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The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; 12(11): 732-735

www.thepharmajournal.com
Received: 21-09-2023
Accepted: 30-10-2023

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A methodology to formulate liquid-based Rhizobium inoculants by using different additives

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Abstract
Conventional carrier-based biofertilizers, despite their advantages over agrochemicals, pose challenges like limited shelf life, risk of contamination, and reduced microbial survival. Consequently, there is a need for alternatives such as liquid biofertilizer as upgraded technology that can enhance the viability of microbial cells. The present study focuses on liquid-based bacterial formulations using polymers such as glycerin, trehalose, polyethylene glycol, polyvinylpyrrolidone (PVP), potato starch and cassava starch and evaluating the effects of various additives on shelf life. *In vitro* experiments demonstrated that the addition of 1.5% PVP was the most effective in maintaining the highest population of *Rhizobium leguminosarum* bv. *viciae* DPR2 (1.11×10^8CFU mL^{-1}) for extended period of time (up to 8 months). Additionally, the study found that combining PVP with low-cost natural additives such as cassava starch further extended the shelf life of *Rhizobium* and maintained a significant bacterial population (1.71×10^8 CFU mL^{-1}) when analyzed after 11 months. The resulting liquid biofertilizer signifies an eco-friendly and cost-effective solution, contributing to sustainable agriculture by reducing the dependency on chemical-based fertilizers. This innovative methodology for developing liquid biofertilizers has gained popularity in Indian agriculture due to its distinctive production methods, addressing the issues associated with traditional carrier-based biofertilizers.

Keywords: Liquid biofertilizer, additives, rhizobium, shelf life, PVP

Introduction
To meet the continuous demand for food supply, the application of fertilizers is indispensable in modern agriculture. These fertilizers rapidly enhance the growth and productivity of food crops, gaining popularity. However, their extensive use raises significant environmental concerns (Ren *et al*., 2021; Upadhayay *et al*., 2022, 2023a, 2023b) [9, 17, 15, 16] and negatively impacts soil biodiversity due to their prolonged presence in soil (Pahalvi *et al*., 2021) [8]. To address these challenges, biofertilizers offer a viable alternative (Khan *et al*., 2020, Upadhayay *et al*., 2021) [4, 17]. While fully replacing chemical fertilizers with biofertilizers may not be realistic, they hold the potential to complement synthetic fertilizers and significantly reduce their use. Biofertilizers, generally defined as preparations containing live or latent cells of efficient strains of N-fixing, P-solubilizing, or cellulolytic microorganisms for application to seeds or soil, are also known as microbial inoculants or bio-inoculants (Roshni *et al*., 2020; Khan *et al*., 2023) [10, 3]. *Rhizobium*, an essential symbiont for legumes, plays a vital role with non-legumes by producing growth hormones. An essential step in this process is the root colonization of beneficial bacteria by plants (Jaggi *et al*., 2023; Khan *et al*., 2023) [2, 3]. *Rhizobia* and *Brady rhizobia* can colonize and survive in the rhizosphere of non-legume plants, acting as plant growth-promoting rhizobacteria (PGPR). The application of PGPR for the development of eco-friendly and cost-effective biofertilizers has gained recognition (Lobo *et al*., 2019; Singh *et al*., 2020) [6, 12]. However, biofertilizers manufactured in India are primarily carrier-based and suffer from challenges like short shelf life, poor survival under adverse environmental conditions, high contamination rates, and inconsistent field performance.

In recent decades, there has been a shift towards carrier-based biofertilizers, utilizing carriers such as rice bran, lignite powder, talc, rock phosphate, paddy straw compost, vermiculite, fly ash, and peat. Unfortunately, this system does not guarantee microbe survival beyond a few months, resulting in issues like short shelf life, poor quality, high contamination, and unpredictable field performance. To overcome these challenges, liquid biofertilizers have been developed as a cost-effective and sustainable agricultural solution.

Liquid biofertilizer technology presents strong justifications for its necessity and specificity, emphasizing the utilization of agriculturally relevant microorganisms as a potent tool for promoting sustainable farming practices. While India has seen significant research on *Rhizobium* strain selection and inoculation response, limited research has focused on inoculant production and formulation technologies. There is a need to enhance formulation for improved field performance.

In this direction, research is ongoing, indicating a shift from solid carrier materials to microbe-friendly liquid formulations. Liquid-based biofertilizers are promising inputs that can effectively address the current challenges in agriculture by improving organism viability, maximizing input efficacy, and ensuring cost-effectiveness. This approach is eco-friendly and economical, offering valuable support for farming. Liquid inoculants are not just common broth cultures, as often perceived. They represent unique liquid formulations containing preferred microorganisms, their nutrients, cell protectants, and supplements to enhance cell survival pre- and post-application. Various additives, such as glycerol, horticultural oil, and saccharides (e.g., glucose and lactose), are used to improve the viability and resilience of microbial cells in biofertilizer formulations (Kumar *et al*., 2022; Valetti *et al*., 2016) [5, 19]. These additives, with their high molecular weight, water solubility, non-toxicity, complex chemical nature, and ability to limit heat transfer, contribute to improved shelf life and overall quality.

