

Original Research Article

<https://doi.org/10.20546/ijcmas.2021.1002.102>

Morphological and Biochemical Characterization – A Comparative Analysis of Non-commercial and Commercial Plant Growth Promoting Microorganisms

A. Muthukumar^{1*}, G. M. Sandhya¹ and G. Dakshayini²

¹Division of Forest Protection, Institute of Wood Science and Technology, Karnataka, India

²Department of Agricultural Microbiology, UAS, GKVK, Bengaluru, Karnataka, India

*Corresponding author

ABSTRACT

Keywords

Bio-fertilizers, Bio-inoculants, P Solubilizer, Nitrogen Fixer, Potash mobilizer

Article Info

Accepted:

10 January 2021

Available Online:

10 February 2021

Bio-fertilizers are cultured microorganisms with suitable carrier material used in broad-spectrum for enhancing the plant growth and productivity. The critical input in Bio-fertilizers is as such the microorganisms. In our study the bio-fertilizer samples of IFGTB (Non-commercial) and IIHR (Commercial) institutions were studied for their Viability, Cultural, Morphological and Biochemical characterization. Serial dilution technique was done to test the viability of Liquid formulation of N-fixers, Phosphobacteria and Potash Mobilizer microorganisms. The results showed that, viability of *Azospirillum*, Phosphobacteria and Potash mobilizer were recorded with highest number of cells in IFGTB developed bio-fertilizers, i.e., $\log_{10}5.43$, $\log_{10}6.13$ and $\log_{10}6.65$ respectively when compared to commercially obtained samples at 10^{-7} dilution and these microorganisms (PGPRs) were studied for their cultural and morphological characters like, size, shape, motility etc. in addition to biochemical characterization. The results showed that, *Azospirillum*, *Azotobacter*, Phosphobacteria and potash mobilizer showed positive results for Amylase production, Catalase and Citrate production tests etc. and negative results for oxidation and fermentation of glucose and peptone utilization.

Introduction

The term bio-fertilizer means the product containing carrier based (solid or liquid) living microorganisms which are agriculturally useful in terms of nitrogen-fixation, phosphorus solubilization or nutrient mobilization to increase the productivity of the soil and/or crop (Malusa and Vassilev, 2014). *Azospirillum* and *Azotobacter* are used as Nitrogen-fixing microorganisms,

Phosphobacteria plays a major role in phosphate solubilization and potash mobilizers converts non-exchangeable K into exchangeable K in the soil and supplies to the plants. The success of microbial inoculants depends on several factors, of which carrier material plays the most important role. The carrier refers to a solid, semisolid or liquid substance, which can sustain a given number of particular bacteria for a given period of time (Khavazi and Rejali, 2000). Liquid

formulations facilitate long shelf life (up to 2 years), minimum contamination, carrier free activity, handling comfort, storage and transport convenience, easy quality control, enhanced export potentials and are preferred by the farmer community as well as manufacturers (Pindi and Sathyanarayan, 2012).

Biochemical tests are the most important methods for microbial identification by differentiating them on the basis of biochemical activities. The differences in protein and fat metabolism, carbohydrate metabolism, enzyme production, compound utilization ability are some factors that aid in bacterial identification. In India most of the microbiological laboratories are depending on the conventional methods to identify and study the diversity of bacteria. The conventional method includes phenotypic characterization (colony morphologies, gram staining, *etc.*) biochemical characterization (nutrient requirements- sugars, enzymatic activities, and metabolic activities).

The idea behind this study was to compare the characters of micro-organisms of Non-commercial and Commercial PGPRs, which is necessary to experiment these PGPR's for their efficacy in field and nurseries.

Materials and Methods

These experiments of Viability, Cultural and Biochemical tests were conducted in the division of Forest Protection, Institute of Wood Science and Technology, Bengaluru.

Collection of liquid bio-fertilizer samples and their maintenance

Liquid formulation of *Azospirillum*, *Azotobacter*, Phosphobacteria and Potash mobilizer were procured from the Institute of Forest Genetics and Tree Breeding (IFGTB),

Coimbatore, Tamil Nadu, and from authorized dealer of product of Indian Institute of Horticulture Research (IIHR), Hesaragatta, Bengaluru. Pure cultures are prepared by using Hi-media of *Azospirillum*, *Azotobacter* mannitol agar (Subba Rao, 1977), Pikovskaya's agar and Aleksandrow's medium (Prajapathi *et al.*, 2012 and Bashan *et al.*, 2013). These cultures were stored as agar slants at 4 °C in a refrigerator and used as mother culture for further work.

