Development of Optimized Liquid Formulations of Azospirillum, Phosphate-Solubilizing Bacteria, and Rhizobium Strains for Enhanced Viability and Shelf Life

ABSTRACT

The aim of this study was to evaluate the stability and shelf-life of *Rhizobium*, *Azospirillum*, and *Pseudomonas striata* a Phosphate Solubilizing Bacterium (PSB) in liquid formulations under ambient conditions, with a focus on the impact of various additives on the viability of these microbes over a 12-month period. Liquid formulations of *Rhizobium* strains NC-92, Sb-120, and Gr-2, *Azospirillum*, and PSB were prepared with different combinations of additives such as polyethylene glycol (PEG), gum arabic (GA), polyvinylpyrrolidone (PVP), and glycerol. The formulations were stored under ambient conditions, and colony-forming unit (CFU) counts were assessed at regular intervals over the year. The results revealed that formulations with higher concentrations of PVP, GA, and PEG maintained significantly higher CFU counts. Azospirillum formulations, particularly F20, exhibited superior viability, maintaining 2.2×10^7 CFU after 12 months compared to rapid declines in other formulations such as F2, F8, and F16. Rhizobium NC-92 in basal medium + 1% PVP (F1) retained 5.2×10^8 CFU after 12 months, while Sb-120 in basal medium + 1% PVP (T1) demonstrated high stability, maintaining 6.9×10^8 CFU over six months. PSB formulations containing combinations of glycerol, PEG, and PVP also performed better, with the best formulation retaining 6.2×10^8 CFU after 12 months. The study highlights the importance of certain additives such as PVP, GA, and PEG in improving the stability and shelf life of microbial

compositions. Optimized formulations significantly increased viability, laying the groundwork for the development of robust biofertilizer solutions.

Keywords: Microbial stability; Rhizobium; Azospirillum; Phosphate Solubilizing Bacterium; Liquid formulations

INTRODUCATION

The expansion of agricultural practices to fulfill the expanding need for food, fiber, and fuel has resulted in a heavy reliance on chemical inputs, which frequently have negative environmental implications(Rilling *et al*., 2023). As global concerns about soil health, water quality, and ecosystem stability grow, there is a pressing need to shift toward more sustainable agriculture practices(Melchior &Newig, 2021). One of the most promising options is the use of microbial biofertilizers, which increase nutrient availability and crop output while lowering the environmental footprint of farming activities(Mahanty*et al*., 2017).

Microbial biofertilizers make use of beneficial microorganisms such as *Azospirillum*, phosphate-solubilizing bacteria (PSB), and *Rhizobium*(Li*et al*., 2024; Nosheen *et al*., 2021). *Azospirillum* is known for its function in nitrogen fixation and the generation of growth-promoting chemicals including auxins and cytokinins, whereas PSB increases phosphorus availability by solubilizing insoluble phosphates(Aasfar*et al*., 2021; Pan & Cai. 2023). *Rhizobium* creates symbiotic relationships with legumes and converts air nitrogen into plant-usable forms(Goyal *et al*., 2021). Together, these microbes provide a comprehensive solution for increasing plant health and productivity. Despite its potential, the widespread use of microbial inoculants has been hampered by concerns about their stability, shelf-life, and

field performance(Elnahal*et al*., 2022). Traditional carrier-based biofertilizers, such as peat or lignite powders, frequently have a short shelf-life, fluctuating microbial populations, and are susceptible to environmental conditions during storage and use(Saif *et al*., 2021) and are generally bulky in nature. Liquid formulations have developed as a preferable option, with benefits including ease of application, increased microbial load, and longer shelf-life(Rai *et al*., 2024). However, establishing stable and effective liquid biofertilizer formulations remains a problem since microbial viability is affected by factors such as basal medium selection, additives, and storage conditions(Elnahal*et al*., 2022).

Existing research has set the framework for understanding the role of microbial inoculants in sustainable agriculture, but considerable gaps remain in the creation of liquid formulations that are widely applicable and effective in the field. Individual strains or single formulation components are frequently studied, but the relationships between media, additives, and microbial physiology are rarely evaluated comprehensively(Khan *et al*., 2023). Furthermore, the shelf-life of liquid biofertilizers under actual storage settings is little characterized, limiting their scalability and uptake(Sharma *et al*., 2023).

The objective of this study is to develop and optimize liquid formulations of *Azospirillum*, PSB, and *Rhizobium* strains to ensure high microbial viability and stability during extended storage times. This study aims to investigate how additives, adjuvants, and surfactants might be tuned to improve the growth and survivability of these microbial strains in liquid formulations. It also investigates which combinations of basal medium and additional components result in the longest shelf-life and microbiological stability under ambient storage settings. Furthermore, it explores how different microbial strains react to formulation changes and the implications for field efficacy.

METHODOLOGY

Microorganisms and Maintenance

Pure cultures of *Azospirillum* ACD-15, *Pseudomonas striata*(PSB), *Rhizobium* NC-92, *Rhizobium* Sb-120, and *Rhizobium* Gr-2 were procured from the Institute of Organic Farming, University of Agricultural Sciences, Dharwad. The strains were initially lyophilized and stored at -20°C at the Institute for long-term preservation. Additionally, the strains were maintained as slants on respective selective media under controlled conditions for regular subculturing and propagation.

Development of Liquid Formulations of Azospirillum ACD-15

To develop the liquid formulations of *Azospirillum* ACD-15, N-free Jensen medium was used as the basal medium, supplemented with Bromothymol Blue as an indicator. Various combinations of additives, adjuvants, and surfactants were tested to optimize the growth and population of *Azospirillum* ACD-15 (Table 1). The additives included polyethylene glycol at concentrations of 0.5% and 1.0%, trehalose at 5 mM and 10 mM, and glycerol at 5 mM and 10 mM. GA was tested as an adjuvant at 0.15% and 0.30%, while polysorbate-20 was evaluated as a surfactant at concentrations of 125 ppm and 250 ppm. Based on their ability to support optimal growth and maintain a high population, the best formulations were identified for further shelf-life analysis. The selected formulations were stored in high-density polypropylene bottles (TARSONS) at room temperature for up to 12 months. Four replications were maintained for each formulation. A control formulation consisting of only the basal medium was included in the study. Any deviations from the standard pH for liquid biofertilizers were corrected using a phosphate buffer (pH 7.0) to maintain the integrity of the formulations.

