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Short Communication

The Effect of Glycerol Use in *Bacillus* Biofertilizer Formulation

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Abstract

Bacillus-based plant growth-promoting rhizobacteria (PGPR) biofertilizer formulations have been extensively studied and are widely used in agricultural applications. Following the identification of PGPR traits and optimization of production processes, the development of formulations with adequate shelf-life remains a critical step. To enhance storage stability, various cell protectants are incorporated into formulations, among which glycerol is one of the most used due to its stabilizing and protective properties. In this study, three *Bacillus subtilis* strains with known PGPR characteristics were evaluated, and an appropriate production medium was selected based on endospore production. Subsequently, *B. subtilis* EGE-B-1.19 was selected for shelf-life studies. The effect of glycerol addition both during cultivation and after production on viable cell counts was monitored over a 12-month storage period. The results demonstrated that glycerol significantly influenced both biomass production and shelf-life stability. Post-production application of glycerol was found to be more effective in maintaining cell viability compared to its addition during cultivation. Among the tested concentrations, 2% glycerol provided the most favorable results in terms of both viable cell count and pH stability. These findings highlight the importance of glycerol application timing and concentration in the development of stable liquid biofertilizer formulations.

Keywords: *Bacillus,* PGPR, glycerol, biofertilizer, formulation

Categories: Biodiversity conservation, Soil microbiology, Use of biostimulants, biofertilizers, bioinoculants in sustainable agriculture

Keywords: bacillus, pgpr, glycerol, biofertilizer, formulation

Introduction

Liquid biofertilizer technology has gained increasing attention due to its potential to deliver agriculturally important microorganisms in stable and effective formulations. These formulations not only contain the desired microorganisms and essential nutrients but also include protective agents that enhance shelf-life and improve tolerance to adverse environmental conditions, making them highly suitable for field applications [1,2]. Various approaches have been developed to optimize liquid formulations, with the common goal of improving storage stability, ease of application, persistence, and protection against environmental stress factors [3,4].

A critical requirement for the successful development of plant-beneficial microbial inoculants is the production of sufficient microbial biomass with high functional efficacy, while maintaining viability during storage. In this context, glycerol has been widely used as a cell-protective agent in microbial formulations, particularly in biofertilizer applications. It plays a key role in preserving cell viability by reducing water activity and stabilizing cellular structures [3-6]. The use of

glycerol-based media is not new, especially in the production of rhizobial inoculants, which represent one of the most widely used biofertilizer groups. In endospore-forming *Bacillus*-based formulations, glycerol is considered to contribute mainly to the preservation of residual vegetative cells and the overall stability of the formulation during storage, rather than directly protecting mature endospores, which are inherently resistant and metabolically dormant structures [1].

B. subtilis strains are among the most used plant growth-promoting rhizobacteria (PGPR) due to their ability to form endospores, which provide enhanced resistance to environmental stress conditions [7-11]. However, despite their inherent resistance, maintaining long-term viability in liquid formulations remains a critical challenge [2,12-14]. Glycerol is therefore considered to support formulation stability by reducing water activity and osmotic stress during storage, which may help limit viability losses in liquid *Bacillus*-based inoculants.

Previous studies have reported the beneficial effects of glycerol on microbial stability; however, the impact of glycerol application timing - during cultivation versus post-production formulation - has not been sufficiently investigated [1,4]. Therefore, this study aims to evaluate the effect of glycerol on both biomass production and shelf-life stability of *Bacillus*-based biofertilizer formulations, with particular emphasis on the timing of its application.

Materials And Methods

Microorganism used

In this study, three *B. subtilis* strains (EGE-B-36.5, EGE-B-3.P.5, and EGE-B-1.19), previously characterized for their PGPR traits and identified as having high biofertilizer potential, were used. These strains were selected based on their demonstrated plant growth-promoting properties, including indole-3-acetic acid production, nitrogen fixation, phosphate solubilization, production of lytic enzymes, and antagonistic activity against various phytopathogens, as reported in previous studies [12,13].