Viability studies

Enumeration of viable cells was done through a standard plate count method. One gram sample was aseptically transferred from each formulation into 9 ml water blank and serially diluted to a dilution of 10^{-5} , 10^{-6} and 10^{-7} . The dilutions were further plated in respective agar media of constituent microorganisms along with replication and incubated at ± 30 °C. After 36-48 hours of incubation plates were observed for the growth of colonies and number of colonies was counted (Chao and Alexander, 1984).

Morphological characterization

Microorganisms were identified based on cultural, morphological and biochemical characteristics as per Bergey's Manual of Systematic Bacteriology (Second edition, 2009). Motility, cell shape, color, texture and Gram stain were described for morphological characterization of microorganisms.

Biochemical tests

Media used for biochemical tests

Nutrient agar, Skim milk agar, Trypticase soy agar, SIM agar, OF glucose agar and Urea agar, Simmon's citrate agar, Nitrate agar media were prepared and autoclaved at 121°C for 30 minutes at 15 psi. Broth cultures of

Azospirillum, *Azotobacter*, Phosphobacteria and Potash Mobilizer were inoculated into the agar slants by using sterilized inoculation loop. Inoculated agar slants were kept for incubation at 35 °C for 24-48 hours. Observations were recorded as positive or negative for the particular organism reaction by seeing visible changes in the media color and enzymatic reactions.

Amylase production test

The ability of isolates to hydrolyse was examined by the procedure of Eckfod (1927). Test tubes containing two per cent starch agar were inoculated with test cultures and incubated at 30 °C for three days. After incubation the plates were flooded with Lugol's iodine solution and allowed for 15-20 minutes.

Casein hydrolysis

Skim milk agar slants were inoculated with the bacterial cultures and incubated the slants for 24-48 hours at 37 °C. After that slants were observed for any clearing around the line of growth.

Catalase test (Blezevic and Ederer, 1975)

Nutrient agar slants were inoculated with test culture and were incubated at 30 °C for 24 hours. After incubation, the tubes were flooded with one ml of 3 per cent hydrogen peroxide and observed for the production of gas bubbles. The occurrence of the gas bubble was taken as positive for catalase activity.

Hydrogen sulfide production test (Cowan and Steel, 1970)

Tubes containing SIM agar were sterilized. The sterilized tubes were stabbed with the test cultures. The tubes were incubated for 48 hours at 28±2°C. after incubation the

development of black color along with the line of the stab was noted and considered as positive for the test.

Oxidation and fermentation of glucose

Oxidation and fermentation glucose agar medium slants were inoculated with test cultures and were incubated at 35 °C for 24-48 hours.

Urease test (James and Natalie, 1992)

The urease activities of the bacterial isolates were determined by inoculating the cultures of five ml of pre-sterilized urea broth containing phenol red as pH indicator. The tubes were incubated for 24-48 hours at 30 °C. The formation of the dark pink color was taken as positive for urease activity.

Citrate utilization test (James and Natalie, 1992)

Bacterial isolates were streaked on citrate agar slants containing bromothymol blue as an indicator and incubated overnight at room temperature. After incubation slants were observed for the formation of blue color in the medium and it was taken as positive for the citrate utilization test

Nitrate reduction

Nitrate agar slants were inoculated with bacterial cultures and incubate the tubes at the optimal temperature 37 °C for 24 hours. Then observe for the reaction (color development).

Media used for biochemical tests

Nutrient agar, Skim milk agar, Trypticase soy agar, SIM agar, OF glucose agar and Urea agar, Simmon's citrate agar, Nitrate agar media were prepared and autoclaved at 121 °C for 30 minutes at 15 psi. Broth cultures of

Azospirillum, *Azotobacter*, Phosphobacteria and Potash Mobilizer were inoculated into the agar slants by using sterilized inoculation loop. Inoculated agar slants were kept for incubation at 35 °C for 24-48 hours. Observations were recorded as positive or negative for the particular organism reaction by seeing visible changes in the media color and enzymatic reactions.

Results and Discussion

Viability test

Observation recorded for the viability of liquid formulation of *Azospirillum*, *Azotobacter*, Phosphobacteria and Potash mobilizer microorganisms are presented in the Table 1.

Azospirillum obtained from IFGTB recorded more number of viable cells ($\log_{10}5.43$ at 10^{-7} dilution) compared to the commercially obtained formulation ($\log_{10}5.00$) and more number of *Azotobacter* viable cells ($\log_{10}5.24$) were found in commercially

obtained bio-fertilizers when compared to that of $\log_{10}5.02$ for IFGTB samples at 10^{-7} dilution.

