Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env. Pharmacol. Life Sci., Spl Issue [3] 2022: 53-58

Online ISSN 2277-1808
Journal's URL:http://www.bepls.com
CODEN: BEPLAD

ORIGINAL ARTICLE **OPEN ACCESS**

Isolation of PGPR From Rhizospheric Soil: Fixing N, Solubilizing P and K, and Formulation Preparation of Liquid Biofertilizer NPK Inoculants

Anna V Varkey[1], Dhwani Upadhyay[2], Indrani Bhattacharya[2], Anjali Thakur[2], Prasad Andhare[3]*
[1]Student, M.Sc Microbiology, Parul Institute of Applied Science, Parul University, Post Limda, Waghodiya, Gujarat
[2] Assistant Professor, Parul Institute of Applied Science, Parul University, Post Limda, Waghodiya , Gujarat
[3] Assistant Professor, Biological Sciences, PDPIAS, Charotar University of Science and Technology, Changa, Anand, Gujarat.
*Corresponding Author: Dr. Prasad Andhare;
E-Mail: prasadandhare.as@charusat.ac.in

ABSTRACT
There is an increase in demand for food production as the world population is growing at pace. Synthetic fertilizers give more yield but they adversely impact the environment and ecosystem. As an alternative biofertilizers are being used, they give yield in an eco-friendly manner. They contain active or dormant microorganisms that are used to treat agricultural seeds or applied directly to the soil. The microbes used for making biofertilizers are mainly PGPR's. They live in rhizospheric soil and flourish in number. They have both synergistic and antagonistic interactions with the soil microbiota and participate in a variety of ecologically important functions. They aid plant development by promoting biotic and abiotic stress tolerance, as well as supporting host plant nutrition. Highest yield is observed when mixture of bacterial inoculants was used in place of using single strain of bacteria in formulation. Liquid formulations give more benefits to farmer as it has longer shelf life and is cost effective compared to solid carrier-based formulations.
Keywords: *Plant Growth Promoting Rhizobacteria (PGPR), Rhizosphere, Nitrogen fixing bacteria (NFB), Phosphate solubilizing bacteria (PSB), Potassium solubilizing bacteria (KSB), Liquid Biofertilizer*

Received 05.08.2022 Revised16.09.2022 Accepted 22.10.2022

INTRODUCTION

As there is an increase in the global population, there is an increase in demand for food production [1]. For plant nutrition and disease management, traditional agricultural practises rely heavily on the widespread use of synthetic fertilizers and pesticides [2]. Because of the careless use of agro-chemicals and their inability to biodegrade, th1ey accumulate below ground, causing adverse changes in soil structure, water holding capacity and fertility. Excessive use of synthetic fertilisers is also connected to eutrophication of water resources, the greenhouse effect, and dangerous levels of heavy metals such as plumbum, arsenic and cadmium [3-5]. As a result, various organic fertilisers that work as natural growth stimulators for plants have been emerged in recent years [6]. The use of natural stimulators has a long history, beginning with compost production on a modest scale, farmers' knowledge is passed down from generation to generation [7]. A subset of this type of fertilizer is made up of substances depending on plant-growth-promoting microorganisms known as Microbial Biostimulants, also known as Biofertilizers, are chemicals that are used to fertilise plants [8]. Biofertilizers are products containing one or more species of microorganisms that can mobilise nutritionally important elements from non-usable to usable form in soil, compost, and other environments through biological processes [9].

Types of Bioformulations

One of the most important elements impacting the quality of biofertilizers containing live microbial cells is formulation. Formulation is defined as the process of combining a chosen microbial strain with a carrier [10]. The active substance is placed in an appropriate carrier, which is commonly supplemented with ingredients that help to stabilise and protect microorganisms during storage and shipping [11]. Formulation should be stable, cost effective, maintain healthy physiological state and enhance activity of microbes thereby increase the crop production [12] Fig.1.

