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The Potentials of *Bacillus thuringiensis* Isolate and Its Extracellular Cuticle-Degrading Enzymes Activity in the Biological Control of *Callosobruchus maculatus* (Cowpea Weevil) in Stored *Vigna unguiculata* L. Walp (Cowpea)

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Abstract

This study assessed the pesticidal effect of *Bacillus thuringiensis* isolate against *Callosobruchus maculatus* (cowpea weevil) infesting stored cowpea seeds. *B. thuringiensis* isolate was obtained through liquid fermentation using sucrose water as a substrate, then transferred onto talc powder for solid formulation. A six-month shelf-life study of the solid formulation was conducted using total microbial plate count. Insect mortality bioassays were performed by applying liquid and solid *B. thuringiensis* formulations to cowpea seeds containing first-generation adult *C. maculatus*, with mortality recorded over 12 and 7 days, respectively. Enzyme activity was assessed using specific enzyme substrates. Results showed a gradual decline in microbial count over time in the solid formulation. The bioassay revealed 100% mortality for the liquid medium and for the solid medium of the *B. thuringiensis* isolate. The *B. thuringiensis* isolate also significantly delayed the first-generation emergence of *C. maculatus*. Enzyme analysis indicated the production of cuticle-degrading enzymes—protease, lipase, and exochitinase with varying activity levels. This study concludes that *B. thuringiensis* isolate effectively controls *C. maculatus*, likely due to its cuticle-degrading enzymes, and has potential as a bio-control agent for stored cowpea pest management. However, higher concentrations are necessary in talc-based formulations to maintain an adequate shelf life.

Keywords: Cowpea, cowpea weevil, shelf-life, biological control, enzyme activity.

Introduction

In the relentless pursuit of sustainable agricultural practices, the search for effective and eco-friendly pest management strategies remains paramount. Among the myriad of challenges faced by farmers, infestation of stored grains and seeds by pests stands as a persistent threat, leading to significant post-harvest losses and economic repercussions. *Cowpea* (*Vigna unguiculata* L. Walp) is an important leguminous crop in many tropical and sub-tropical regions, serving as a significant source of protein and income for many people, especially in Nigeria, which is considered the largest producer of cowpea [1]. However, its production and post-harvest storage are severely hampered by the cowpea weevil, *Callosobruchus maculatus* [2]. Infestation by *C. maculatus* causes considerable losses in yield and quality, posing a challenge to food security and economic stability in the affected regions [3]. The conventional methods of controlling *C. maculatus*, such as synthetic chemical pesticides, have drawbacks, particularly regarding the food safety of cowpea, as the produce contains high levels of chemical pesticides considered dangerous to human health [4; 5]. Other drawbacks include environmental pollution, biodiversity disruption due to toxicity to non-target organisms, and the development of pesticide resistance [6]. As a result, there is an increasing interest in exploring alternative eco-friendly approaches, such as biological methods, for managing post-harvest cowpea pest populations of *C. maculatus*. Among these biological methods is the use of microbial agents, notably bacteria and fungi, offering health- and environmentally-benign alternatives to conventional chemical pesticides [7]. *Bacillus thuringiensis*, a naturally occurring bacterium, is a Gram-positive, spore-forming bacterium known for its insecticidal properties against a wide range of agricultural pests [8]. Its efficacy, specificity to target pests, and minimal impact on non-target organisms, coupled with its favorable safety profile for humans and the environment, make it an attractive candidate for integrated pest management (IPM) programs. *B. thuringiensis* produces pesticidal toxins through several molecular mechanisms, most of which are from the Cry family of crystalline proteins produced in the parasporal crystals and encoded by the *cry* genes [9]. These Cry proteins are responsible for the death of insect pests when they ingest the Cry protoxins or absorb them through the insect body cell membrane. The protoxins solubilize, releasing a protease-resistant biologically active endotoxin [10]. This disrupts the osmotic balance through the formation of transmembrane pores, eventually causing cell lysis in the gut wall and leakage of gut contents [11].

Microbial isolates that have insecticidal potential, such as *B. thuringiensis*, can also be pathogenic to their host by penetrating the insect cuticle and sporulating on desiccated cadavers, thereby facilitating the onset and spread of epizootics [12]. Penetration of the insect cuticle is typically the initial stage of infection, relying on mechanical pressure, cuticle-degrading enzymes (lipase, chitinase, protease, etc.), and specialized infection structures (appressoria) produced by the hyphae, which breach the host cells and subsequently proliferate [13]. Cuticle-degrading enzymes are likely key virulence factors in entomopathogens, as their substrates contain structural components of the insect cuticle that provide carbon and nitrogen sources to support microbial growth and facilitate host penetration [14]. The spore propagates in response to chemical signals on the cuticle and subsequently develops an appressorium, the specialized structure responsible for penetration. For many years since identifying the pesticidal properties of *B. thuringiensis*, its preparations have usually been sprayed directly on plants to shield them against different orders of insect pests [10]. In current pest management innovations, the genes encoding the *B. thuringiensis* pesticidal proteins have been successfully transferred into plants through genetic engineering; such plants are now referred to as transgenic plants [15]. These transgenic plants containing *B. thuringiensis* genes, specifically for Lepidoptera and Diptera insect pests, were produced to provide protection against pre-harvest pests without the need for spraying [16].

