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Enhancing the effectiveness and stability of biopesticides *Bacillus thuringiensis* against *Spodoptera frugiperda* J.E Smith (Lepidoptera: Noctuidae) by lyophilization freeze-drying

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Abstract

The Gram-positive spore-forming bacterium Bacillus thuringiensis (Bt) Berliner produces crystal proteins as its main characteristic. The agricultural industry uses this bacteria because its insecticidal proteins established it as an effective environment-friendly biopesticide. The investigation into using freeze drying to produce the biopesticides *Bacillus thurigiensis* as a powder for control *Spodoptera frugiperda* damage on maize in Vietnam. This study assessed the stability of this product as pure biopesticides on *Spodoptera frugiperda* during storage. The results showed that the viability of the formulated *B. thuringiensis* spore-crystal complex stored at 4°C and 25°C was maintained at 22.9 × 10^{22} and 19.5 × 10^{22} CFU/g, respectively, after one month of drying storage. Furthermore, freeze-drying supported the viability of the *B. thuringiensis* spore-crystal complex for up to 18 months, with 10.2 × 10^{22}CFU/g and 1.01 x 10^{22} CFU/g in both temperature conditions during storage, respectively. Our study demonstrated the potential of the freeze-dried *Bacillus thuringiensis* spore-crystal complex powder as a more effective biopesticide. The efficiency against *Spodoptera frugiperda* second instar larvae showed that after 12 months during storage at two conditions, 4°C and 25°C, the *B. thuringiensis* spore-crystal complex powder killed 94.7% and 88% in the highest concentration of 35 mg/L. This suggests that the *B. thuringiensis* spore-crystal complex powder, when formulated by the freeze-drying method, could significantly improve its activity as a biopesticide under laboratory and net-house conditions. This promising result offers a bright future for pest control, with the potential for more effective and stable biopesticides.

***Keywords**: Fall armyworm, Spodoptera frugiperda, lyophilization, freezer drying, bacillus thuringiensis*

INTRODUCTION

Maize serves as a crucial agricultural commodity in Vietnam, while insects cause multiple problems that affect the corn crops. FAW Spodoptera frugiperda (J.E. Smith) (Lepidoptera: Noctuidae) caused its first appearance in April 2019 as a significant pest in the country. Since its original introduction, fall armyworm has spread quickly throughout Vietnam, while estimates show that 35,000 hectares of corn fell victim to FAW infestation in 40 provinces [1] [2]. Spodoptera frugiperda originally inhabited the Americas as it continues to be the primary agricultural pest for southern America and the United States and disrupts essential crop species such as corn and rice and sorghum and cotton [3] [4]. Scientific research shows that FAW larvae prefer feeding on maize foliage, yet they sometimes consume

ear tissue [5] [6]. The FAW-caused damage in Vietnam leads to reduced crop yields and increased costs for insecticides and labour expenses [7]. Extensive migrations based on the remarkable mobility levels of FAW create difficulties during its management because the insect moves between regions and countries throughout its range [8] [9]. The use of chemical insecticides creates resistance in this insect species, yet entomopathogenic bacteria demonstrate promise as ACP and IPM agents to promote clean food in Vietnam and other countries [10] [11]

During bacterium sporulation, Bacillus thuringiensis (Berliner) (Bacillales: Bacillaceae) (Bt) produces insecticidal crystal proteins (ICPs) as its major Gram-positive bacterial characteristic. The bacteria produce crystals, which include pesticide crystal proteins as its main identifying feature [12] [13]. The substance called endotoxin functions as a biopesticide primarily against Lepidoptera insects as well as Coleoptera types and Diptera species [14]. It stands as one of the main commercial biopesticide products used for insect biological control because the studies describe it as representing more than 90% of all biopesticide sales worldwide [15] [16].

Hundreds of Bt strains originated from natural environments or dead insects, but few have been brought to market because Bt crystal toxin species show distinct infection patterns [17]. The Bt products sold in markets show low insect infection rates because their availability period is shorter due to instability and lethal characteristics which harm Insecticidal Crystal Proteins during storage [18].