Solid Carrier Formulation

Microorganisms are bound with a delivery vehicle in solid-carrier based biofertilizers, which is responsible for moving microbial strains from the laboratory to the rhizosphere [13]. They have a number of advantages, they are inexpensive, simple to manufacture, and do not necessitate large financial investments [14].

Liquid Formulation

Liquid formulations have longer shelf life compared to carrier-based formulations. Based on broth cultures, mineral oils, organic oils, or oil-in-water suspensions, these biofertilizers are also classified as flowable or aqueous suspensions [15]. The most commonly used bacterial inoculants for formulations are *Bacillus, Psedumonas, Azotobacter, Rhizobium, Azospirillum* etc. species of PGPR [17].

MATERIAL AND METHODS

Materials and Glass wares

Clean petri-dishes, Test-tube, Test-tube stand, Conical flask, Beaker, Measuring cylinder, Pipettes, Spatula, Nichrome wire loop, Media, Distilled water, Gram staining reagent [18]. UV of laminar air flow chamber is kept ON prior and switched OFF after 15 minutes, done to keep the aseptic lab environment. The air flow is kept on so sterile air flow is there inside the chamber. Glass wares were sterilized in autoclave at 121°C, 15 p.s.i for 30-60 minutes and directly kept in laminar air flow chamber [19].

Collection of soil sample

Soil sample was collected from Una, Junagadh district (20°50'N, 71°03'E). It was collected in sterile plastic zip locker from healthy rhizosphere region of *Arachis hypogaea L.* (Groundnut) at the depth of 20-30 cm and it was kept at 4°C in refrigerator prior to usage.

Preparation of soil sample

For serial dilution 9 test tubes and 1 sugar tube was taken. 9 ml water was added in each tube and in sugar tube suspension was prepared by adding 1 gm of soil sample to 9 ml of water. Mixture was allowed to homogenize and serial dilution of suspension was done up to 10^{-10} [20].

Media preparation and procedure

Respective media were autoclaved and poured in petri dishes (Table.1). It was allowed to cool and solidify.100µl of suspension was spread on the respective media plates and were incubated for 3-4 days at 32°C to observe the bacterial colonies. The bacteria were sub-cultured from Azotobacter Agar Mannitol plates on same media. Bacterial colonies that showed clear zones i.e., halo zone was selected from Pikovskaya and Aleksandrow Agar plates and were sub-cultured on same media. Pure culture was obtained by four flame streak method both on nutrient agar plates and selective media plates [18]. The morphological and cultural properties of the plates were observed using macroscopy (Fig.2,3 and 4).

RESULTS

The colony morphology, cell shape, and Gram's staining reaction of each of the selected isolates were investigated (Table.2 and 3).

Screening of bacteria solubilizing phosphate and potassium

Streaking of AV2[PSB] and AV3[KSB] was done on respective selective media plates. For screening, bromothymol blue dye was used. Dye was poured to the plate as such covering the culture [24]. Clear halo zone was seen as shown in Fig.8(a), (b).

Compatibility test of culture and NPK consortia preparation

Bacterial cultures were streaked over nutrient agar plates in such a way that a single bacterial culture was streaked in the centre of the plate, with additional cultures radiating outwards [25]. The plates were incubated for 48 hours at 37°C, and the inhibitory zone was seen and recorded. It was observed that AV1, AV2 and AV3 were compatible as no zone formation was seen Fig.9. This means there is no inhibition and isolates are compatible thereby showing positive result. The bacterial culture was used together to form a formulation. Microbial consortia were made using 3 bacterial inoculants containing AV1[NFB], AV2[PSB] and AV3[KSB].