Table 1: Presentation of various formulations tested for Azospirillum ACD-15 growth

Formulation ID	Components

F2	Basal media + 0.5% Polyethylene glycol + 5 mM Trehalose + 5 mM Glycerol + 0.15% Gum arabica + 250 ppm Polysorbate-20
F7	Basal media + 0.5% Polyethylene glycol + 5 mM Trehalose + 10 mM Glycerol + 0.3% Gum arabica + 125 ppm Polysorbate-20
F8	Basal media + 0.5% Polyethylene glycol + 5 mM Trehalose + 10 mM Glycerol + 0.3% Gum arabica + 250 ppm Polysorbate-20
F11	Basal media + 0.5% Polyethylene glycol + 10 mM Trehalose + 5 mM Glycerol + 0.3% Gum arabica + 125 ppm Polysorbate-20
F12	Basal media + 0.5% Polyethylene glycol + 10 mM Trehalose + 5 mM Glycerol + 0.3% Gum arabica + 250 ppm Polysorbate-20
F14	Basal media + 0.5% Polyethylene glycol + 10 mM Trehalose + 10 mM Glycerol + 0.15% Gum arabica + 250 ppm Polysorbate-20
F16	Basal media + 0.5% Polyethylene glycol + 10 mM Trehalose + 10 mM Glycerol + 0.3% Gum arabica + 250 ppm Polysorbate-20
F18	Basal media + 1% Polyethylene glycol + 5 mM Trehalose + 5 mM Glycerol + 0.15% Gum arabica + 250 ppm Polysorbate-20
F20	Basal media + 1% Polyethylene glycol + 5 mM Trehalose + 5 mM Glycerol + 0.3% Gum arabica + 250 ppm Polysorbate-20

Basal Media: N-free malate medium

Development of Liquid Formulations of PSB

To develop liquid formulations of PSB, a basal medium containing tricalcium phosphate was used. Different combinations of additives, adjuvants, and surfactants were tested, including glycerol at concentrations of 5 mM and 10 mM, polyethylene glycol at 0.5% and 1%, carboxymethyl cellulose at 0.05% and 0.1%, GA at 0.15% and 0.3%, and polysorbate 20 at concentrations of 125 ppm and 250 ppm (Table 2). The best formulations were identified based on their ability to support growth and population density and were subsequently used for shelf-life studies. The selected formulations were stored in high-density polypropylene bottles (TARSONS) at room temperature for up to 12 months. Four replications were maintained for each formulation, and a control consisting of only the basal medium was included. pH of the formulations was monitored and adjusted as necessary with a phosphate buffer (pH 7.0).

Table 2: Presentation of various formulations tested for PSB growth

Formulation ID	Components
F1	Basal media + 0.5% Polyethylene glycol + 5 mM Trehalose + 10 mM Glycerol +

	0.3% Gum Arabic + 250 ppm Polysorbate-20
F2	Basal media + 0.5% Polyethylene glycol + 10 mM Trehalose + 5 mM Glycerol + 0.3% Gum Arabic + 125 ppm Polysorbate-20
F3	Basal media + 1% Polyethylene glycol + 5 mM Trehalose + 5 mM Glycerol + 0.15% Gum Arabic + 250 ppm Polysorbate-20
F4	Basal media + 1% Polyethylene glycol + 5 mM Trehalose + 10 mM Glycerol + 0.15% Gum Arabic + 250 ppm Polysorbate-20
F5	Basal media + 1% Polyethylene glycol + 5 mM Trehalose + 10 mM Glycerol + 0.3% Gum Arabic + 125 ppm Polysorbate-20
F6	Basal media + 1% Polyethylene glycol + 10 mM Trehalose + 5 mM Glycerol + 0.15% Gum Arabic + 125 ppm Polysorbate-20
F7	Basal media + 1% Polyethylene glycol + 10 mM Trehalose + 5 mM Glycerol + 0.15% Gum Arabic + 250 ppm Polysorbate-20
F8	Basal media + 1% Polyethylene glycol + 10 mM Trehalose + 5 mM Glycerol + 0.3% Gum Arabic + 125 ppm Polysorbate-20
F9	Basal media + 1% Polyethylene glycol + 10 mM Trehalose + 5 mM Glycerol + 0.3% Gum Arabic + 250 ppm Polysorbate-20

Basal Media: tricalcium phosphate

Development of Liquid Formulations of Rhizobium NC92, Rhizobium Sb-120, and Rhizobium Gr-2

A basal medium was developed for preparing liquid formulations of *Rhizobium* NC92, *Rhizobium* Sb-120, and *Rhizobium* Gr-2. The basal medium contained the following components: Mannitol (10 g/L), K_2HPO_4 (0.5 g/L), $MgSO_4$ (0.2 g/L), NaCl (0.1 g/L), Yeast Extract (1 g/L), Glucose (1 g/L), and $CaCO_3$ (3 g/L). Various additives, adjuvants, and surfactants were incorporated into this medium to optimize the growth and survival of the strains in the liquid formulation. Eleven different treatments, as listed previously, were prepared by adding different concentrations of additives, adjuvants, and surfactants to the basal medium. The cultures were inoculated at 5% (w/v) of each *Rhizobium* strain and incubated in a rotary shaker. Viability was assessed at 5, 10, 15, and 30-day intervals through serial dilution and pour plate methods. Viable populations were measured on CRY-YEMA medium under standard incubation conditions. The treatments involved modifying the basal medium with various additives. Treatment T1 consisted of basal medium with 1% Polyvinylpyrrolidone (PVP), while T2 included 2% PVP. Treatment T3 was prepared by adding

1% Polyethylene Glycol (PEG) to the basal medium, and T4 contained 0.5% PEG. Treatment T5 consisted of basal medium with 0.15% GA, and T6 contained 0.3% GA. For treatments T7 and T8, the basal medium was amended with 200 µM Fe EDTA, glycerol, and 2% PVP, but T7 contained 1 mL of glycerol and 2 mM trehalose, whereas T8 included 4 mL glycerol and 2% PVP. Treatment T9 was amended with 0.025% Tween 20, and T10 contained 0.05% Tween 20. Treatment T11 served as the control with just the basal medium.Each treatment was prepared by adding the specified amendments to the basal medium in 250 mL Erlenmeyer flasks containing 100 mL of the broth. The cultures were inoculated with 5% (w/v) inoculum of *Rhizobium* strains and incubated in a rotary shaker under optimal conditions for microbial growth.

Viability Assessment of Rhizobium Strains

The viability of the *Rhizobium* strains was assessed at various time intervals (5, 10, 15, and 30 days) using the standard serial dilution and pour plate methods. The dilutions were plated on CRY-YEMA medium, and the plates were incubated at room temperature. Colony counts were recorded after 3 days of incubation for each dilution (10^7, 10^8, and 10^9). Population dynamics were monitored to identify the formulations that maintained the highest viable populations of the strains.

