A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Construction and Characterization of Dual-functional *Bacillus subtilis* Strain against Phytopathogenic Fungi and Insect Pests

살균 활성과 살충 활성을 동시에 가지는 재조합 *Bacillus subtilis* 균주의 제작 및 특성 구명

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UNDER THE DIRECTION OF ADVISER YEON HO JE
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF
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ABSTRACT

Biocontrol in agriculture is a crucial method for the management of insects and plant diseases. It reduces the use of various synthetic pesticides which cause many harmful effects on the environment. *Bacillus subtilis* (Bs) is known as a biocontrol agent against plant diseases due to its ability to produce a range of antimicrobial compounds such as iturines, fengicines and surfactines. Also, *Bacillus thuringiensis* (Bt) produces δ-endotoxins used to control insects. This study aimed to construct the dual-functional biocontrol agent against insects and plant fungal diseases by introducing the pMBD-ABC

encoding *cry1Ac*, *cry1Be*, and *cry1C* Bt insecticidal proteins genes into the Bs strain, which controls the growth of certain plant pathogenic fungi. The improved Bs strain was also evaluated for its insecticidal activities and antifungal activities in this study. These results suggested that the improved Bs strain could be useful for the development of microbiome pesticides which could be applied for simultaneous control of both insect and plant diseases.

Key words: biocontrol agent, *Bacillus thuringiensis*, *Bacillus subtilis*, antifungal activity, insecticidal proteins

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INTRODUCTION

Plant diseases and insects are crucial problems in agriculture. Plants must face these infections or attacks of pests and which cause losses in crop production. Plant diseases occupy the loss of at least 10% of global agriculture production (Strange & Scott, 2005). To increase the yields of agriculture, the use of pesticides is inevitable. Chemical pesticides have been used frequently because of their price competitiveness and effectiveness. However, the excessive use of fungicides and insecticides leads to accumulating residual pesticides. It develops resistance to pests and several environmental problems. For this reason, there have been many attempts to replace chemical pesticides with biopesticides. Biopesticides are less toxic to non-target species and decompose more quickly in the environment (Thakore, 2006). Therefore, biopesticides have been widely used in the world in recent decades as part of an integrated pest management approach.

Biopesticides could be classified into three types: Substances found in nature, Plant-Incorporated Protectants (PIPs), and microbes. Substances found in nature include plant materials like black pepper and plant essential oil, and these also include insect hormones. PIPs are materials plants produce to protect themselves from pests by genetic engineering or introducing genes into plants. The crystal toxin genes from *Bacillus thuringiensis* are most frequently used to insert because of their insecticidal activities. Microbial pesticides are biopesticides based on living microorganisms like bacteria, fungi, and viruses (Kaleh et al., 2022).

Microorganism-based products amount to 30% of total sales of biopesticides, and

bacterial products are the majority of these products (Cawoy et al., 2011). Among them, the *Bacillus* genus is the primary microbial biocontrol agent. *Bacilli* are gram-positive, spore-forming bacteria. *Bacilli* exist throughout environments ranging from soil to aquatic ecosystems, even found in extreme environments like hot springs (Priest, 1993). When the *Bacilli* face unfavorable environmental conditions, they produce endospores to resist extreme conditions.

Bacillus subtilis (Bs) are well-studied, non-pathogenic, and industrially-used strains. Bs have several reasons that can be used as biocontrol agents. They compete with other bacteria for ecological niches, produce inhibitory allelochemicals, and colonize the phytosphere. Based on these characteristics, they benefit plants by protecting them from infection by plant pathogens. Also, members of *Bacilli* such as *B. subtilis*, *B. cereus*, *B. megaterium*, and *B. pumilus* are known to produce antibiotic molecules. Bs are known to have an average of 4-5% of their genome devoted to antibiotic synthesis. *B. subtilis* RB14, for example, was known to produce iturin A that control damping-off the tomato caused by *Rhizoctonia solani* (Asaka & Shoda, 1996). Besides, there are several reports of various antibiotic substances produced by Bs.

