Evaluation of different oil formulations to study the shelf life of Trichoderma viride

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***Abstract*: The present study focused on optimizing the production medium and oil-based formulation to enhance the shelf life and efficacy of Trichoderma viride, a widely used biological control agent in sustainable agriculture. Seven different liquid media were evaluated for their ability to support fungal growth and sporulation. Among them, Molasses Yeast Broth (MYB) and Molasses Soy Broth (MSB) supported the highest biomass and spore production, with MYB yielding 800.00 ± 4.90 mg biomass and 12.00 ± 0.07 × 10^8 CFU/mL spores. Spearman correlation analysis revealed a strong positive correlation (ρ = 0.955, p = 0.0008) between mycelial weight and spore count. Based on these results, MYB was selected for large-scale inoculum generation.**

To assess long-term viability, T. viride formulations were prepared using different oil combinations: paraffin alone, soybean oil alone, and their mixtures with glycerol. The viability and CFU/mL were monitored over 180 days. Paraffin oil alone exhibited the best shelf-life performance, retaining 9.0 × 10^4 ± 0.51×10^4 CFU/mL and showing only a 55.0 ± 1.65% reduction in viability. In contrast, formulations with glycerol, especially soybean + glycerol, showed rapid decline in spore viability (99.5 ± 1.83% reduction), attributed to glycerol's hygroscopicity and the nutrient-rich nature of soybean oil. These conditions likely favored premature germination or spore degradation under ambient temperatures.

The results highlighted the importance of selecting nutrient-rich media like MYB for optimal production and inert carriers like paraffin oil for long-term stability. This dual optimization approach could significantly improve the field applicability and commercial viability of T. viride based biocontrol formulations.

***Index Terms*—Oil formulation, Shelf life, Spearman correlation, Trichoderma viride.**

I. INTRODUCTION

Modern agriculture faces immense pressure to increase output sustainably. By 2050, global food production must rise by roughly 50% to meet demand. Yet a significant portion of current production is lost to pests and diseases up to 20–40% of global crop yield is destroyed annually by plant pests and pathogens [1]. Reducing such losses is crucial for improving agricultural productivity and food security. Traditionally, heavy use of chemical pesticides helped boost 20th-century yields, but this approach raises concerns about environmental and health impacts. In response, there is growing interest in sustainable disease management solutions, particularly biological control agents (BCAs). Biological control harnesses beneficial microbes to suppress plant pathogens, offering an eco-friendly alternative to chemical pesticides. Beneficial microorganisms such as Trichoderma, Bacillus, Pseudomonas, and others have gained prominence as natural antagonists of crop pathogens [2]. Studies show that microbial BCAs can effectively reduce pathogen pressure and even bolster plant defense mechanisms, thereby protecting yields in a safer, sustainable manner.

These organisms can curb diseases through mechanisms like competition, parasitism, and induction of plant resistance, ultimately safeguarding crop performance [3]. For example, deploying BCAs in integrated pest management can significantly reduce disease incidence, which translates into higher effective yields. In one case, a Trichoderma-based treatment cut disease incidence of *Rhizoctonia solani* by over 80% and correspondingly increased crop yield by ~69% under greenhouse conditions [4]. *Trichoderma viride*, in particular, is a well-known antagonistic fungus naturally present in soils worldwide. Because of these benefits, *T. viride* and related species are commercially formulated as biofungicides or soil inoculants; in fact, *T. harzianum* and *T. viride* are two of the most extensively exploited BCAs, reported to be used on over 80 different crops against dozens of soil-borne and foliar diseases [5].

A critical challenge in developing microbial biocontrol products is formulating them for long shelf life and consistent field performance [6]. Many microbial bioagents that work well in laboratory tests fail to perform in field conditions due to rapid decline in their viable populations. Traditional formulations for Trichoderma include talc-based powders, peat/charcoal carriers, and granular inoculants. While these dry carrier formulations are relatively easy to handle and apply, they often support limited shelf stability. For instance, a typical talc-based Trichoderma product maintains effective spore viability for about 3–6 months under ambient conditions.