Shelf-Life Assessment of Liquid Inoculant Formulations

Based on their ability to support the growth and viability of Rhizobium strains, six formulations were selected for shelf-life testing. These formulations included F1, which consisted of basal medium with 1% PVP; F2, which was prepared by amending the basal medium with 200 µM Fe EDTA, 4 mL of glycerol, and 2% PVP; F3, which combined basal medium with 1% PEG and 0.025% Tween 20; and F4, which contained basal medium with 0.3% GA and 0.025% Tween 20.

The selected formulations were stored in high-density polypropylene bottles (TARSONS) at room temperature for up to 12 months. A control formulation containing only the basal medium was included in the study. Four replications were maintained for each formulation, and viable population counts were recorded monthly to evaluate the stability and shelf-life of the formulations. The shelf-life was determined by assessing the period during which the formulations retained a viable population of *Rhizobium* at or above 10^7 CFU/mL.

RESULTS

The study observed the viable count of *Azospirillum* in various liquid formulations over a 12-month period (Table 3). Formulation F20 demonstrated the highest initial viable count of 1.3×10^{10} CFU and maintained relatively better viability, with a count of 2.2×10^7 CFU at the end of 12 months. In contrast, formulations such as F2, F8, and F16 showed rapid population decline, with final counts dropping to 1.1×10^6, 5.3×10^5, 2.5×10^4 CFU, respectively. Notably, F7 maintained moderate stability with a decline from 6.9×10^{10} to 1.7×10^7 CFU over the year. Formulations with higher additive concentrations generally retained better viability, as observed in F20 and F12, while lower additive levels, as in F11 and F14, resulted in faster reductions in viable counts. For example, F11 exhibited a sharp decline from 1.8×10^9 to 2.5×10^4, and F14 dropped from 5.4×10^9 to 3.2×10^4 CFU. The un-amended basal media, lacking additives, showed poor viability retention, decreasing from 1.8×10^{10} to 1.5×10^6 CFU by the 12th month.

Table 3: *Azospirillum* colony forming units studies over the period of 12 months.

Sl.No	Formulations	Population in MPNE at monthly intervals											
		1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th	12th
1	F2: Basal media+A1+B1+ C1+D1+E2	4.2x 10^{10}	9.7x 10^9	1.2x 10^8	2.8x 10^8	3.5x 10^8	1.1x 10^8	1.4x 10^8	1.7x 10^7	1.8x 10^8	0.95 x10^9	2.1x 10^8	0.11x 10^7
2	F7: Basal media+ A1+B1+C2+D2	6.9x 10^{10}	9.9x 10^9	7.1x 10^8	5.1x 10^8	3.0x 10^8	1.4x 10^8	2.2x 10^8	2.1x 10^8	2.8x 10^8	0.9x 10^8	0.8x 10^8	1.7x10^7

	+E1												
3	F8: Basal media+A1+B1+ C2+D2+E2	4.3x 10^9	1.1x 10^9	1.7x 10^8	4.4x 10^7	5.5x 10^7	0.2x 10^7	0.3x 10^7	0.3x 10^8	0.2x 10^8	0.1x 10^7	0.2x 10^7	0.53x10^6
4	F11: Basal media+A1+B2+ C1+D2+E1	1.8x 10^9	4.0x 10^8	4.5x 10^7	3.7x 10^7	1.1x 10^6	0.49 x10^6	0.7x 10^6	0.4x 10^6	0.5x 10^6	0.3x 10^5	0.3x 10^5	0.25x10^5
5	F12: Basal media+A1+B2+ C1+D2+E2	7.2x 10^9	3.0x 10^8	1.0x 10^6	2.2x 10^6	2.3x 10^6	3.5x 10^6	2.8x 10^6	1.8x 10^6	0.6x 10^6	0.6x 10^6	0.6x 10^5	0.56x10^5
6	F14: Basal media+A1+B2+ C2+D1+E2	5.4x 10^9	2.0x 10^8	2.3x 10^7	4.4x 10^6	2.9x 10^6	0.1x 10^6	0.1x 10^6	0.2x 106	0.1x 106	0.1x 106	0.2x 10^5	0.32x10^5
7	F16: Basal media+A1+B2+ C2+D2+E2	5.2x 10^9	7.9x 10^8	3.0x 10^6	1.3x 10^6	1.0x 10^6	0.2x 10^6	0.3x 10^6	0.2x 10^6	0.2x 10^6	0.20 x 10^6	0.48 x 10^5	2.5x10^5
8	F18: Basal media+A2+B1+ C1+D1+E2	7.1x 10^9	1.2x 10^8	3.3x 10^6	1.3x 10^6	4.3 x10^6	16x 10^6	1.7x 10^6	0.31 x 10^6	0.36 x 10^6	0.39 x10^6	0.45 x 10^6	0.36x10^5
9	F20: Basal media+A2+B1+ C1+D2+E2	1.3x 10^{10}	1.0x 10^{10}	8.7 x10^9	2.1x 10^8	4.1x 10^8	1.1x 10^8	1.3x 10^7	2.2x 107	1.5x 108	0.49 x 10^9	1.8x 10^8	2.2x 10^7
10	Un-amended basal media	1.8x 10^{10}	7.1x 10^9	2.6x 10^8	1.1x 10^8	3.3x 10^7	0.70 x 10^7	1.2x 10^7	0.78 x 10^7	0.72 x 10^7	2.2x 10^6	1.7x 10^6	1.5x10^6

Note: **A1** – 0.5% Poly ethylene glycol, **A2 -** 1 % Poly ethylene glycol, **B1** - 5 mM Trehalose, **B2** – 10 mM Trehalose, **C1** - 5 mM Glycerol, **C2** – 10 mM Glycerol, **D1** - 0.15% Gum arabica, **D2** - 0.3 % Gum arabica, **E1** - 125 ppm Polysorbate-20, **E2** - 250 ppm Polysorbate-20, **Basal media** – N-free malate media.

All formulations of PSB exhibited a decline in CFU over 12 months (Table 4). For instance, Basal media + 10 mM Glycerol + 0.5% PEG + 0.05% Corboxy methyl cellulose + 0.15% GA + 250 ppm Polysorbate 20 started with 1.26×10^{11} CFU and ended at 6.2×10^8 CFU. Similarly, Basal media + 5 mM Glycerol + 0.1% Corboxy methyl cellulose + 0.3% GA + 250 ppm Polysorbate 20 began with 2.2×10^{10} CFU and dropped to 1×10^6 CFU. In contrast, formulations such as Basal media + 10 mM Glycerol + 1% PEG + 0.05% Corboxy methyl cellulose + 0.3% GA + 250 ppm Polysorbate 20 showed a decrease from 1.8×10^{10} CFU to 2.1×10^6 CFU by the end of the study. The largest initial populations were observed in formulations with 10 mM Glycerol and 1% Polyethylene glycol, but the rate of decline was similar across most formulations, stabilizing around 10^6 to 10^8 CFU by the 12th month.

