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ORIGINAL ARTICLES

Effect of Temperature-Induced Stress on Sporulation and Crystallization in an Entomopathogenic Bacterium, *Bacillus thuringiensis*

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Abstract *Bacillus thuringiensis* (*Bt*), a widely used biopesticide, is known to produce insecticidal crystal (Cry) proteins and spores. This study investigated the effects of temperature stress on sporulation, crystallization, and insecticidal efficacy of *Bt* strains, *BtA* and *BtK*, on *Plutella xylostella* larvae. Subsequent *Bt* culture (at 0°C for 72 h) significantly enhanced both spore and crystal yields compared to 28°C and 70°C as temperature stress following pre-culture (at 28°C for 48 h). *BtK* and *BtA* both rapidly exhibited 100% larval mortality rates. This insecticidal activity was fully achieved earlier than at other subsequent temperature treatments after 48 h of culture at 0°C. The improved efficacy of the *Bt* insecticide is attributed to increased crystal production under cold stress. Production of these crystals is strongly correlated with spore formation. The findings of this study suggest that low-temperature treatment after pre-culture can enhance *Bt* performance and provide a practical approach for optimizing biopesticide formulation. Additional field studies under variable environmental conditions are needed to assess Bt stability and efficacy as a dependable insecticide.

Key words: *Bacillus thuringiensis*, Biopesticide, Endotoxin protein, Pesticide resistance, Spore

Introduction

The long-term and excessive use of chemical pesticides for controlling insect pests has led to the development of pesticide resistance, which has made pest management increasingly challenging in agriculture (Satyanarayana et al., 2024). There is growing interest in identifying pesticides with novel modes of action and in employing biological control agents to improve pest control and avoid problems caused by resistance as resistance to chemical pesticides continues to rise (Satyanarayana et al., 2024).

Bacillus thuringiensis (*Bt*), an entomopathogenic bacterium, has been widely utilized as a biopesticide due to its insecticidal activity and safety for humans and other vertebrates (Noppun et al., 1984; Grisolia et al., 2009). *Bt* is a Gram-positive, aerobic, rod-shaped bacterium with peritrichous flagella and is commonly found in soil environments (Nair et al., 2018). *Bt* forms endospores, during which it produces insecticidal crystal (Cry) proteins, also known as δ-endotoxins under adverse environmental conditions (Roh et al., 2007; Schnepf et al., 1998). Although *Bt* shares similar biochemical traits with other similar species in the *Bacillus* genus, such as *B. cereus* and *B. subtilis*, *Bt* can only kill insects and classified as a separate species (Chen et al., 2022).

Bt exhibits toxicity against more than 130 insect species across orders such as Lepidoptera, Coleoptera, and Diptera, while demonstrating no toxicity to vertebrates or plants (Schnepf, 1995; Schnepf et al., 1998). *Bt*'s host specificity to target insects makes it a safer and effective alternative to chemical pesticides (Höfte and Whiteley, 1989; Rosas-García, 2009). The Cry proteins produced by *Bt* are referred to as Cry toxins and are classified into different groups based on their structure and specificity (Pendleton and Morrison, 1966). Among these, *B. thuringiensis* subsp. *kurstaki* (*BtK*), subsp. *aizawai* (*BtA*), and subsp. *sotto* produce Cry I class proteins, such as CryIA, CryIB, and CryIC. These proteins have a characteristic bipyramidal shape and exhibit high toxicity

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against lepidopteran insects (Loutfi et al., 2021). Although Cry II toxins are also toxic to lepidopteran insects, CryIIA toxin additionally exhibits insecticidal activity against dipteran larvae such as *Aedes aegypti* (Höfte and Whiteley, 1989). Cry III and IV toxins are more specific to coleopteran and dipteran species, respectively (Höfte and Whiteley, 1989).

The insecticidal effect of *Bt* primarily occurs during the larval stage when the Cry toxins are ingested. These toxins are solubilized by the alkaline pH in midgut and are initially converted into protoxins. The protoxins are then further processed by digestive enzymes into active Cry toxins (Yang et al., 2023; Talaei-Hassanloui et al., 2014). The active Cry toxins bind to receptors on the midgut epithelial cells, leading to pore formation in the cell membrane and triggering apoptosis. This disruption allows bacteria in gut to enter the hemocoel, causing septicemia and ultimately leading to insect death (Caccia et al., 2016).

The aim of this study is to identify the optimal stress conditions that enhance sporulation and crystallization *BtA* and *BtK* strains, which are commonly used in commercially available *Bt* biopesticides in Korea. To achieve this, both strains were first subjected to a pre-culture under controlled temperature conditions, followed by exposure to various low and high temperature treatments. The numbers of bacterial cells, spores, crystals were measured under different temperature conditions and the insecticidal effect were compared accordingly.