Greenhouse farmers can add commercial bacterial products directly to their sprayers since these bacteria arrive as powdered substances [19]. Freeze drying, spray drying, vacuum drying, and fluidized bed drying represent standard operations in creating bacterial powder production [20]. Freeze drying stands out among different drying methods for biological material preservation and maintains bacteria properties and toxins at greater than 98% levels [21]. The process of freeze drying demands up to three times the operational energy consumption compared to other drying methods, such as oven drying or spray drying, according to studies [22] [23], but yields superior bacterial inoculant-drying outcomes [25] [26]. Therefore, industrial-scale freeze drying needs development since stability and efficiency levels are high. The production of microbial pesticides through freeze-drying enables storage in various conditions while making the devices both convenient for transport and straightforward to operate. Freeze drying serves well in biological pest management since it allows the removal of cold storage requirements and creates longer-lasting products. The discovery creates new prospects for utilizing bacterial endotoxin due to its laboratory-proven virulent properties. The capability to develop freezer drying formulations with high concentrations of B. thuringiensis spore-crystal complex for biopesticide manufacturing appears promising as a plant-based production facility. The current advancement will help develop better methods to control FAW pests from damaging maize crops in Vietnam.

In this present study, after lyophilization by freeze-dried, the *B. thurigensis* evaluated the viability, efficacy, stability, or viability of *Bacillus thurigensis* (Bt) against larvae of fall armyworm in laboratory conditions during storage at cold and room temperate.

MATERIAL AND METHODS

Research Site

The Biological Control Laboratory of the Plant Protection Department, operating within the College of Agricultural at Can Tho University in Vietnam, served as the research location.

For this study, researchers utilized the Bacillus thuringiensis var. Aizawa (Bta-VN116) strain originates from the culture collection of the Plant Protection Department at Can Tho University [27]. The researchers extracted the Bta-VN116 bacterium from Fall armyworm S. frugiperda larvae intestinal tissue through multiple separation processes. The isolated bacterium required Luria Bertani (LB), which contained Tryptone (10 g) and yeast extract (5 g) with NaCl (5 g) and Glucose (1 g) and agar (20 g) and 1000 mL distilled water at pH 7.0 and 4 °C as a stock culture [27] until it reached use.

Preparation of the liquid (seed) culture

This study employed the preferred cultivation medium, which Bta systematically used to achieve maximum sporulation and endotoxin production, as based on Içygen et al. (2002) and Mazmira et al. (2012). The researchers inoculated a single Petri dish colony of Bta-VN116 [27], which grew on nutrient agar (LB) medium for 48 hours at 25°C. The TheNSTC laboratory subjected 500 mL flasks containing 250 ml of nutrient broth, including (NH4)2SO4 at 2 g/L together with K2HPO4.3H2O at 0.5 g/L, MgSO4.7H2O at 0.2 g/L and The MnSO4.4H2O The at The 0.05 The g/L The as The well The as The CaCl2.2H2O The at The 0.08 The g/L The and The yeast The extract The at The 2.0 The g/L The and Glucose at 1.0 g/L to bacterial inoculation. The pH value was set to 6.5 for medium sterilization at 15 psi for 15 minutes using 121°C temperature, and the final solution was used as culture medium for 24-hour incubation at 25°C with 250 rpm on an incubator shaker (IKAR KS 260 basis GMBH and Co. KG, Germany). Laboratory staff moved cultures from the middle of their logarithmic growth period, where cells reached 107 cells/mL, into 1,000 mL flasks containing 500 mL nutrient broth solutions that ran at 250 rpm under 25°C conditions for 48 to 72 hours. This process allowed most spores and crystals to be released. A measurement process determined the spore-to-crystal ratio present in Bt nutrient broth. A solution containing Bt spores crystals and vegetative cells in the broth was gathered through centrifugation (Ortoaleresa Digicen 21R centrifuge) at 4°C at 10,000 rpm for 10 minutes. Distilled water combined with 0.01% tween 80 and mixed with 0.5% alginate and 0.5% xanthan gum was added to the pellet. One millilitre of the sample received equal distribution into sterile brown glass vials before being frozen at -600 C for 24 hours and subjected to a vacuum freezer-drying machine (BioBase) for three days of drying until complete dryness at 12 mTorr pressure was achieved.

