



Mass Multiplication and Shelf-life Study of Talc-Based Bioformulation of *Bacillus subtilis* B4: in Hot and Semi-Arid Zone

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Abstract: Biocontrol agents such as *Bacillus subtilis* are widely used against soil-borne plant pathogens. Mass production and shelf life are crucial for the commercial success of bioformulation against plant pathogens. The microbial cfu count declined with an increase in the storage time. The talc-based bioformulation of *Bacillus subtilis* B4 was evaluated for shelf life at different temperatures (ambient, 25°C, 4°C and 0°C) in the polythene bags. The initial microbial count for the bacterial antagonist i.e. *B. subtilis* B4 the initial inoculum density at zero day was 8.3×10^{10} which gradually decreased to 3.4×10^4 after 360 days at ambient temperature. Still, the decline was not so prominent at 0°C after 360 days (7.1×10^5). There was a significant interaction between temperature and storage time Treatment combinations (T: Temperature; M: Month) T₁M₀, T₂M₀, T₃M₀ and T₄M₀ i.e. 8.3×10^{10} cfu/g, exhibited significant counts as compared to other treatments combinations. Storage of talc-based bioformulation of *B. subtilis* B4 at 4°C and 0°C was more suitable than storage at 25°C and ambient temperature. The current findings indicated that to maintain the spore viability and effective threshold density above 10^6 Cfug (colony forming units/g of formulation) at ambient for six months. However, the effective cfu count (10^6 Cfug) of *B. subtilis* was maintained for 11 and 9 months at freezing (0°C) and at 4°C temperatures, respectively.

Keywords: *Bacillus subtilis*, Shelf life, Talc, Bioformulation

During the Green Revolution, an intensification in agriculture was done to meet the increasing demands for food and fibre globally (Sharma et al 2014). Because of this in the last two to three decades, rampant chemical fertilization posed a threat to the health of all living beings and destroyed the soil health which led to the reduction of Indigenous beneficial soil microbes (Pathak et al 2022) and unstable crop responses coupled with the development of resistance in the pathogens (Choudhary et al 2022). An alternative green approach requires time to maintain farm productivity without chemical dependency. In view of organic farming and sustainable agriculture, there is a need to investigate for successful biological control (Gade and Lad 2018). Biological control is the use of unambiguous microbes that intervene with various mechanisms of plant pathogens and pests (Choudhary et al 2023). Till date, several bacterial and fungal biocontrol control agents (BCAs) are registered and are available as commercial products (Choudhary et al 2021). Microbial bio formulations offer an alternative way to attain better plant growth and productivity (Ayilara et al 2023). The use of beneficial microbes in the greenhouse is fine but under field conditions, their viability issue gets enhanced. To get positive results from a bioformulation, a significant number of living microbial cells ($\approx 10^6$) is needed (Vassilev et al 2020) which leads to the commercial success of the formulated product through consistent field responses (Saikia et al 2023). Therefore, a potent bioagent must possess propagules that must remain viable with a good

shelf life. The shelf life varies widely with microbial species, water availability, storage time and temperature and production methods etc. *Bacillus subtilis*, is known for the production of bioactive metabolites and siderophores, in addition to nutrient solubilization (Bora and Bora 2021). *Bacillus* spp. are effective against various diseases of crops like wheat, sugarbeet, sweet potato, etc. are widely reported (Bora et al 2024). In the present study, the objective was to isolate *Bacillus* species with potent antagonistic activity, to prepare bioformulations and to study their shelf-life for 360 days from the date of manufacturing at a regular interval of 30 days.

MATERIAL AND METHODS

Site specifications: This study was conducted at Biocontrol Laboratory, Department of Plant Pathology, Punjab Agricultural University, Ludhiana during 2021-22. The site is located in the South-Western zone of Punjab having latitude and longitude of 30°54' N, 75°48' E, respectively -above the mean sea level of 247 meters.

Isolation and identification: *Bacillus subtilis* B4 was isolated from soil sample adhered to potato roots by dilution plate technique and was maintained on MYP Agar media (Mannitol egg Yolk Polymyxin agar, HiMedia). The identification of species was done using *Bacillus* spp. specific primers Bsub5F and Bsub3R (Singh and Chaudhari 2012) and the sequence was submitted to NCBI GenBank to obtain its accession number (ON479713).

