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Shelf-life study of *Bacillus* isolates B1 and B3 in talc based bioformulation at different storage temperature

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Abstract
The formulation of a biological control agent for plant pathogens is essential to its commercial success. Two potent *Bacillus* spp. from rhizosphere of healthy watermelon plants with potent antifungal activity against Fusarium spp. were used to prepare bioformulations. In this study, shelf life of the talc based bioformulation of *Bacillus subtilis* B1 and *Bacillus cereus* B3 isolates was observed for one year at different storage temperatures viz 30 °C, 25 °C, 15 °C and 5 °C in various packing materials like polythene, cloth and paper bags. There was a gradual decrease in the shelf life of *Bacillus* bioformulation in different storage conditions. The cfu of bioformulation decreased less at 5 °C storage temperature as compared to bioformulation which were stored at 30 °C temperature.

Keywords: Bioformulations, *Bacillus* spp., talc, temperature, shelf life

Introduction
Agriculture directly or indirectly supports more than half of the population in India. Phytopathogens, such as pests, herbs, insects, and microorganisms such as bacteria, fungi, algae, viruses, cause significant economic losses to Indian farmers each year due to weather irregularities and crop diseases (Shahcheraghi *et al* 2015; Owen *et al* 2015; Iqbal *et al* 2017) [17, 15, 17]. Managing plant diseases through overuse of chemical pesticides or fungicides are potential threat for the environment and all kind of life on earth. Thus equivalent solutions to replace chemical in crop disease management need to be strengthened. Profitable measure for controlling this disease is not available. Use of resistant varieties was an effective approach against the pathogens but the new virulent races of Fusarium appears within a short period of time (Ling *et al* 2010) [10]. Therefore an effective non-chemical and environment - friendly method of management of microorganisms is through valuable method of disease control. Biological control through application of antagonistic microbes of plant pathogen is one among the foremost favourable options (Nagorska *et al* 2007; Khan *et al* 2017) [13, 9].
There are a wide range of biocontrol products available in the form of powders, pellets, and wet formulations that can be used in seed coatings, soil amendments, and foliar sprays. *In vitro* testing of antagonists from disease suppressive soils against phytopathogens is the first step toward preparing biocontrol formulations (Sharma *et al* 2013; Shaikh *et al* 2015; Gotor-Vila *et al* 2017) [19, 18, 5]. In secondary screening, high-potency antagonists are selected and used to produce a large amount of biomass. In order to prepare bioformulations of biomass from talcum powder, farmyard manure, dried fecal pellets, and gram shells, suitable carrier materials are needed (Sandikar *et al* 2010; Mercado-Flores *et al* 2014) [3, 12]. In the present study, the objective was to isolate Bacillus species with potent antagonistic activity, prepare biocontrol formulations, and study their shelf-life and efficiency as biocontrol agents.

Material and Method
Isolation of *Bacillus* spp.
Bacillus spp. was isolated from rhizospheric soils of watermelon growing districts of Punjab region, during crop seasons of 2018-19. Heat treatment method described by Walker *et al* (1998) [20] was used for isolation of *Bacillus* to bias the selection towards spore-forming bacteria. For enrichment of spores from the soil samples, 1g of soil was suspended in 9ml sterile distilled water and incubated in water bath at 80 °C for 10 min. Suspension of 100µl was spread on the Mannitol yolk polymixin agar medium plates. The plates were incubated at 28-30 °C for 24-40 h.

After 2 days, bacterial colonies were transferred to Nutrient agar media and incubated at 27 °C.