Materials and Methods

Isolation of *Bt* strains

BtA and *BtK* strains were isolated from commercially available *Bt*-based biopesticides, Zentari WG (NongHyup Chemical, Seongnam, Korea) and Nabangmyul WP (Daeyu, Seoul, Korea), respectively. A 0.5 g of each biopesticide formulation was transferred into a 1.5 ml E-tube (Eppendorf, Seoul, Korea) containing 1 ml of sterile distilled water and the mixture was vortexed to homogenize. The E-tubes containing the suspensions were heat-treated at 60°C for 1 h using a heating block (Allsheng, Hangzhou, China). Following heat treatment, 100 μl of each suspension was spread onto Tryptic Soy Agar (TSA) (BD, Franklin, USA). The plates were incubated at 28°C for 72 h to isolate single colonies of *BtA* and *BtK*, which were subsequently selected, streaked using a loop and stored at -80°C in 10% glycerol stocks for accurate identification.

Identification of isolated *Bt* strains

For molecular identification of the isolated strains, each strain was cultured on TSA medium for 48 h, followed by 16S rRNA gene analysis. Genomic DNA was extracted using a 5% Chelex solution (BIO-RAD, Seoul, Korea). PCR amplification was performed using the CFX Duet Real-Time PCR System (BIO-RAD, Seoul, Korea). The PCR reaction mixture consisted of AccuPower® HotStart PCR PreMix (Bioneer, Daejeon, Korea), 1 μl each of the 27F and 1492R primers (Bioneer, Daejeon, Korea), and template DNA, adjusted to a final volume of 20 μl with sterile distilled water. The PCR was performed under the followng conditions: An initial denaturation at 95°C for 4 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. A final extension was performed at 72°C for 5 min. The amplified PCR products were sequenced by a commercial service provider (Macrogen, Seoul, Korea), and sequence homologies were analyzed using the NCBI BLAST tool (https://blast.ncbi.nlm.nih.gov). Using the MEGA 11.0 program (www.megasoftware.net), the phylogenetic trees were built using the Neighbor-joining approach and the Poisson correction model (1,500 bootstrap repetitions to support branching clusters). In addition, biochemical characteristics based on carbon source utilization of each isolated strain were analyzed using GEN III MICROPLATE (Biolog, Hayward, CA, USA).

Measurement of growth curves of *BtA* and *BtK* strains

Each strain previously cultured on TSA plates was inoculated into 50 ml of Tryptic Soy Broth (TSB) (BD, Franklin, USA) from frozen glycerol stocks and incubated at 28°C with shaking at 120 rpm for 48 h to measure the growth rates of *BtA* and *BtK* strains. Subsequently, 10 μl of each *Bt* culture (1×10^5 colony forming units (CFU)/μL) was transferred into 100 ml of fresh TSB and subcultured under the same incubation conditions. To generate growth curves, the optical density (OD) at 600 nm was measured at 4 h intervals using a spectrophotometer (Ubi-600 UV/Vis, MicroDigital, Seongnam, Korea), and bacterial cell counts were determined using a hemocytometer (Paul Marienfeld, Lauda-Königshofen, Germany). The experiment was conducted using three independent TSB cultures for each strain.

Measurement of sporulation in *BtA* and *BtK*

Each *Bt* strain was inoculated into 100 ml of TSB and incubated at pre-culture condition (28°C for 48 h) under

the same conditions used in the growth curve experiment based on previous study (Seo and Kim, 2011; Eom et al., 2014). Cultures were incubated for 96 h at various temperatures (0, 5, 10, 20, 28, 40, 50, 60, and 70°C). Time-course experiments also were conducted at 28°C for various time points (0, 6, 12, 24, 48, 72, and 96 h). Effects of temperature stress on sporulation of *BtA* and *BtK* were measured by count the numbers of spores.

Effect of temperature stress on *Bt* sporulation and crystallization

Based on the previous experiment, 2 temperature conditions (0°C and 70°C), which showed the highest levels of spore formation, were selected for further time-course analysis. Spore and crystal formations were examined at various time points (0, 6, 12, 24, 48, 72, and 96 h) under these 2 temperatures. The numbers of spores and crystals were determined using a hemocytometer. The experiments were conducted using three independent TSB cultures per strain, and the measurements of spores and crystals were performed in triplicate for each culture.

Insecticidal effect of *BtA* and *BtK* by temperature stress

Plutella xylostella, which had been reared for more than 10 generations under laboratory conditions, were used for bioassay. The insect was obtained from the Insect Molecular Physiology Laboratory at Gyeongkuk National University. The *P. xylostella* population was maintained at 26±2°C, with a photoperiod of 16:8 h (L:D) and 60% of RH. Sterilized cabbage leaves were provided as the larval diet after washing with tap water. *BtA* and *BtK* cultures were first incubated under pre-culture condition in TSB and then subjected to temperature treatment at 0°C and 70°C for 96 h. Cabbage leaves were cut into 4 cm diameter leaf disks and immersed in the treated culture broth for 20 min. Subsequently disks were air-dried for 10 min in 9 cm of Petri dishes lined with filter paper. Each dried leaf disk was then exposed to 2nd instar larvae after 6 h starvation. Larval mortality rate was recorded every 24 h for 96 h. The control group was treated under the same conditions using cabbage leaves immersed in sterile TSB without bacterial inoculation. Each experiment was conducted with 10 larvae per replicate, and three replicates were performed.