Highest number of Phosphobacteria was recorded in IFGTB bio-fertilizer at 10^{-7} dilution *i.e.*, $\log_{10}6.13$ when compared to commercially obtained formulation ($\log_{10}6.11$). Potash mobilizer liquid formulation was recorded highest number of cells in IFGTB developed bio-fertilizers ($\log_{10}6.65$) when compared to that of $\log_{10}5.12$ in commercially obtained samples at 10^{-7} dilution.

Results are in conformity with the Gupta and Sahu, 2017 where viable cell count of *Azospirillum* ranges 5×10^7 CFU/g of carrier material or 1×10^8 cell /ml of liquid. Similar results were obtained from Rama *et al.*, 2015; Sridhar *et al.*, 2004; Dayamani, 2010; Veleneni and Brahma Prakash, 2011 that the liquid formulation also provides longer shelf life and higher number of CFU per ml. Findings shows that approximately 10^8 cells per ml by the end of one year.

Table.1 Viability of microbial population in IFGTB and Commercially procured liquid bio-fertilizer formulation under ambient room temperature

Microorganisms	Population density (log ₁₀ CFU)		
	10-5	10-6	10-7
I. IFGTB Products			
<i>Azospirillum</i>	8.12	6.69	5.43
<i>Azotobacter</i>	7.98	6.10	5.02
<i>Phosphobacteria</i>	8.23	6.91	6.13
Potash mobilizer	8.11	7.14	6.65
II. Commercial Products			
Lipoterum	7.89	6.17	5.00
Tropicalis	8.21	6.33	5.24
Aryabhatai	7.80	7.03	6.11
Taiwanensis	8.41	7.70	5.12

Table.2 Morphological characteristics of bio-fertilizer samples

Microorganism	Size (diameter)	Shape	Color	Texture	Elevation	Edge	Motility	Gram staining reaction
Azospirillum	≤ 2.0 mm	Straight rods	Pink with congo red	rigid	flat	undulating	Motile	Gram negative
Azotobacter	≤ 5.0 mm	Flat to circular	Dark brown/green	slimy	convex	undulating	Motile	Gram negative
Phosphobacteria	≤ 2.0 mm	Rod-like	Fluorescent greenish-yellow	Paste-like	flat	undulating	Motile	Gram positive
Potash mobilizer	≤ 2.0 mm	Short rods	white	mucous	convex	undulating	Motile	Gram negative

Table.3 Biochemical characterization of bio-fertilizer samples

Biochemical tests	Microorganisms			
	Azospirillum	Azotobacter	Phosphobacteria	Potash mobilizer
Amylase production	+	+	+	+
Casein hydrolysis	-	-	+	-
Catalase test	+	+	+	+
Hydrogen sulfide production test	+	+	-	+
Oxidation and fermentation of glucose	-	-	-	-
Urease test	+	+	+	-
Citrate test	+	+	+	+
Nitrate reduction	+	+	+	-
Microorganisms confirmed	<i>Azospirillum sp.</i>	<i>Azotobacter sp.</i>	<i>Pseudomonas sp.</i>	<i>Bacillus sp.</i>

Morphological characterization

Morphological characters *viz.* size, shape, color, texture, elevation, edge, motility and Gram staining reactions were done for all the procured plant growth promoting microorganisms.

Azospirillum cells were found be ≤ 2.0 mm in diameter, straight rods and motile in nature. These are the gram negative bacteria and pink with Congo red in color. Microscopic examination of *Azotobacter* revealed that they were dark brown/green colour, ≤ 5.0 mm

diameter in size, gram negative and motile in nature. Phosphobacteria cells size found to be ≤ 2.0 mm in diameter, rod-like structures, motile in nature and they were gram positive, looks like fluorescent greenish-yellow color under microscope. And potash mobilizers observed to be short rods with ≤ 2.0 mm diameter in size. These are the gram negative cells and white in color and motile in nature (Table 2).

These characteristics of microorganisms were comparable with the studies of Hegazi *et al.*, 1998 where they stated that rigid deep red

colour colonies of *Azospirillum* clearly appeared on agar plates supplemented with Congo red. *Azotobacter* sp. easily distinguished by deep brown pigmented and rather confined colonies. *Bacillus* sp. grew well and the colonies were tear-like, slimy and transparent. Bacterial colonies appeared on the culture medium showed similar morphological characters as that of *Azotobacter* sp. such as cream colored colonies, Gram negative bacilli, large and short, in pairs or in chains (Rueda *et al.*, 2016).