Preparation of the bioformulation: During bioformulation preparation, the sterilization of talc (magnesium silicate) was done by autoclaving at the temperature of 121°C for 30 min (Khan et al 2023). Bacterial suspensions were produced on NB (Nutrient Broth). 500ml of this bacterial broth culture was added to the 1kg of sterilized talc powder under aseptic conditions. 1% CMC (carboxymethylcellulose) was added to the mixture as an adjuvant before packing it in the sterilized polythene bags (Choudhary et al 2021) and stored at ambient (5-45°C), 25°C and 4°C and 0°C for checking the shelf life of bioformulation.

Shelf life of bioformulation: The vivacity of bacterial cultures in the formulation was confirmed by the serial dilution plating method. One gram of formulation was added to 9 ml of sterilized water. Dispersing agents like Tween-20 were added for their uniform distribution (Singh et al 2021). One ml formulated bioproduct which was serially diluted to 10⁻¹³ was transferred to the freshly prepared Nutrient Agar

plates and then incubated at 28 ± 1°C. The population counts of *B. subtilis* B4 were made at zero day and then at every one-month interval for 12 months. Plating was done in triplicates and the final CFU/mL was the average of three readings used to represent viable bacterial count (Maheshwari et al 2015). The shelf life of the formulations during the storage period was expressed as log₁₀ CfU g⁻¹ (Mulatu et al 2021).

Statistical analysis: CfU values of *B. subtilis* was analysed using IBM SPSS Statistics 29.0.2.0

RESULTS AND DISCUSSION

The population of *B. subtilis* B4 remained at a higher density and the gradual decrease was observed at different storage conditions. Significantly higher value was in M₀ (8.3×10¹⁰ cfu/g) followed by M₁T₁ (6.2×10⁹ cfu/g) and the lowest count was recorded in M₁₂T₁ (3.4×10⁴ cfu/g) (Table 1). The interaction of temperature and storage time (T×M) on microbial count was also significant. Treatment combinations

Table 1. Shelf life of *Bacillus subtilis* B4 isolates in talc powder-based bioformulation

Storage period*(Days)	Cfu/g of bioformulation /temperature				Mean
	T ₁ (Ambient) 5°C - 45°C	T ₂ 25°C	T ₃ 4°C	T ₄ 0°C	
M ₀ (0 days)	8.3 × 10 ¹⁰ (25.14)	8.3 × 10 ¹⁰ (25.14)	8.3 × 10 ¹⁰ (25.14)	8.3 × 10 ¹⁰ (25.14)	8.3 × 10 ¹⁰ (25.14)
M ₁ (30 days)	6.2 × 10 ⁹ (22.55)	6.9 × 10 ⁹ (22.65)	8.8 × 10 ⁹ (22.90)	7.3 × 10 ⁹ (25.01)	2.3 × 10 ¹⁰ (23.28)
M ₂ (60 days)	5.8 × 10 ⁸ (20.18)	6.7 × 10 ⁸ (20.32)	7.2 × 10 ⁹ (22.70)	6.1 × 10 ¹⁰ (24.83)	1.7 × 10 ¹⁰ (22.01)
M ₃ (90 days)	7.1 × 10 ⁷ (18.08)	7.4 × 10 ⁷ (18.12)	6.9 × 10 ⁸ (20.35)	8.8 × 10 ⁹ (22.90)	2.4 × 10 ⁹ (19.86)
M ₄ (120 days)	6.9 × 10 ⁶ (15.75)	7.2 × 10 ⁷ (18.09)	6.3 × 10 ⁸ (20.26)	8.6 × 10 ⁹ (22.88)	2.3 × 10 ⁹ (19.24)
M ₅ (150 days)	5.4 × 10 ⁶ (15.50)	4.6 × 10 ⁷ (17.64)	3.2 × 10 ⁸ (19.58)	7.9 × 10 ⁹ (22.79)	2.0 × 10 ⁹ (18.88)
M ₆ (180 days)	3.2 × 10 ⁶ (14.98)	2.4 × 10 ⁷ (16.99)	2.7 × 10 ⁸ (19.41)	4.1 × 10 ⁹ (22.13)	1.0 × 10 ⁹ (18.38)
M ₇ (210 days)	8.0 × 10 ⁵ (13.59)	4.2 × 10 ⁶ (15.25)	1.2 × 10 ⁸ (18.60)	8.8 × 10 ⁸ (20.60)	2.5 × 10 ⁸ (17.01)
M ₈ (240 days)	4.7 × 10 ⁵ (13.06)	3.9 × 10 ⁶ (15.18)	7.7 × 10 ⁷ (18.16)	4.7 × 10 ⁸ (19.97)	1.3 × 10 ⁸ (16.59)
M ₉ (270 days)	8.3 × 10 ⁴ (11.33)	1.9 × 10 ⁶ (14.46)	5.4 × 10 ⁶ (15.50)	6.5 × 10 ⁷ (17.99)	1.8 × 10 ⁷ (14.82)
M ₁₀ (300 days)	5.9 × 10 ⁴ (10.99)	4.8 × 10 ⁵ (13.08)	6.7 × 10 ⁵ (13.42)	7.6 × 10 ⁶ (15.84)	2.2 × 10 ⁷ (13.33)
M ₁₁ (330 days)	4.6 × 10 ⁴ (10.74)	7.4 × 10 ⁴ (11.21)	4.2 × 10 ⁵ (12.95)	2.9 × 10 ⁶ (14.88)	8.6 × 10 ⁶ (12.44)
M ₁₂ (360 days)	3.4 × 10 ⁴ (10.43)	6.6 × 10 ⁴ (11.10)	3.9 × 10 ⁵ (12.87)	7.1 × 10 ⁵ (13.47)	3.1 × 10 ⁶ (11.97)
Mean	9.3 × 10 ⁹ (15.56)	1.0 × 10 ¹⁰ (16.86)	1.1 × 10 ¹⁰ (18.60)	2.6 × 10 ¹⁰ (20.65)	-
LSD _{0.05}	T (Temperature)		0.18		
	M (Month)		0.08		
	T × M		0.14		