Mass culturing and preparation of bioformulation

Potent isolates of *Bacillus* spp. were selected and formulated individually in commercial talc powder. The talc powder (carrier) was autoclaved each for both the antagonists for 30 minutes at 15 psi at 121 °C. The *Bacillus* culture of bacterial suspension were produced on Nutrient broth. 500ml of this bacterial broth culture was added to the 1 kg of talcum powder. The mixture was then dried under shade. One per cent Carboxy Methylcellulose (CMC) was added in the mixture as an adhesive before packing in bags made of cloth, paper and polythene and these bags were stored at 30 °C, 25 °C, 15 °C and at low temperature (5 °C) to check the shelf life of the bioformulation in different packaging materials at different temperatures (Ardakani *et al* 2009) [2].

Results and Discussion

The initial population density of these bacterial formulations was 10^9 cfu /g. It is evident from the data shown in the Tables 1, initial inoculum density was 6.1×10^9 cfu/g, which was 4.0×10^9, 6.0×10^9, 4.1×10^9 and 3.0×10^9 cfu/g at temperature of 30 °C, 25 °C, 15 °C and 5 °C, respectively at 30 days of storage. A declined trend was observed after interval of each 60 days upto 360 days after storage of bioformulation. So it was observed that cfu/g of bioformulation count was maximum (4.2×10^7) at 5 °C even at 360 days of storage. From the data of Table 2, it was observed that inoculum density or cfu/g of bioformulation decreased gradually at different storage temperatures and with passage of time. The initial inoculum density was 6.1×10^9 cfu/g. At 360 days the paper bags packed bioformulation exhibited cfu/g count of 1.6×10^5, 4.4×10^6, 4.6×10^6 and 3.4×10^6 at temperature of 30 °C, 25 °C, 15 °C and 5 °C, respectively. The final Cfu count was less, when the bioformulation was stored in paper bags as compared to the bioformulation stored in polythene bags. Data in Table 3, initial inoculum density was 6.1×10^9 cfu/g. At 60 days the cfu/g get decreased to inoculum density of 4.2×10^8, 3.8×10^9, 4.5×10^9 and 3.1×10^9 cfu/g at 30 °C, 25 °C, 15 °C and 5 °C, respectively. The final Cfu count was found minimum when the bioformulation was stored in cloth bags package than the cfu count stored in polythene bags and paper bags respectively.

Results in the Table 4, showed that initial inoculum density of *Bacillus cereus* B3 isolate bioformulation was 7.2×10^9 cfu/g at all storage temperatures which remained same to 6.7×10^9, 4.4×10^9, 5.3×10^9 and 3.5×10^9 cfu/g of formulation at 30 °C, 25 °C, 15 °C and 5 °C respectively at 30 days of storage. The maximum survivability of *Bacillus cereus* B3 isolate was recorded at 5 °C storage condition and it was 3.4×10^7 cfu/g of bioformulation. When the bioformulation packed in the paper bags (table 5). The maximum survivability of B3 isolate bioformulation was recorded at 5 °C storage conditions and it was 3.4×10^6 cfu/g of bioformulation. But still it was less than cfu count when the bioformulation was stored in polythene bags package.

Our results were similar to Amer and Utkhede (2000) [1] who were formulated the *Bacillus subtilis* based on various carrier material such as vermiculite, talc, kaolin, and peat moss. In all carrier formulations, *B. subtilis* strain BACT0 survived up to 95 days, the populations was significantly higher in vermiculite, kaolin, and bacterial broth carriers as compared to other carriers. Chung *et al* (2010) [4] found that powder based bioformulation of *Bacillus subtilis* strain AH8 and *Bacillus licheniformis* strain K11showed cfu count 5.8×10^9 which remained same upto 60 days at 45 °C. Zhan *et al* (2012) [21] prepared freezed dryed *Bacillus cereus* strain AR156 and showed their survival rates upto 50 % after 12 months. Mendizabel *et al* (2012) [11] observed the 3.3×10^9 Cfu of *Bacillus* spp. which stored at 4 °C ± 1 °C and 20 °C ± 1 °C temperatures indicated good shelf life till 6 months, and its viability was maintained or slightly decreased. Gupta and Dohroo (2014) [6] found that the talc based formulations of *Bacillus subtilis* having initial count 2.0 x 10^8 at zero day which was reduced to 8.3 x 10^6 cfu/g after 80 days of storage at ambient temperature. Narsimhan *et al* (2015) [14] revealed that the population level of the *Bacillus subtilis* was stabled in talc based formulation with cfu count 1.6×10^8 at 30 °C and it was remained same till the 180 days of storage. Martinez *et al* (2016) [22] reported a talc-based powder formulation of *Bacillus cereus* strain B 25 spores with cfu 1.1×10^9 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) [8] also reported vermiculite and talc based bioformulation of *Bacillus subtilis* strain KK-9A recorded the highest number of colonies forming unit examined at fifteen days interval up to three months of storage.