Bacillus thuringiensis (Bt) were the best-known *Bacillus* species used as biopesticides. Bt produce insecticidal proteins, such as Cry and Cyt proteins. The best advantage of these Bt toxins is that they have high insecticidal activities for target species, but not for non-target species and the environment. The Bt toxin was first applied in 1938 and has been used as a significant biological pesticide in the past few decades. When insects take Bt toxins, they move toward the insect's midguts. The insect's midguts are alkaline environments with pH 11, and Bt toxins are activated by protease present in insect guts.

The activated Bt toxins act through pore formation in the midgut wall. This cascade is only effective in the presence of specific receptors in the insect's gut, which is why Bt toxin has specific target species. Bt toxin's major target insect families are Lepidoptera, Diptera, and Coleoptera. It has been many reports on insecticidal genes derived from Bt. Also, the various biopesticides based on Bt toxins were developed and are still used in agriculture.

There is a high probability of plant diseases and pests occurring after the rainy season, and if they are not adequately controlled, crop damage may worsen. It is necessary to develop an extensive control system in the case of simultaneously outbreak of plant diseases and pests. In this study, it was aimed to produce the improved Bs strain showing both fungicidal and insecticidal activity by introducing the *cry* genes of Bt into the Bs strain known to have antifungal activity. To improve the expression of Bt toxins in Bs, four types of promoters were used to express *cry* genes, and the optimal promoter was selected. The improved Bs strain's dual activities have been evaluated against plant pathogenic fungi and lepidopterans (Fig. 1).

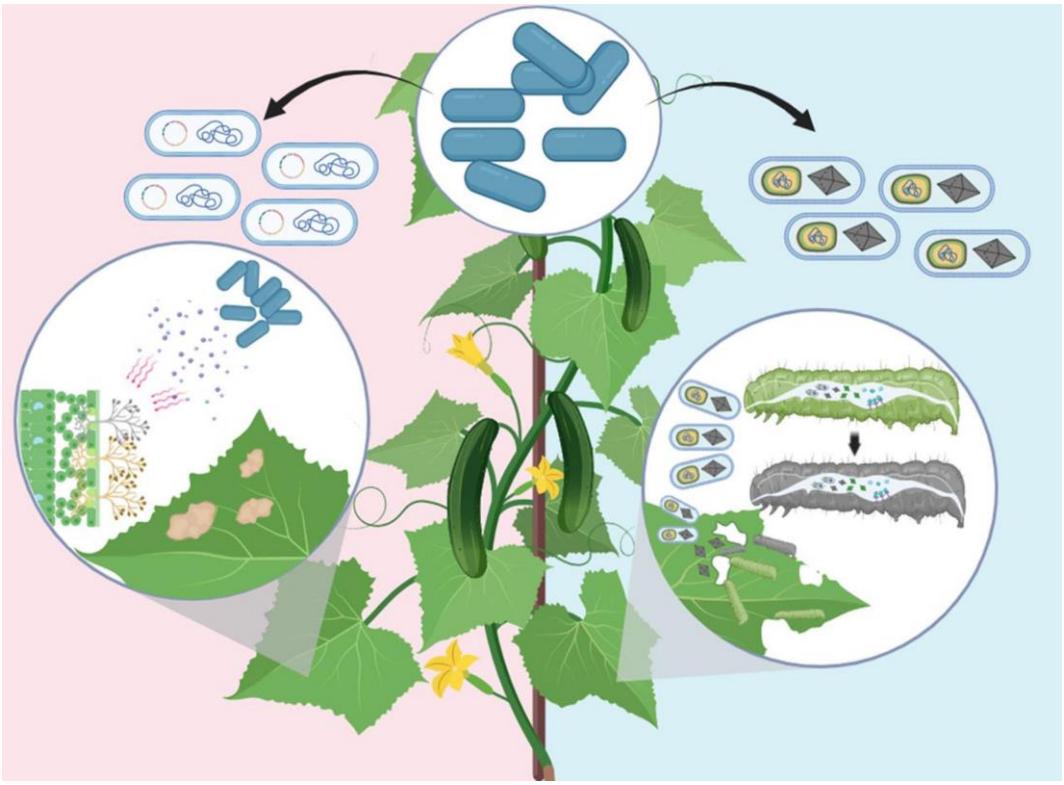

Fig. 1. The graphical abstract of this study.