The results were similar to the results of Andriani *et al.*, 2017, they stated that *Bacillus megaterium* are concave, smooth and milk white. Our results were parallel with the studies of Bhattacharyya *et al.*, 2016 they stated that Potash mobilizing isolates were gram negative, rod-shaped, motile and creamish in appearance.

Biochemical tests

Biochemical tests *viz.*, Amylase production, Casein hydrolysis, Catalyst test, Hydrogen sulfide production, Oxidation and fermentation of glucose, Urease test, Citrate test and Nitrate reduction test were carried out to all the PGPRs to identify the microorganisms (Table 3).

Azospirillum and *Azotobacter* showed positive results for Amylase production, catalase, hydrogen sulphide, citrate and Nitrate reduction test. And negative results for Casein hydrolysis and Oxidation and fermentation of glucose tests. These results are comparable with the studies of Sulaiman *et al.*, 2019 and Shubha *et al.*, 2014.

Citrate was utilized as a sole carbon source by *Azotobacter* microorganism and showed positive results for Nitrate reduction test. These studies were parallel with the studies of Apte and Shende, 1981.

Phosphobacteria hydrolyses the casein, utilizes citrate and reduces the nitrate and shows negative result for Hydrogen sulfide and OF glucose tests. The results obtained were parallel with the studies of Patel *et al.*, 2016.

Biochemical characterization of Potash mobilizing bacteria shows positive results for Amylase production, catalase, and citrate utilization test. And negative results for casein hydrolysis, Oxidation and fermentation of glucose, urease and nitrate reduction tests. The same outcomes were observed from the research findings of Kammar *et al.*, 2016.

In conclusion according to the morphological, cultural and biochemical characteristics it is confirmed that the bio-inoculants obtained from IFGTB and of Commercial ones were *Azospirillum* sp., *Azotobacter* sp., *Pseudomonas* sp. (*Phosphobacteria*) and *Bacillus* sp. (Potash mobilizer). This selected Non-commercial PGPRs are found to be reliable for its application or use to enhance plant growth.

Conflict of interest

The research findings in this article do not have any conflict of interest.

Acknowledgement

We thank our HOD, Division of Forest Protection, and Institute of Wood Science and Technology, Bengaluru, Karnataka, India, to provide all the facilities to carry out the work.

References

Andriani, Y., Rochima, E., Safitri, R., and Rahayuningsih, S. R., 2017, Characterization of *Bacillus megaterium* and *Bacillus mycoides* Bacteria as Probiotic Bacteria in Fish and Shrimp Feed, ICSAFS Conference

- Proceedings 2nd International Conference on Sustainable Agriculture and Food Security.
- Apte, R. and Shende, S. T., 1981, Studies on *Azotobacter chroococcum* I. Morphological, Biochemical and Physiological Characteristics of *Azotobacter chroococcum*, *Zbl. Bakt. II. Abt.* 136: 548-554.
- Bashan, Y., Kamnev, A. A., and De-Bashan, L. E., 2013, A proposal for isolating and testing phosphate-solubilizing bacteria that enhance plant growth. *Biol. Fertil. Soils.* 49:1–2.
- Bhattacharyya, P. N., Dutta, P., Madhab, M., Phukan, I. K., Sarmah, S. R. and Pathak, S. K., 2016, Isolation of potash mobilizing microorganisms in tea soil and evaluation of their efficiency in potash nutrition in tea: a novel approach, *Two and a Bud*, 63(1): 8-12.
- Blezevic, D. J. and Ederer, G. M., 1975, Principles of biochemical tests in diagnostic microbiology. Wiley and Company, New York: 13-45.
- Chao, W. L. and Alexander, M., 1984, Mineral soils as carriers for Rhizobium inoculants. *Appl. Environ. Microb.* 47: 947.
- Cowan, S. T. and Steel, K. J., 1970, Manual for the identification of medical bacteria. *Lowe and Brydon*, London.
- Dayamani, K. J., 2010, formulization and determination of effectiveness of liquid inoculants of plant growth promoting rhizobacteria, *PhD thesis, Univ. Agril. Sci. Blr.*
- Eckford, M. D., 1927, Thermophilic bacteria in milk. *Am. J. Hyg.*, 7: 200-201.
- Gupta, A. and Sahu, P. K., 2017, Azospirillum: Bioformulations, Product Quality and Survivability, *Int. J. Res. Appl. Sci. & Eng. Tech*, ISSN: 2321-9653.
- Hegazi N.A., Hamza M.A., Osman A., Ali S., Sedik M.Z., Fayez M. (1998) Modified combined carbon N-deficient medium for isolation, enumeration and biomass production of diazotrophs. In: Malik K.A., Mirza M.S., Ladha J.K. (eds) Nitrogen Fixation with Non-Legumes. Developments in Plant and Soil Sciences, vol 79. Springer, Dordrecht. https://doi.org/10.1007/978-94-011-5232-7_28
- James, G. C. and Natalie, S., 1992, Microbiology and laboratory manual. Rockland community college, Suffern, New York, Third edition. The Benjamin/ Cummings Publishing Co. Inc., Redwood city California.
- Kammar, S. C., Gundappagol, R. C., Santosh, G. P., Shubha, S., and Ravi, M. V., 2016, Isolation, Morphological and Biochemical Characterization of Potassium Solubilizing Bacteria (KSB) Isolated from Northern Part of Karnataka, *J. Pure and Appl. Microbio.*, 10(1): 471-477.
- Khavazi, K. and Rejali, F., 2000, Perlite as a carrier for soybean inoculant. Paper presented in Xth International colloquium for the optimization of plant nutrition. Cairo, Egypt from 12-14 Feb. 1999.
- Malusa, E. and Vassilev, N., 2014, A contribution to set a legal framework for bio-fertilizers. *Appl. Microbiol. Biotechnol.*, 98(15): 6599–6607.
- Patel, G., Singh, S., Saxena, S. K., and Kaur, K. J., 2016, Isolation, Biochemical Characterization and Production of Bio-fertilizer from *Bacillus megaterium*, *Int. J. Life. Sci. Res.*, 2(6): 749-752.
- Pindi, P. K., and Sathyanarayan, S. D. V., 2012, Liquid Microbial Consortium - A Potential Tool for Sustainable Soil Health, *J. Bio-fertilizers & Biopesticides.* 3:4.
- Prajapathi, K. and Modi, H. A., 2012, Isolation and characterization of potassium solubilizing bacteria from ceramic industry soil. *CIB Tech J.*