Statistical analysis

All experimental results were analyzed by ANOVA and treatment means were compared using PROC GLM in SAS (SAS Institute Inc., 1989).

Results

Identification of *BtA* and *BtK*

The amplified PCR products obtained from the DNA of the isolated strains yielded sequences of 1,404 bp for *BtA*

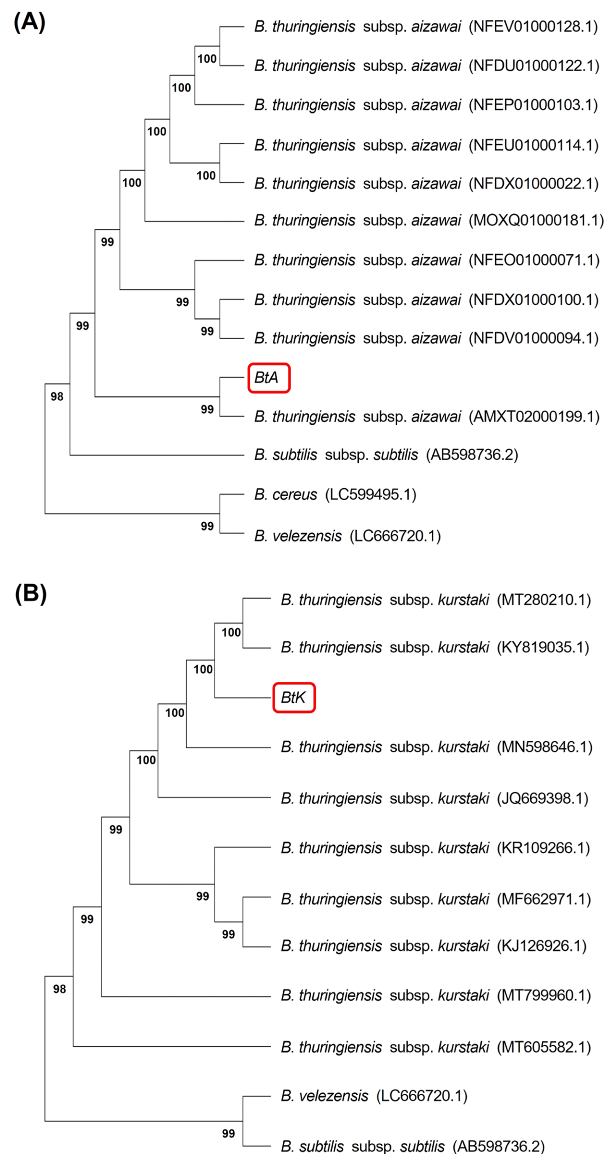

Fig. 1 Phylogenetic analysis of 16S rRNA of the isolated *Bacillus thuringiensis* with other *Bacillus* genus. (A); Isolate from commercial *Bt* pesticide (Xentari) is clustered by *B. thringiensis* subsp. *aizawai* (*BtA*). (B); Isolate from commercial *Bt* pesticide (Nabangmyeol) is clustered by *B. thringiensis* subsp. *kurstaki* (*BtK*). The phylogenetic tree was generated by Neighbor-joining method using the software package MEGA11.0. Each node contains bootstrap value after 1,500 replications to support branching and clustering. Accession numbers follow the scientific names.

Table 1. Characters of carbon source utilization of isolated two *Bacillus thuringiensis* subsp. *aizawai* (*BtA*), *B. thuringiensis* subsp. *kurstaki* (*BtK*)

Carbon sources	Reaction[a)]		
	BtA	BtK	*B. thuringiensis*[b)]
Sucrose	+	+	+
Stachyose	+	+	+
D-Raffinose	+	+	++
α-D-Lactose	+	+	++
D-Melibiose	+	+	++
N-Acetyl-β-D-Mannosamine	+	+	+
N-Acetyl-β-D-Galactosamine	+	+	+
N-Acetyl Neuraminic acid	-	-	-
1% NaCl	++	++	++
4% NaCl	++	++	++
α-D-Glucose	+	+	++
D-Mannose	+	+	++
D-Fructose	+	+	++
L-Fucose	+	+	++
L-Rhamnose	+	+	++
Inosine	+	+	+
1% Sodium Lactate	++	++	++
Fusidic acid	-	-	-
D-Serine	++	++	++
D-Sorbitol	+	+	++
D-Mannitol	+	+	++
D-Arabitol	+	+	++
D-Aspartic acid	+	+	+
D-Serine	+	+	+
Troleandomycin	-	-	-
Rifamycin SV	-	-	-
Gelatin	+	+	++
Glycyl-L-Proline	+	+	++
L-Alanine	+	+	++
L-Arginine	+	+	++
L-Serine	+	+	+
Lincomycin	-	-	-
Guanidine HCl	++	++	++
Niaproof 4	-	-	-
Vancomycin	-	-	-
Lithium Chloride	++	++	++
Sodium Butyrate	+	+	++
Acetoacetic acid	+	+	++
Propionic acid	+	+	++
Acetic acid	+	+	++
Formic acid	+	+	++
Aztreonam	++	++	++
Sodium Bromate	+	+	+