Formulation preparation of liquid biofertilizer inoculants

Firstly, loopful of bacterial cultures AV1[NFB], AV2[PSB] and AV3[KSB] were individually inoculated in 100ml nutrient broth in three conical flasks and incubated overnight at 32°C on shaker Fig.10. Next day it was seen that the broth turned turbid and O.D. readings were taken in Spectrophotometer. Nutrient broth readings were taken at different timings and after 48 hrs sufficient growth of bacteria was obtained Fig.11. Materials which were used to prepare the formulation mixture were 5gm Guar gum, 5ml glycerol and 100ml distilled water [26]. 5ml AV1[NFB] + 5ml AV2[PSB] + 5ml AV3[KSB] from each inoculated

broth culture was added to conical flask containing formulation mixture Fig.12. It was kept overnight at 32°C on shaker, the O.D. readings were taken for several days to check the bacterial growth.

Growth curve of liquid biofertilizer

O.D. readings were taken daily till stationary phase was attained. The no. of cells was gradually increasing on daily basis. It was in log phase and after few days stationary phase was achieved as shown in Fig.13.

Plate Counting Technique

Colony Forming Unit is calculated by: cfu/ml = (no. of colonies x dilution factor) / volume of culture plate Serial dilution of 10^{-9} was done and 0.1ml volume of culture was spread on the nutrient agar plate Fig.14 [27].

cfu/ml = (146 x 10^9) / 0.1 = 1.46 x 10^{12} **Total Colony Forming Unit** = 1.46 x 10^{12} per ml

DISCUSSION

The soil sample taken from rhizosphere had numerous bacteria and where isolated using serial dilution technique by growing them on respective medias such as Azotobacter Mannitol Agar, Pikovskaya Agar and Aleksandrow Agar. Out of which three isolates were selected from each media and grown as pure culture on the media plates. Characterization of *Azotobacter sp.* was done and were found to be gram negative, small, round/oval, white and transclucent (Azotobacter Mannitol Agar Media). Characterization of Phosphate Solubilizing Bacteria was done and were found to be gram negative, medium, round, opaque and whitish (Pikovskaya Agar); it seemed to be *Pseudomonas sp.* Characterization of Potassium Solubilizing Bacteria was done and were found to be gram positive, medium, round, opaque and white (Aleksandrow Agar); it seemed to be *Bacillus sp.* Using these three bacteria of interest, formulation was made by adding gaur gum, glycerol and distilled water. Gaur gum was used to retain maximum number of bacteria when inoculated to seed it acts as an adhesive while glycerol acts as a preservative also as a carbon source. Thus, increasing the shelf life of bacterial formulation.

CONCLUSION

Steady growth curve of bacteria was observed. After some days stationary phase was maintained in the prepared liquid biofertilizer formulation. It should be used by diluting with distilled water. Live bacteria were seen on spread plate, this showed that the liquid biofertilizer had longer shelf life. The purpose of this study was to isolate the PGPRs from the soil sample of *Arachis hypogaea L.* (Groundnut). The isolated bacteria AV1, AV2, AV3 as Nitrogen fixers, Phosphate and Potassium solubilizers showed efficient growth when added to formulation mixture. This demonstrates that the PGPRs can be used as biofertilizer for sustainable agriculture.

Table.1. Medias for different PGPR

PGPR	Media used	References
Nitrogen-fixing bacteria	Azotobacter Agar Mannitol	[21]
Phosphate solubilizing bacteria	Pikovskaya Agar	[22]
Potassium solubilizing bacteria	Aleksandrow Agar	[23]

Table. 2. Colony characterization of isolates

Isolate	Size	Shape	Margin	Elevation	Texture	Consis-tency	Opacity	Pig-mentation
AV1 (NFB)	small	round / oval	entire	convex	smooth	viscous	transluce nt	white
AV2 (PSB)	mediu m	round	entire	raised	mucoid	viscous	opaque	whitish yellow
AV3 (KSB)	mediu m	round	entire	raised	mucoid	viscous	opaque	white