Determination of production medium

To evaluate biomass production performance, commercial media including nutrient broth (NB) and lysogeny broth (LB), as well as two previously optimized endospore production media (EPM) for *Bacillus* strains, were tested [15,16]. The component ratios were adopted from previously optimized formulations reported in the literature [13,17], where these concentrations were shown to support efficient *Bacillus* growth and sporulation.

EPM 1 consisted of dry yeast (1.7 g/L), glucose (4.4 g/L), soy flour (13.3 g/L), $(NH_4)_2SO_4$ (0.2 g/L), and 5 mL/L of a stock salt solution. The stock salt solution contained $MgCl_2{\cdot}6H_2O$ (20.3 g/L), $CaCl_2{\cdot}2H_2O$ (10.2 g/L), and $MnCl_2{\cdot}4H_2O$ (1.0 g/L). The pH of the medium was adjusted to 7.0 [13].

EPM 2 consisted of glucose (5 g/L), soy flour (23.5 g/L), corn steep liquor (9.5 g/L), and 0.6 g/L $ZnSO_4$, along with the same stock salt solution described above [17].

All media were sterilized at 121°C for 15 min prior to use. The cultures were inoculated at a rate of 2% (*v*/*v*) and incubated at 30°C with shaking at 180 rpm for 6 days. Following cultivation, viable cell counts were determined using the pour plate method [12,13].

Effect of glycerol on biomass production

To evaluate the effect of glycerol on biomass production, glycerol was added to the production medium at concentrations of 1%, 2%, and 3% (*v*/*v*). Following incubation at 30°C and 180 rpm for 6 days, viable cell counts were determined using the pour plate method.

Preparation of glycerol-based formulations and shelf-life study

To investigate the effect of glycerol application timing on the shelf-life of *Bacillus*-based biofertilizer formulations, two different approaches were applied:

• Glycerol addition during cultivation [3].

• Glycerol addition after production (formulation stage) [4].

Following cultivation, microbial suspensions were standardized to an initial concentration of 10^8 CFU/mL. The pH of all formulations was adjusted to 5.0 using acetic acid. This pH value was selected based on a previously published formulation study in which the effect of pH on shelf-life stability was evaluated [13].

For post-production application, sterile glycerol solutions at concentrations of 1%, 2%, and 3% (*v*/*v*) were prepared and mixed with microbial suspensions in 100 mL amber-colored bottles.

All formulations were stored at room temperature (22 ± 2°C) under dark conditions. Potassium sorbate (0.2% *w*/*v*) was added to prevent fungal contamination. Shelf-life stability was monitored over a 12-month period. Viable cell counts and pH values were measured at monthly intervals.

Endospore counting

Viable cell counts were evaluated based on endospore counts. For endospore counting, 1 mL of the sample was transferred into 9 mL of sterile distilled water and subjected to heat treatment at 80°C for 20 min in a water bath to eliminate vegetative cells. Following heat treatment, serial dilutions were prepared and plated using the pour plate method [18].

Statistical analysis

All experiments were conducted in triplicates. Results are presented as mean ± standard deviation. Statistical differences between groups were evaluated using one-way analysis of variance followed by Tukey's post hoc test. A significance level of $p < 0.05$ was considered statistically significant.

Results And Discussion

Determination of production medium

The effects of different culture media on biomass production of *B. subtilis* strains are presented in Table *1* and Figure *1*. Among the tested media, EP Medium 1 yielded the highest viable cell counts for all three strains.

Specifically, viable cell counts reached 6.5×10^8 CFU/mL for EGE-B-36.5, 8.7×10^8 CFU/mL for EGE-B-3.P.5, and 4.6×10^8 CFU/mL for EGE-B-1.19 in EP Medium 1. In contrast, commercial media such as NB and LB resulted in comparatively lower biomass production.