Table 4: Phosphate solubilizing bacterial count (colony forming units)over12 months of incubation.

Formulations & Composition	Colony forming units (CFU) at monthly intervals											
	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th	12th
Basal media+ A1+B1+C2+D2+E2	2.2x 10^{10}	1.6 x 10^{10}	2x 10^{9}	7.0x 10^{8}	6x 10^{8}	1.8x 10^{8}	2.6x 10^{8}	2.3x 10^{8}	2x 10^{7}	2.5x 10^{6}	2.1x 10^{6}	1x10^{6}
Basal media+ A1+B2+C1+D2+E1	1.2x 10^{10}	0.9 x 10^{10}	4.3x 10^{9}	2.67 x 10^{9}	5x 10^{8}	4x 10^{8}	5.1x 10^{8}	4.9x 10^{8}	4.2x 10^{8}	3.3x 10^{7}	3.5 x 10^{7}	5.1x 10^{6}
Basal media+ A2+B1+C1+D1+E2	126.6 x 10^{10}	109 x 10^{10}	73x 10^{10}	28.6 x 10^{10}	23.3 x 10^{10}	15x 10^{10}	16x 10^{9}	7x 10^{8}	7.5x 10^{8}	8x 10^{8}	7.6x 10^{8}	6.2x 10^{8}
Basal media+ A2+B1+C2+D1+E2	2.6x 10^{10}	1.4 x 10^{10}	7x 10^{9}	4x 10^{9}	2.6x 10^{9}	6.3x 10^{8}	4.1x 10^{8}	5.5x 10^{7}	1.2x 10^{7}	3.0x 10^{6}	1.8x 10^{6}	1.5x 10^{6}
Basal media+ A2+B1+C2+D2+E1	2.3x 10^{10}	1.5 x 10^{10}	1.4x 10^{10}	2.6 x 10^{10}	9x 10^{9}	8.6x 10^{9}	7.4x 10^{8}	15.7 x 10^{8}	9.4x 10^{8}	1.3x 10^{8}	3.6x 10^{7}	1.3x 10^{8}
Basal media+ A2+B2+C1+D1+E1	18.6 x 10^{10}	3.7 x 10^{10}	2.6x 10^{10}	2.6x 10^{10}	6x 10^{9}	2.2x 10^{9}	44.3 x 10^{9}	15x 10^{8}	4x 10^{7}	1.3x 10^{6}	3.6x 10^{5}	44.3x 10^{5}
Basal media+ A2+B2+C1+D1+E2	32x 10^{10}	10. 6x 10^{10}	3.9x 10^{10}	2.3x 10^{10}	5x 10^{8}	1.7x 10^{8}	8.8x 10^{8}	6.8x 10^{8}	4.4x 10^{8}	3.2x 10^{7}	1.5x 10^{7}	7.7x 10^{6}
Basal media+ A2+B2+C1+D2+E1	52.3x 10^{10}	44. 3x 10^{10}	15x 10^{10}	4x 10^{10}	1.3x 10^{10}	3.6x 10^{9}	3.1x 10^{9}	2.4x 10^{9}	3.8x 10^{8}	4.1x 10^{8}	2.8x 10^{8}	1.5x 10^{8}
Basal media+ A2+B2+C1+D2+E2	18.3x 10^{10}	12x 10^{10}	.8x 10^{10}	1.6x 10^{10}	2x 10^{9}	5.2x 10^{8}	7.9x 10^{8}	9.1x 10^{8}	15.2 x 10^{8}	5.8x 10^{8}	8.5x 10^{8}	3.1x 10^{8}
Basal media+ A2+B2+C2+D1+E1	6.7x 10^{10}	6x 10^{9}	6x 10^{9}	4.7x 10^{9}	3.8x 10^{9}	3.3x 10^{9}	6x 10^{9}	8.6x 10^{8}	6.7x 10^{8}	7.2x 10^{7}	7.7x 10^{7}	8.6x 10^{7}
Basal media+ A2+B2+C2+D1+E2	33.7x 10^{10}	20. 6x 10^{10}	1.3x 10^{10}	3.3x 10^{10}	5.3x 10^{9}	4.4x 10^{9}	4.3x 10^{9}	15x 10^{8}	4x 10^{8}	1.3x 10^{8}	3.6x 10^{8}	3.5x 10^{8}
Basal media+ A2+B2+C2+D2+E2	22.3x 10^{10}	13. 6x 10^{10}	8.5x 10^{10}	2.0x 10^{10}	2.5x 10^{9}	2.4x 10^{8}	5.8x 10^{7}	8x 10^{6}	5.7x 10^{5}	6x 10^{5}	1.6x 10^{5}	4.6x 10^{4}

A1 - 5 mM Glycerol; A2 - 10 mM Glycerol;B1 - 0.5% Poly ethylene glycol; B2 - 1% Poly ethylene glycol; C1 - 0.05% Corboxy methyl cellulose; C2 - 0.1% Corboxy methyl cellulose; D1 - 0.15% Gum arabica; D2 - 0.3% Gum arabica; E1 - 125 ppm Polysorbate 20; E2 - 250 pp m Polysorbate 20; Basal media – Pikovskaya's broth.

Rhizobium NC92 showed the highest viable count of 4×10^9 CFU in the basal medium amended with 1% PEG (T3) at 15 days, followed by 0.3% GA (T6) with 1.4×10^9 CFU and 1% PVP (T1) with 1.3×10^9 CFU. Basal medium with Tween 20 at 0.025% (T9) maintained a viable count of 1×10^9 CFU, which was higher than Tween 20 at 0.05% (T10), where the viable count was 1×10^8 CFU.Treatment T11 which contained only basal medium, exhibited a significant decline in viable population, reducing to 6×10^7 CFU (Figure 1). The viable counts in the other treatments were statistically comparable.Combination amendments of additives with 0.025% Tween 20 further enhanced the population, with the basal medium + 0.3% GA + 0.025% Tween 20 (T14) showing the maximum viable population of 3.2×10^9 CFU (Figure 2).