Table. 3. Morphological characterization of isolates

Isolate	Cell shape	Arrangement	Gram's reaction
AV1 (NFB)	rod	paired	-ve
AV2 (PSB)	rod	paired / scattered	-ve
AV3 (KSB)	rod	paired / scattered	+ve

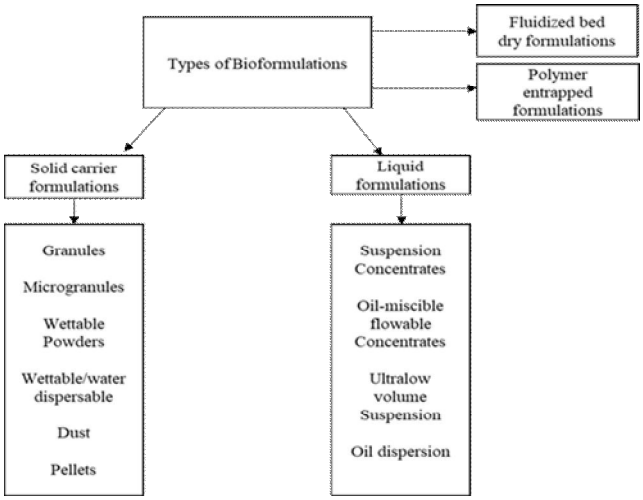


Fig. 1. Types of Biofertilizer formulations [16]

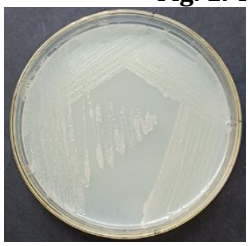
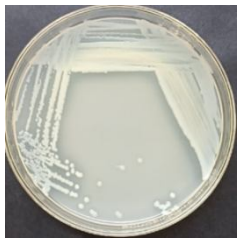
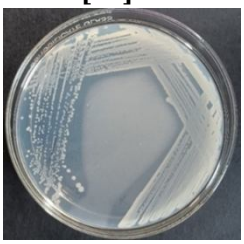

(2)

(3)

(4)

Fig. 2. Isolation of N, Fig. 3. Isolation of P, Fig. 4. Isolation of K


(8.a)

(8.b)

Fig. 8. (a)Halo zone formation AV2[PSB], (b) Halo zone formation AV3[KSB],

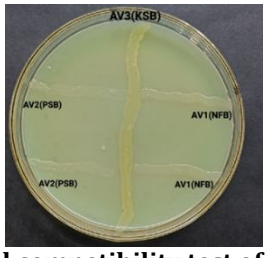


Fig. 9. Cultural compatibility test of rhizobacteria

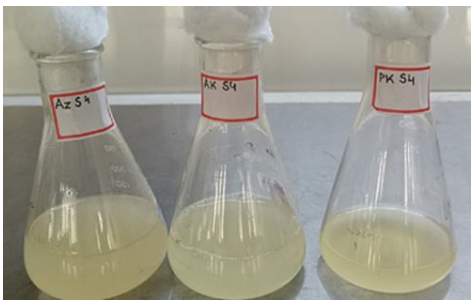


Fig. 10. Inoculated bacterial cultures [NSB-Az, KSB-Ak, PSB-Pk] in Nutrient broth