[a)]++: strong positive reaction, +: positive reaction, −: negative reaction
[b)]Standard *B. thuringiensis* recorded in the Biolog System (Biolog, Hayward, USA)

and 1,360 bp for *BtK*. Sequence analysis using NCBI BLAST revealed high genetic similarity of isolated bacteria compared with *B. thuringiensis* (Fig. 1). Two isolates showing 99.86% identity with *B. thuringiensis* subsp. *aizawai* (Accession No. KY819034.1) for *BtA* (Fig. 1A) and 100% identity with *B. thuringiensis* subsp. *kurstaki* (Accession No. EF117854.1) for *BtK* (Fig. 1B). Additionally, carbon utilization profiling using Biolog GEN III plates showed that both strains metabolized key carbon sources such as D-serine, guanidine HCl, and aztreonam, demonstrating identical patterns to *B. thuringiensis* as standard in the Biolog Station database. Therefore, both isolates were finally identified as *BtA* and *BtK* (Table 1).

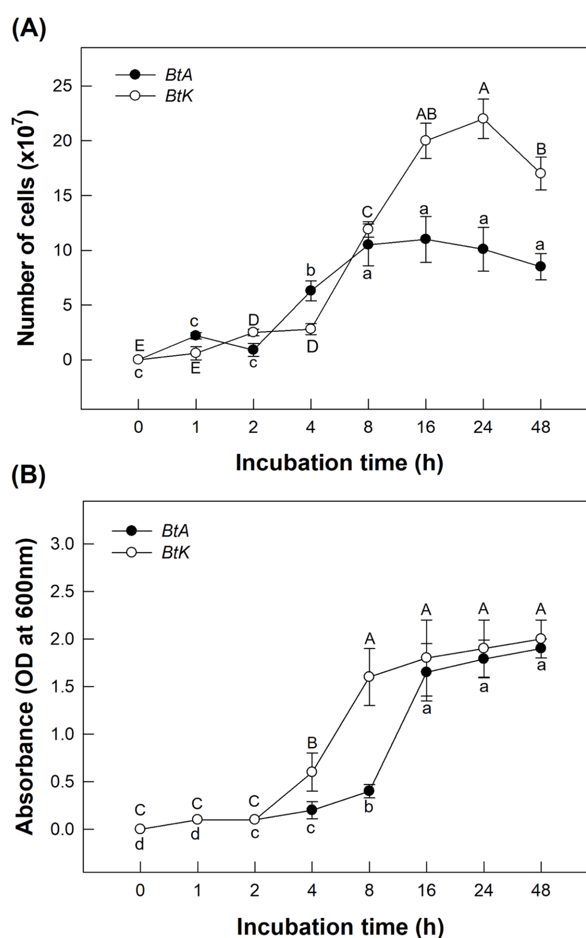

Fig. 2 Cell growth of *Bacillus thuringiensis* subsp. *aizawai* (*BtA*) and *B. thringiensis* subsp. *kurstaki* (*BtK*). Bacteria were inoculated in TSB medium at 28°C in shaking incubator (120 rpm). (A); Number of live cells of *BtA* and *BtK*. (B); Absorbance of *BtA* and *BtK* and optical density (OD) were measured at 600 nm. Each treatment was replicated three times per replication. Different letters indicate significant differences among means at (Type I error = 0.05, LSD test). Uppercase and lowercase letters indicate statistical analysis results for *BtA* and *BtK*, respectively.

Growth curves of *Bt* strains

Both *BtA* and *BtK* strains followed the typical bacterial growth curve model (Fig. 2). In bacterial cell count measurements using a hemocytometer, *BtA* entered the exponential phase from 2 to 16 h after the lag phase, reaching a peak of 10.6×10^7 cells, followed by a decline in cell number. *BtK* showed exponential growth from 4 to 24 h. Both strains then transitioned into the stationary phase, during which the rates of cell proliferation and death were balanced, and subsequently entered the death phase after 24 h of incubation, showing a decrease in viable cell numbers (Fig. 2A). Statistically significant differences in bacterial counts over incubation time were observed for both *BtA* ($F = 24.23$; df: 7,16; $P < 0.0001$) and *BtK* ($F = 219.58$; df: 7,16; $P < 0.0001$). In optical density analysis using a spectrophotometer, *BtA* showed a continuous increase in absorbance, reaching 1.576 at 16 h post-inoculation, after which it plateaued during the stationary phase. *BtK* showed a rapid increase in absorbance up to 8 h, with no further significant change after 16 h (Fig. 2B). There are significant differences in absorbance on incubation time for both *BtA* ($F = 45.52$; df: 7,16; $P < 0.0001$) and *BtK* ($F = 116.22$; df: 7,16; $P < 0.0001$), indicating that the increase in optical density corresponded to an increase in bacterial cell numbers.