Strain Sb-120 exhibited the maximum population of 2.8×10^9 CFU in T1 (basal medium + 1% PVP) and T7 (200 µM Fe EDTA + glycerol + 2 mM trehalose + 2% PVP). A decline was noted in T3 (1% PEG) and T8 (200 µM Fe EDTA + 4 mL glycerol + 2% PVP), with the lowest CFU of 8×10^6 CFU observed in T11 (unamended basal medium) (Table 5).

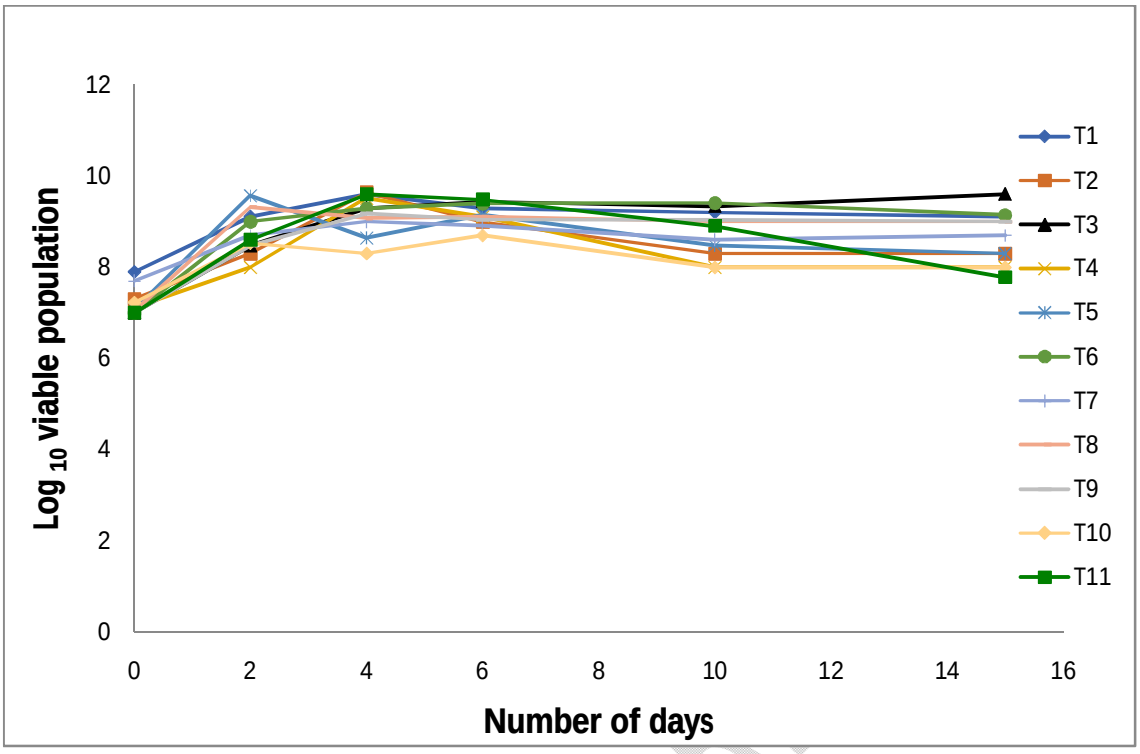

Figure 1: Effect of additives, adjuvants, and surfactants on viability of *Rhizobium* NC 92

Table 5: Effect of different concentrations of additives, adjuvants and surfactants on growth and survival of Rhizobium Sb 120

Treatments	Population in colony forming units		
	0 Days	**5 Days**	**10 Days**
T1	$1x\ 10^9$	$1.3x\ 10^9$	$2.8x10^9$
T2	$3x\ 10^9$	$4x\ 10^9$	$1.7x\ 10^9$
T3	$2\ x\ 10^9$	$6x\ 10^8$	$3.7x\ 10^8$
T4	$1\ x\ 10^9$	$9\ x\ 10^8$	$1.3\ x\ 10^9$
T5	$9x\ 10^8$	$1\ x\ 10^9$	$1.4\ x\ 10^9$
T6	$3\ x\ 10^9$	$2\ x\ 10^9$	$2.4\ x\ 10^9$
T7	$3\ x\ 10^9$	$1\ x\ 10^9$	$2.8x\ 10^9$
T8	$3\ x\ 10^9$	$7\ x\ 10^8$	$2.5\ x\ 10^8$
T9	$2\ x\ 10^9$	$5x\ 10^9$	$1.8\ x\ 10^9$
T10	$1x\ 10^9$	$4\ x\ 10^9$	$2.2\ x\ 10^9$
T11	$3\ x\ 10^9$	$7\ x\ 10^8$	$8x\ 10^6$

The shelf-life studies of Rhizobium formulations under ambient conditions revealed distinct trends across different strains and compositions. In the case of Rhizobium NC-92 formulations (Table 6), F1 (Basal medium + 1% PVP) demonstrated relatively stable CFU

levels, starting at 8 × 10^8 and maintaining 5.2 × 10^8 CFU by the 12th month. Similarly, F4 (Basal medium + 0.3% GA + Tween 20) exhibited consistent performance, starting at 4.1 × 10^8 CFU and reaching 7.5 × 10^8 CFU at the end of the study. However, the unamended medium showed a drastic decline, starting at 1.1 × 10^6 CFU and reducing to 1 × 10^4 CFU by the 12th month, highlighting the significance of additives in improving stability.

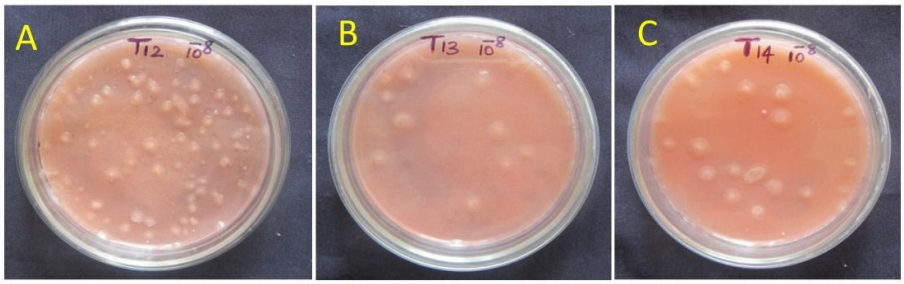

Figure 2. Viable population in different combinations of additives, adjuvants and surfactants. **A**- Basal medium + 1% PEG+ 0.025% Tween 20; **B**- Basal medium +1% PVP+ 0.025% Tween 20; and **C**- Basal medium + 0.3%+ 0.025% Tween 20

Table 6: Shelf-life study of liquid formulations of *Rhizobium* NC-92 under ambient conditions up to 12 months.

