 : 1213- 1217. Doudoroff, M. and Stanier, R. Y.. (1959). Role of poly-β-hydroxybutyric acid in the assimilation of organic carbon by bacteria. Nature, 183 : 1440-1442.
27. Goebel, E. M. and Kreig, N. R. (1984). Fructose catabolism in Azospirillum brasilense and Azospirillum lipoferum. J. Bacteriol. 159 : 86-92.
28. Haward, A.C., Forsyth, W.G.C. and Roberts, J.B. (1959). Synthesis and break down of poly-β-hydroxybutyric acid by bacteria. J. Gen. Microbiol. 20 : 510-518.
29. Haward, A.C. (1959). Poly-β-hydroxybutyrate inclusions in the classification of aerobic gram negative bacteria. J. Gen. Microbiol. 21 : 550-556.
30. Eklund, C., Pope, L. M. and Wyss, O.. (1966). Relationship of encapsulation and encystment in Azotobacter. J. Bacteriol. 92 : 1828-1830.