Table 1: Shelf life of *Bacillus subtilis* B1 talc powder based bioformulation packed polythene bags stored at different temperatures regimes

Storage period (Days)	cfu/g of bioformulation / temperature (°C)			
	30	25	15	5
0	6.1×10^9	6.1×10^9	6.1×10^9	6.1×10^9
15	4.6×10^9	5.6×10^9	5.3×10^9	4.6×10^9
30	4.0×10^9	6.0×10^9	4.1×10^9	3.0×10^9
45	2.7×10^9	4.7×10^9	2.7×10^9	2.5×10^9
60	6.1×10^8	5.1×10^9	5.4×10^9	4.1×10^9
75	2.5×10^8	4.5×10^9	2.5×10^9	1.5×10^9
90	5.3×10^8	4.3×10^9	5.3×10^9	4.3×10^9
105	3.3×10^8	5.5×10^9	9.0×10^9	1.4×10^9
120	1.5×10^8	6.5×10^8	1.5×10^9	5.5×10^9
135	1.6×10^8	2.4×10^8	5.9×10^9	7.2×10^9
150	4.2×10^7	4.2×10^8	4.2×10^9	6.2×10^9
165	5.8×10^7	6.6×10^8	7.8×10^8	1.3×10^9
180	2.2×10^7	3.2×10^8	2.2×10^8	4.2×10^9
195	9.0×10^7	1.7×10^8	3.6×10^8	4.7×10^9
210	8.4×10^7	6.4×10^7	8.4×10^8	2.4×10^8
225	2.9×10^7	4.5×10^7	6.9×10^8	8.4×10^8
240	3.1×10^7	5.1×10^7	3.1×10^8	2.1×10^8
255	5.3×10^6	1.1×10^7	2.6×10^7	3.5×10^8
270	6.7×10^6	4.7×10^7	6.7×10^7	2.7×10^8
285	4.9×10^6	5.0×10^7	6.5×10^7	7.6×10^8
300	5.2×10^6	3.2×10^7	5.2×10^7	3.5×10^8
315	2.0×10^6	3.7×10^7	5.9×10^7	7.3×10^7
330	3.6×10^6	2.6×10^6	3.6×10^7	2.9×10^7
345	3.3×10^6	5.2×10^6	6.1×10^6	6.3×10^7
360	2.6×10^6	4.6×10^6	2.6×10^6	4.2×10^7

*****Mean of three replications

Table 2: Shelf life of *Bacillus subtilis* isolate B1 talc powder based formulation packed in paper bags stored at different temperatures regimes