Oil-based formulations have emerged as a promising strategy to improve the shelf life and efficacy of *Trichoderma viride* [7]. In these formulations, fungal spores (conidia) are suspended in oils or emulsions (water-oil mixtures) instead of traditional dry powders [8]. This approach offers several stability advantages: the oil carrier forms a protective barrier around spores, shielding them from environmental stress like UV radiation and high temperatures. Oils also reduce desiccation by retaining moisture around the propagules and improve adhesion of the biocontrol agent to seeds or plant surfaces upon application [9]. As a result, Trichoderma in oil-based preparations is released more gradually and survives longer in storage and after application. For example, an oil emulsion of *T. asperellum* retained ~2.8×10^9 CFU/mL after 180 days, whereas a comparable vegetable oil formulation had ~1.8×10^9 CFU/mL, both indicating high viability at 6 months [10]. Conidia preserved with coconut oil as the primary carrier showed enhanced shelf life in ambient storage, outperforming traditional formulations in viability over time The present study aimed to evaluate oil-based formulations for *Trichoderma viride* to enhance its shelf life and efficacy. By optimizing oil and adjuvant combinations, we seek to develop formulations that offer longer spore viability and improved biocontrol performance. The results could lead to more stable, cost-effective, and scalable biocontrol solutions, crucial for sustainable agricultural practices.

II. MATERIALS AND METHODS

A. Collection of fungal cultures

The standard cultures of *Trichoderma viride* strain-ITCC6914 (1 % WP) were obtained from Tamil Nadu Agricultural University (TNAU) with an initial concentration of 2 x 10^6 CFU/mL. The experiments were conducted at M/s Pravara Biotech Laboratory, Chikali, Taluka Sangamner, District Ahmednagar, Maharashtra.

B. Optimization of liquid medium for production of Trichoderma viride

Trichoderma viride was grown on Potato Dextrose Agar (PDA) for 7 days to obtain a fully sporulated culture [11]. The inoculum was prepared by scraping the surface of the culture with a sterile scalpel and transferring the spores into sterile deionized water containing 0.1% Tween 20. The spore concentration was adjusted to 10^6 CFU/mL. One mL of the inoculum was added to 100 mL of each of the following media in 250 mL Erlenmeyer flasks: Czapek Broth (CzB), Potato Dextrose Broth (PDB), Molasses Yeast Broth (MYB), Richard's Broth (RB), Molasses Soy Broth (MSB), Water Dextrose Broth (WDB), and Sabouraud Dextrose Broth (SDB). The inoculated flasks were incubated at 28 ± 2°C for 72 hours. After incubation, the entire contents of each flask were homogenized in a blender. One liter of the homogenized inoculum was added to 9 liters of the respective fermenter medium for mass production. After 10 days of inoculation, the mycelial mats were harvested on pre-weighed Whatman No. 42 filter paper and washed with warm (40°C) distilled water. Dry mycelial weight and spore count were recorded to compare the efficiency of growth and sporulation in each medium.

C. Optimization of oil formulation to increase shelf life of Trichoderma viride

The shelf life and viability of *Trichoderma viride* were studied using different oil formulations: paraffin + glycerol (1:1), soybean + glycerol (1:1), paraffin + soybean (1:1), paraffin alone, and soybean alone. The produced *Trichoderma viride* was transferred into a mixing tank for spore and mycelium harvesting. The mixed *Trichoderma viride* formulation was poured into pre-sterilized 150 ml conical flask. Each treatment contained glycerol (10 mL), surfactant (3 mL), and suspender (3 mL). Treatment T6 contained a departmental culture, and T7 was a liquid formulation market product. The bottles were capped and stored at 27 ± 1°C for a period of six months [11].

To assess the shelf life and viability of the formulations, serial dilutions of the samples were prepared, ranging from 10^{-1} to 10^{-8}. One milliliter of each dilution was plated onto PDA plates under laminar flow and spread evenly using a sterile L-shaped glass rod. The plates were left to dry completely before incubation at 25°C for 2-3 days. Three replicate plates were used for each dilution. After incubation, the CFU of Trichoderma were counted, and the total CFU/mL was calculated by multiplying the number of colonies by the dilution factor. The viability of spores and CFU/mL were monitored over a six-month period, with evaluations conducted at monthly intervals to assess population dynamics.

D. Pour Plate Method for CFU Count

Serial dilution was performed by transferring 1 mL of suspension to the first water blank, followed by successive dilution to obtain a 10^7 dilution factor. The desired dilution (10^8) was transferred to sterilized Petri plates, and 20 mL of melted medium was poured into each plate, which was then gently rotated and allowed to solidify. The plates were incubated for three days. After incubation, the total number of colonies was counted, and the CFU was calculated using the formula:

$$\frac{\text{CFU}}{\text{mL}} = \frac{\text{number of colonies} * \text{dilution factor}}{\text{volume plated (mL)}}$$

E. Statistical analysis

The experiments were conducted in triplicates and the standard error of the mean was calculated. Analysis of variance was performed at $p<0.05$ level of significance by Tukey's HSD post hoc analysis. Spearman correlation was studied between mycelial weight and spore count for the optimization of liquid media for the *Trichoderma viride* production.