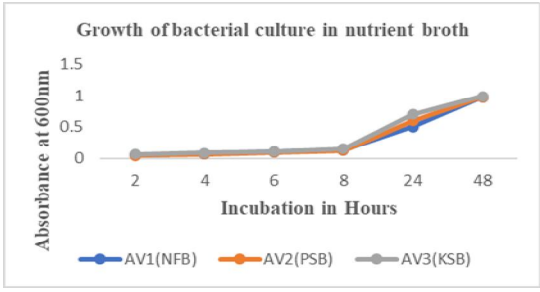


Fig. 11. Growth curve of Bacteria in Nutrient Broth

Fig. 12. Liquid Biofertilizer Formulation

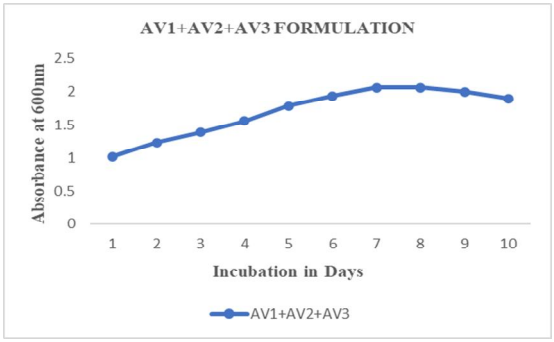


Fig. 13. Growth curve of NPK consortia

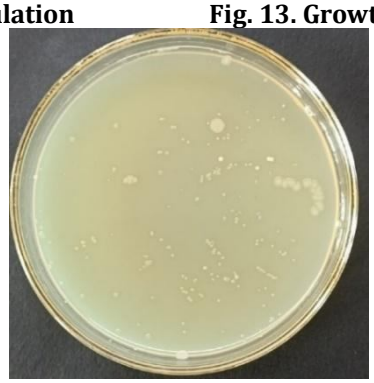


Fig. 14. Spread plate of inoculant formulation

ACKNOWLEDGEMENT

It is our privilege and honour to express our sincerest gratitude to the Parul University, Vadodara, Gujarat for providing all the necessary support and giving facilities including state of the art infrastructural facilities with advanced technological scientific laboratories and everything else that was required to carry out this research work under the guidance of Dr. Prasad Andhare.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

1. Ehrlich, P. R., & Harte, J. (2015). Opinion: To feed the world in 2050 will require a global revolution. Proceedings of the National Academy of Sciences, 112(48), 14743-14744.
2. Vasile, A. J., Popescu, C., Ion, R. A., & Dobre, I. (2015). From conventional to organic in Romanian agriculture–Impact assessment of a land use changing paradigm. Land Use Policy, 46, 258-266.
3. Savci, S. (2012). An agricultural pollutant: chemical fertilizer. International Journal of Environmental Science and Development, 3(1), 73.
4. Goenadi, D. H., Mustafa, A. B., & Santi, L. P. (2018, August). Bio-organo-chemical fertilizers: a new prospecting technology for improving fertilizer use efficiency (FUE). In IOP Conference Series: Earth and Environmental Science (Vol. 183, No. 1, p. 012011). IOP Publishing.
5. Atafar, Z., Mesdaghinia, A., Nouri, J., Homaee, M., Yunesian, M., Ahmadimoghaddam, M., & Mahvi, A. H. (2010). Effect of fertilizer application on soil heavy metal concentration. Environmental monitoring and assessment, 160(1), 83-89.
6. Al Abboud, M. A., Ghany, T. A., & Alawlaqi, M. M. (2014). Role of biofertilizers in agriculture: a brief review. Mycopath, 11(2).