LITERATURE REVIEW

1. General characteristics of *Bacillus thuringiensis*

B. thuringiensis (Bt) is a ubiquitous, spore-forming, Gram-positive bacterium which can be readily isolated from a variety of environmental sources including soil, water, plant surfaces, grain dust, dead insects, and insect feces. Bt is belonging to the Bacillaceae family and first described by Berliner (1911). Originally, Bt was considered a danger to lepidopteran silkworms and it was several decades before serious research was aimed at utilizing Bt for insect controls. Using Bt for the control of the European cornborer (*Ostrinia nubilalis*) was initiated since 1928, and the first field applications was recorded by Husz (1929) against these lepidopteran pests. The commercial production utilizing Bt were released in 1938, named as Sporeine (Lambert & Peferoen, 1992).

Bt was characterized by its ability to produce a parasporal protein crystal during sporulation. Under the extreme conditions, Bt sporulates producing a spore and parasporal body, and then composes primarily of one or more insecticidal proteins in the form of crystalline inclusions (Federici et al., 2010). These proteins are highly toxic to a wide variety of important agricultural and health-related insect pests. As their high specificity and their safety for the environment, crystal proteins are a valuable alternative to chemical pesticides for control of insect pests in agriculture. Insecticidal crystal proteins from Bt have been used intensively as biopesticides for the several decades.

Crystal protein

Insecticidal proteins are typically produced during sporulation, with the expression of their encoding genes being driven by one or two sporulation dependent promoters (Wong et al., 1983). Transcription from these promoters, referred to as BtI, and BtII, is initiated by RNA polymerase complexes that contain, sigma-E and sigma-K like factors, respectively (Agaisse & Lereclus, 1995; Baum & Malvar, 1995; Wong et al., 1983). Individual Cry proteins are toxic to insects and classified as CryI, CryII, CryIII and CryIV, etc., according to their activity spectra (H. Höfte & H. Whiteley, 1989). These insecticidal activities are usually restricted to a species within particular order of Lepidoptera, Diptera, Coleoptera, and Hymenoptera. The most common endotoxin proteins that produced in Bt are those of the CryI class. These are 130 to 135 kDa protoxins, which form a single large bipyramidal crystal about the size of a spore in each cell.

Currently, the toxins are classified on the basis of amino acid sequence homology, where each protoxin acquired a name consisting of the Cry (or Cyt) and four hierarchically ranks consisting of numbers depending on its place in a phylogenetic tree. The first level represented the primary rank, and toxins that shared at least 45% sequence identity were given the same number. The second rank was used to distinguish sequences sharing between 45% and 78% identity. After that, toxins shared between 78% and 95% identity were distinguished at the level of the tertiary rank. The quaternary rank was used to distinguish between different clones that shared at least 95% sequence identity (Jurat-Fuentes & Crickmore, 2017).