- Microbiol.* 1: 8-14.
- Rama, A. A., Mahadevaswamy, K., Naik, N. and Kuruber, A. R., 2015. Influence of Efficient Strain of PSB on Growth and Yield of Maize (*Zea mays* L.) Under Black Cotton Soil Condition. *Journal of Pure and Applied Microbiology*, 9(2), pp.1179-1184.
- Rueda, D., Valencia, G., Soria, B. N., RUEDA, B., Manjunatha, B., Rajesh R. Kundapur and Selvanayagam, M., 2016, Effect of *Azospirillum* spp. and *Azotobacter* spp. on the growth and yield of strawberry (*Fragaria vesca*) in hydroponic system under different nitrogen levels, *J. Appl. Pharm. Sci.*, 6(01): 048-054.
- Shubha, S., V. P. Savalagi, and A. Anantha Rama and GB Santoshagowda., 2014, Occurrence and microbiological characteristics of azospirillum strains associated with wheat rhizosphere and endorhizosphere. *Biochemical and Cellular Archives*, 14.1: 81-88.
- Sridhar, V., Brahmaaprakash, G. P. and Hegde, S. V., 2004, Development of a liquid inoculant using osmoprotectants for Phosphate solubilizing bacteria. *Karnataka J. Agri. Sci.* 17: 251–257.
- Subba Rao, N. S., 1977, Soil Microorganisms and Plant Growth, Oxford and IBH Publishing Co., New Delhi.
- Sulaiman, K. H., Al-Barakah, F. N., Assaeed, A. M. and Dar, B. A. M., 2019, Isolation and identification of *Azospirillum* and *Azotobacter* species from *Acacia* spp. at riyadh, Saudi Arabia, *Bangladesh J. Bot.* 48(2): 239-251.
- Velineni, S. and Brahmaaprakash, G. P., 2011, Survival and phosphate solubilizing ability of *Bacillus megaterium* in liquid inoculants under high temperature and desiccation stress. *J. Agr. Sci. Tech.*, 13: 795-802.
- William, B. and Whitman, 2009, Bergey's Manual Trust, Department of Microbiology, *Biological Sciences Building University of Georgia Athens*, GA 30602-2605, USA.

How to cite this article:

Muthukumar, A., G. M. Sandhya and Dakshayini, G. 2021. Morphological and Biochemical Characterization – A Comparative Analysis of Non-commercial and Commercial Plant Growth Promoting Microorganisms. *Int.J.Curr.Microbiol.App.Sci.* 10(02): 867-874.
doi: <https://doi.org/10.20546/ijcmas.2021.1002.102>