Sl. No	Formulations & Composition	Colony forming units (CFU) at monthly intervals											
		1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th	12th
1	F1: Basal medium+ 1%PVP	8x 10^8	9.9 x 10^9	4.1 x 10^8	5.4 x 10^8	3.2 x 10^9	3x 10^8	4.2 5 x 10^9	3.2 1 x 10^9	4.1 x 10^9	5 x 10^8	4.9 x 10^8	5.2 x 10^8
2	F2: Basal medium+Glycerol +FeEDTA+2%PVP	7.7 x 10^8	8x 10^8	2.1 x 10^8	2.5 x 10^8	2x 10^8	2.5 x 10^8	4 x 10^8	7 x 10^8	6 .2x 10^8	3 x 10^8	2.5 x 10^8	2 x 10^8
3	F3:Basal Medium+ 1%PEG+ Tween 20	5.4 x 10^8	7x 10^9	3.2 x 10^8	2x 10^8	2.1 x 10^8	2.3 x 10^8	2.6 x 10^8	3x 10^8	3.3 x 10^8	3.8 x 10^8	3.5 x 10^8	3.2 x 10^8
4	F4:Basal medium+ 0.3%GA+Tween 20	4.1 x 10^8	5.3 x 10^9	3.1 x 10^8	6x 10^8	3.3 x 10^8	3x 10^8	3.7 x 10^9	4.5 x 10^8	6.8 x 10^8	7.2 x 10^8	6.9 x 10^8	7.5 x 10^8
5	Unamended media	1.1 x 10^6	2x 10^9	1.7 x 10^8	3x 10^7	1x 10^8	5x 10^7	1.3 x 10^6	6.9 x 10^4	5x 10^4	4x 10^4	3x 10^4	1x 10^4

For Rhizobium (Sb-120) formulations (Table 7), T1 (Basal medium + 1% PVP) maintained a high initial CFU of 43 × 10^9 but showed a decline to 6.9 × 10^8 CFU over six months. Meanwhile, T7 (Basal medium + Fe EDTA + Glycerol + Trehalose + 2% PVP) exhibited consistent stability, with CFU levels reducing from 5.1 × 10^9 to 4.3 × 10^8 CFU by the sixth month. The unamended basal media, while starting at 8 × 10^9 CFU, showed a significant reduction to 2 × 10^7 CFU, emphasizing the importance of supplementation.

Table 7: Shelf-life study of liquid formulations of *Rhizobium* Sb-120 under ambient conditions up to 12 months.

Sl. No	Formulations & Composition	Colony forming units (CFU) at regular intervals					
		1st Month	2nd Month	3rd Month	4th Month	5th Month	6th Month

1	T1: Basal medium+ 1%PVP	43 x 10^9	7.6 x 10^8	8 x 10^8	7.5 x 10^8	8.3 x 10^8	6.9x 10^8
2	T6:Basal medium+ 0.3% GA	2.3 x 10^9	2.7 x 10^8	2.2 x 10^8	1.8 x 10^8	1.7 x 10^7	2x 10^8
3	T7:Basal medium+ Fe EDTA+ Glycerol+ Trehaolse +2%PVP	5.1 x 10^9	4.6 x 10^8	5.5 x 10^8	5 x 10^8	5.8 x 10^8	4.3x 10^8
4	T11:(Un-amended basal media)	8 x 10^9	7.7 x 10^8	5 x 10^8	5 x 10^7	3 x 10^7	2x 10^7

In the case of *Rhizobium* Gr-2 formulations (Table 8), T5 (Basal medium + 0.15% GA) displayed superior performance, beginning at 41.2 × 10^9 CFU and retaining 9.9 × 10^8 CFU after six months. Similarly, T6 (Basal medium + 0.3% GA) showed promising stability, with CFU levels declining from 40.9 × 10^9 to 2.5 × 10^9 CFU. However, unamended basal media exhibited a sharp decline from 26.8 × 10^9 CFU to 1 × 10^7 CFU, reinforcing the critical role of additives like GA and PVP in maintaining viability over time.

Table 8: Shelf-life study of liquid formulations of *Rhizobium* (Gr-2) under ambient conditions up to 12 months.

Sl. No	Formulations & Composition	Colony forming units (CFU) at regular intervals					
		1st Month	2nd Month	3rd Month	4th Month	5th Month	6th Month
1	T1:Basal media+ 1%PVP	37.9 x 10^9	1.69 x 10^9	2.2 x10^8	2.8 x 10^8	3 x 10^8	4.2 x 10^8
2	T4:Basal media+0.5% PEG	38.2 x 10^9	1.69 x 10^9	2x10^8	2.3 x 10^9	4 x 10^9	2 x 10^9
3	T5:Basal media+0.15% GA	41.2 x 10^9	2.36 x10^9	2.2 x10^9	4 x 10^9	3.8 x 10^8	9.9 x 10^8
4	T6:Basal media+0.3% GA	40.9 x 10^9	2.39 x10^9	2x10^8	1.5 x 10^8	1 x 10^9	2.5 x 10^9
5	T11:Un-amended basal media	26.8 x 10^9	1.51 x 10^9	8 x 10^7	5 x 10^7	1 x 10^7	1 x 10^7

DISCUSSION

Liquid formulations are crucial as they enhance microbial growth and stability. Our study indicated that formulation F20 exhibited the highest initial viable count of 1.3×10^{10} CFU and relatively superior viability throughout the study, maintaining a count of 2.2×10^{7} CFU by the end of the 12th month. This formulation's ability to sustain higher populations may be attributed to the presence of effective stabilizers that support the microbial cells in maintaining their activity over extended periods (Thakral *et al*., 2021). Conversely, formulations such as F2, F8, and F16, which showed a rapid decline in viable counts, support the hypothesis that the absence or insufficient concentration of stabilizing agents can lead to poor viability retention (Lewis *et al*., 2021). Specifically, these formulations dropped to 1.1×10^{6}, 5.3×10^{5}, and 2.5×10^{4} CFU, respectively, indicating the susceptibility of *Azospirillum* populations under suboptimal conditions (Bocatti*et al*., 2022). A balance of additive offers some level of protection to the microbial populations without causing excessive decline (Bhat *et al*., 2023). Notably, formulations with higher additive concentrations, such as F20 and F12, were more successful in maintaining higher viable counts, supporting the importance of optimizing the media components for microbial growth (Breig *et al*., 2021). Imbalanced media formulations or their concentrations have the negative effect on the viability of microbes. This results in failure to protect the microorganisms from environmental stresses or degradation over time. Balanced media nutritional components prolong the shelf life of microbial inoculants, particularly in protecting microbial populations from degradation due to environmental factors such as temperature fluctuations or nutrient depletion (Mazzucotelli*et al*., 2016). Similarly, when observing the viability of PSB formulations, all formulations showed a decline in CFU over the 12-month period. For example, the formulation containing Basal media + 10 mM Glycerol + 0.5% PEG + 0.05% Corboxy methyl cellulose + 0.15% GA + 250 ppm Polysorbate 20 demonstrated a high initial