The potential of *B. thuringiensis* based bio-pesticides in controlling Lepidopteran field pests has been demonstrated in several studies, but its effectiveness against Coleoptera: bruchids such as *C. maculatus* remains relatively unexplored. While the molecular mechanism of *B. thuringiensis* in infecting hosts through Cry proteins, inducing cell lysis in the gut wall and resulting in leakage of gut contents, has been extensively studied, the production of extracellular cuticle-degrading enzymes by *B. thuringiensis* isolates for degrading the cuticle of Coleoptera: bruchids like *C. maculatus*-a critical barrier to infection and death—remains markedly underexplored. This knowledge gap is especially striking given that adult *C. maculatus* do not require food, unlike the larvae that feed and develop exclusively on seed legumes, thereby providing a scientific opportunity to assess the potential of cuticle-degrading enzymes to breach the adult *C. maculatus* exoskeleton and cause pathogenicity. Hence, the cuticle-degrading pathway may represent a pivotal, yet overlooked, component of the *B. thuringiensis* entomopathogenic arsenal, particularly against coleopteran species. This study aimed to evaluate the bio-pesticidal capabilities of a *B. thuringiensis* isolate and the implications of its extracellular cuticle-degrading enzyme activity on the control of *C. maculatus* under laboratory conditions. The study hypothesized that *B. thuringiensis* isolate, applied in both liquid and solid talc-based formulations, would cause significant mortality in adult *C. maculatus*; its efficacy would be concentration-dependent; the solid formulation would be more effective; and the pesticidal activity is correlated with the production of extracellular cuticle-degrading enzymes like protease, lipase, and chitinase. Considering the relevance of this study in addressing the pressing issue of *C. maculatus* infestation in stored cowpea, it aims to contribute to the development of sustainable and environmentally friendly strategies for cowpea protection and storage, thereby offering valuable insights for ensuring cowpea food safety, improving food security, and guaranteeing livelihoods in major cowpea-growing regions.

Materials and methods

Collection of *B. thuringiensis* isolate

B. thuringiensis isolate was obtained from the Reference Laboratory section of Biocrops Biotechnology Limited, Abuja, Nigeria, originally sourced from the soil within the Abuja environment and identified and confirmed through molecular analysis. Bacterial cell culture was performed using Potato Nutrient Agar containing penicillin G. Culture plates were incubated at room temperature (28°C ± 2°C) for 72 hours. The emerging colonies after the incubation period were discretely isolated and sub-cultured repeatedly on freshly prepared Nutrient Agar to obtain pure isolates. The pure isolates were subjected to morphological and biochemical tests for confirmation. The confirmed pure isolates were maintained on agar slant bottles, stored at 4°C, and subsequently subjected to liquid fermentation to enhance microbial proliferation, with sucrose water serving as the carbon-rich substrate and liquid growth medium for the isolate.

Collection of cowpea seeds and *C. maculatus*

Cowpea seeds (Black-eye pea, Sampea 10-Nigeria) were harvested directly from the experimental farm at the National Biotechnology Research and Development Agency (NBRDA), Abuja, Nigeria, to eliminate the possibility of prior pesticide exposure during storage. The seeds were aseptically sealed in sterile bags, placed in tightly covered containers, and stored at freezing temperature until further use. Cowpea seeds already infested with cowpea weevils were collected locally from cowpea storage facilities in the Federal Capital Territory, Abuja, Nigeria, and also from traders of the cowpea commodity. Adult weevils were isolated from visibly infested seeds through selective picking for subsequent experimental use.

Table 1. Shelf-life of *B. thuringiensis* isolate on talc powder medium

Concentration of *B. thuringiensis* in Talc Powder	CFU count of *B. thuringiensis* (per g) ($\times 10^4$)					
Months (June to November)	1	2	3	4	5	6
10 ml/50 g	63.5 ± 0.14^a	53.4 ± 0.07^a	37.9 ± 0.05^a	18.7 ± 0.21^a	6.15 ± 0.09^a	2.45 ± 0.02^a
15 ml/50 g	213.4 ± 0.81^b	92.5 ± 0.18^b	55.6 ± 0.31^b	26.2 ± 1.18^b	7.87 ± 0.22^b	4.54 ± 0.05^b
20 ml/50 g	272.3 ± 0.24^c	143.3 ± 0.40^c	80.9 ± 0.44^c	47.9 ± 0.56^c	12.8 ± 0.06^c	8.16 ± 2.11^c

Values are in mean ± S.E. Values between experimental treatments on a column bearing the same superscript are not significantly different at the 5% level ($P > 0.05$). S.E = Standard error of mean. CFU: Colony Forming Unit, X: concentration.

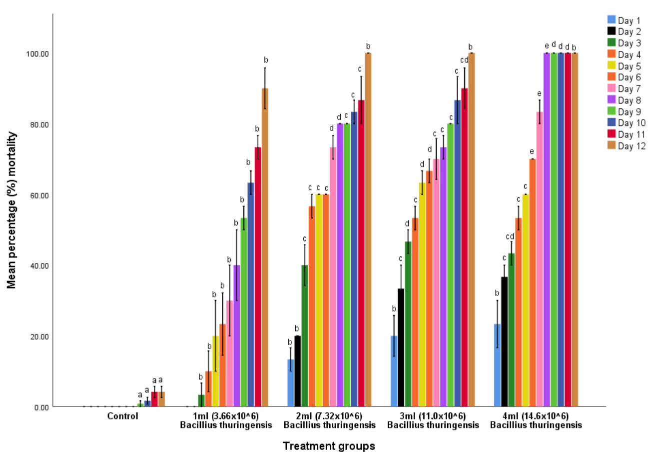

Figure 1. Percentage (%) mortality of *C. maculatus* treated with the liquid medium of *B. thuringiensis* isolate

Culturing of the *C. maculatus*

Collected cowpea weevils were reared on freshly collected cowpea seeds under laboratory conditions to facilitate acclimatization, following the method described by [17]. Batches of about one thousand seeds were each distributed into five prewashed, sterilized, and dried containers. Thirty weevils were introduced into each transparent plastic container and covered with mesh nets fastened tightly with rubber bands and masking tape to permit ventilation and prevent escape of the pest. The cowpea seed-weevil mixtures were incubated under laboratory conditions for 10 days to permit mating and oviposition. Parent weevils were then removed, and rearing continued until adult emergence. First filial generation (F_1) adult weevils were used in the subsequent experiments.