Molecular biology of *cry* genes expression

In the *Bacillus* species, endospores occur in a sporangium consisting of two cell compartments, known as the mother cell and the forespore. The development process is temporally regulated at the transcriptional level by the successive activation of sigma factors such as σ^A, σ^H, σ^F, σ^E, σ^G, and σ^K. The σ^A and σ^H factors are active in the predivision cell, on the other hand σ^E and σ^K are active in the mother cell, and σ^F, σ^G are active in the forespore (Agaisse & Lereclus, 1995). The *cry1Aa* gene is a typical example of a sporulation-dependent cry gene expressed only in the mother cell compartment of Bt. Two transcription start sites found (BtI and BtII), defining two overlapping, and sequentially activated promoters (Wong et al., 1983). BtI is active between about t2 and t6 of sporulation, and BtII is active from about t5 onwards (tn is n hours after the end of the exponential phase). BtI is initiated by a form of RNA polymerase containing an alternative sigma factor, σ^{35} and BtII is initiated by σ^{28}. The deduced amino acid sequences of σ^{35} and σ^{28} show 88% and 85% identity with σ^{35} and σ^{28} of *B. subtilis* respectively (Adams et al., 1991).

2. General characteristics of *Bacillus subtilis*

Bacillus subtilis (Bs) is a gram-positive, rod-shaped bacterium. It could be isolated in a wide variety of environments, including soil and the gastrointestinal tracts of animals (Earl et al., 2008). Bs secretes numerous enzymes to degrade a variety of substrates, enabling the bacterium to survive in a continuously changing environment. Bs is also known for its ability to fix nitrogen in the soil. These remarkable secretion abilities of Bs make them important hosts for the production of industrial enzymes. Bs has been widely used to produce heterologous proteins and moreover, it has great physiological

characteristics and highly adaptable metabolism, which makes it easy to cultivate on cheap substrates (Su et al., 2020). Furthermore, prominent expression systems with good genetic stability are available for this organism, and it has no strong codon preference. Different from *Escherichia coli*, Bs has a single cell membrane, which facilitates protein secretion, simplifies downstream processing, and reduces the process costs. Finally, this species is generally recognized as safe (GRAS) (Westers et al., 2004; Zweers et al., 2008).

Besides of its rapid growth, low nutritional requirements, safety to humans and animals, and high antibacterial activity, Bs is one of the most widely used microorganisms industrially to produce active secondary metabolites. It often used as a microbial additive to improve intestinal function in animals, promoting animal growth and preventing diseases (Lee et al., 2019). Additionally, Bs can produce polypeptides that have an antagonistic effect against intestinal pathogens and can secrete a variety of antimicrobial peptides and bacteriocins, such as bacilysin (Afsharmanesh et al., 2018), subtilin (Corvey et al., 2003), and lipopeptides: surfactin (Kim et al., 2010), fengycin (Vanittanakom et al., 1986), and iturin (Dunlap et al., 2019).

Lipopeptides

The lipopeptides are secondary metabolites synthesized by various microorganisms. Lipopeptides are small compounds contained hydrophobic fatty acid chains and hydrophilic cyclic peptides. Some lipopeptides have antifungal activity at high concentration to elicit the formation of pores in the cell membrane and at low concentration to induce apoptosis. Also, lipopeptides inhibit cell wall generation and affect the adhesion

of microorganisms by partitioning the interface of fluid phases with distinct polarities and hydrogen bonding.

Bacillus species are one of the bacteria producing the lipopeptides. It could be divided into three families according to the structure of the cyclic peptides: surfactin, fengycin, and iturin. The lipopeptides have multiple bio-activities including anti-bacterial, anti-fungal, anti-viral, and anti-tumor activities. Also, lipopeptides have low toxicity, high biodegradability and environment friendly characteristics compared with chemical pesticides. For this reason, lipopeptides have been used to the chemical, agricultural, pharmaceutical, and food industries (Jiang et al., 2014). Surfactin and fengycin act as elicitors of induced systemic resistance in plants (Palanisamy, 2008). Some of the lipopeptides of Bs could be effectively inhibit the growth of microorganisms in foods and can be used as biological preservatives for food preservation (Meena & Kanwar, 2015).