Optimal sporulation conditions of *Bt* strains under different temperatures and incubation times

The isolated *BtA* and *BtK* strains showed a positive correlation between treatment duration and sporulation under both low and high-temperature conditions following constant temperature incubation (Fig. 3). In the experiment conducted to determine the optimal temperature for spore formation after 72 h, *BtA* produced 4.5×10^6 spores at 28°C, whereas it produced 1.4×10^7 and 1.5×10^7 spores at 0°C and 70°C, respectively, indicating the highest spore counts at the extreme temperatures. The number of spores formed over time increased until 48 h and plateaued thereafter (Fig. 3A and 3B). For *BtA*, temperature ($F = 174.38$; df: 8,18; $P < 0.0001$) and incubation time ($F = 191.88$; df: 6,14; $P < 0.0001$) had statistically significant effects on spore formation. *BtK* produced the highest number of spores as 1.5×10^7 each at both 0°C and 70°C, and spore numbers increased with longer incubation times. Statistically significant differences were also found for *BtK* based on temperature ($F = 131.28$; df: 8,18; $P < 0.0001$) and time ($F = 12.06$; df: 6,14; $P < 0.0001$). Spores formed by both *BtA* and *BtK* strains were observed within the bacterial cell wall under microscope (Fig. 3C).

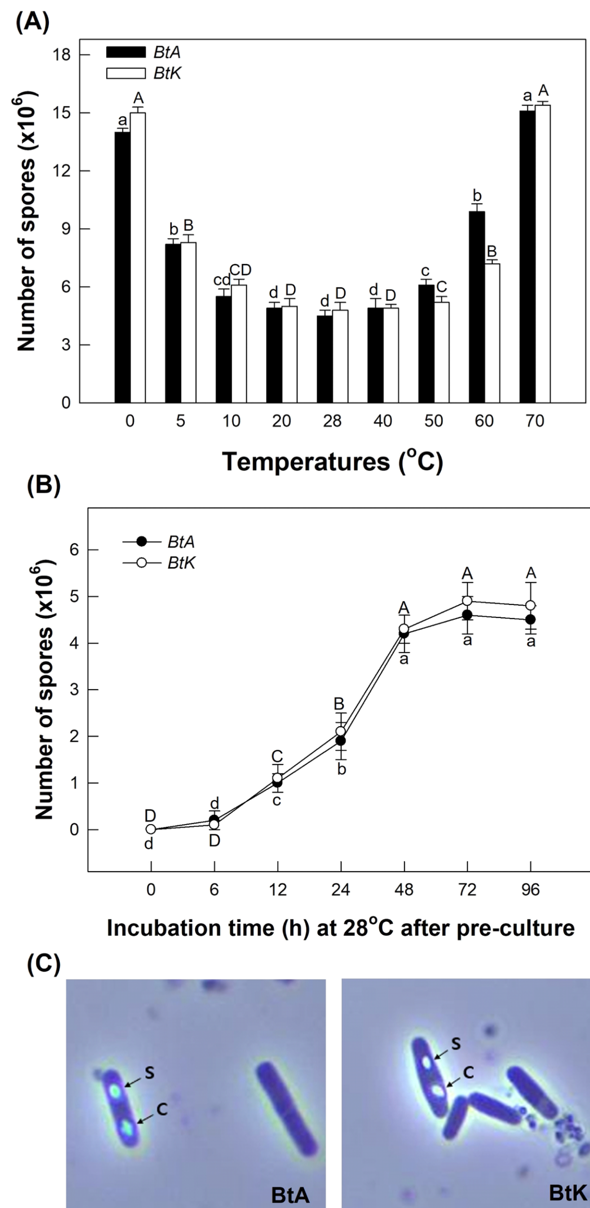

Fig. 3 Sporulation of *Bacillus thuringiensis* subsp. *aizawai* (*BtA*) and *B. thringiensis* subsp. *kurstaki* (*BtK*) by incubation temperatures and times. (A); The numbers of spores of *BtA* and *BtK* after subsequent incubation at different temperatures for 96 h following pre-culture (28°C, 48 h). (B); The numbers of spores of *BtA* and *BtK* after subsequent incubation at 28°C for 96 h following pre-culture (28°C, 48 h). (C); Spore and crystal in *BtA* and *BtK* strains after subsequent incubation at 28°C for 96 h following pre-culture. S and C mean spore and crystal, respectively. Each treatment was replicated three times per replication. Different letters indicate significant differences among means at (Type I error = 0.05, LSD test). Uppercase and lowercase letters indicate statistical analysis results for *BtA* and *BtK*, respectively.

Sporulation of *Bt* strains at different incubation times under high and low temperatures

After initial pre-culture, subsequent incubation at 0°C and 70°C showed that *BtA* produced over 3 times, and *BtK* over 4 times more spores compared to subsequent incubation at 28°C (Fig. 4). For *BtA*, spore numbers at 0°C and 70°C reached 5.5×10^6 and 4.3×10^6, respectively, within the first 6 h of subsequent incubation (F = 29.40; df: 2,6; P < 0.001), and peaked at 1.4×10^7 and 1.3×10^7 at 48 h (F = 111.00; df: 2,6; P < 0.0001). However, no further increase in spore numbers was observed after 48 h (Fig. 4A). *BtK* formed 5.3×10^6 and 4.5×10^6 spores at 0°C and 70°C, respectively, at 6 h (F = 44.33; df: 2,6; P < 0.001), with a maximum of 1.5×10^7 spores at 48 h under both conditions (F = 52.55; df: 2,6; P < 0.001). No significant increase in spore numbers was observed similar to *BtA* (Fig. 4B).