7. Halim, N. A. (2009). Effects of using enhanced biofertilizer containing N-fixer bacteria on patchouli growth (Doctoral dissertation, UMP).
8. Khosro, M., Gholamreza, H., Mohammad, T. K. N., Salah, G., & Yousef, S. (2012). Contrasting soil microbial responses to fertilization and tillage systems in canola rhizosphere, Saudi J. In Biol. Sci (Vol. 19, pp. 337-383).
9. Kumar, R., Kumawat, N., & Sahu, Y. K. (2017). Role of biofertilizers in agriculture. Pop Kheti, 5(4), 63-66.
10. Bargaz A, Lyamlouli K, Chtouki M, Zeroual Y, Dhiba D.(2018). Soil microbial resources for improving fertilizers efficiency in an integrated plant nutrient management system. Frontiers in microbiology. ;9:1606.
11. Namasivayam, S. K. R., Saikia, S. L., & Bharani, A. R. S. (2014). Evaluation of persistence and plant growth promoting effect of bioencapsulated formulation of suitable bacterial bio-fertilizers. Biosci Biotech Res Asia, 11(2), 407-415.
12. Nehra, V., & Choudhary, M. (2015). A review on plant growth promoting rhizobacteria acting as bioinoculants and their biological approach towards the production of sustainable agriculture. Journal of Applied and Natural Science, 7(1), 540-556.iology, 9, 1606.
13. Patil, N. N., Jadhav, S., Ghorpade, S. S., & Sharma, A. B. (2013). Isolation and enrichment of sugar press mud (SPM) adapted microorganism for production of biofertilizer by using sugar press mud. Intl J Adv Biotech Res, 14(1), 96-104.
14. Mishra, J., & Arora, N. K. (2016). Bioformulations for plant growth promotion and combating phytopathogens: a sustainable approach. In Bioformulations: For sustainable agriculture (pp. 3-33). Springer, New Delhi.
15. Bharti, N., Sharma, S. K., Saini, S., Verma, A., Nimonkar, Y., & Prakash, O. (2017). Microbial plant probiotics: problems in application and formulation. In Probiotics and plant health (pp. 317-335). Springer, Singapore.
16. Mącik, M., Gryta, A., & Frąc, M. (2020). Biofertilizers in agriculture: An overview on concepts, strategies and effects on soil microorganisms. Advances in agronomy, 162, 31-87.
17. Vessey, J. K. (2003). Plant growth promoting rhizobacteria as biofertilizers. Plant and soil, 255(2), 571-586.
18. Onyeze, R. C., Onah, G. T., & Igbonekwu, C. C. (2013). Isolation and characterization of nitrogen-fixing bacteria in the soil. International Journal of Life Sciences Biotechnology and Pharma Research, 2, 438-445.
19. Bhojwani SS, Dantu PK. (2013). General Requirements and Techniques. InPlant Tissue Culture: An Introductory.(pp. 11-25). Springer, India.
20. Reynolds J. (2005). Serial dilution protocols. Am Soc Microbiol. :1-7.
21. Subba Rao NS. (1977). Soil microorganisms and plant growth Oxford and IBH publishing co. RVT. LTD pp.:192-207.
22. Bashan Y, Kamnev AA, de-Bashan LE. (2013). A proposal for isolating and testing phosphate-solubilizing bacteria that enhance plant growth. Biology and Fertility of Soils. 49(1):1.
23. Prajapati KB, Modi HA. (2012). Isolation and characterization of potassium solubilizing bacteria from ceramic industry soil. CIBTech J Microbiol. 1(2-3):8-14.
24. Etesami H, Emami S, Alikhani HA.(2017). Potassium solubilizing bacteria (KSB):: Mechanisms, promotion of plant growth, and future prospects A review. Journal of soil science and plant nutrition. 17(4):897-911.
25. Prasad, A. A., & Babu, S. (2017). Compatibility of Azospirillum brasilense and Pseudomonas fluorescens in growth promotion of groundnut (Arachis hypogea L.). Anais da Academia Brasileira de Ciências, 89, 1027-1040.
26. Santhosh, G. P. (2015). Formulation and shelf life of liquid biofertilizer inoculants using cell protectants. IJRBA T, II, (7), 243-247.
27. Rani MU, Arundhathi A, Reddy G. (2012). Screening of rhizobacteria containing plant growth promoting (PGPR) traits in rhizosphere soils and their role in enhancing growth of pigeon pea. African Journal of Biotechnology. 1(32):8085-91.

CITATION OF THIS ARTICLE

Anna V Varkey, D Upadhyay, I Bhattacharya, A Thakur, P Andhare. Isolation of PGPR From Rhizospheric Soil: Fixing N, Solubilizing P and K, and Formulation Preparation of Liquid Biofertilizer NPK Inoculants. Bull. Env.Pharmacol. Life Sci., Vol Spl Issue [3] 2022: 53-58