These findings indicate that EP Medium 1 provides a more suitable nutrient composition for *Bacillus* growth and was therefore selected for subsequent experiments.

Medium	Viable cell count (CFU/mL)		
	EGE-B-36.5	EGE-B-3.P.5	EGE-B-1.19
NB	$(1.2 \pm 0.1) \times 10^{7c}$	$(3.4 \pm 0.4) \times 10^{8b}$	$(2.5 \pm 0.4) \times 10^{7c}$
LB	$(6.4 \pm 0.4) \times 10^{7c}$	$(8.4 \pm 0.9) \times 10^{7c}$	$(1.2 \pm 0.1) \times 10^{8b}$
EPM 1	$(6.5 \pm 0.5) \times 10^{8a}$	$(8.7 \pm 1.2) \times 10^{8a}$	$(4.6 \pm 0.3) \times 10^{8a}$
EPM 2	$(2.5 \pm 0.7) \times 10^{8b}$	$(1.7 \pm 0.5) \times 10^{7c}$	$(4.2 \pm 0.5) \times 10^{8a}$

TABLE 1: Effect of different culture media on the biomass production of B. subtilis strains.

Values are presented as mean ± SD ($n = 3$). Means sharing the same letter within a column are not significantly different according to Tukey's test ($p > 0.05$). Pairwise comparisons revealed that for EGE-B-36.5, NB and LB did not differ significantly ($p = 0.491$), whereas EPM 2 differed from NB ($p = 0.001$) and LB ($p = 0.003$), and EPM 1 differed from all other media ($p < 0.001$). For EGE-B-3.P.5, EPM 2 and LB ($p = 0.592$) as well as LB and NB ($p = 0.050$) were not significantly different, while all other comparisons were significant ($p \leq 0.001$). For EGE-B-1.19, EPM 1 and EPM 2 did not differ significantly ($p = 0.417$), whereas LB and NB differed significantly ($p = 0.013$); all remaining comparisons were significant ($p < 0.001$).
CFU/mL, Colony-forming units per milliliter
NB, nutrient broth; LB, lysogeny broth; EPM, endospore production media.

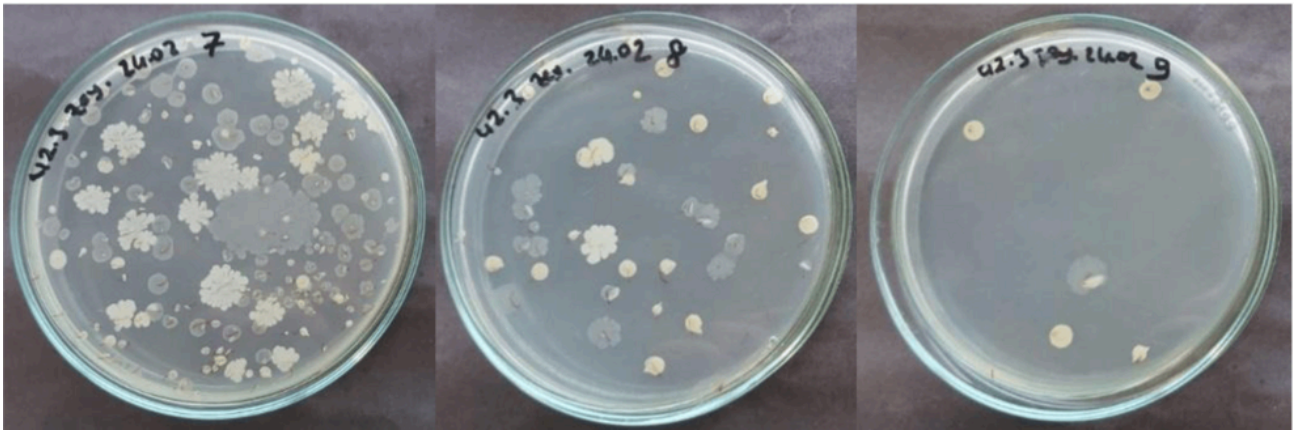


FIGURE 1: Pour plate assay showing bacterial colony formation.