III. RESULTS AND DISCUSSION

A. Optimization of liquid medium for Trichoderma viride production

The growth and sporulation of *Trichoderma viride* were assessed in seven different liquid media by measuring both mycelial biomass and spore count (Table 1). The results revealed significant variability in biomass accumulation and sporulation efficiency among the tested media. Molasses Yeast Broth (MYB) and Molasses Soy Broth (MSB) demonstrated the highest biomass yields, recording 800.00 ± 4.90 mg and 780.00 ± 4.78 mg, respectively, owing to their rich carbon and nitrogen content, which supported robust fungal growth. Richard's Broth (RB) also supported substantial growth, with a biomass of 700.00 ± 4.29 mg. In contrast, Water Dextrose Broth (WDB) yielded the lowest biomass (110.00 ± 0.67 mg), likely due to its inadequate nutrient composition. Similarly, Czapek's Dox Broth (CB) produced relatively low biomass (260.00 ± 1.59 mg), suggesting that the nitrogen source provided in this medium may be suboptimal for supporting T. viride growth.

Spore production followed a similar trend. MSB and MYB yielded the highest spore counts (12.00 ± 0.07 × 10^8 CFU/mL). Potato Dextrose Broth (PDB) supported moderate sporulation (2.80 ± 0.02 × 10^8 CFU/mL), while RB (0.60 ± 0.00 × 10^8 CFU/mL) and SDB (0.22 ± 0.00 × 10^8 CFU/mL) produced significantly fewer spores. The lowest spore count was observed in WDB (0.02 ± 0.00 × 10^8 CFU/mL). similar results were obtained by Sorathiya, et al. [12] and Khan, et al. [11] who reported good production of *T. viride* in molasses yeast broth.

Analysis of variance (ANOVA) followed by Tukey's post-hoc test revealed statistically significant differences in dry mycelial weight among the tested media. However, no significant difference was observed in spore production between Molasses Yeast Broth (MYB) and Molasses Soy Broth (MSB), both of which exhibited the highest sporulation levels. Furthermore, Spearman's rank correlation analysis demonstrated a strong positive correlation between mycelial biomass and spore count ($\rho = 0.955$, $p = 0.0008$), suggesting that increased mycelial growth is closely associated with enhanced spore production. Based on these findings, MYB was identified as the most suitable medium and selected for subsequent inoculum development.

Table 1 Mycelial weight and spore count of *Trichoderma viride* in different liquid media

Sr. No.	Liquid media	Mycelial Weight (mg)*	Spore Count (×10^8 CFU/mL)
1	Molasses Soy Broth (MSB)	780.00 ± 4.78	12.00 ± 0.07c

2	Potato Dextrose Broth (PDB)	440.00 ± 2.70	2.80 ± 0.02
3	Richard's Broth (RB)	700.00 ± 4.29	0.60 ± 0.00
4	Sabouraud Dextrose Broth (SDB)	300.00 ± 1.84	0.22 ± 0.00ad
5	Czapek's Dox Broth (CB)	260.00 ± 1.59	0.10 ± 0.00ab
6	Molasses Yeast Broth (MYB)	800.00 ± 4.90	12.00 ± 0.07c
7	Water Dextrose Broth (WDB)	110.00 ± 0.67	0.02 ± 0.00bd

'*' is that all means are significantly different from each other, means followed by similar means are not significantly different from each other.