Crystallization of *Bt* strains under different temperatures

The production of crystals in *BtA* and *BtK* under various temperatures showed a similar trend to sporulation, with higher levels observed as the incubation time increased under both low and high temperatures (Fig. 5). After pre-culture, *BtA* showed 6 times and 4 times higher crystal production at 0°C compared to 28°C and 70°C, respectively, during 12 h of subsequent culture. At 48 h, *BtA* produced 1.4×10^7 crystals

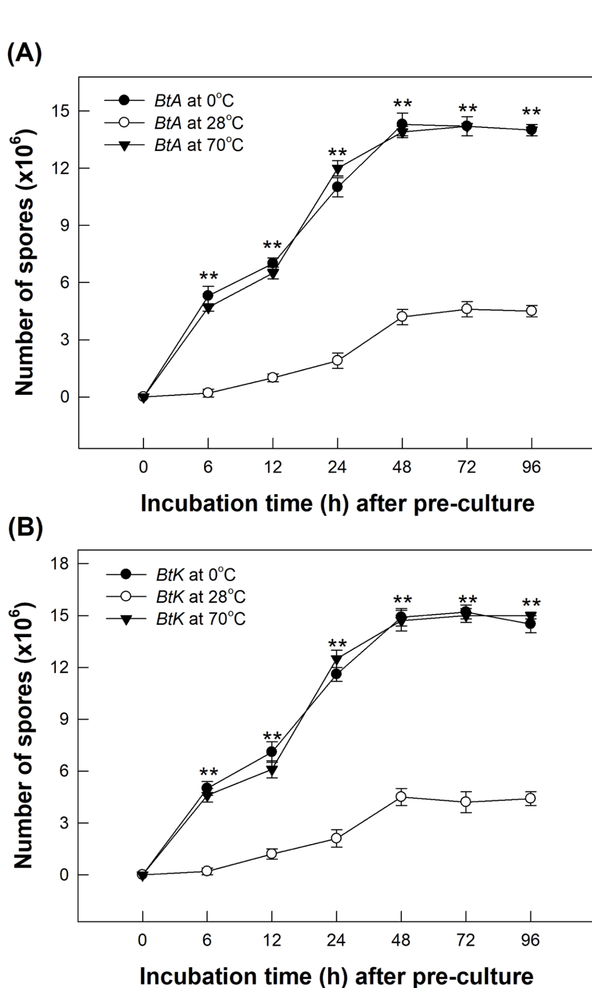

Fig. 4. Sporulation of *Bacillus thuringiensis* subsp. *aizawai* (*BtA*) and *B. thringiensis* subsp. *kurstaki* (*BtK*) by high and low temperatures. (A); The numbers of spores of *BtA* after subsequent incubation at 0°C and 70°C for 96 h following pre-culture (28°C, 48 h). (B); The numbers of spores of *BtK* after subsequent incubation at 70°C for 96 h following pre-culture (28°C, 48 h). Each treatment was replicated three times per replication. Two asterisks above the bars indicate statistically significant differences between temperatures at each incubation time (P < 0.0001).