Storage period (Days)	cfu/g of bioformulation / temperature(°C)			
	30	25	15	5
0	6.1×10^9	6.1×10^9	6.1×10^9	6.1×10^9
15	5.6×10^9	6.6×10^9	3.6×10^9	4.0×10^9
30	5.0×10^9	4.1×10^9	4.0×10^9	3.9×10^9
45	4.7×10^9	4.5×10^9	3.7×10^9	4.5×10^9
60	3.8×10^8	3.1×10^9	4.1×10^9	3.4×10^9
75	1.5×10^8	3.8×10^9	5.5×10^9	3.5×10^9
90	3.3×10^8	4.3×10^9	5.5×10^9	3.3×10^9
105	3.6×10^8	6.0×10^8	9.2×10^9	3.3×10^9
120	4.5×10^8	1.9×10^8	3.5×10^9	5.2×10^9
135	2.8×10^8	4.6×10^8	5.2×10^9	7.4×10^9
150	3.2×10^7	2.2×10^8	5.2×10^9	5.8×10^9
165	6.3×10^7	6.7×10^8	7.9×10^8	2.2×10^9
180	1.2×10^7	3.7×10^8	4.2×10^8	3.2×10^8
195	9.4×10^7	3.3×10^7	4.2×10^8	5.2×10^8
210	4.4×10^7	3.4×10^7	6.4×10^8	1.4×10^8
225	6.5×10^6	4.7×10^7	7.2×10^8	8.8×10^8
240	2.1×10^6	4.1×10^7	3.1×10^7	2.9×10^7
255	6.4×10^6	1.1×10^7	2.7×10^7	4.4×10^7
270	4.7×10^6	3.7×10^7	5.7×10^7	2.5×10^7
285	6.1×10^6	6.3×10^7	6.6×10^7	7.9×10^7
300	3.2×10^6	2.2×10^7	3.2×10^7	3.8×10^7
315	3.7×10^6	6.0×10^6	6.8×10^7	8.5×10^7
330	2.6×10^6	1.6×10^6	2.6×10^6	1.9×10^6
345	5.3×10^6	6.4×10^6	7.9×10^6	3.5×10^6
360	1.6×10^5	4.4×10^6	4.6×10^6	3.4×10^6

*****Mean of three replications

Table 3: Shelf life of *Bacillus subtilis* isolate B1 talc powder based formulation packed in cloth bags stored at different temperature regimes

Storage period (Days)	cfu/g of bioformulation / temperature (°C)			
	30	25	15	5
0	6.1×10^9	6.1×10^9	6.1×10^9	6.1×10^9
15	4.8×10^9	5.4×10^9	4.6×10^9	4.2×10^9
30	3.0×10^9	4.4×10^9	4.3×10^9	4.9×10^9
45	3.7×10^8	4.2×10^9	4.7×10^9	3.5×10^9
60	4.2×10^8	3.8×10^9	4.5×10^9	3.1×10^9
75	2.5×10^8	3.5×10^8	3.5×10^9	4.5×10^9
90	3.8×10^8	4.7×10^8	4.5×10^9	4.3×10^9
105	7.9×10^7	4.1×10^8	8.6×10^9	3.0×10^9
120	4.2×10^7	2.9×10^8	3.8×10^9	3.2×10^9
135	2.0×10^7	3.2×10^8	4.3×10^8	1.4×10^8
150	3.7×10^7	2.6×10^8	4.2×10^8	5.4×10^8
165	3.7×10^7	1.1×10^7	3.0×10^8	4.3×10^8
180	2.2×10^7	4.7×10^7	3.7×10^8	2.2×10^8
195	3.1×10^6	8.4×10^7	2.9×10^8	5.6×10^8
210	3.4×10^6	4.4×10^7	5.4×10^7	1.6×10^7
225	1.7×10^6	5.4×10^7	9.3×10^7	1.8×10^7
240	1.1×10^6	3.3×10^7	2.1×10^7	2.5×10^7
255	5.7×10^6	1.9×10^7	4.0×10^7	4.2×10^7
270	5.7×10^6	3.0×10^7	3.7×10^7	1.5×10^7
285	1.8×10^6	3.6×10^6	7.6×10^7	6.3×10^7
300	4.2×10^6	2.8×10^6	3.9×10^6	2.8×10^6
315	5.0×10^6	6.3×10^6	1.5×10^6	4.4×10^6
330	3.6×10^5	3.6×10^6	4.6×10^6	1.7×10^6
345	8.1×10^5	4.6×10^6	6.1×10^6	3.8×10^6
360	2.6×10^5	2.4×10^5	3.6×10^6	2.4×10^6