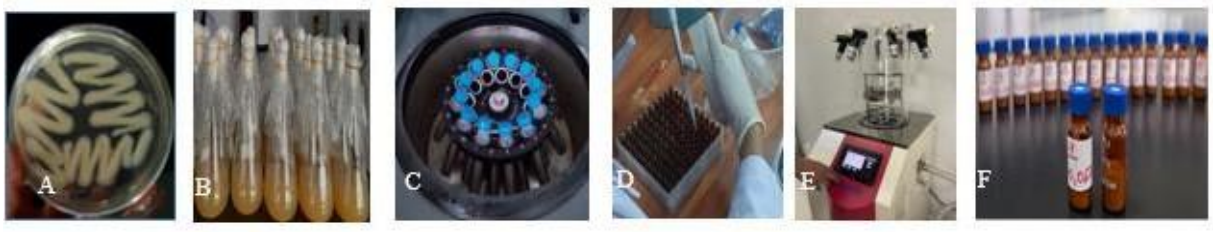

Figure 1: The B. thuringiensis spore-crystal complex was produced using freeze-drying

A: *B. thuringiensis* cultivated on LB medium; B: *B. thuringiensis* in seed culture; C: Centrifugation at 10,000 rpm at 4 degrees Celsius; D: aliquoted into brown glass vials of 1.0 mL; E: prepared for lyophilization using a vacuum freeze-drying apparatus; F: *B. thuringiensis* spore-crystal complex post-drying

The Bt rehydration, shelf life and viability assay took place after drying by evaluating samples that were kept in storage for 18 months at 40 and 25°C at room temperature. Routine freeze-dried sample

collection at random intervals underwent rehydration with distilled water until their original size was reached before plating suitable diluted cultures onto nutrient agar (LB) through the drop count method. Observations of the plates occurred at 24 hours and 48 hours incubation at 37°C to determine bacterial population through the following calculation method.

PB = A x C

PB = The population of bacteria (CFU mL^{-1});

A = The colony number of bacteria develop on Petri dishes;

C = The dilution factor.

The survival rate was calculated using the formula described by Peiren et al., (2016)

Survival (%) = (Log10 (CFU/mL) after lyophilization)/ (Log10 (CFU/mL) before lyophilization) × 100

Bioassays against larvae FAW

S. frugiperda larvae were collected from maize fields in the Mekong Delta of Vietnam and propagated in the laboratory at the Department of Plant Protection, College of Agriculture, Can Tho University. The larvae fed on an artificial diet [7] in an environmentally controlled growth chamber (at temperature 25 $\pm 2^0$C; relative humidity of 75%, photoperiod of 18hrs light/6hrs dark) until the pupal stage.

The effectiveness of the toxicity of the spore crystal complex formed during storage was assessed by a diet-feeding bioassay. Subsequent to freeze-drying, the spore crystal complex powder contained within the brown glass tube was partitioned into two segments: one stored in darkness at 25 ± 2^0C in the laboratory, and the other and the other at 4 ± 2^0C in a laboratory refrigerator. The insecticidal efficacy of both subsamples was assessed against second instar larvae of *S. frugiperda* at concentration of 25 mg/L, 27 mg/L, 29 mg/L, 31 mg/L, 33 mg/L, and 35 mg/L. The toxicity assessment using a bioassay and feed dipping method [27]. The test feeds an artificial diet (8 mm in diameter, 1.2 mm in thickness), prepared by dipping them into the formulation according to the treatment. The B.t suspension (30 µL) was applied to the surface of artificial diet disks. Each experiment was conducted in triplicate, with 25 larvae per dosage for each replication. The larvae were incubated in a 25°C chamber with an 18-hour light and 6-hour dark photoperiod. Larval mortality was observed on several days post-treatment (DAT).

Data analysis

Statistical measurements of mean differences used the student t-test at 5% significance threshold. The data underwent a correction using the Abbot Formula stated in 1987. The calculation shows corrected mortality equals to the treatment group percentage subtracted from the control group percentage that is divided by the control group percentage and then multiplied by 100. A statistical evaluation of the gathered data occurred using SPSS Statistical Software while LSD demonstrated variations between treatment means at $p<0.05$.

RESULTS AND DISCUSSION

The result from Table 1 showed that following cultivation in a liquid medium, bacteria are subjected to centrifugation and freeze-drying, after which their population is assessed by enumerating viable bacteria on a petri dish and conducting T-test statistical analysis. The comparison of the population

before and after drying indicates that there is no statistically significant difference in the population of Bta bacteria, as evidenced by a t probability of 0.2529>0.05. The bacterial population prior to drying was 24.7 x10^{22} CFU/g, whereas post-freezer drying, it was 24.0 x 10^{22} CFU/g, demonstrating that the freezer drying technique does not impact the bacterial population and effectively preserves it.