population of 1.26 × 10^{11} CFU, but this decreased to 6.2 × 10^{8} CFU by the end of the study. Similarly, another formulation (Basal media + 5 mM Glycerol + 0.1% Corboxy methyl cellulose + 0.3% GA + 250 ppm Polysorbate 20) started with 2.2 × 10^{10} CFU but dropped to 1 × 10^{6} CFU. Although the formulations with higher concentrations of glycerol and polyethylene glycol (such as those with 10 mM glycerol and 1% PEG) showed the largest initial populations, the overall decline in CFU was comparable across most formulations, stabilizing around 10^{6} to 10^{8} CFU by the 12th month. These findings suggest that while some formulations might contribute to higher initial populations, their effect on long-term viability might be limited, indicating the need for further optimization of formulation composition for improved shelf life (Berninger *et al*., 2018).

The viability of *Rhizobium* strain NC92 in various liquid formulations revealed that specific amendments significantly enhanced microbial populations. Among the treatments, the basal medium amended with 1% PEG (T3) demonstrated the highest viable count of 4 × 10^{9} CFU at 15 days, highlighting PEG's effectiveness in maintaining microbial stability. Similarly, 0.3% GA in T6 supported a viable count of 1.4 × 10^{9} CFU, followed closely by 1% polyvinylpyrrolidone (PVP) in T1, which achieved 1.3 × 10^{9} CFU. The additives likely mitigate osmotic stress, enhance nutrient availability, and create favorable microenvironments, contributing to the higher CFU counts observed in these treatments (Zvinavashe *et al*., 2021). In the tested formulations, PEG at an optimal concentration demonstrated its ability to sustain microbial populations over extended periods, reflecting its role as a hydrating agent that prevents cellular desiccation. Similarly, the performance of GA and PVP indicated their capacity to stabilize microbial cells by forming protective matrices and preventing aggregation or sedimentation, as evidenced by the relatively higher viable counts in these treatments (Biradar *et al*., 2018).

Furthermore, the findings suggest that lower concentrations of surfactants like Tween 20 are more beneficial than higher concentrations, possibly due to reduced cellular stress and membrane damage at lower levels (Reitermayer*et al*., 2018). For instance, addition of Tween 20 contributed to population stability, with 0.025% Tween 20 (T9) maintaining a viable count of 1×10^9 CFU, outperforming the higher concentration of 0.05% Tween 20 (T10), which resulted in a decline to 1×10^8 CFU. These findings suggest that lower concentrations of Tween 20 are more favorable for microbial survival. The unamended basal medium consistently showed the poorest performance. This outcome aligns with the understanding that basal media alone often lack the buffering and protective capabilities required for extended storage of liquid formulations (Wang*et al*., 2024).

Rhizobium strains respond differently to formulations, with NC92 exhibiting higher CFU counts in PEG-amended treatments, while Sb-120 showed better stability in formulations containing PVP and complex additive combinations. Particularly, for strain Sb-120 the basal medium supplemented with 1% PVP (T1) and a combination of 200 µM Fe EDTA, glycerol, 2 mM trehalose, and 2% PVP (T7) yielded the maximum population of 2.8×10^9 CFU. Treatments with 1% PEG (T3) and 200 µM Fe EDTA + glycerol + 2% PVP (T8) demonstrated a decline in CFU, while the unamended basal medium (T11) resulted in the lowest viable count of 8×10^6 CFU. This strain-specific response towards media components is based on the physiological needs of the microbial strain (Stieir*et al*., 2024). Additionally, the role of combination formulations in enhancing stability is evident. Formulations containing synergistic blends of additives, such as GA with Tween 20 or PVP with Fe EDTA and glycerol, consistently maintained higher CFU counts. This suggests that the combined effects of these additives enhance protective mechanisms, including stress tolerance and nutrient

stabilization (Rouphael&Colla, 2020; Asfra*et al*., 2021), though limitations are associated with these additives (Table 9).

Table 9: Summary of Advantages and Limitations of Tested Additives for Microbial Viability

Additive	Advantages	Limitations
Polyvinyl Pyrrolidone	High CFU retention over extended periods; enhances microbial stability across strains.	Relatively expensive compared to other additives.
Gum Arabic	Promotes moderate stability; enhances CFU retention when combined with Tween 20.	Effectiveness varies with concentration; less effective alone.
Polyethylene Glycol	Provides long-term stability in specific formulations.	Performance decreases at lower concentrations.
Glycerol	Stabilizes initial populations; improves performance when combined with other additives.	Limited long-term effectiveness as a standalone additive.
Tween 20	Improves CFU retention in combination with other stabilizers (e.g., GA).	Less effective at higher concentrations.

FUTURE PROSPECTS

The outcomes of this work pave the way for future advances in the development of stable and effective liquid formulations for agriculturally beneficial microbes such as Rhizobium, Azospirillum, and PSB. Future study can focus on optimizing additive combinations for varied environmental circumstances and crop systems, assuring microbial survival and effectiveness in field applications. Furthermore, modern biotechnological methods, such as encapsulation and nanotechnology, may improve the shelf life and functional delivery of these bioinoculants. Beyond formulation stability, studying the interaction of these bioformulations with various soil microbiomes and how they affect nutrient uptake efficiency may provide useful insights for sustainable agriculture operations. Scaling up production techniques and testing these formulations under various agro-climatic situations will be critical for validating their performance and encouraging widespread acceptance among farmers. These advances will not only contribute to enhancing agricultural output,

but also align with global initiatives to minimize chemical fertilizer use and promote eco-friendly farming alternatives.

CONCLUSION

The study demonstrated the significant role of media formulation and additives in enhancing the shelf-life and microbial viability of agriculturally important microorganisms like Rhizobium, Azospirillum, and PSB. Various formulations, particularly those containing specific additives such as PVP, GA, PEG, and Polysorbate 20, showed improved stability and better retention of viable counts over extended periods. These findings highlight the critical role of formulation composition in prolonging the viability of microbial strains, which is crucial for their potential use in agricultural and environmental applications. The study warrants for further exploration of optimal formulation strategies to maximize the effectiveness and shelf-life of microbial inoculants.