Effect of glycerol on biomass production

The impact of glycerol supplementation on biomass production is shown in Table 2. The addition of glycerol to the production medium resulted in a noticeable increase in viable cell counts for all strains.

The most significant enhancement was observed in *B. subtilis* EGE-B-1.19, where cell counts increased from 4.6×10^8 CFU/mL (control) to 2.2×10^{10} CFU/mL with 3% glycerol supplementation, corresponding to approximately a 2-log increase. Similarly, EGE-B-3.P.5 exhibited a substantial increase, reaching 4.2×10^9 CFU/mL at 3% glycerol concentration. In contrast, EGE-B-36.5 showed a relatively modest increase.

These results demonstrate that glycerol supplementation enhances biomass production, although the magnitude of this effect is strain dependent. Based on its superior performance, *B. subtilis* EGE-B-1.19 was selected for further shelf-life studies.

Medium	Viable cell count (CFU/mL)		
	EGE-B-36.5	EGE-B-3.P.5	EGE-B-1.19
EPM 1	$(6.5\pm0.1) \times 10^{8ab}$	$(8.7\pm1.2) \times 10^{8b}$	$(4.6\pm0.3) \times 10^{8c}$
With 1% glycerol	$(6.2\pm0.4) \times 10^{8b}$	$(1.5\pm0.2) \times 10^{9b}$	$(5.0\pm1.5) \times 10^{9b}$
With 2% glycerol	$(7.7\pm0.7) \times 10^{8ab}$	$(3.5\pm0.9) \times 10^{9a}$	$(2.1\pm0.4) \times 10^{10a}$
With 3% glycerol	$(8.2\pm1.2) \times 10^{8a}$	$(4.2\pm0.2) \times 10^{9a}$	$(2.2\pm0.3) \times 10^{10a}$

TABLE 2: Effect of glycerol supplementation in the production medium on biomass production of B. subtilis strains.

Values are presented as mean ± SD ($n = 3$). Means sharing the same letter within a column are not significantly different according to Tukey's test ($p > 0.05$). For strain EGE-B-1.19, no significant differences were observed between 1% glycerol and EPM 1 ($p = 0.168$) or between 2% and 3% glycerol ($p = 0.962$), while all other pairwise comparisons were statistically significant ($p < 0.001$). For strain EGE-B-3.P.5, significant differences were observed between 1% and 2% glycerol ($p = 0.004$), 1% and 3% glycerol ($p = 0.001$), and 2% glycerol and EPM 1 ($p = 0.001$), whereas no significant differences were detected between EPM 1 and 1% glycerol ($p = 0.419$) or between 2% and 3% glycerol ($p = 0.338$), and all remaining comparisons were significant ($p < 0.001$). For strain EGE-B-36.5, most pairwise comparisons were not significant, including EPM 1 vs 1% glycerol ($p = 0.955$), 1% vs 2% glycerol ($p = 0.128$), 2% vs 3% glycerol ($p = 0.832$), and 2% glycerol vs EPM 1 ($p = 0.255$), while a significant difference was observed between 1% and 3% glycerol ($p = 0.039$), and a non-significant trend was observed between 3% glycerol and EPM 1 ($p = 0.080$).
EPM, Endospore production media; CFU, Colony-forming units per milliliter

Effect of glycerol addition during cultivation on shelf-life

The long-term stability of formulations prepared with glycerol addition during cultivation is presented in Table *3*. All formulations were initially standardized to 10^8 CFU/mL and pH 5.0. Over the 12-month storage period, a gradual decline in viable cell counts was observed in all groups.