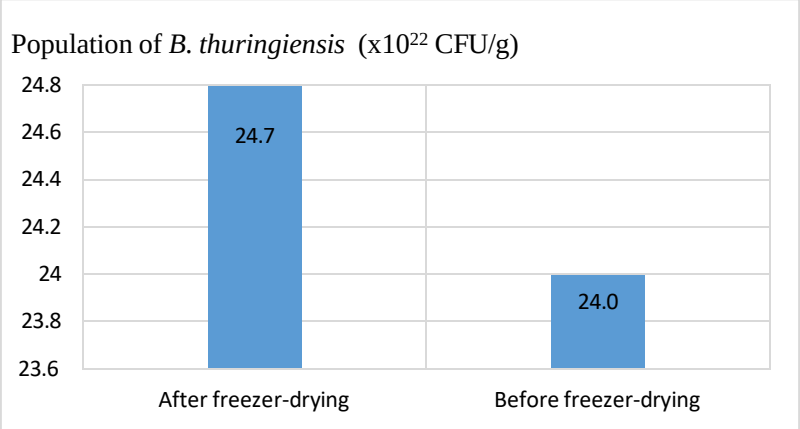

Fig.1 Population of *B. thuringiensis* before and after freezer- drying

Bacillus thuringiensis subsp. kurstaki cultivated on a wheat bran medium enriched with carbon and nitrogen yielded the maximum efficiency in terms of toxin concentration and spore production (x 10^{11} CFU/g). In a wheat bran medium devoid of supplementary carbon and nitrogen, it yields 6.6 x 10^{10} CFU/g. The findings indicate that standard production methods yield a density of approximately 10^{11} CFU/g, but in this study after centrifugation and freezer- drying achieves a significantly higher density of up to 10^{22} CFU/g.

Shelf life of bacterial viability

The stability of a viable bacterial population during storage is important for its commercial application in controlling insects [40] [41]. In this study, the storage life of the bacterial viability test was carried out to determine the survival ability of bacteria during storage in different conditions. The results presented in **Fig. 2** show that the storage life of *B. thuringiensis gradually* declined from the date of storage to 9 months of storage. The population that was dried by freeze and kept at 4^0C showed the viability of *B. thuringiensis* with a population of 22.9 × 10^{22} CFU/g after one month of storage and which has become 10.2 × 10^{22} CFU/g after 18 months of storage. This indicated that the bacteria in the population of 10.2× 10^{22} CFU/g did not decrease and remained at a concentration of 10^{22} CFU/g and could survive during storage. The viability of *B. thuringiensis* dried through freezer drying and kept at room conditional 25^0C drastically reduced from 19.5 × 10^{22} CFU/g to 1.01 × 10^{22} CFU/g than the kept at 4-degree cells. The above results showed that after the freeze-drying method, keeping it at 4^0C can support the viability of *B. thuringiensis* with a longer shelf life than 25^0C. The storage time also significantly affected both temperate storages to keep the bacteria.

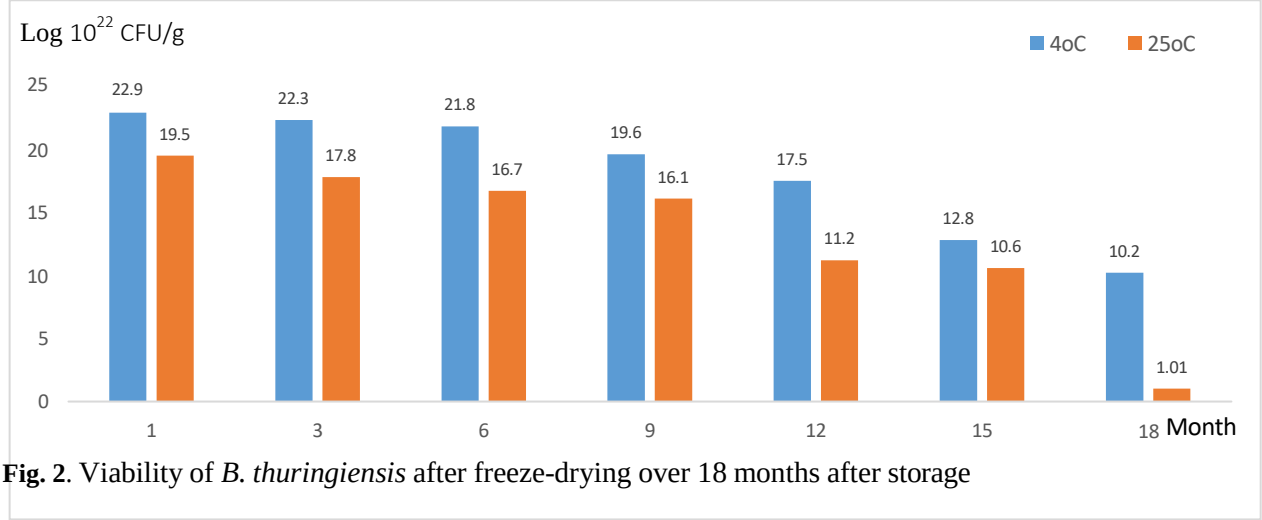

Fig. 2. Viability of *B. thuringiensis* after freeze-drying over 18 months after storage