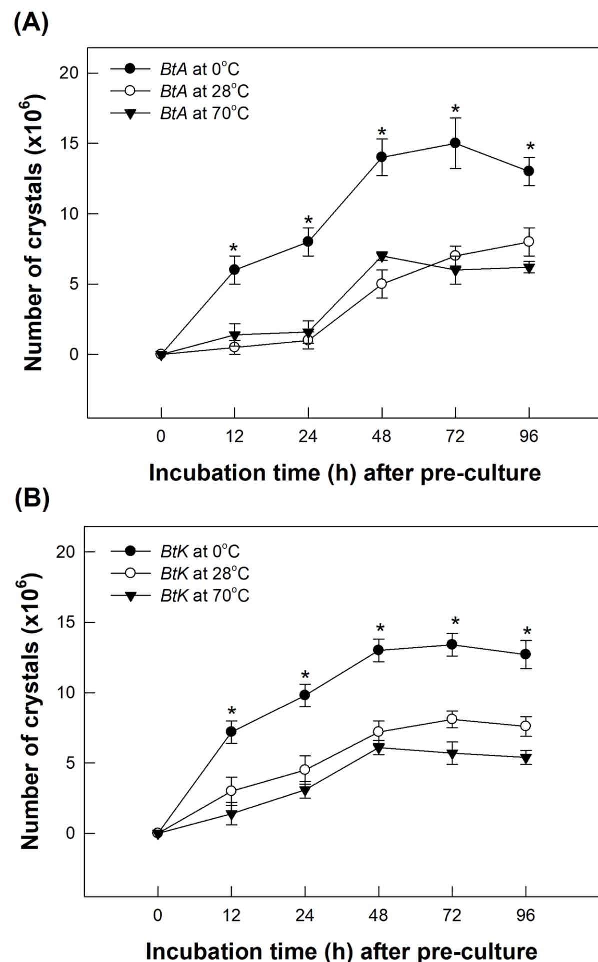

Fig. 5. Crystallization of *Bacillus thuringiensis* subsp. *aizawai* (*BtA*) and *B. thringiensis* subsp. *kurstaki* (*BtK*) by high and low temperatures. (A); The numbers of crystals of *BtA* after subsequent incubation at 0°C and 70°C for 96 h following pre-culture (28°C, 48 h). (B); The numbers of crystals of *BtK* after subsequent incubation at 70°C for 96 h following pre-culture (28°C, 48 h). Each treatment was replicated three times per replication. Asterisk above the bars indicate statistically significant differences between temperatures at each incubation time (P < 0.001).

at 0°C, which was higher than the 4.8×10^6 at 28°C and 5.8×10^6 at 70°C ($F = 490.74$; df = 2,6; $P < 0.0001$) (Fig. 5A). *BtK* produced 5-fold and 2-fold higher levels of Cry proteins at 0°C compared to 28°C and 70°C, respectively, during 12 h of subsequent incubation. After 48 h at 0°C, *BtK* produced 1.3×10^7 crystal, which was higher than 6.8×10^6 at 70°C and 6.2×10^6 at 28°C ($F = 46.87$; df = 2,6; $P < 0.001$) (Fig. 5B). Additional incubation beyond 48 h did not further increase crystal production in either strain.

Insecticidal effect of *Bt* strains under different temperatures

When *BtA* and *BtK* strains were pre-culture at 28°C for 48 h and then subjected to subsequent culture at 0°C, both strains showed rapid and high insecticidal activity to 2nd instar larvae of *P. xylostella* (Fig. 6). After subsequent culture at 0°C, *BtA* showed over 60% higher mortality rate at 24 h compared to those incubated at 28°C and 70°C ($F = 84.69$; df = 2,6; $P < 0.0001$), and reached to 100% mortality rate by 48 h. However, *BtA* under subsequent culture at 28°C reached 100% mortality rate at 96 h, while subsequent culture at 70°C showed 80% mortality rate (Fig. 6A). For *BtK*, subsequent culture at 0°C showed about 80% and 40% higher mortality rate at 24 h compared to subsequent culture at 28°C and 70°C, respectively ($F = 198.02$; df = 2,6; $P < 0.0001$). *BtK* required to reach 100% mortality rate was 24 h faster than that of the incubated at 28°C (Fig. 6B). Notably, *BtK* showed approximately 13% higher mortality rate than *BtA* at 24 h after treatment, and differences in insecticidal activity between the two strains at 96 h.

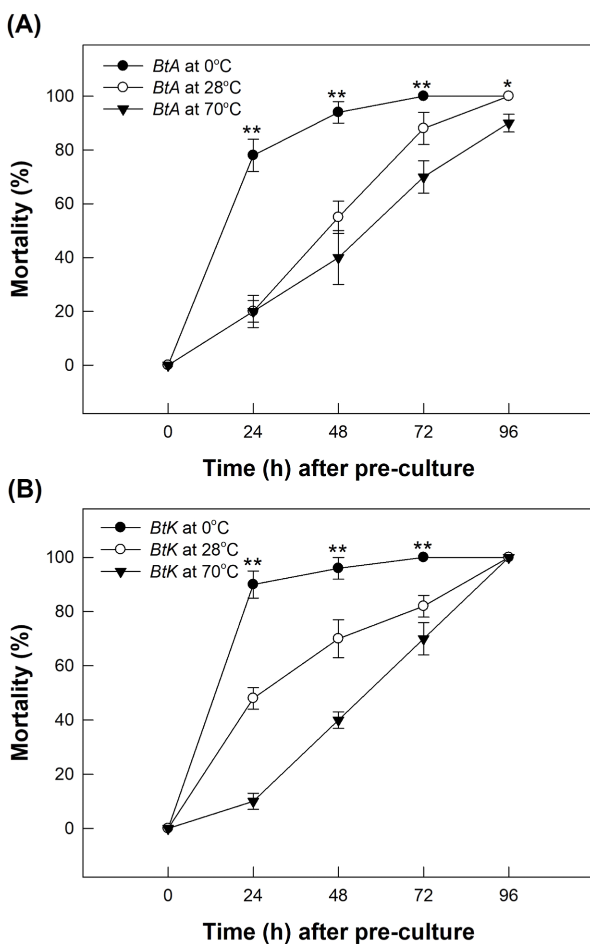

Fig. 6. Insecticidal effect of *Bacillus thuringiensis* subsp. *aizawai* (*BtA*) and *B. thringiensis* subsp. *kurstaki* (*BtK*) to *Plutella xylostella* 2nd instar larvae. (A); mortality rate of *Bt*A after subsequent incubation at 0°C and 70°C for 96 h following pre-culture (28°C, 48 h). (B); mortality rate of *BtK* after subsequent incubation at 70°C for 96 h following pre-culture (28°C, 48 h). Each treatment was replicated three times with 10 larvae per replication. Two asterisks above the bars indicate statistically significant differences between temperatures at each incubation time ($P < 0.0001$).