Inoculation of the *B. thuringiensis* isolate onto solid medium (talc powder) and shelf-life study

The talc stones were collected from Ejiba town in Yagba West Local Government Area of Kogi State, Nigeria and were processed into fine powder using the mechanical grinding machines. The talc powder was packed into tightly sealed waterproof nylons and then sterilized appropriately using autoclave with a holding time of 15 minutes at 121 ^{o} C. The sterilized talc powder was stored at room temperature. Talc powder was used as the solid medium because of its cost-effectiveness considering its local availability, ability of being chemically inert as it doesn't react with other substances and its good adhesion capacity due to its fine particle size and high surface area. The *B. thuringiensis* isolate with microbial load of 3.66×10^6 CFU/ml was at different quantity (10 ml, 15 ml and 20 ml) each inoculated onto 50 g of sterilized talc powder, appropriately sealed and stored at room temperature. The talc powder containing *B. thuringiensis* isolates at varying concentrations, were used for mortality study after one month to allow for sporulation. The shelf-life of the *B. thuringiensis* isolate on the talc powder at different concentrations were assessed at one-month interval for a period of six months using Total Microbial Plate Count (Colony Forming Unit per gram) as described by [18].

Effect of *B. thuringiensis* isolate on mortality of *C. maculatus*

Ten-fold serial dilution and calculation of Total Microbial Plate Count of the microbial isolates was done as described by [18].

a) In triplicate, the *B. thuringiensis* isolate was assayed for pesticidal activity using the method described by [19]. Twenty gram of the cowpea seed were introduced each into sterilized transparent containers with small openings on the cover to allow ventilation. Ten cowpea weevils were introduced each into the containers followed by spraying 1 to 4 ml of the liquid formulation of the *B. thuringiensis* isolate with 3.66×10^6 CFU/ml, 7.32×10^6 CFU/ml, 11.0×10^6 CFU/ml and 14.6×10^6 CFU/ml concentrations respectively using small (10 ml) plastic spray bottles.

b) Two gram from each of the prepared 10 ml/50 g, 15 ml/50 g, 20 ml/50 g talc powder-*B. thuringiensis* isolates mixture with 8.99×10^5 CFU/g, 10.5×10^5 CFU/g, 12.6×10^5 CFU/g concentrations, respectively, were introduced into a sterilized transparent container with small openings on the cover that allows ventilation, containing 20 g of cowpea seed and 10 cowpea weevils by dusting over the seeds.

c) Distilled water treatment was used as the control for the *B. thuringiensis* isolate liquid medium assay while distilled water-talc powder mixture was used as control for the *B. thuringiensis* isolate solid medium assay.

Mortality study of the *C. maculatus*

Adult cowpea weevil mortality was recorded at 24-hour intervals over an exposure period of 12 and 7 days for the liquid and solid formulation of the *B. thuringiensis* isolates respectively. Dead weevils were carefully removed with the help of a magnifying lens and forceps to reveal the hidden pests in the grains. Weevils that failed to respond to gentle prodding with the forceps were considered dead and subsequently removed. The percent mortality was calculated using the following formula:

$$\% \text{ Mortality} = \frac{\text{Number of Dead Weevils}}{\text{Number of Weevils Introduced}} \times 100 \quad (1)$$

Assessment of the percentage of emergence of first generation of *C. maculatus*

Emergence of first-generation (F_1) adult *C. maculatus* was determined after 40 days in the same samples used to assess percent mortality. The percentage of emergence (PE) was estimated using:

$$\text{PE} = \frac{X}{Y} \times 100 \quad (2)$$

where:

X = Number of insects that emerged in the treatment

Y = Number of insects that emerged in the control treatment Treatments where first-generation emergence is ≤ 50% are considered as promising [20].

Cuticle-degrading enzyme activity assay

Liquid culture for the extracellular enzyme production of the B. thuringiensis isolate

The nutrient broth was prepared according to the manufacturers specifications. For the production of extracellular enzymes, 1 mL of the liquid medium of the isolate containing 3.66×10^6 CFU/mL was inoculated into the nutrient broth and incubated at room temperature (28 ± 2°C) for 7 days. After incubation, the contents of the test tube were centrifuged at 10,000 rpm for 20 minutes at 4°C. Finally, the supernatants were used in enzymatic assays.

Protein concentration assay

The total soluble protein concentration in *B. thuringiensis* isolate was determined using the method of [21], with bovine serum albumin (BSA, 1 mg/mL) as the standard. A stock BSA solution was prepared by dissolving 0.25 g of BSA in distilled water and making up the volume to 250 mL in a volumetric flask. Aliquots of 0.2, 0.4, 0.6, 0.8, and 1.0 mL of the BSA solution were dispensed into test tubes and made up to a volume of 1 mL with distilled water. From each tube, 0.5 mL was transferred to a new tube, followed by the addition of 0.5 mL of reagent (50 mL of 2% Na_2CO_3 in 0.1 M NaOH) and 1.0 mL of 0.5% $CuSO_4$ in 1% sodium-potassium (Na–K) tartrate. The mixtures were thoroughly mixed and allowed to stand for 10 minutes at room temperature. Subsequently, 0.5 mL of Folin–Ciocalteu reagent was rapidly added to each tube and mixed immediately. The tubes were again allowed to stand for 10 minutes at room temperature, after which absorbance was measured at 625 nm using a UV–VIS spectrophotometer (1800-series, Shimadzu). A calibration curve was generated by plotting absorbance against BSA concentration. For the determination of sample protein concentration, 0.5 mL of the enzyme solution was processed in the same manner, and protein concentrations were extrapolated from the calibration curve based on the corresponding absorbance values.

Cuticle-degrading enzyme assay

The cuticle-degrading enzymes; total protease, exochitinase, and lipase assays were performed as described by [14] in triplicates. The total protease was assayed using casein as the substrate, while the exochitinase was assayed using *p*-nitrophenyl-*N*-acetyl-*β*-*D*-glucosaminide as the substrate, and the lipase activity was assayed using *p*-nitrophenyl butyrate as the substrate.