Surfactin was first identified from the culture medium of Bs by Arima et al. (Arima et al., 1968). Typical surfactin contains a heptapeptide of amino acid sequence: (L-)Glu-(L-)Leu-(D-)Leu-(L-)Val-(L-)Asp-(D-)Leu-(L-)Leu. The peptide is linked via a lactone bond to a beta-hydroxy fatty acid of 13-15 C atoms. Iturin is also a cycle lipoheptapeptide. The seventh position of the heptapeptide is a Ser that is linked by a β-amino acid residue of a fatty acid chain with 14-17 C atoms. The amino acid sequence of the heptapeptide is (L-)Asn-(D-)Tyr-(D-)Asn-(L-)Gln-(L-)pro-(D-)Asn-(L-)Ser (BESSON & Michel, 1987; Maget-Dana & Peypoux, 1994). Fengycin is another kind of anti-fungal lipopeptide complex. Also to the hydroxy fatty acid with 15-19 C atoms, fengycin is typically composed of the peptide chain (L-)Glu-(D-)Orn-(L-)Tyr-(D-)Thr-(L-)Glu-(D-)Ala-(Val)-(L-)Pro-(L-)Gln-(D-)Tyr-(L-)Ile. The Tyr at position 3 is bonded to the Ile at position 10

to form a cycle peptide via a lactone bond. There are two main variants of fengycin, fengycin A and B, that differ in the identity of the amino acid at position 6 (Schneider et al., 1999; Tang et al., 2014).

Chapter 1.

Optimization for expression of Cry proteins in *Bacillus subtilis* strain

INTRODUCTION

Bacillus thuringiensis (Bt) normally synthesize diverse insecticidal proteins. Crystal (Cry) and cytolytic (Cyt) toxins known as δ-endotoxins were synthesized at the onset of sporulation. These toxins are formed as parasporal crystalline inclusions during the stationary phase of growth (H. Höfte & H. R. Whiteley, 1989). In addition, Bt produce the different kind of insecticidal proteins during the vegetative growth phase which are Vegetative insecticidal proteins (Vip) (Estruch et al., 1996) and Secreted insecticidal protein (Sip) (Donovan et al., 2006).

There are more than 700 *cry* genes encoding Cry proteins identified. Cyt proteins were classified into three different families (Cyt1, Cyt2, and Cyt3). Cyt proteins synergize the insecticidal activity of other Cry or Vip3 toxins (Pardo-López et al., 2013). Vip proteins were classified into four different families (Vip1, Vip2, Vip3, and Vip4) (Crickmore et al., 1998). These three types of Bt toxins express under the control of different promoters. The promoters from various insecticidal toxin genes have been identified that control the binding of RNA polymerase to DNA when the transcription of genes initiates.

Each promoter was regulated by a series of sigma factors and different sigma factors were associated according to the expression timing of each Bt toxin. The *cry* and *cyt* genes were transcribed in the sporulation stage, controlled by sigma factors σ^{35} and σ^{28} (Bravo et al., 1996), which are homologous to the *Bacillus subtilis* (Bs) sporulation-stage-specific sigma factors σ^{E} and σ^{K} (Adams et al., 1991).

In this study, the similarities of the sigma factors between Bt and Bs were confirmed.

Additionally, it was demonstrated that the operability of Bt promoters in the Bs by recombinant strain. In order to develop an improved Bs strain with optimized promoter, four different types of Bt promoters were cloned to EGFP expression vector respectively. The EGFP expression vectors were introduced into Bs, and then the fluorescent expression level was compared for the optimized promoter.

MATERIALS AND METHODS

1. Bacterial strains and growth media

The *Escherichia coli* TOP 10 strain (Thermo Fisher Scientific, Waltham, MA, USA) was used for transformation and amplification of the plasmid DNA. An acrystalliferous strain of Bt subsp. *israelensis* 4Q7 was used for the targeted cry toxin-producing recombinant Bt strain. *B. subtilis* subsp. *subtilis* (KACC 17109) was deposited from the Korean agricultural culture collection (KACC). *E. coli* was grown at 37℃, 220 rpm in LB medium for plasmid preparation. The LB medium was also used as a primary culture of *Bacillus* strains. Brain heart infusion (BHI, Difco Co., MD, USA) medium was used to culture Bt competent cells. GYS medium was used to culture *Bacillus* strains to express crystal proteins. All of the restriction endonucleases used in this study were purchased from New England Biolabs (Ipswich, MA, USA). Media compositions are described in Table 1.