The present study focuses on conducting experiments to improve the shelf life and quality parameters of microbial inoculants by adding appropriate additives such as glycerin, trehalose, polyethylene glycol, polyvinylpyrrolidone (PVP), gum arabic, cassava starch and more. Furthermore, it offers a new dimension for the development and evaluation of new liquid-based bacterial formulations.

Materials and Methods

Bacterial strains: The bacterial strains used in the present study, i.e., *Rhizobium leguminosarum* bv. *viciae* DPR2 was obtained from the Microbiology Department of the College of Basic Sciences & Humanities, Rajendra Prasad Central Agricultural University, Pusa, Samastipur, Bihar.

Growth medium

The standard media such as ‘Yeast extract mannitol media (YEM)’ (10.0g/l mannitol; 0.5 g/l K_2HPO_4; 0.2 g/l $MgSO_4.7H_2O$; 0.1 g/l NaCl and 0.5 g/l yeast extract was used for *Rhizobium leguminosarum*.

Liquid inoculant formulation

In the present experiments, liquid inoculant formulations have been modified to assess the shelf-life persistence. It was performed in 125 ml Erlenmeyer flasks containing 50 ml of YEM amended with different concentrations of additives as follows: glycerol @ 0, 2.0, 4.0 and 6.0 ml for YEM Trehalose @ 0, 2.5, 5.0 and 10.0 mM (w/v) for YEM, PVP @ 0, 1.5, 2.0 and 2.5% (w/v) for YEM, PEG @ 0, 0.25, 0.5, 1.0(w/v) for YEM. Late log phase cultures of *Rhizobium leguminosarum* bv. *viciae* DPR2 was inoculated into YEM and supplemented with additives *viz*. glycerol, trehalose, PVP and PEG, respectively. After incubation at 28 °C (200 rpm) for 6 days, the effect of various concentrations of different additives was assessed by determining viable cell populations by plate counts on respective agar medium.

Shelf life studies of liquid inoculant formulation

Liquid inoculant formulation containing an appropriate concentration of each additive that showed higher viable cell counts were stored at room temperature and evaluated for shelf life at monthly intervals up to 12 months. The best additives, selected based on higher cellular population, were further evaluated with the addition of natural and low-cost biopolymer-based stabilizing agents such as cassava starch at 0%, 0.5%, 1.0%, and 1.5% (w/v) for YEM, and potato starch at 0%, 0.25%, 0.5%, and 1.0% (w/v) for YEM.

Results and Discussion

The effects of different concentrations of four additives to YEM on the final cell concentration of *Rhizobium leguminosarum* bv. *viciae* DPR2 are presented in Table 1. After 12 months of storage, the viable count of all liquid *Rhizobium* inoculants prepared in amended media was higher than that of inoculants prepared in the control medium YEMB. The population of *Rhizobium leguminosarum* bv. *viciae* DPR2 was the highest at 1.5% PVP compared to other additives. In the case of PVP (1.5%), the highest population of *Rhizobium leguminosarum* (1.11×10^8 CFU mL^{-1}) was maintained for an extended period (up to 8 months), after which it declined to 10^5 cfu mL^{-1} when measured after 12 months. Therefore, PVP at a concentration of 1.5% was the most suitable supplement for enhancing the shelf life of *Rhizobium leguminosarum* bv. *viciae* DPR2, with 10^8 CFU mL^{-1}, meeting the quality standard for liquid bacterial inoculant formulations. The highest cell population in the PVP-supplemented medium demonstrates the remarkable characteristics of this supplement in terms of its effective cell protection and maintenance of bacterial cell metabolism by providing adequate water around the cells (Maitra *et al*., 2021; Sehrawat *et al*., 2017; Surendra Gopal and Baby, 2016) [7, 11, 13].