By the 12th month, the control group showed a marked reduction in viable cell counts, decreasing from 1.1×10^8 CFU/mL to 0.1×10^7 CFU/mL. In contrast, formulations containing glycerol exhibited improved stability over the storage period. Among these, the 3% glycerol treatment showed the highest protective effect, with viable cell counts decreasing only from 1.2×10^8 CFU/mL to 0.8×10^8 CFU/mL. Additionally, pH values in all groups increased over time. The control group showed a rapid increase, reaching approximately pH 8.1-8.6 within the first few months. Glycerol-containing formulations exhibited a slower pH increase, particularly at higher concentrations.

These findings suggest that glycerol addition during cultivation provides a partial protective effect, improving both cell survival and pH stability during storage.

A previous study investigated the effect of various cell protectants added to the production medium on the shelf-life of *Rhizobium*, *Azotobacter*, *Azospirillum*, and *Bacillus megaterium* strains. The study reported that formulations containing a combination of 0.5% polyvinylpyrrolidone (PVP) and 0.5% glycerol retained the highest viable cell counts across all tested strains. In particular, the viable cell counts of *B. megaterium* decreased from 2×10^{10} to 0.96×10^{10} CFU/mL after 6 months of storage under this formulation [3]. These findings demonstrate that the combined use of cell protectants can significantly improve microbial survival during storage. In line with this, future studies based on the results of the present work may explore the addition of different cell protectants during the production stage to further enhance formulation stability.

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Month	Control		With 1% glycerol		With 2% glycerol		With 3% glycerol	
	Viable cell count ($\times 10^8$ CFU/mL)	pH	Viable cell count ($\times 10^8$ CFU/mL	pH	Viable cell count ($\times 10^8$ CFU/mL	pH	Viable cell count ($\times 10^8$ CFU/mL	pH
0	1.1 ± 0.1	5.0 ± 0.3	1.1 ± 0.05	5.0 ± 0.1	1.3 ± 0.1	5.0 ± 0.1	1.2 ± 0.3	5.0 ± 0.2
1	1.5 ± 0.3	6.8 ± 0.6	1.9 ± 1.0	5.6 ± 0.2	1.2 ± 0.4	5.9 ± 0.4	1.5 ± 0.1	5.7 ± 0.5
2	1.7 ± 0.2	7.9 ± 0.5	2.1 ± 0.9	5.9 ± 0.5	1.6 ± 0.3	5.9 ± 0.5	1.5 ± 0.3	5.7 ± 0.5
3	1.8 ± 0.6	7.8 ± 1.2	2.0 ± 0.4	6.0 ± 0.2	1.7 ± 0.8	5.9 ± 0.2	2.1 ± 0.8	5.7 ± 0.4
4	2.1 ± 0.9	8.4 ± 1.1	1.5 ± 0.4	6.6 ± 0.6	1.5 ± 0.4	6.1 ± 0.1	1.9 ± 0.3	5.8 ± 0.1
5	1.8 ± 1.1	8.7 ± 1.1	1.5 ± 0.3	7.1 ± 0.4	1.3 ± 0.1	6.3 ± 0.6	1.8 ± 0.5	5.9 ±0 .4
6	1.2 ± 0.1	8.2 ± 0.9	1.4 ± 0.4	7.2 ± 0.3	1.3 ± 0.1	6.5 ± 0.6	1.9 ± 0.9	6.1 ± 0.7
7	0.8 ± 0.1	8.3 ± 0.8	1.1 ± 0.2	7.4 ± 0.5	1.0 ± 0.3	6.9 ± 0.5	1.9 ± 0.5	6.2 ± 0.5
8	0.8 ± 0.4	8.1 ± 1.1	0.9 ± 0.1	7.3 ± 0.5	1.1 ± 0.2	6.8 ± 0.7	1.7 ± 0.5	6.3 ± 0.4
9	0.7 ± 0.2	8.2 ± 1.1	0.7 ± 0.3	7.5 ± 0.4	1.2 ± 0.5	7.1 ± 0.3	1.1 ± 0.4	6.3 ± 0.3
10	0.5 ± 0.1	8.2 ± 1.2	0.5 ± 0.1	7.9 ± 0.3	1.1 ± 0.1	7.6 ± 0.9	1.0 ± 0.3	6.6 ± 0.5
11	0.2 ± 0.1	8.1 ± 1.1	0.5 ± 0.2	8.1 ± 1.2	0.9 ± 0.1	7.9 ± 0.8	0.8 ± 0.2	6.8 ± 0.4
12	0.1 ± 0.05^{c}	8.2 ± 1.0^{A}	$0.3 \pm 0.1b^{c}$	8.1 ± 0.9^{A}	0.5 ± 0.05^{b}	7.7 ± 0.5^{A}	0.8 ± 0.1^{a}	6.7 ± 0.3^{A}