*****Mean of three replications

Table 4: Shelf life of *Bacillus cereus* isolate B3 talc powder based formulation packed in polythene bags stored at different temperature regimes

Storage period (Days)	cfu/g of bioformulation / temperature (°C)			
	30	25	15	5
0	7.2×10^9	7.2×10^9	7.2×10^9	7.2×10^9
15	2.1×10^9	5.4×10^9	6.6×10^9	4.0×10^9
30	6.7×10^9	4.4×10^9	5.3×10^9	3.5×10^9
45	1.9×10^9	4.2×10^9	5.7×10^9	3.3×10^9
60	5.2×10^9	3.8×10^9	6.5×10^9	2.1×10^9
75	3.5×10^8	3.5×10^9	4.5×10^9	3.5×10^9
90	4.8×10^8	4.7×10^9	5.5×10^9	4.7×10^9
105	4.3×10^8	6.9×10^9	9.7×10^9	3.4×10^9
120	4.7×10^8	2.9×10^9	4.8×10^9	4.2×10^9
135	2.9×10^8	4.9×10^8	6.6×10^9	3.4×10^9
150	2.7×10^8	2.6×10^8	3.2×10^9	3.4×10^9
165	7.0×10^7	7.6×10^8	8.3×10^9	2.7×10^9
180	3.2×10^7	4.7×10^8	2.7×10^8	4.1×10^9
195	9.5×10^7	4.8×10^8	5.2×10^8	6.3×10^9
210	3.8×10^7	4.4×10^8	4.4×10^8	2.6×10^9
225	8.6×10^7	5.5×10^7	8.5×10^8	9.2×10^8
240	1.7×10^7	3.3×10^7	2.9×10^8	2.0×10^8
255	6.9×10^7	1.9×10^7	4.3×10^8	5.9×10^8
270	4.7×10^6	3.0×10^7	3.3×10^7	2.5×10^8
285	8.0×10^6	6.5×10^7	7.7×10^7	8.2×10^8
300	4.0×10^6	2.8×10^7	3.7×10^7	1.8×10^8
315	4.1×10^6	6.0×10^7	7.2×10^7	9.9×10^7
330	3.3×10^6	3.6×10^6	2.6×10^7	2.7×10^7
345	5.1×10^6	7.9×10^6	8.7×10^7	3.9×10^7
360	2.3×10^5	2.4×10^6	4.6×10^6	3.4×10^7

*****Mean of three replications

Table 5: Shelf life of *Bacillus cereus* isolate B3 talc powder based formulation packed in paper bags stored at different temperature regimes

Storage period (Days)	cfu/g of bioformulation / temperature (°C)			
	30	25	15	5
0	7.2×10^9	7.2×10^9	7.2×10^9	7.2×10^9
15	5.1×10^9	4.4×10^9	3.9×10^9	4.5×10^9
30	4.7×10^9	3.4×10^9	4.3×10^9	2.5×10^9
45	3.9×10^9	2.2×10^9	3.7×10^9	2.3×10^9
60	2.2×10^8	5.8×10^8	5.5×10^9	2.9×10^9
75	4.5×10^8	4.5×10^8	2.5×10^9	3.7×10^9
90	3.8×10^8	3.7×10^8	3.5×10^9	2.7×10^9
105	3.6×10^8	6.7×10^8	9.1×10^9	2.4×10^9
120	4.1×10^8	1.9×10^8	4.1×10^9	3.2×10^9
135	2.0×10^8	3.9×10^8	6.6×10^9	8.1×10^9
150	2.2×10^7	1.6×10^8	2.2×10^8	3.9×10^9
165	6.8×10^7	7.6×10^8	8.0×10^8	1.7×10^9
180	4.2×10^7	3.7×10^8	3.7×10^8	4.8×10^9
195	9.4×10^7	2.7×10^7	4.7×10^8	5.3×10^8
210	1.8×10^7	2.4×10^7	3.4×10^8	3.6×10^8
225	3.3×10^7	5.1×10^7	7.2×10^8	8.8×10^8
240	2.7×10^6	3.8×10^7	4.9×10^8	2.6×10^8
255	5.9×10^6	1.4×10^7	3.7×10^7	4.6×10^8
270	5.7×10^6	2.0×10^7	2.3×10^7	2.4×10^8
285	6.0×10^6	6.4×10^7	7.4×10^7	8.2×10^7
300	4.3×10^6	2.4×10^6	1.7×10^7	3.8×10^7
315	2.2×10^6	5.7×10^6	7.1×10^7	8.4×10^7
330	3.7×10^6	4.6×10^6	2.8×10^6	1.7×10^7
345	8.6×10^6	6.6×10^6	8.5×10^6	7.6×10^6
360	1.6×10^5	3.4×10^6	3.6×10^6	2.4×10^6