Survival of bacterial after freeze-dried and during storage

The survival rate of *Bacillus thuringiensis* was calculated as the total number of bacteria after freeze-drying divided by the total number of bacteria before freeze-drying. Fig.3 shows the rate of bacteria after lyophilisation; according to the data presented after freeze-drying and storage at 40C and 250C was 95.4% and 81.3%, respectively, during the 6 months of storage at 4-degree cells, the survival decreased to 90.8%; significant differences in bacterial survival were observed at 25-degree cells (Fig. 3). These results indicate that when bacteria were freeze-dried showed higher survival after storage.

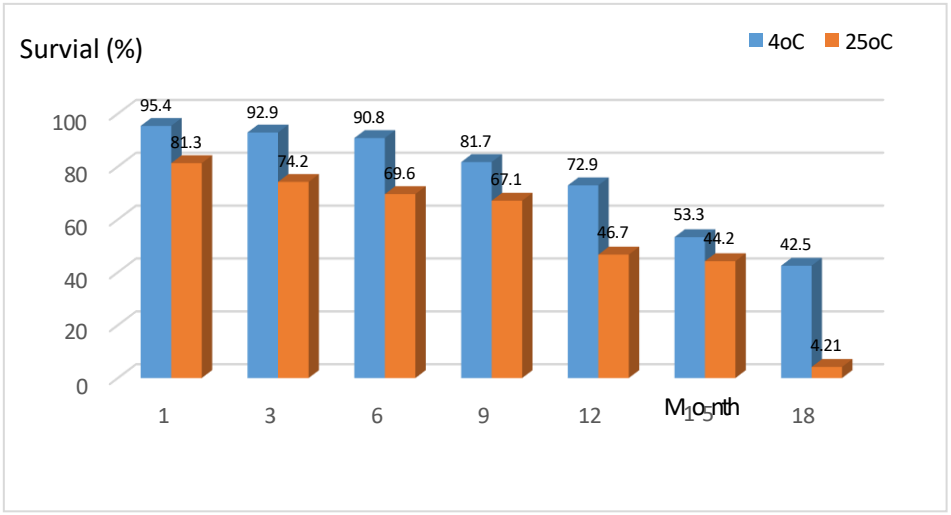

Fig. 3. Survival values of *B. thuringiensis* after freezer dried over 18 months after storage

Pathogenicity of freeze-dried *B. thuringiensis* against fall armyworm

To determine the shelf life of *B. thuringiensis* after freeze-drying at six concentrations after storage at room (25±2^{0}C) and cold (4±2^{0}C) temperatures. The mortality of *S. frugiperda* on second instar larvae was observed in feeding bioassay using doses 25, 27, 29, 31, 33, and 35 mg/L of *B. thuringiensis* after freeze-dried. The present data in Table 1 showed that the bacteria stored for 1, 3, and 6 months gave a positive correlation of concentrations and mortality as expected, independent of storage temperature. The *B. thuringiensis* stored at 4^{0}C resulted in 98.7%, 85.3%, and 100%, and *B. thuringiensis* stored at 25^{0}C had 93.3%, 96.0%, and 100% at concentrations 31, 33, and 35 mg/L, respectively.

Similarly, the results were measured using *B. thuringiensis* stored at 4^0C for 12 months, resulting in 82.7%, 85.3%, and 94.7%, and *B. thuringiensis* stored at 25^0C had 74.7%, 78.7%, and 88.0% at concentrations 31, 33 and 35 mg/ L, respectively.