Disclaimer (Artificial intelligence)

Author(s) hereby declares that no generative AI technologies such as Large Language Models and text-to-image generators have been used during the writing or editing of this manuscript.

REFERENCES

Aasfar, A., Bargaz, A., Yaakoubi, K., Hilali, A., Bennis, I., Zeroual, Y., et al. (2021). Nitrogen fixing Azotobacter species as potential soil biological enhancers for crop nutrition and yield stability. *Frontiers in microbiology*, 12, 628379.

Berninger, T., González López, Ó., Bejarano, A., Preininger, C., &Sessitsch, A. (2018). Maintenance and assessment of cell viability in formulation of non-sporulating bacterial inoculants. *Microbial biotechnology*, 11(2), 277-301.

Bhat, M. A., Mishra, A. K., Jan, S., Bhat, M. A., Kamal, M. A., Rahman, S., et al. (2023). Plant growth promoting rhizobacteria in plant health: a perspective study of the underground interaction. *Plants*, 12(3), 629.

Biradar, B. P., & Santhosh, G. P. (2018). Role of polymeric additives in formulation, shelf-life and bioefficacy of liquid inoculant of Pseudomonas fluoresens. *Int. J. Pure Appl. Biosci*, 6, 123-133.

Bocatti, C. R., Ferreira, E., Ribeiro, R. A., de Oliveira Chueire, L. M., Delamuta, J. R. M., Kobayashi, R. K. T., et al. (2022). Microbiological quality analysis of inoculants based on Bradyrhizobium spp. and Azospirillumbrasilense produced “on farm” reveals high contamination with non-target microorganisms. *Brazilian Journal of Microbiology*, 1-14.

Breig, S. J. M., & Luti, K. J. K. (2021). Response surface methodology: A review on its applications and challenges in microbial cultures. *Materials Today: Proceedings*, 42, 2277-2284.

Elnahal, A. S., El-Saadony, M. T., Saad, A. M., Desoky, E. S. M., El-Tahan, A. M., Rady, M. M., et al. (2022). The use of microbial inoculants for biological control, plant growth promotion, and sustainable agriculture: A review. *European Journal of Plant Pathology*, 162(4), 759-792.

Goyal, R. K., Mattoo, A. K., & Schmidt, M. A. (2021). Rhizobial–host interactions and symbiotic nitrogen fixation in legume crops toward agriculture sustainability. *Frontiers in Microbiology*, 12, 669404.

Khan, A., Singh, A. V., Gautam, S. S., Agarwal, A., Punetha, A., Upadhayay, V. K., et al. (2023). Microbial bioformulation: a microbial assisted biostimulating fertilization technique for sustainable agriculture. *Frontiers in Plant Science*, 14, 1270039.

Lewis, W. H., Tahon, G., Geesink, P., Sousa, D. Z., &Ettema, T. J. (2021). Innovations to culturing the uncultured microbial majority. *Nature Reviews Microbiology*, 19(4), 225-240.

Mahanty, T., Bhattacharjee, S., Goswami, M., Bhattacharyya, P., Das, B., Ghosh, A., et al. (2017). Biofertilizers: a potential approach for sustainable agriculture development. *Environmental Science and Pollution Research*, 24, 3315-3335.

Mazzucotelli, C. A., Agüero, M. V., del Rosario Moreira, M., &Ansorena, M. R. (2016). Optimization of medium components and physicochemical parameters to simultaneously enhance microbial growth and production of lypolitic enzymes by Stenotrophomonas sp. *Biotechnology and Applied Biochemistry*, 63(3), 407-418.

Melchior, I. C., &Newig, J. (2021). Governing transitions towards sustainable agriculture—taking stock of an emerging field of research. *Sustainability*, 13(2), 528.

Nosheen, S., Ajmal, I., & Song, Y. (2021). Microbes as biofertilizers, a potential approach for sustainable crop production. *Sustainability*, 13(4), 1868.

Pan, L., & Cai, B. (2023). Phosphate-solubilizing bacteria: advances in their physiology, molecular mechanisms and microbial community effects. *Microorganisms*, 11(12), 2904.

Rai, S., Mago, Y., Aggarwal, G., Yadav, A., & Tewari, S. (2024). Liquid bioformulation: a trending approach towards achieving sustainable agriculture. *Molecular Biotechnology*, 66(10), 2725-2750.

Reitermayer, D., Kafka, T. A., Lenz, C. A., & Vogel, R. F. (2018). Interrelation between Tween and the membrane properties and high pressure tolerance of Lactobacillus plantarum. *BMC microbiology*, 18, 1-14.

Rillig, M. C., Ågerstrand, M., Bi, M., Gould, K. A., & Sauerland, U. (2023). Risks and benefits of large language models for the environment. *Environmental Science & Technology*, 57(9), 3464-3466.

Rouphael, Y., & Colla, G. (2020). Biostimulants in agriculture. *Frontiers in plant science*, 11, 40.

Saif, S., Abid, Z., Ashiq, M. F., Altaf, M., & Ashraf, R. S. (2021). Biofertilizer formulations. *Biofertilizers: Study and Impact*, 211-256.

Wang, A., & Zhong, Q. (2024). Drying of probiotics to enhance the viability during preparation, storage, food application, and digestion: A review. *Comprehensive Reviews in Food Science and Food Safety*, 23(1), e13287.

Sharma, B., Tiwari, S., Kumawat, K. C., & Cardinale, M. (2023). Nano-biofertilizers as bio-emerging strategies for sustainable agriculture development: Potentiality and their limitations. *Science of The Total Environment*, 860, 160476.

Li, C., Chen, X., Jia, Z., Zhai, L., Zhang, B., Grüters, U., (2024). Meta-analysis reveals the effects of microbial inoculants on the biomass and diversity of soil microbial communities. *Nature Ecology & Evolution*, 1-15.

Thakral, S., Sonje, J., Munjal, B., & Suryanarayanan, R. (2021). Stabilizers and their interaction with formulation components in frozen and freeze-dried protein formulations. *Advanced drug delivery reviews*, 173, 1-19.

Steier, V., Prigolovkin, L., Reiter, A., Neddermann, T., Wiechert, W., Reich, S. J., (2024). Automated workflow for characterization of bacteriocin production in natural producers Lactococcus lactis and Latilactobacillussakei. *Microbial Cell Factories*, *23*(1), 74.

Zvinavashe, A. T., Mardad, I., Mhada, M., Kouisni, L., & Marelli, B. (2021). Engineering the plant microenvironment to facilitate plant-growth-promoting microbe association. *Journal of Agricultural and Food Chemistry*, 69(45), 13270-13285.