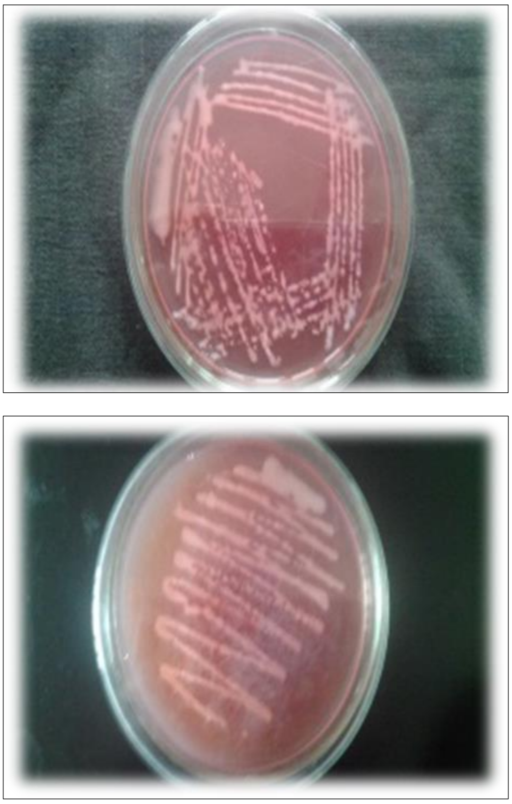

Fig 1: *Rhizobium leguminosarum* bv. *viciae* DPR2

Table 1: Effect of different chemical additives on the population of *Rhizobium leguminosarum* bv. *viciae* DPR2 at monthly intervals

Chemical Additives	Months											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
4ml Gly	1.58×10^{8}	1.0×10^{8}	0.6×10^{8}	1.0×10^{7}	1.8×10^{6}	5.7×10^{6}	2.5×10^{5}	0.65×10^{5}	0.4×10^{5}	0.45×10^{4}	0.8×10^{3}	0.33×10^{3}
8ml Gly	1.66×10^{8}	0.6×10^{8}	0.5×10^{8}	2.5×10^{8}	2.6×10^{7}	1.5×10^{7}	1.1×10^{7}	0.82×10^{6}	0.61×10^{6}	0.63×10^{5}	1.0×10^{4}	$2. 5\times10^{4}$
12ml Gly	1.72×10^{8}	1.5×10^{8}	1.24×10^{8}	1.52×10^{7}	1.65×10^{8}	1.51×10^{8}	1.45×10^{7}	1.3×10^{7}	3.51×10^{6}	0.27×10^{6}	4.2×10^{5}	1.36×10^{4}
2.5mM Treh.	0.15×10^{9}	0.75×10^{9}	1.15×10^{7}	1.28×10^{7}	0.30×10^{7}	1.45×10^{6}	0.55×10^{6}	0.41×10^{6}	0.45×10^{5}	0.32×10^{5}	0.32×10^{4}	0.21×10^{4}
5mM Treh.	0.41×10^{9}	0.45×10^{9}	0.98×10^{8}	0.71×10^{8}	1.16×10^{7}	0.19×10^{7}	0.21×10^{6}	0.12×10^{6}	0.32×10^{5}	0.16×10^{5}	0.08×10^{5}	0.55×10^{4}
10mM Treh.	0.55×10^{9}	1.30×10^{9}	1.15×10^{9}	0.84×10^{8}	0.56×10^{7}	0.71×10^{7}	0.30×10^{7}	0.10×10^{6}	0.21×10^{5}	0.18×10^{5}	0.15×10^{5}	0.83×10^{5}
1% PVP	1.52×10^{9}	1.37×10^{9}	1.50×10^{9}	0.83×10^{9}	0.74×10^{8}	0.75×10^{8}	1.07×10^{7}	0.64×10^{6}	0.19×10^{6}	0.02×10^{6}	0.64×10^{5}	0.55×10^{5}
1.5% PVP	1.30×10^{9}	1.09×10^{9}	1.70×10^{9}	1.62×10^{9}	2.87×10^{8}	1.70×10^{8}	1.36×10^{8}	1.11×10^{8}	2.58×10^{7}	1.47×10^{6}	1.33×10^{6}	0.95×10^{5}
2% PVP	0.11×10^{10}	0.48×10^{10}	0.20×10^{9}	1.3×10^{7}	1.62×10^{7}	0.8×10^{7}	0.6×10^{7}	1.21×10^{6}	1.10×10^{6}	0.65×10^{6}	0.29×10^{6}	0.80×10^{5}
0.25% PEG	0.75×10^{9}	1.02×10^{9}	1.51×10^{8}	0.54×10^{8}	0.44×10^{8}	0.07×10^{8}	0.72×10^{7}	0.43×10^{6}	1.42×10^{5}	1.32×10^{5}	0.49×10^{5}	1.11×10^{4}
0.5% PEG	0.15×10^{10}	1.06×10^{9}	0.9×10^{9}	1.26×10^{8}	1.09×10^{8}	1.09×10^{7}	1.49×10^{6}	1.19×10^{6}	1.15×10^{5}	1.01×10^{5}	0.59×10^{5}	0.5×10^{5}
1% PEG	1.06×10^{9}	1.38×10^{8}	1.2×10^{8}	0.65×10^{8}	0.05×10^{8}	0.63×10^{7}	1.67×10^{6}	1.51×10^{6}	1.31×10^{5}	1.25×10^{5}	1.16×10^{4}	1.09×10^{4}