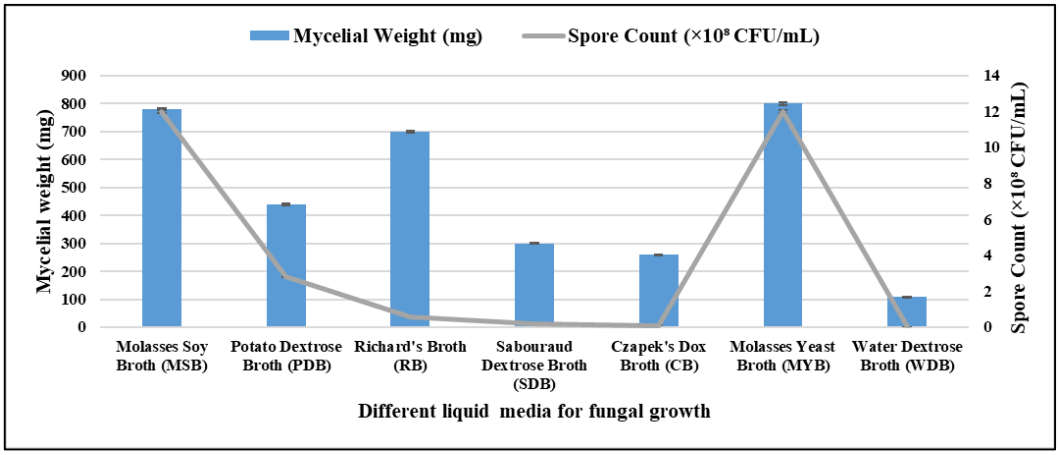

Figure 1 Comparison of mycelial weight and sporulation in Trichoderma viride using different liquid media

B. Optimization of oil formulation to increase shelf life of Trichoderma viride

The study evaluated the effect of various oil formulations on the shelf life of *Trichoderma viride* by monitoring the sporulation count (CFU/mL) and viability reduction (%) over a 180-day storage period (Table 2 and Table 3). Initially, all treatments exhibited a sporulation count of 2×10^6 CFU/mL. Over time, paraffin maintained relatively higher viability, with a gradual decline to $9.0 \times 10^4 \pm 0.51 \times 10^4$ CFU/mL at 180 days and a corresponding viability reduction of 55.0 ± 1.65%. Soybean oil alone showed a slightly sharper decline to $7.0 \times 10^4 \pm 0.40 \times 10^4$ CFU/mL with 65.0 ± 1.44% viability reduction. Among the combinations, the paraffin + soybean (1:1) formulation showed a moderate decline in CFU/mL to $5.0 \times 10^4 \pm 0.28 \times 10^4$ and a 75.0 ± 3.69% viability reduction. The paraffin + glycerol (1:1) formulation performed comparatively inefficient, dropping to $3.0 \times 10^4 \pm 0.17 \times 10^4$ CFU/mL with an 85.0 ± 2.98% reduction in viability. Similar results were obtained by Kajal Mane [5]. Many studies have reported better shelf life of T. viride in paraffin compared to other oils like soybean and glycerol [10, 13]. The inert, non-nutritive nature of paraffin limits metabolic activity and preserves dormancy [14], whereas the unsaturated, nutrient-rich profile of soybean oil tends to promote faster viability loss due to oxidation and possible premature germination.

The most significant reduction in both sporulation count and viability was observed in the soybean + glycerol (1:1) treatment, which declined to $1.0 \times 10^3 \pm 0.015 \times 10^4$ CFU/mL and exhibited a 99.5 ± 1.83% viability reduction in 180 days. The control treatment followed a similar trend, with sporulation decreasing to $9.0 \times 10^3 \pm 0.012 \times 10^4$ CFU/mL and a viability reduction of 96.0 ± 3.8%. Based on the data, paraffin alone proved to be the most stable formulation for preserving *T. viride* over long-term storage, showing the least reduction in both CFU count and viability. Formulations involving glycerol, particularly in combination with soybean oil, significantly compromised the shelf life, that indicated their unsuitability for maintaining long-term viability of *T. viride* spores.

Although glycerol is widely reported as an effective cryoprotectant for microbial preservation, especially under low-temperature storage (such as –20°C or –

80°C) [15], the current study observed a decline in spore viability when glycerol was combined with oil-based carriers and stored at room temperature (25°C). This discrepancy may be due to the fact that glycerol is usually used alone and under refrigerated or frozen conditions, which inhibit microbial metabolism and degradation. In contrast, at room temperature, glycerol's hygroscopic nature may create microenvironments that support partial hydration or even germination of spores, particularly when used in nutrient-rich oil matrices.