Figures in parentheses are log-transformed values

T₁M₀ (T: Temperature; M: Month), T₂M₀, T₃M₀, and T₄M₀ (8.3×10¹⁰ cfu/g) exhibited higher counts as compared to other T × M combinations. The minimum count was noticed in T₁M₁₂ (3.4 ×10⁴ cfu/g). The population decline continued for 12 months, with fast decline observed at ambient and 25°C and slow decline at 4 °C and 0°C. The freezing temperature acted as best for long-term storage throughout the year with a last count of 7.1 × 10⁵ cfu/g of bioformulation. Chung *et al* (2010) also observed that bioformulation of *B. subtilis* strain AH8 and *B. licheniformis* strain K11 showed cfu count of 5.8 × 10⁹ which remained the same up to 60 days at 45°C. Similarly, Gupta and Dohroo (2014) found that the talc-based formulations of *Bacillus subtilis* had an initial count of 2.0 × 10⁸ at zero day which was reduced to 8.3×10⁶ cfu/g after 80 days of storage at ambient temperature. Narasimhan and Shivakumar (2015) revealed that the population level of *B. subtilis* was stabled in talc-based formulation with cfu count of 1.6×10⁸ at 30°C and remained the same until 180 days of storage. Martinez *et al* (2016) proved that a talc-based powder formulation of *B. cereus* strain B25 spores with cfu 1.1×10⁹ spore count and its viability in the powder formulation decreased slowly over time after 360 days of storage at room temperature. Jayasudha *et al* (2017) reported vermiculite and talc-based bioformulation of *B. subtilis* strain KK-9A recorded the highest number of colonies forming unit examined at fifteen days intervals up to three months of storage. Naveesh *et al* (2022) and Sinha *et al* (2004) demonstrate the suitability of talc as a carrier for maintaining the efficacy of microorganisms during storage. Successful management of plant disease by biocontrol agents depends on the availability of effective bioformulations, their rapid multiplication, colonization after inoculation and most importantly survival during storage conditions.

CONCLUSION

Successful management of plant disease by biocontrol agents directly depends upon the availability of effective bioformulations, their rapid multiplication, colonization after inoculation and most importantly survival during storage conditions. The present study concluded that there was a general decline in the number of cfu's with increasing storage time at different temperatures. The storage of talc-based bioformulation of *Bacillus subtilis* at 4°C and 0°C was more suitable than storage at 25°C and ambient temperature..

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