*****Mean of three replications

Table 6: Shelf life of *Bacillus cereus* isolate B3 talc powder based formulation packed in cloth bags stored at different temperature regimes

Storage period (Days)	cfu/g of bioformulation / temperature (°C)			
	30	25	15	5
0	7.2×10^9	7.2×10^9	7.2×10^9	7.2×10^9
15	6.2×10^9	5.4×10^9	4.9×10^9	5.5×10^9
30	5.7×10^9	4.4×10^9	3.3×10^9	3.5×10^9
45	4.9×10^8	3.2×10^8	4.7×10^9	4.3×10^9
60	3.2×10^8	4.8×10^8	5.4×10^9	3.9×10^9
75	5.5×10^8	3.5×10^8	4.5×10^9	3.1×10^9
90	4.8×10^8	2.7×10^8	2.5×10^9	3.7×10^9
105	2.1×10^8	5.7×10^8	9.8×10^9	3.4×10^9
120	3.1×10^7	2.9×10^8	4.1×10^9	2.2×10^9
135	3.1×10^7	4.0×10^8	6.3×10^8	1.8×10^9
150	1.2×10^7	3.6×10^8	3.2×10^8	3.5×10^8
165	1.6×10^7	3.7×10^7	5.5×10^8	6.1×10^8
180	3.2×10^7	3.0×10^7	3.9×10^8	4.3×10^8
195	3.3×10^7	9.4×10^7	2.9×10^8	6.8×10^8
210	2.8×10^6	2.9×10^7	2.4×10^8	2.6×10^8
225	1.7×10^6	5.9×10^7	1.0×10^8	2.3×10^8
240	3.7×10^6	3.3×10^7	4.5×10^7	2.1×10^7
255	6.4×10^6	1.8×10^7	6.9×10^7	5.6×10^7
270	4.7×10^6	2.6×10^6	2.7×10^7	1.4×10^7
285	2.0×10^6	5.4×10^6	5.2×10^7	1.4×10^7
300	4.3×10^6	2.4×10^6	1.2×10^7	2.8×10^7
315	3.2×10^6	4.7×10^6	6.5×10^6	4.4×10^7
330	1.7×10^6	3.6×10^6	1.8×10^6	4.7×10^6
345	4.1×10^5	5.3×10^6	7.3×10^6	8.1×10^6
360	1.9×10^5	4.4×10^5	4.6×10^6	3.4×10^6

Conclusion

It can be concluded that talc based bioformulation of *Bacillus subtilis* B1 and *Bacillus cereus* B3 were viable enough after 6 months storage at ambient temperature and almost for one year when stored at 5 °C. Shelf life of both the antagonists were found maximum when the bioformulation was stored in polythene bags at temperature of 5 °C as compared to other package materials (paper and cloth bags) stored at temperatures 30 °C, 25 °C and 15 °C respectively.

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