Discussion

This study investigated how varying temperature treatments and incubation durations affect spore and Cry protein production in *B. thuringiensis* strains used as biopesticides, and evaluated the resulting insecticidal activity against *P. xylostella* larvae.

Bt has played a significant role in the biopesticide market due to its diverse modes of action and high target specificity (Ragasruthi et al., 2024). For nearly two decades after its commercialization, there were no reports of resistance in insect populations. However, the first *Bt* resistance case was reported in *Plodia interpunctella*, a stored product pest, in 1985 (McGaughey, 1985), and subsequent cases have since been documented in various Lepidopteran species (Heckel et al., 2007). *BtK*-based biopesticides have long been utilized for the control of key pests such as *P. xylostella*, whereas *BtA* formulations were introduced later (Liu et al., 1996). Although early studies showed that insect populations with high resistance to *BtK* displayed relatively lower resistance to *BtA* (Wright et al., 1997), later research conducted in Brazil reported stronger resistance to *BtA* (Zago et al., 2014), possibly due to lower *BtK* usage in the region or stronger selection pressure imposed by *BtA*. In this study, both *BtA* and *BtK* strains, when applied to *P. xylostella* populations maintained for more than 10 generations in laboratory, resulted in 100% mortality rate by 5 days after treatment. However, *BtK* exhibited higher insecticidal activity (64%) compared to

BtA (30%) by 3 days after treatment. These differences could be attributed to the types and compositions of Cry proteins produced by each strain. Further study is needed for the susceptibility and interactions of specific Cry proteins with target insect midgut receptors.

Bt strains inherently differ in spore yield and Cry protein production depending on their genetic background, even under identical culture conditions (Mohan and Gujar, 2001). For example, *BtA* strain HD-137 produces Cry1Aa, Cry1Ab, Cry1C, and Cry1D toxins, while *BtK* strain HD-1 produces Cry1Aa, Cry1Ab, Cry1Ac, Cry2A, and Cry2B toxins. In contrast, strains like *BtK* HD-73, which produce only Cry1Ac, have been reported to exhibit lower toxicity (Dilawari et al., 1996). Likewise, *B. thuringiensis* subsp. *israelensis* HD-14, which is specific to mosquito larvae, is ineffective against *P. xylostella* (Chilcott and Ellar, 1988), indicating that insecticidal activity is determined by the types and combinations of Cry proteins produced (Höfte and Whiteley, 1989).

This study confirmed that temperature treatments significantly affect both sporulation and crystallization in *Bt* strains. Crystals are synthesized during spore formation inside the bacterial cell (Rudd et al., 2023), and spore number is positively correlated with Cry protein yield (Aronson, 2002). Additionally, sporulation is regulated not only by nutrient composition but also by environmental stressors such as temperature (Park et al., 1997; Stanford, 1989), consistent with the findings of this study. *BtA* and *BtK* strains achieved 90% and 100% mortality rate within 48 h after treatment respectively, by subsequent culture at 0°C for 48 h. These results suggest that low-temperature treatments positively influence spore and Cry protein biosynthesis, indicating their potential as effective subsequent culture conditions in *Bt* formulation processes. Although spore number increased over time under high-temperature conditions, insecticidal efficacy was delayed by 2 days for *BtA* and 3 days for *BtK* compared to low-temperature treatments. This delay may be attributed to structural denaturation of Cry proteins at high temperatures, impairing their ability to bind to midgut receptors. Moustafa et al. (2018) reported that heat-labile Cry proteins exhibit reduced insecticidal activity due to structural degradation at elevated temperatures.

To enhance the insecticidal activity of *Bt*, previous studies have examined its combination with immunosuppressive substances derived from the entomopathogenic bacterium *Xenorhabdus nematophila* (*Xn*) (Eom et al., 2014). When *Bt* was applied together with secondary metabolites produced by *Xn*, the insecticidal efficacy against *P. xylostella* and *S. exigua* larvae increased up to two times. Among the *Xn*-derived compounds that enhance the insecticidal effect of *Bt*, benzylideneacetone and oxindole have been identified. Therefore, combining these beneficial compounds with the *Bt* cultivation process established in this study may further improve its insecticidal efficacy against pest insects.

In conclusion, this study demonstrated the effectiveness of low-temperature as subsequent culture as a physical strategy to enhance the insecticidal activity of *Bt*-based biopesticides. It offers a practical approach to promote early Cry protein synthesis during industrial-scale formulation. However, further studies are needed to assess the influence of environmental factors such as soil pH, temperature fluctuations, and microbial interactions on *Bt* efficacy in field settings since the experiments were conducted under controlled laboratory conditions. The findings of this study contribute to the technological foundation for optimizing *Bt*-based biopesticide formulations and broadening their application in sustainable pest management strategies.

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Conflict Interest

The authors declare that they have no conflict of interest.

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