2. Sigma factor genes

To compare sigma factor sequences between Bt and Bs, four sigma factor genes of Bs σ^{E}, σ^{K} and Bt σ^{35}, σ^{28} associated with sporulation were amplified by PCR using specific primers (Table 2). The σ^{A} and σ^{H} of both Bs and Bt detected in the growth phase were also amplified by PCR using specific primers (Table 3). All sequences were aligned and compared their similarity (CLC Main Workbench 7.9.1).

Table 1. Composition of culture media for a strain of *Bacillus* strains.

Medium*	Components	%(g/L)
LB	Tryptone	1
	Yeast extract	0.5
	NaCl	1
GYS	Glucose	0.1
	Yeast extract	0.2
	K_2HPO_4	0.05
	$(NH_4)_2SO_4$	0.2
	$MgSO_4$	0.002
	$MnSO_4$	0.005
	$CaCl_2$	0.008

*LB: Luria-Bertani; GYS: Glucose-yeast extract salt medium.

Table 2. The primer lists for the PCR of sigma factors detected in sporulation phase of *Bacillus* strains.

Genes	NCBI Accession no.	Primer	Sequence (5'-3')	Target size (bp)
Bs σ^H	CP053102.1	Bss-sigH-F Bss-sigH-R	GTGAATCTACAGAACAACAAGGG TTACAAACTGATTTCGCGAATTTCC	657
Bs σ^A	CP053102.1	Bss-sigA-F Bss-sigA-R	ATGGCTGATAAACAAACCCAC TTATTCAAGGAAATCTTTCAAACGTTTAC	1116
Bt σ^H	CP037890.1	Bt-sigH-F Bt-sigH-R	GTGGAAGCAGGCTTCGTAA TTATGAATTTAAAGTTGTACTTTCTCGC	660
Bt σ^A	CP037890.1	Bt-sigA-F Bt-sigA-R	ATGGCTGACAAACCAGCTC CTATTCTAAGAAATCCTTAAGACGCTTAC	1128

Table 3. The primer lists for the PCR of sigma factors detected in growth phase of *Bacillus* strains.

Genes	NCBI Accession no.	Primer	Sequence (5'-3')	Target size (bp)
Bs σ^{K}	CP032860.1	Bss-sK-F	AAGAGCTTGTCTTTTTAGTATC	672
		Bss-sK-R	TCTTCCGCTTTTCCTTCTCT	
Bs σ^{E}	CP053102.1	Bss-sE-F	ACCTCTGGTATAAGCTGC	692
		Bss-sE-R	TTACACCATTTTGTTGAACTC	
Bt σ^{28}	JEOC01000009.1	Bt4Q7-s28-F	TTACTCTTTCGCTTTTTTCTC	714
		Bt4Q7-s28-R	TTGAGTCTATTCGCCGCAA	
Bt σ^{35}	AY083614.1	Bti-s35-F	ATGATGAAATTAAAATTTTATTTAGTATACC	720
		Bti-s35-R	TTACACCATTTTATTAAATTCTTTTCG	

3. Construction of pMkE expressing EGFP vectors

To develop EGFP expression vector, a pMk plasmid vector containing the *Mre*I restriction enzyme site was used to modify. This vector contains both *E. coli* and Bt origins of replication and erythromycin and ampicillin resistance genes. Total 4 types of pMkE-X vector were constructed. pMkE-1 vector contains *cry3A* sporulation-independent promoter. pMkE-2 vector contains *vip* sporulation-independent promoters. pMkE-3 vector contains *cry1C* sporulation-dependent promoters. pMkE-4 vector contains *cyt* sporulation-dependent promoters (Fig. 2). Each promoter was amplified and then cloned between the *Eco*RI and *Sac*I site. The 720 bp fragments of EGFP genes were amplified with the specific primers (EGFP-*Bam*H1-F: 5'-GCAGGATCCGCCATGGTGAGCAAGGGCGAG-3', EGFP-*Sal*1-R: 5'-GCAGTCGACGGCTTACTTGTACAGCTCGTCCATGCC-3') and then cloned between the *Bam*H1 and *Sal*1 site of the pMk vector to generate pMkE-X vectors.