TABLE 3: Effect of glycerol supplementation during cultivation on the shelf-life (viable cell count and pH) of B. subtilis formulations.

Values are presented as mean ± SD ($n = 3$). Different lowercase letters indicate significant differences in viable cell counts, while different uppercase letters indicate significant differences in pH values among treatments at the 12th month ($p < 0.05$). Values sharing the same letter are not significantly different ($p > 0.05$). At month 12, viable cell counts differed significantly among treatments. No significant differences were observed between 1% and 2% glycerol ($p = 0.058$) or between 1% glycerol and the control ($p = 0.058$). In contrast, 2% glycerol differed significantly from 3% glycerol ($p = 0.007$) and the control ($p = 0.001$), while all remaining pairwise comparisons were highly significant ($p < 0.001$). Regarding pH, no significant differences were detected among any of the treatments, including comparisons between 1% and 2% glycerol ($p = 0.906$), 1% and 3% glycerol ($p = 0.168$), 1% glycerol and the control ($p = 0.998$), 2% and 3% glycerol ($p = 0.396$), 2% glycerol and the control ($p = 0.837$), and 3% glycerol and the control ($p = 0.123$).
CFU/mL, Colony-forming units per milliliter

In experiments where glycerol was added to the production medium, statistical analysis of the 12th-month data revealed no significant differences among the groups in terms of pH. However, a statistically significant difference in viable cell count was observed, with the highest value obtained in the formulation containing 3% glycerol.

Effect of post-production glycerol addition on shelf-life

The results of shelf-life analysis for formulations with post-production glycerol addition are presented in Table *4*.

Compared to the control group, glycerol-containing formulations exhibited significantly higher viable cell counts throughout the storage period. The most notable effect was observed at 1% glycerol concentration, where cell counts remained at approximately 2.9 log units after 12 months. Similarly, 2% and 3% glycerol formulations maintained higher viability compared to the control, although slight fluctuations were observed during the storage period.

In contrast to the cultivation-stage application, post-production glycerol addition resulted in markedly improved cell survival, indicating a stronger protective effect. Regarding pH, all formulations exhibited an increasing trend over time. However, glycerol-containing groups maintained relatively lower pH values (approximately 6.0-7.0 for most of the storage period), compared to the control group, which rapidly reached alkaline levels (~pH 8-9).

An increase of approximately 1 log unit in viable cell counts was observed at the second month in formulations containing 1% glycerol, and at the third month in formulations containing 2% and 3% glycerol. Since all samples were subjected to heat-shock treatment prior to plating, this temporary increase is unlikely to be associated with germination or metabolic activity. Instead, it may be attributed to improved dispersion of spore aggregates and enhanced recovery efficiency during plating in glycerol-containing formulations.

Although no significant overall decline in viable cell counts was observed at the end of the 12-month storage period, the initial increase followed by a subsequent decrease indicates potential instability within the formulation. Such fluctuations in cell viability may negatively affect the stability and consistency of the biofertilizer product.