Calculation of the specific enzyme activity

The specific protease, exochitinase, and lipase activity of the *B. thuringiensis* isolate was calculated using the following formula:

$$\text{Specific Activity (U/mg)} = \frac{\text{Enzyme Activity (U/mL)}}{\text{Protein Concentration (mg/mL)}} \quad (3)$$

Statistical Analysis Data were expressed as mean value ± standard error of the mean (S.E.M) and analyzed using ANOVA. Significant differences between the control and treatment groups were determined by Duncan's Multiple Range Test (DMRT) in SPSS (version 26).

Results

The six-month shelf-life study of the *B. thuringiensis* isolate inoculated onto talc powder as a solid medium **(Table 1)** revealed that *B. thuringiensis* experienced a gradual reduction in colony-forming units (CFU) per gram over time, with the initial CFU count in the range of 63.5 to 272.3×10^4 CFU/g and the final count in the range of 2.45 to 8.16×10^4 CFU/g, respectively. The toxicity assay of the *B. thuringiensis* isolate evaluated its efficacy against *C. maculatus*, a major pest affecting stored cowpea grains. This study assessed the effectiveness of *B. thuringiensis* in inducing mortality in *C. maculatus* when applied in liquid and solid forms. The results, presented in **(Figures 1 and 2)**, illustrate

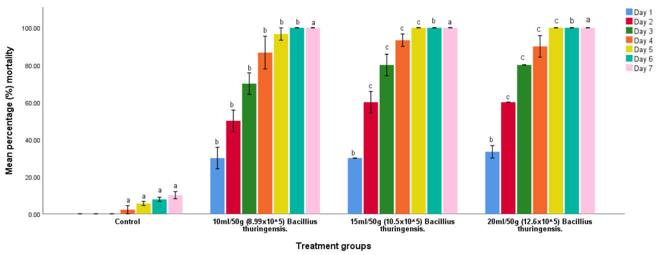

Figure 2. Percentage (%) mortality of *C. maculatus* treated with the *B. thuringiensis* isolate in solid medium (Talc powder)

the insecticidal activity exhibited by the *B. thuringiensis* isolates across different concentrations and durations of treatment.

The results presented in (**Tables 2** and **3**) show the mean emergence of first-generation (F_1) adult *C. maculatus* treated with the *B. thuringiensis* isolate in liquid and solid formulations, respectively, and further reveal the percentage emergence of F_1 adults, highlighting the efficacy of each treatment concentration in reducing pest reproduction. Treatments where F_1 emergence is $\leq$ 50% are considered promising for pest suppression [22; 20]. This study also evaluated the enzymatic activity of *B. thuringiensis* to determine their contribution to the toxicity potential of the isolate against *C. maculatus*. The results presented in (**Figure 3**) illustrate the specific activities of protease, lipase, and exochitinase of the *B. thuringiensis* isolate, considering their potential to degrade the structural components of the insect exoskeleton [23].

Discussion

The development of *B. thuringiensis*-based pesticides for the control of *C. maculatus* in stored cowpea represents a significant advancement in sustainable agricultural practices. Microbial-based pesticides, derived from entomopathogenic bacteria such as *B. thuringiensis*, offer an eco-friendly alternative with promising potential for pest management in cowpea production [24]. These microbial pesticides are target-specific, biodegradable, and pose minimal risks to non-target organisms and human health [25].

The shelf-life study of the *B. thuringiensis* isolate inoculated onto talc powder provides crucial insights into the long-term viability of the isolate and its potential as a bio-pesticide. (**Table 1**) revealed a significant decline in microbial counts over six months, with variations depending on the concentration, confirming that the gradual decrease in microbial load was significantly influenced by the concentration formulations. The highest concentration (20 mL/50 g) generally maintained higher microbial counts for a longer duration compared to lower concentrations, suggesting that a higher initial inoculation helps sustain viability. In other words, the higher the quantity of *B. thuringiensis* isolate inoculated onto talc powder, the higher the CFU count and the longer the shelf life. The declining trend observed is also similar to that reported by [26].

Other studies have shown a similar declining trend in microbial load of *Bacillus* species when inoculated onto talc-based powder. The study of [27] reported a talc-based powder formulation of *Bacillus cereus* strain B25 spores, in which CFU count of 1.1×10^9 gradually declined during 360 days of storage at room temperature. Similarly, [28] reported that talc-based formulations of *Bacillus subtilis* with an initial count of 2.0×10^8 CFU/g at zero day was reduced to 8.3×10^6 CFU/g after 80 days of storage at room temperature.

Any inorganic solid carrier material should offer a protective environment for microbial growth and, when applied for seed treatment, should possess strong adhesion capacity to maximize efficacy and guarantee successful release of bacterial cells after application [29]. In this study, the good efficacy of talc powder–*B. thuringiensis* isolate may have resulted from strong adhesion capacity to cowpea seeds. Talc powder as a solid carrier for microbial isolates has also been reported to be cost-effective, easy to process, chemically stable, with good moisture absorption and buffering capacity, non-toxic to both plants and microbes, and able to ensure bacterial cell viability for at least 2–3 months [30]. Studies have shown that *Bacillus* species in talc-based formulations remain viable at a density of 1.0×10^6 CFU/g after 45 days of storage [31].