4. Transformation of Bt

Bt 4Q7 and Bs (KACC 17109) strains were used to construct *Bacillus* transformants. For the transformation Bt cells were grown in 20 ml LB medium with shaking at 30℃, 220 rpm overnight. Two hundred microliter of the primary culture was inoculated into 20 ml BHI, and the cells were incubated with shaking at 30℃, 220 rpm to an OD_{600} of 0.4 ~ 0.7. The cells were harvested, washed twice in 10 ml of distilled water at 4℃ and then resuspended in 2 ml of electroporation buffer (400 mM sucrose, 1 mM $MgCl_2$, 7 mM

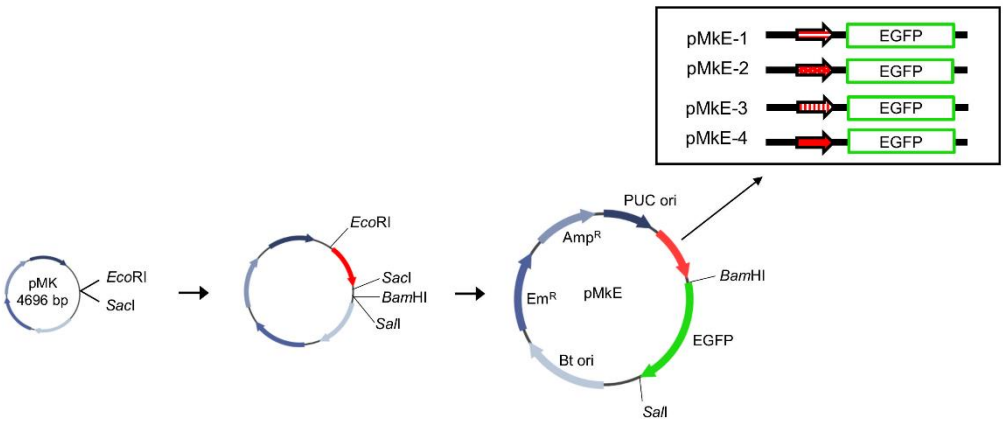

Fig. 2. Construction map of EGFP producing vectors pMkE-X. The PCR-amplified promoter genes were inserted into *Eco*RI and *Sac*I digested pMk, and the PCR-amplified EGFP gene was inserted into *Bam*HI and *Sal*I.

K_2HPO_4) at 4℃. Then, a 0.2 ml aliquot of the cell was mixed with 2 μg of plasmid DNA and electroporated once in a 0.2 cm electroporation cuvette (Bio-Rad, Hercules, CA, USA) at 4°C using a Gene Pulser II (Bio-Rad, Hercules, CA, USA) under the following conditions: volts, 1.6 kV; resistance, 400 Ω; capacitance, 25 μF. Following electroporation, the cells were diluted in 1 ml of prewarmed BHI medium and incubated at 30℃, 220 rpm for 2 h. The cells were then plated on a nutrient agar medium (Difco Co., MD, USA) containing erythromycin (50 μg/ml).

5. Transformation of Bs

Transformation of the Bs strain was done by modifying a method described by Bennallack et al. (Bennallack et al., 2014). Bs (KACC 17109) strain was cultured in LB medium overnight for the primary culture. The next day, Bs was inoculated in 10 ml of SM1 medium and incubated at 37℃ for 3 hours. After 3 hours, 10 ml of SM2 medium was added to the culture medium, and the culture was incubated for 90 minutes. The 2 μg of plasmid was added in 500 μl of cell culture and incubated at 37℃ for 50 min. Afterward, 500 μl of LB was added and incubated for 3 hours at 37℃ rotating. After incubating, the 200 μl of cell culture was spread on nutrient agar (NA) containing 50 μg/ml erythromycin.