A previous study evaluated the effects of different cryoprotectants and storage conditions on the viability of probiotic bacteria over a 30-day period. The results indicated that the effectiveness of cryoprotectants decreased with increasing storage time. However, *B. subtilis* preserved in a 1:1 mixture of glycerol and mineral oil exhibited a higher survival rate (38.1%) compared to glycerol alone (30.95%), demonstrating a synergistic effect between the cryoprotectants [4].

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Month	Control		1% Glycerol		2% Glycerol		3% Glycerol	
	Viable cell count ($\times 10^8$ CFU/mL	pH	Viable cell count ($\times 10^8$ CFU/mL	pH	Viable cell count ($\times 10^8$ CFU/mL	pH	Viable cell count ($\times 10^8$ CFU/mL	pH
0	1.1 ± 0.1	5.0 ± 0.3	1.2 ± 0.1	5.0 ± 0.4	1.1 ± 0.05	5.0 ± 0.1	1.2 ± 0.4	5.0 ± 0.3
1	1.5 ± 0.3	6. ± 0.6	3.0 ± 0.5	6.6 ± 0.8	2.9 ± 1.2	6.4 ± 0.4	3.0 ± 0.2	6.6 ± 0.4
2	1.7 ± 0.2	7.9 ± 0.5	11.0 ± 1.4	6.1 ± 0.4	3.8 ± 1.2	6.4 ± 0.2	3.4 ± 0.6	6.3 ± 0.3
3	1.8 ± 0.6	7.8 ± 1.2	6.0 ± 0.8	6.4 ± 0.3	8.8 ± 1.1	6.5 ± 0.3	9.4 ± 0.9	6.0 ± 0.2
4	2.1 ± 0.9	8.4 ± 1.1	6.5 ± 0.5	7.1 ± 0.2	5.4 ± 1.4	6.7 ± 0.1	4.7 ± 0.3	6.5 ± 0.5
5	1.8 ± 1.1	8.7 ± 1.1	4.2 ± 1.1	8.1 ± 0.1	2.6 ± 0.2	6.2 ± 0.1	3.4 ± 0.2	6.4 ± 0.3
6	1.2 ± 0.1	8.2 ± 0.9	5.8 ± 1.2	7.8 ± 0.1	5.1 ± 0.9	6.3 ± 0.4	2.5 ± 0.1	6.3 ± 0.6
7	0.8 ± 0.1	8.3 ± 0.8	4.3 ± 0.9	7.7 ± 0.5	3.1 ± 0.5	6.4 ± 0.5	2.9 ± 0.9	6.5 ± 0.2
8	0.8 ± 0.4	8.1 ± 1.1	3.6 ± 1.2	7.2 ± 0.3	1.2 ± 0.4	6.3 ± 0.2	1.9 ± 0.2	6.2 ± 0.1
9	0.7 ± 0.2	8.2 ± 1.1	2.4 ± 0.4	7.3 ± 0.6	2.5 ± 0.3	6.3 ± 0.2	3.5 ± 0.6	6.3 ± 0.2
10	0.5 ± 0.1	8.2 ± 1.2	4.3 ± 0.3	7.6 ± 0.4	3.2 ± 0.2	6.2 ± 0.5	3.7 ± 0.8	6.1 ± 0.1
11	0.2 ± 0.1	8.1 ± 1.1	3.5 ± 0.7	7.7 ± 0.5	3.2 ± 0.6	5.8 ± 0.2	0.8 ± 0.05	6.2 ± 0.1
12	0.1 ± 0.05^{c}	8.2 ± 1.0^{A}	2.9 ± 0.1^{a}	8.1 ± 0.8^{A}	2.5 ± 0.5^{ab}	6.0 ± 0.1^{B}	1.8 ± 0.5^{b}	6.7 ± 0.1^{AB}

TABLE 4: Effect of post-production glycerol addition on the shelf-life (viable cell count and pH) of B. subtilis formulations.