Table 2 Effect of different oil formulations on the sporulation count of *Trichoderma viride*

Treatment	Oil Formulation	0 days ($10^6 \pm 10^4$)	30 days ($10^5 \pm 10^4$)	60 days ($10^5 \pm 10^4$)	90 days ($10^5 \pm 10^4$)	120 days ($10^5 \pm 10^4$)	150 days ($10^5 \pm 10^4$)	180 days ($10^4 \pm 10^4$)
1	Paraffin	2.0 ±1.2	1.9 ± 1.1	1.8 ± 1.1	1.6 ± 0.92	1.3 ± 0.75	1.1 ± 0.63	90 ± 0.51
2	Soybean	2.0 ± 1.1	1.8 ± 1.1	1.6 ± 0.92	1.4 ± 0.80	1.1 ± 0.63	8.0 ± 0.46	70 ± 0.40
3	Paraffin + Soybean (1:1)	2.0 ± 1.2	1.6 ± 0.92	1.3 ± 0.75	1.1 ± 0.63	9.0 ± 0.51	7.0 ± 0.40	50 ± 0.28
4	Paraffin + Glycerol (1:1)	2.0 ± 1.1	1.4 ± 0.90	1.1 ± 0.63	9.0 ± 0.51	7.0 ± 0.40	5.0 ± 0.28	30 ± 0.17
5	Soybean + Glycerol (1:1)	2.0 ± 1.2	1.2 ± 0.62	9.0 ± 0.51	7.0 ± 0.40	5.0^b ± 0.28	3.0 ± 0.17^a	1.0 ± 0.02
6	Control	2.0 ± 1.0	1.5 ± 0.86	8.0 ± 0.46	8.0 ± 0.46	5.0^b ± 0.28	3.0 ± 0.17^a	9.0 ± 0.01

The readings are given in CFU/mL. The values followed by means are standard error of the mean. The values are significantly different from each other at $p<0.05$ level of significance. The values with common superscripts denote similar means.

Table 3 Viability reduction percentage of different oil formulations in *Trichoderma viride*

Treatment No.	Oil formulation	0 days	30 days	60 days	90 days	120 days	150 days	180 days
1	Paraffin	0 ± 0	5 ± 0.23	10 ± 0.36	20 ± 0.7	35 ± 0.77	45 ± 1.66	55.0 ± 1.65
2	Soybean	0 ± 0	10 ± 0.47	20 ± 0.75	30 ± 0.79	45 ± 1.68	60 ± 2.62	65.0 ± 1.44
3	Paraffin + Soybean (1:1)	0 ± 0	20 ± 0.77	35 ± 1.06	45 ± 1.36	55 ± 1.54	65 ± 2.96	75.0 ± 3.69
4	Paraffin + Glycerol (1:1)	0 ± 0	30 ± 0.71	45 ± 1.41	55 ± 1.94	65 ± 1.17	75 ± 1.44	85.0 ± 2.98
5	Soybean + Glycerol (1:1)	0 ± 0	40 ± 1.45	55 ± 0.74	65 ± 2.71	75a ± 2.73	85b ± 2.14	99.5 ± 1.83
6	Control	0 ± 0	33 ± 0.9	60 ± 2.94	60 ± 2.42	75a ± 1.3	85b ± 3.49	96.0 ± 3.8

The values followed by means are standard error of the mean. The values are significantly different from each other at $p<0.05$ level of significance. The values with common superscripts denote similar means.

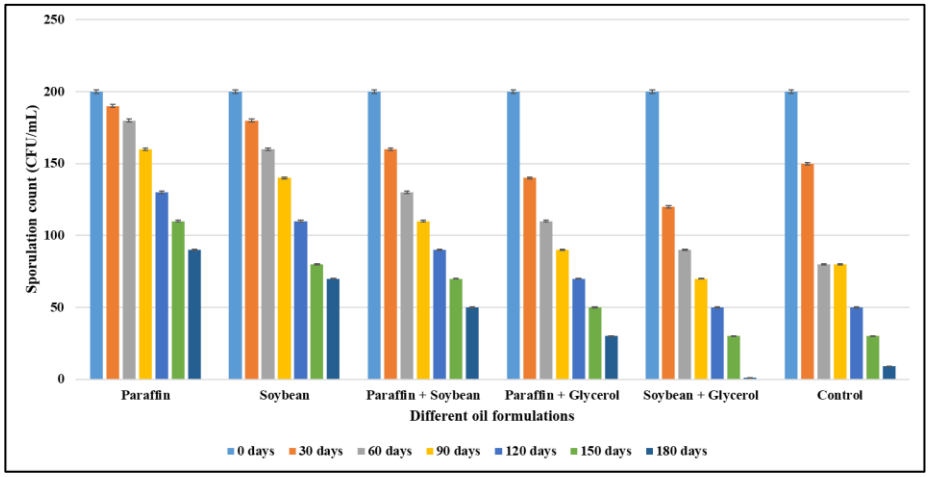

Figure 2 Effect of different oil formulations on the sporulation count of *Trichoderma viride*

IV. CONCLUSION

This study optimized both the production medium and oil-based formulation for enhancing the shelf life of *Trichoderma viride*. Molasses Yeast Broth (MYB) showed the highest biomass and spore yield, making it ideal for inoculum preparation. Among oil formulations, paraffin alone best-preserved spore viability over 180 days. In contrast, combinations with glycerol, especially soybean + glycerol, led to rapid viability loss. This is likely due to glycerol's hygroscopic nature and the nutritive profile of soybean oil promoting spore degradation at room temperature. Overall, MYB and paraffin oil proved most effective for producing and storing *T. viride*.