Each value represents a mean of three replication

Fig 2: Formulation of liquid biofertilizers with different additives of *Rhizobium sps.*

In addition, Table 2 illustrates the effect of PVP supplemented with a stabilizing agent (potato starch at 0, 0.25, 0.5, and 1.0% (w/v) and cassava starch at 0, 0.5, 1.0, and 1.5% (w/v)) added to YEMB on the final cell population of *Rhizobium leguminosarum* bv. *viciae* DPR2. YEMB supplemented with 1.5% PVP + 1.5% cassava starch maintained the highest population of *Rhizobium* inoculant (1.71×10^{8} CFU mL^{-1}) for 11 months. The results clearly indicate that the combination of PVP as a supplement (1.5% for *Rhizobium*) and cassava starch (1.5%) depicted a higher cellular population of bacterial strains. In addition to PVP, the augmented CFU can be attributed to the additional supplement (cassava starch) due to its ability to limit heat transfer, possess good rheological properties, and have higher water activities. Moreover, cassava starch possesses a high molecular weight biopolymer complex that provides stabilizing properties. Likewise, cassava starch also exhibits a high water-binding ability, which may retain water around the cells for their metabolism (Trimurtulu *et al*., 2014) [14]. Furthermore, it is utilized as a thickener and a low-cost starch ingredient, making it easy for farmers to adopt.

Table 2: Effect of 1.5% PVP supplemented with different concentrations of potato starch (PS) and cassava starch (CS) on the population of *Rhizobium leguminosarum* bv. *viciae* DPR2 at monthly intervals

Concentration	Months											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
0.25%PS	1.62×10^{9}	1.16×10^{8}	1.12×10^{8}	0.80×10^{8}	1.25×10^{7}	1.27×10^{7}	0.43×10^{7}	1.18×10^{6}	0.54×10^{6}	1.62×10^{5}	1.51×10^{5}	1.31×10^{5}
0.5%PS	1.82×10^{9}	1.08×10^{9}	0.96×10^{9}	1.68×10^{8}	1.80×10^{8}	1.89×10^{8}	1.56×10^{8}	1.62×10^{7}	1.68×10^{7}	1.76×10^{7}	1.70×10^{6}	1.64×10^{5}
1%PS	1.16×10^{9}	1.12×10^{9}	1.86×10^{9}	1.70×10^{8}	1.56×10^{9}	1.77×10^{8}	0.61×10^{8}	0.32×10^{8}	1.26×10^{7}	1.40×10^{6}	1.44×10^{6}	1.41×10^{5}
0.5%CS	1.84×10^{8}	1.16×10^{9}	1.12×10^{9}	0.88×10^{9}	$1.82\times\times10^{8}$	1.89×10^{8}	1.56×10^{8}	1.62×10^{8}	1.67×10^{8}	1.68×10^{7}	1.57×10^{6}	0.38×10^{7}
1%CS	1.68×10^{9}	2.60×10^{9}	2.52×10^{9}	1.08×10^{8}	1.76×10^{9}	1.79×10^{8}	1.67×10^{8}	0.78×10^{8}	1.76×10^{7}	1.48×10^{7}	1.15×10^{7}	1.39×10^{6}
1.5%CS	1.56×10^{9}	2.52×10^{9}	2.60×10^{9}	1.84×10^{9}	1.72×10^{9}	1.80×10^{9}	2.18×10^{8}	2.1×10^{8}	1.93×10^{8}	1.85×10^{8}	1.71×10^{8}	1.34×10^{7}

Each value represents a mean of three replication

Conclusion

The development of liquid biofertilizers as a modern technology in the context of Indian agriculture is gaining popularity due to its unique production methods. To enhance their efficacy, various polymeric additives such as glycerol, trehalose, PVP, and PEG are being used. These polymers help to better adhere the inoculants to the seeds. In this study, the developed liquid biofertilizer exhibited a noteworthy enhancement in the shelf life of *Rhizobium* when supplemented with a combination of PVP and cost-effective natural additives like cassava starch. This liquid biofertilizer, enriched with eco-friendly additives, offers a cost-effective and sustainable approach to agriculture, reducing dependence on chemical fertilizers. The adoption of this innovative liquid biofertilizer production methodology has the potential to overcome the limitations associated with conventional carrier-based biofertilizers, such as low shelf life and lower persistence in field conditions, contributing to more sustainable and environmentally friendly farming practices.

Acknowledgement

This experiment is financially supported by University funded Project, Directorate of Research, RPCAU, Pusa.

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