In this study, despite the decrease in shelf-life of the *B. thuringiensis* isolate inoculated on talc powder as storage time increased, it still showed that *B. thuringiensis* cell viability was maintained even at the 4th month (120 days), with maximum viability at $47.9 \pm 0.56 \times 10^4$ CFU/g and minimum viability at $18.7 \pm 0.21 \times 10^4$ CFU/g, indicating that talc powder can be a veritable carrier for inoculating *B. thuringiensis* isolate for commercial production. This study has confirmed that talc powder effectively supports *B. thuringiensis* isolates for bio-pesticide purposes by providing a stable medium for storage and application. While talc-based formulations can sustain microbial viability for some months, a decline is unavoidable. Considering that the rate of decline varies among organisms, there is a need to explore options that could further enhance microbial longevity. For example, [29] reported that talc was an effective carrier, but additional stabilizers such as glycerol improved shelf life beyond six months. Therefore, this study emphasizes the importance of considering optimized storage conditions (e.g., low humidity, cool temperatures) and incorporating stabilizers such as glycerol, silica gel, or oil-based formulations to further enhance microbial persistence over time.

The toxicity assay, which assessed the efficacy of *B. thuringiensis* isolate against *C. maculatus*, as shown in (**Figures 1** and **2**), revealed that at all treatment concentrations, a significant percent mortality was recorded compared with the control, indicating the high pesticidal potential of the *B. thuringiensis* isolate against adult *C. maculatus*. The study further showed that the isolate caused mortality in a concentration-dependent manner, with effectiveness increasing over time and at higher microbial concentrations, and demonstrated stronger effects through solid medium exposure.

Table 2. Percentage emergence of first generation of *C. maculatus* after treatment with liquid medium of the *B. thuringiensis* isolate

Microbial isolate	Treatment volume (ml)	Treatment concentration (CFU/ml)	Mean emergence of treatment (X)	Mean emergence of control (Y)	PE (%)	Remarks
B. thuringiensis	1	3.66×10^6	2.33 ± 0.01^a	11.3 ± 0.04^b	20.61	Promising
	2	7.32×10^6	1.33 ± 0.03^c	9.66 ± 0.11^d	13.76	Promising
	3	11.0×10^6	0.67 ± 0.02^c	7.67 ± 0.05^e	8.73	Promising
	4	14.6×10^6	0.33 ± 0.01^a	6.33 ± 0.03^b	5.21	Promising

Values are in mean ± S.E. Values between experimental treatments on a row bearing the different superscript are significantly different at the 5% level ($P > 0.05$). S_E = Standard error of Mean. PE: Percentage emergence. CFU: Colony Forming Unit. X = Number of insects that emerged in the treatment, and Y = number of insects that emerged in the control treatment. Treatments where first-generation emergence is ≤ 50% are considered as promising.

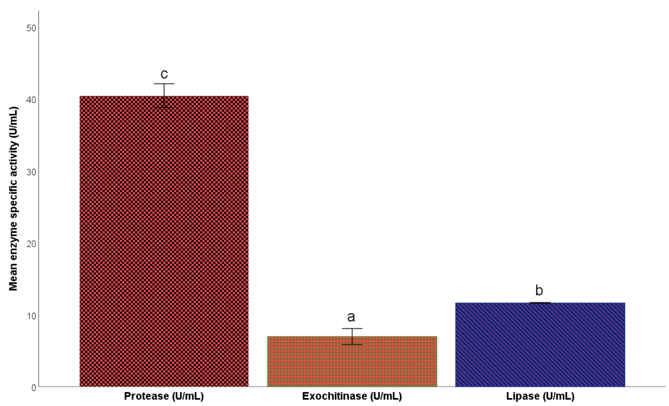

Figure 3. The cuticle-degrading enzyme activity of *B. thuringiensis* isolate

The 12-day treatment with the liquid medium of *B. thuringiensis* isolate showed 100% mortality on the 12th day at concentrations of 7.32×10^6 CFU/mL and 11.0×10^6 CFU/mL, and on the 8th day at 14.6×10^6 CFU/mL. A similar toxicity trend was observed in the 7-day treatment study using the solid carrier, where 100% mortality was recorded on the 6th day at the lowest concentration (8.99×10^5 CFU/g) and on the 5th day at higher concentrations (10.5×10^5 CFU/g and 12.6×10^5 CFU/g). These results indicate that for the commercial production of *B. thuringiensis*-based bioinsecticides for the control of *C. maculatus*, concentrations above 10^6 CFU/g or CFU/mL will be required for faster, early, and effective pest suppression. The control group, which was not treated with any microbial suspension, showed negligible mortality, confirming that the observed insect deaths were substantially due to the microbial treatments rather than external environmental factors.

According to [11], insect mortality caused by *B. thuringiensis* is usually a result of insecticidal toxins (Cry toxins), often referred to as δ-endotoxins, which are somewhat specific to certain insect families. The adult *C. maculatus* may have absorbed the *B. thuringiensis* isolates through its cuticle as a result of the direct application of the isolate in liquid and solid carriers. These findings are consistent with previous research on microbial biocontrol agents against *C. maculatus*. For example, [32] reported that *Bacillus flexus* biofilms exhibited significant insecticidal effects on *C. maculatus*, suggesting that bacterial biofilm extracellular matrices could be leveraged as bio-pesticides for cowpea storage protection. Similarly, [33] examined the impact of *Bacillus flexus* S13 on *C. maculatus* and reported LC_{50} values indicating moderate toxicity, supporting the effectiveness of bacterial isolates in insect pest control. In comparison, the current study suggests that *B. thuringiensis* exhibits slightly higher potency than *B. flexus* against *C. maculatus*, though further comparative analysis is needed.