Values are presented as mean ± SD ($n = 3$). Different lowercase letters indicate significant differences in viable cell counts, while different uppercase letters indicate significant differences in pH values among treatments at the 12th month ($p < 0.05$). Values sharing the same letter are not significantly different ($p > 0.05$). At month 12, viable cell counts differed significantly among treatments. No significant differences were observed between 1% and 2% glycerol ($p = 0.550$) or between 2% and 3% glycerol ($p = 0.155$). However, 1% and 3% glycerol differed significantly ($p = 0.023$). The 3% glycerol treatment also showed significantly higher viable cell counts than the control ($p = 0.002$), while all remaining pairwise comparisons were highly significant ($p < 0.001$). Regarding pH, no significant differences were observed between 1% and 3% glycerol ($p = 0.107$), 2% and 3% glycerol ($p = 0.571$), 3% glycerol and the control ($p = 0.082$), or 1% glycerol and the control ($p = 0.997$). In contrast, significant differences were detected between 1% and 2% glycerol ($p = 0.017$) and between 2% glycerol and the control ($p = 0.013$).
CFU/mL, Colony-forming units per milliliter

At the 12th month, no statistically significant differences in viable cell counts were observed between 1% and 2% glycerol-containing formulations, although the highest numerical value was recorded in the 1% glycerol treatment. However, this treatment exhibited a greater pH increase compared to 2% glycerol. Although 2% glycerol resulted in slightly lower viable cell counts, the differences in CFU were not statistically pronounced, while it provided better pH stability throughout the storage period. Considering that pH stability is a critical parameter for maintaining long-term formulation integrity and preventing microbial imbalance, post-production addition of 2% glycerol appears to be more suitable for achieving overall formulation stability.

Conclusions

This study demonstrated that the timing of glycerol application plays a critical role in determining the stability of *Bacillus*-based biofertilizer formulations. While glycerol supplementation during cultivation enhanced biomass production, its contribution to long-term stability was limited. In contrast, post-production glycerol addition significantly improved both cell viability and pH stability during the 12-month storage period. Among the tested conditions, 2% glycerol applied after production provided the most promising formulation in terms of overall stability, considering both pH maintenance and viable cell counts at month 12. However, the observed mid-storage fluctuations in colony-forming units per milliliter indicate that further optimization is required to ensure consistent viability throughout the storage period. Overall, these findings indicate that glycerol functions more efficiently as a post-production stabilizing agent rather than as a cultivation additive for enhancing shelf-life. The results highlight the importance of optimizing both the concentration and application stage of protective agents in the development of stable liquid biofertilizer formulations. Overall, the use of 2% post-production glycerol represents a promising and scalable formulation strategy that may improve shelf-life stability and support the commercial deployment of *Bacillus*-based biofertilizers with more consistent field performance.

Additional Information

Author Contributions

All authors have reviewed the final version to be published and agreed to be accountable for all aspects of the work.

Concept and design: Kemal Karaca, Rengin Eltem

Acquisition, analysis, or interpretation of data: Kemal Karaca

Drafting of the manuscript: Kemal Karaca

Critical review of the manuscript for important intellectual content: Kemal Karaca, Rengin Eltem

Supervision: Rengin Eltem

Disclosures

Plant subjects: All authors have confirmed that this study did not involve any herbarium specimen. **Human subjects:** All authors have confirmed that this study did not involve human participants or tissue. **Animal subjects:** All authors have confirmed that this study did not involve animal subjects or tissue. **Conflicts of interest:** In compliance with the ICMJE uniform disclosure form, all authors declare the following: **Payment/services info:** This study was supported by the "TÜBİTAK 1512 Entrepreneurship Support Program (BİGG)". . **Financial relationships:** All authors have declared that they have no financial relationships at present or within the previous three years with any organizations that might have an interest in the submitted work. **Other relationships:** All authors have declared that there are no other relationships or activities that could appear to have influenced the submitted work.

Data Availability Statements

The datasets (and/or code) supporting this study are available from the corresponding author upon reasonable request.

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