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REFERENCES

[1] FAO. (2024). *About | Plant Production and Protection | Food and Agriculture Organization of the United Nations*. Available: https://www.fao.org/plant-production-protection/about/en

[2] M. T. El-Saadony, A. M. Saad, S. M. Soliman, H. M. Salem, A. I. Ahmed, M. Mahmood, *et al.*, "Plant growth-promoting microorganisms as biocontrol agents of plant diseases: Mechanisms, challenges and future perspectives," *Frontiers in plant science*, vol. 13, p. 923880, 2022.

[3] M. C. Enebe and O. O. Babalola, "The impact of microbes in the orchestration of plants' resistance to biotic stress: a disease management approach," *Applied microbiology and biotechnology*, vol. 103, pp. 9-25, 2019.

[4] K. M. Ghoneem, A. A. Al-Askar, and W. I. Saber, "A Simple Formula of the Endophytic Trichoderma viride, a Case Study for the Management of Rhizoctonia solani on the Common Bean," *Life*, vol. 13, p. 1358, 2023.

[5] D. G. Kajal Mane, SR Potdukhe, BD Sonune, VS Wargane, VS Gavade, Hemalata Khobragade and VD Pawar, "Comparison of different oil formulations on shelf life of Trichoderma asperellum," *Journal of Pharmacognosy and Phytochemistry*, vol. 10, pp. 171-174, 2021.

[6] N. Teixidó, J. Usall, and R. Torres, "Insight into a successful development of biocontrol agents: Production, formulation, packaging, and shelf life as key aspects," *Horticulturae*, vol. 8, p. 305, 2022.

[7] W. R. Mahadeo, "INVESTIGATION OF DIFFERENT OIL FORMULATIONS ON SHELF LIFE OF TRICHODERMA HARZIANUM," 2022.

[8] H. D. Burges, "Formulation of mycoinsecticides," in *Formulation of microbial biopesticides: Beneficial microorganisms, nematodes and seed treatments*, ed: Springer, 1998, pp. 131-185.

[9] S. Green, S. M. Stewart-Wade, G. J. Boland, M. P. Teshler, and S. H. Liu, "Formulating microorganisms for biological control of weeds," *Plant-microbe interactions and biological control*, pp. 249-280, 1998.

[10] L. Kolombet, S. Zhigletsova, N. Kosareva, E. Bystrova, V. Derbyshev, S. Krasnova, *et al.*, "Development of an extended shelf-life, liquid formulation of the biofungicide Trichoderma asperellum," *World Journal of Microbiology and Biotechnology*, vol. 24, pp. 123-131, 2008.

[11] S. Khan, N. Bagwan, M. A. Iqbal, and R. Tamboli, "Mass multiplication and shelf life of liquid fermented final product of Trichoderma viride in different formulations," *Advances in Bioresearch*, vol. 2, pp. 178-182, 2011.

[12] K. Sorathiya, K. Sorathiya, S. Kalariya, and L. Patel, "Development and Evaluation of a Liquid Formulation of Trichoderma viride as a Bio-pesticide for Pest Management," *International Journal of Applied Sciences and Biotechnology*, vol. 11, pp. 60-65, 2023.

[13] V. Ahamedemujtaba and S. Kulkarni, "Shelf life of Trichoderma harzianum an antagonist in different oil based formulations," *IRA Int J Appl Sci*, vol. 6, pp. 34-40, 2017.

[14] Y. A. Nikolaev, E. V. Demkina, E. A. Ilicheva, T. A. Kanapatskiy, I. A. Borzenkov, A. E. Ivanova, *et al.*, "Ways of Long-Term Survival of Hydrocarbon-Oxidizing Bacteria in a New Biocomposite Material—Silanol-Humate Gel," *Microorganisms*, vol. 11, p. 1133, 2023.

[15] Z. Hubalek, "Protectants used in the cryopreservation of microorganisms," *Cryobiology*, vol. 46, pp. 205-229, 2003.