Table 1. Efficacy of *B. thuringiensis* after 12 months storage against FAW in laboratory

Treatments	**Temperate storage**	**Corrected mortality (%)**											
		1 month			**3 months**			**6 months**			**12 months**		
		2 DAT	**3 DAT**	**4 DAT**	**2 DAT**	**3 DAT**	**4 DAT**	**2 DAT**	**3 DAT**	**4 DAT**	**2 DAT**	**3 DAT**	**4 DAT**
Control	4°C	0.00 e	0.00 e	0.00e	0.00 h	0.00 g	0.00d	0.00 g	0.00 h	0.00 f	0.00 g	0.00f	0.00 h
25 mg/l		77.3 d	85.3 d	85.3d	60.0 fg	76.0 ef	89.3b	60.0 cde	61.3 fg	65.3 de	41.3 def	57.3 cde	61.3 g
27 mg/l		85.3 c	89.3 c	89.3c	66.7 ef	80.0 de	97.3a	64.0 cd	73.3 ef	81.3 c	45.3 de	69.3 bc	73.3 de
29 mg/l		89.3 c	94.7 b	94.7b	73.3 de	86.7 c	98.7a	66.7 bc	74.7 ef	82.7 c	50.7 d	70.7 bc	78.7 cd
31 mg/l		94.7 b	96.0 b	96.0b	86.7 bc	94.7 b	100 a	81.3 a	90.7 c	98.7 ab	66.7 c	73.3 b	82.7 bc
33 mg/l		98.7 a	100 a	100 a	88.0 b	100 a	100 a	86.7 a	97.3 ab	100 a	72.0 bc	76.0 b	85.3 b
35 mg/l		100 a	100 a	100 a	94.7 a	100 a	100 a	89.3 a	100a	100 a	85.3 a	90.7 a	94.7 a
25 mg/l	25°C	44.0 d	62.5 d	79.3d	50.7 g	70.7 f	78.7c	41.3f	53.3 g	58.7 e	33.3 f	46.7 e	58.7 g
27 mg/l		62.7 c	72.7 c	79.3c	60.0 fg	73.3 f	81.3c	45.3 ef	60.0 fg	64.0 de	37.3 ef	52.0 de	64.0 fg
29 mg/l		80.0 b	85.9 b	92.5b	653 3ef	85.3 cd	88.0b	50.7 def	68.0 efg	74.7 cd	40.0 ef	65.3 bcd	68.0 ef
31 mg/l		84.0 ab	84.0 ab	95.0b	68.0 ef	93.3 b	97.3a	78.7 ab	80.0 de	93.3 b	68.0 c	70.7 bc	74.7 d
33 mg/l		95.3 ab	85.3 ab	100 a	78.7 cd	96.0 b	100 a	80.0 ab	88.0 cd	96.0 ab	69.3 c	73.3 b	78.7 cd
35 mg/l		92.0 a	92.0 a	100 a	88.0 b	100 a	100 a	85.3 a	93.3 bc	100 a	77.3 b	82.0 a	88.0 a

Data with the same letter within a column are non-significantly different at P<0.05.

DISCUSSION

Freeze-drying is the optimal method for preserving microorganisms (Morgan et al., 2006). Many of the reports by several authors [28] [29] [30] [32] [33] have found that microorganisms from various species and strains may exhibit differing sensitivities to freeze-drying. This work examined the survivability of *B. thuringiensis* following freeze-drying. It assessed its storage under two settings for 18 months, confirming the long-term stability of Bt endotoxin proteins after prolonged storage. Conversely, numerous data on enduring toxicity in the field or under controlled conditions suggest that these proteins swiftly diminish their insecticidal efficacy due to various factors, including heat drying [34] [3] [36]. Limited research exists regarding the shelf life stability of Bt endotoxins and their commercial formulations. Their notable biodegradability is widely recognized as a characteristic of natural materials [37]. Consequently, these imported products often exhibit limited efficacy in local and environmental settings [38] [39]. This research evaluated the effectiveness of freeze-drying as a method for manufacturing *B. thuringiensis* as a biocontrol agent targeting *Spodoptera frugiperda* also demonstrated the efficacy of the freeze-drying method in preparing *B. thuringiensis* as a biocontrol agent against *Spodoptera frugiperda*, achieving 100% mortality at concentrations of 33 and 35 mg/L after six months of storage at cool temperatures.

CONCLUSION

The production of functional biopesticides requires B. thuriginensis VN116 powder to maintain bacterial culture viability at its highest level. A freezing-drying methodology that preserves the high viability of B. thuriginensis VN116 spore crystals was developed while protecting the composition of both the drying and storage phases. A robust spray drying process depends on understanding the volatile nature of B. thuriginensis because such knowledge shows critical importance for system development. The storage duration did not affect the preserved quality of freeze-dried Bta product after eighteen months. The B. thuriginensis VN116 spores and crystal mixture retain its maintenance capability to effectively fight fall armyworm infection in laboratory settings over 90%.

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Disclosure statement

The authors declare that there is no competing interest regarding the publication of manuscripts.

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