The high toxicity of *B. thuringiensis* isolates on adult *C. maculatus* recorded with the solid medium treatment, where even the lowest concentration achieved 100% mortality before the 7th day, may be due to the increased surface area exposure provided by talc powder as a carrier. This level of mortality was not achieved with liquid medium treatments, even at the highest concentration used in the study. Therefore, in microbial control of *C. maculatus* in stored cowpea, application methodologies that enhance surface area exposure of the microbial isolate should be adopted for effective control. These findings align with recent research demonstrating the efficacy of entomopathogenic bacteria on solid carriers for pest control. For instance, [34] reported that *B. thuringiensis* on a solid carrier significantly impacted stored-product insects, particularly *Trogoderma granarium*, through its endotoxins. The current study also found *B. thuringiensis* on talc powder to be highly potent against *C. maculatus*, with the highest mean mortality (over 80%) in the 20 mL/50 g treatment. While these results are consistent with past research, certain variations exist due to environmental factors, strain differences, and formulation methods. For instance, [35] emphasized that formulation type (liquid vs. solid) influences microbial persistence and virulence, which may explain minor differences in mortality rates

Table 3. Percentage emergence of first generation of *C. maculatus* after treatment with the *B. thuringiensis* isolate in solid medium (Talc powder)

Microbial isolate	Treatment volume (ml/50g)	Treatment concentration (CFU/g)	Mean emergence of treatment (X)	Mean emergence of control (Y)	PE (%)	Remarks
B. thuringiensis	10	8.99×10^5	2.00 ± 0.01^a	9.33 ± 0.18^b	21.43	Promising
	15	10.5×10^5	1.67 ± 0.01^a	7.67 ± 0.12^b	21.77	Promising
	20	12.6×10^5	0.67 ± 0.01^a	5.33 ± 0.22^b	12.57	Promising

Values are in mean ± S.E. Values between experimental treatments on a column bearing the different superscript are significantly different at the 5% level ($P > 0.05$). S_E = Standard error of Mean. PE: Percentage emergence. CFU: Colony Forming Unit. X = Number of insects that emerged in the treatment, and Y = number of insects that emerged in the control treatment. Treatments where first-generation emergence is ≤ 50% are considered as promising.

observed across different studies.

Adjuvants such as inert carriers, like talc powder for loading microorganisms as active ingredients of microbial pesticides, possess greater adsorption capacity and enhanced dispersion properties, enabling controlled release of active components at appropriate intervals [36]. When microbial isolates penetrate the host cell, they proliferate, providing a pathway for epizootics [13]. At relatively low quantities, these crystalline proteins exhibit high insecticidal activity [37].

The emergence of the first-generation (F_1) adult *C. maculatus* serves as a crucial indicator of the long-term effectiveness of microbial bio-pesticides. While initial mortality rates provide insight into the immediate insecticidal effects of microbial isolates, F_1 emergence evaluates their residual impact on pest populations by determining whether microbial treatments can suppress reproductive success and prevent re-infestation. In this study, F_1 emergence was assessed 40 days after treatment application, following the mortality evaluation. The study showed a significant delay in the F_1 emergence period of adult *C. maculatus* at all treatment concentrations compared with the control. The significant delay observed in both liquid and solid medium treatments was expressed in a concentration-dependent manner, with greater delays recorded at higher concentrations. The percentage emergence further validated the efficacy of *B. thuringiensis* treatments. In both formulations, *B. thuringiensis* isolates at higher concentrations resulted in F_1 emergence percentages well below the 50% threshold.

The results from this study align with previous research on microbial bio-pesticides for stored-product pest management. For instance, [38] confirmed the efficacy of *B. thuringiensis* in reducing weevil emergence and suggested that the persistence of *B. thuringiensis* spores in treated grains may provide long-term protection against re-infestation, reinforcing the results of this study. Similarly, [39] investigated *Trichoderma* spp. for cowpea beetle control and found that while microbial spores significantly reduced F_1 emergence, they were not as effective as *B. thuringiensis*. Their findings align with the present study, where *B. thuringiensis* demonstrated high efficiency in reducing F_1 emergence, suggesting that bacterial bio-pesticides such as *B. thuringiensis* may offer more immediate control compared to fungal alternatives.

The findings of this study underscore the significance of bio-pesticide concentration, microbial strain selection, and storage conditions in optimizing pest suppression. Higher concentrations of *B. thuringiensis* isolate resulted in a significant reduction in F_1 emergence, suggesting its potential as a leading candidate for the biological control of *C. maculatus*. Additionally, the superior performance of solid formulations in limiting F_1 emergence suggests that formulation type plays a critical role in bio-pesticide effectiveness. Furthermore, the calculation of the percentage F_1 emergence suggests that the concentration levels used in the study are promising for pesticidal activity.

The assessment of cuticle-degrading enzyme activity in *B. thuringiensis* isolate demonstrated the presence of protease, lipase, and exochitinase, suggesting their role in the toxicity mechanisms of *B. thuringiensis* against *C. maculatus*. These enzymes are crucial for the pathogenicity of microbial bio-pesticides, as they degrade the structural components of the insect exoskeleton, facilitating microbial penetration and systemic infection [23]. Beyond mechanical pressure, enzymatic breakdown of insect cuticles can aid in the penetration of microbial isolates through the degradation of proteins, chitins, and lipids that make up the insect cuticle [40]. The present study revealed the secretion of different cuticle-degrading enzymes, such as proteases, lipases, and chitinases.

The findings indicate that the *B. thuringiensis* isolate exhibited measurable enzymatic activity, although at varying levels, suggesting differential enzymatic strategies in their pathogenicity. Protease activity was observed to display the highest activity, exceeding that of lipase and exochitinase. The protease, exochitinase, and lipase enzyme activity of the *B. thuringiensis* isolate may have synergistically contributed to the pathogenicity against adult *C. maculatus*. The significant differences in the extracellular enzyme activities per milliliter of the liquid culture of the *B. thuringiensis* isolate could be attributed to variability in virulence at different medium concentrations. Hence, this study suggests that the role of these cuticle-degrading enzymes in the pathogenicity of *C. maculatus* may be concentration-dependent.

Proteases are regarded as pivotal catalysts in the infection processes by cleaving peptide bonds in proteins and breaking them into small peptides and amino acids [14]. Exochitinase plays a vital role in cleaving *N*-acetylglucosamine in cuticular layers, while lipases break down ester bonds in lipoproteins, fats, and waxes found in the interior part of the insect integument to release (un)saturated fatty acids [41]. The higher protease activity of the *B. thuringiensis* isolate may have acted as the key catalyst for the infection of adult *C. maculatus*. Supporting the findings of this study, [42] reviewed hydrolytic enzymes in integrated pest management and highlighted the role of bacterial entomopathogens such as *B. thuringiensis* in producing proteases that degrade insect cuticle proteins. This complements the current study, which observed significant protease activity in *B. thuringiensis*, reinforcing its role as an effective microbial pesticide. Similarly, [43] examined microbial hydrolytic enzymes and found that bacterial mechanisms of cuticle degradation differ from fungal entomopathogens, as bacterial enzymes often exhibit species-specific targeting, a factor that may influence their efficacy.

The production of protease, exochitinase, and lipase by the *B. thuringiensis* isolate might be the key virulence factor against adult *C. maculatus*, considering that adult weevils do not require food or water within their limited lifespan and therefore could not have ingested the treated cowpea. Drawing on the results of this study, the relationship between cuticle-degrading enzymes and virulence could serve as a diagnostic criterion for selecting effective microbial control agents for *C. maculatus*. Future studies are required to elucidate the mechanistic role of cuticle-degrading enzymes in the significant delay in adult *C. maculatus* emergence observed in this study.

Conclusion

This study demonstrated that *Bacillus thuringiensis* isolate is highly effective against adult *Callosobruchus maculatus* and holds significant potential as a bio-control agent within integrated pest management strategies for stored cowpea. Its application can promote food security and enhance cowpea safety by providing an alternative to toxic chemical pesticides. Furthermore, the study confirmed that the *B. thuringiensis* isolate secretes key cuticle-degrading enzymes-protease, exochitinase, and lipase which likely serve as major virulence factors. These enzymes facilitate cuticle degradation, enabling the bacterium to penetrate the insect host, ultimately causing infection and mortality.

The findings underscore the promise of *B. thuringiensis* not only as a microbial bio-pesticide for controlling bruchids but also as a candidate for developing genetically engineered cowpea varieties with plant-incorporated protectants for bruchid resistance. For commercial production using talc powder as a solid carrier, the study recommends maintaining higher microbial concentrations above 10^4 CFU/g to ensure adequate shelf life and sustained efficacy.

Reference

1. Nwagboso C, Andam KS, Amare M, Bamiwuye T, Fasoranti A. The economic importance of cowpea in Nigeria trends and Implications for achieving agri-food system transformation. Intl Food Policy Res Inst; 2024.
2. Akbar R, Khan IA, Alajmi RA, Ali A, Faheem B, Usman A, et al. Evaluation of insecticidal potentials of five plant extracts against the stored grain pest, Callosobruchus maculatus (Coleoptera: Bruchidae). Insects. 2022;13(11):1047.
3. Jehajo N, Shah N. The damage caused by cowpea weevil Callosobruchus maculatus (Fab.)(Coleoptera: Bruchidae) to some stored pulses at different temperature and humidity levels. 2023.
4. Ritz BR, Paul KC, Bronstein JM. Of pesticides and men: a California story of genes and environment in Parkinsons disease. Current environmental health reports. 2016;3(1):40-52.
5. Nwosu OK, John A. Chemical pesticides and food safety. 2022.
6. Zaller JG, Brühl CA. Non-target effects of pesticides on organisms inhabiting agroecosystems. Frontiers Media SA; 2019.
7. Pathak D, Yadav R, Kumar M. Microbial pesticides: development, prospects and popularization in India. In: Plant-Microbe Interactions in Agro-Ecological Perspectives: Volume 2: Microbial Interactions and Agro-Ecological Impacts. Springer; 2017. p. 455-71.
8. Kumar P, Kamle M, Borah R, Mahato DK, Sharma B. Bacillus thuringiensis as microbial biopesticide: uses and application for sustainable agriculture. Egyptian Journal of Biological Pest Control. 2021;31(1):95.
9. Roh JY, Choi JY, Li MS, Jin BR, Je YH, et al. Bacillus thuringiensis as a specific, safe, and effective tool for insect pest control. Journal of microbiology and biotechnology. 2007;17(4):547.
10. Malaikozhundan B, Vinodhini J. Biological control of the Pulse beetle, Callosobruchus maculatus in stored grains using the entomopathogenic bacteria, Bacillus thuringiensis. Microbial pathogenesis. 2018;114:139-46.
11. Heckel DG. How do toxins from Bacillus thuringiensis kill insects? An evolutionary perspective. Archives of insect biochemistry and physiology. 2020;104(2):e21673.

12. Pathak D, Kumar M. Microbial inoculants as biofertilizers and biopesticides. In: Microbial Inoculants in Sustainable Agricultural Productivity: Vol. 1: Research Perspectives. Springer; 2016. p. 197-209.

13. Huarte-Bonnet C, Paixao FR, Ponce JC, Santana M, Prieto ED, Pedrini N. Alkane-grown Beauveria bassiana produce mycelial pellets displaying peroxisome proliferation, oxidative stress, and cell surface alterations. Fungal Biology. 2018;122(6):457-64.

14. Golzan SR, Talaei-Hassanloui R, Homayoonzadeh M, Safavi SA. Role of cuticle-degrading enzymes of Beauveria bassiana and Metarhizium anisopliae in virulence on Plodia interpunctella (Lepidoptera, Pyralidae) larvae. Journal of Asia-Pacific Entomology. 2023;26(2):102038.

15. Nwosu OK, Ubaoji KI. Genetic modification as a control mechanism to plant pest attack. In: Natural Remedies for Pest, Disease and Weed Control. Elsevier; 2020. p. 203-8.

16. Fernández-Chapa D, Ramírez-Villalobos J, Galán-Wong L. Toxic potential of Bacillus thuringiensis: an overview. Protecting rice grains in the post-genomic era. 2019.

17. Sowunmi E. Effect of storage length and insecticide treatment on cowpea (Vigna unguiculata L. Walp). 1982.

18. Agu K, Igweoha C, Umeh C. Antimicrobial activity of the ethanolic and petroleum ether extracts of tangerine seed on selected bacteria. 2013.

19. Tiroesele B, Thomas K, Seketeme S. Control of cowpea weevil, Callosobruchus maculatus (F.)(Coleoptera: Bruchidae), using natural plant products. Insects. 2014;6(1):77-84.

20. Rendón-Huerta JA, Juárez-Flores BI, Aguirre-Rivera JR, Álvarez F. Insecticide effect of wild plant powders on bean weevil (Zabrotes subfasciatus Boheman; Coleoptera: Bruchidae) in vitro. African Journal of Agricultural Research. 2013;8(11):971-7.

21. Lowry OH, Rosebrough NJ, Farr A, Randall R. Protein measurement with the folin phenol reagent. Journal of Biological Chemistry. 1951;193:256-75.

22. Lagune TA. Vegetable extracts and mineral powders for combating maize and bean pest in subsistence agriculture. Montecillo, Edo, Mexico: College of Postgraduate Agricultural Sciences Press; 1994.

23. Ortiz-Urquiza A, Keyhani NO. Molecular genetics of entomopathogenic fungi. Annual Review of Microbiology. 2000;74:127-50.

24. Sharma P, Tyagi V, Srivastava P. Potential of microbial pesticides in insect pest management. International Journal of Current Microbiology and Applied Sciences. 2018;7(4):2589-601.

25. Lacey LA, Grzywacz D, Shapiro-Ilan DI, Frutos R, Brownbridge M, Goettel MS. Insect pathogens as biological control agents: Back to the future. Journal of Invertebrate Pathology. 2015;132:1-41.

26. Geetika G, Buttar DS, Singh N. Shelf life study of Bacillus isolates B1 and B3 in talc based bioformulation at different storage temperature. Pharma Innovation Journal. 2023;12(7):2106-10.

27. Martinez N, Sinedino LD, Bisinotto RS, Daetz R, Risco CA, Galvão KN. Effects of oral calcium supplementation on productive and reproductive performance in holstein cows. Journal of Dairy Science. 2016;99(10):8417-30.

28. Gupta M, Dohroo NP. Shelf life study of formulation of fungal and bacterial antagonist as bioinoculant. Agricultural Science Digest. 2014;34:281-4.

29. Singh J, Singh AV, Upadhayay VK, Khan A. Comparative evaluation of developed carrier based bioformulations bearing multifarious PGP properties and their effect on shelf life under different storage conditions. Environment and Ecology. 2020;38(1):96-103.

30. Khan A, Singh AV, Gautam SS, Agarwal A, Punetha A, Upadhayay VK, et al. Microbial bioformulation: A microbial assisted biostimulating fertilization technique for sustainable agriculture. Frontiers of Plant Science. 2023;14:1270039.

31. Amer GA, Utkhede RS. Development of formulations of biological agents for management of root rot of lettuce and cucumber. Canadian Journal of Microbiology. 2000;46(1):809-16.

32. Reda FM, Hassanein WA, Moabed S. Potential exploitation of Bacillus flexus biofilm against the cowpea weevil, Callosobruchus maculatus (F.) (Coleoptera: Bruchidae). Egyptian Journal of Biological Pest Control. 2020;30:18.

33. Hassanein AW, Reda FM, Moabed S, El Shafiey SN. Insecticidal impacts of Bacillus flexus S13 biofilm extracellular matrix on cowpea weevil, Callosobruchus maculatus. Biocatalysis and Agricultural Biotechnology. 2021;31:101898.

34. Riaz T, Masoom A, Virk UY, Raza M. Impacts of Metarhizium anisopliae on mortality, energy reserves, and carbohydrase of Trogoderma granarium. Journal of Stored Products Research. 2022;99:102013.

35. Mantzoukas S, Kitsiou F, Lagogiannis I, Eliopoulos PA. Potential use of Fusarium isolates as biological control agents: Helicoverpa armigera (Hübner) (Lepidoptera: Noctuidae) case study. Applied Sciences. 2022;12(17):8918.

36. Lin F, Mao Y, Zhao F, Idris AL, Liu Q, Zou S, et al. Towards sustainable green adjuvants for microbial pesticides: Recent progress, upcoming challenges, and future perspectives. Microorganisms. 2023;11(2):364.

37. Rajadurai G, Anandakumar S, Raghu R. Bacillus thuringiensis in pest management. Plant Health Archive. 2023;1(1):11-3.

38. Mohammed NAG, Hassan AAH, Mohammed MO. The effect of pyrrolidinium bromide salt in the life of the southern cowpea beetle Callosobruchus maculatus (Fab.). IOP Conference Series: Earth and Environmental Science. 2021;910:012140.

39. Rodríguez-González Campelo MP, Lorenzana A, Mayo-Prieto S, González-López Álvarez García S, et al. Spores of Trichoderma strains sprayed over Acanthoscelides obtectus and Phaseolus vulgaris L. beans: Effects in the biology of the bean weevil. Journal of Stored Products Research. 2020;88:101666.

40. Lu HL, St Leger RJ. Insect immunity to entomopathogenic fungi. In: Lovett B, St Leger RJ, editors. Advances in Genetics. vol. 94. Academic Press; 2016. p. 251-85.

41. Mondal S, Baksi S, Koris A, Vatai G. Journey of enzymes in entomopathogenic fungi. Pacific Science Review A: Natural Science and Engineering. 2016;18(2):85-99.

42. Ahmad T, Rasool A. Hydrolytic enzymes and integrated pest management. In: Khan MA, Ahmad W, editors. Microbes for sustainable insect pest management. vol. 17. Springer, Cham; 2021. p. 25-36.

43. Lopes FC, Martinelli AHS, John EBO. Microbial hydrolytic enzymes: Powerful weapons against insect pests. In: Khan MA, Ahmad W, editors. Microbes for sustainable insect pest management. vol. 17. Springer, Cham; 2021. .