6. Detection of EGFP expression

To clarify the introduction of pMkE-X vectors, *Bacillus* colonies grown on the NA plate containing the erythromycin (50 μg/ml) were cultured in 10 ml of LB medium containing the same concentration of erythromycin. The pellet of cells was harvested by

centrifuge at 13500 rpm for 5 min. Then the pellet was diluted in 100 μl of TE buffer, and boiled at 100℃ for 10 min, and it was used in PCR to amplify the vector portion, from promoter to EGFP genes.

To observe the EGFP expressing by Bt/pMkE-X and Bs/pMkE-X, these strains were cultured in 20 ml LB medium at 30℃ for 3 days and 7 days with shaking at 220 rpm then washed with DDW and analyzed by confocal laser scanning microscopy (SP8X STED, Leica, Wetzlar, Germany).

RESULTS

1. Similarities of sigma factors detected in *Bacillus* strain

To compare the sigma factors of Bt and Bs, the polymerase chain reaction (PCR) was conducted in Bt 4Q7 and Bs (KACC 17109). The primer design was based on the Bt 4Q7 genome sequence and the Bs 168 strain. PCR products were identified by electrophoresis and purified for DNA sequence analysis (Fig. 3, Fig. 4). Sequence alignment was used to identify the region of similarity. Sigma factors detected in the vegetative growth phase were exhibited with high similarity. Their σ^A showed 82.09% similarity (Fig. 5A), and σ^H showed 90.28% (Fig. 5B). Also, the amino acid sequence similarity of Bs σ^E with Bt σ^{28} was about 89.40% (Fig. 6A), and the similarity of Bs σ^K with Bt σ^{35} was 89.50% (Fig. 6B). All of these sigma factors showed high similarities, and this result suggested the probability of expression of Bt promoter in Bs.

2. Generation of recombinant Bt and Bs strains expressing EGFP

To develop EGFP-producing vectors, pMkE-X plasmid vectors containing *cry3A*, *vip*, *cry1C*, and *cyt* genes own promoters which are pMkE-1, pMkE-2, pMkE-3 and pMkE-4 respectively were constructed and confirmed by restriction enzyme pattern analysis (Fig. 7) and DNA sequencing. These vectors contain both *E. coli* and Bt origins of replication and erythromycin and ampicillin resistant genes for selection. Strains of Bt 4Q7/pMkE-X,

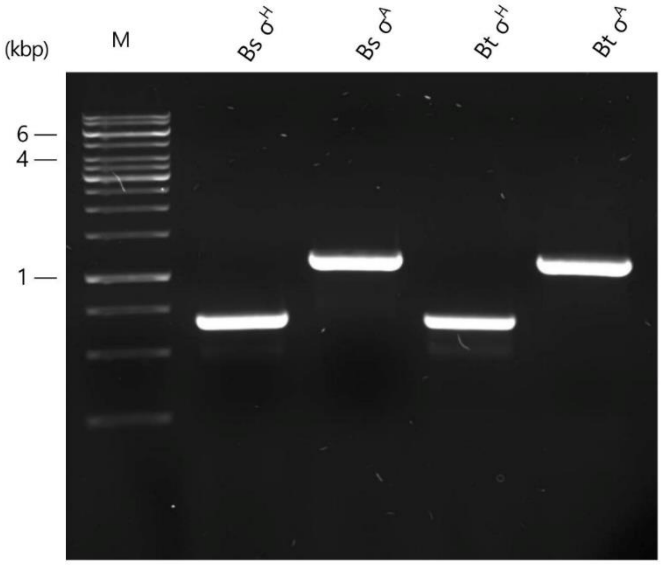

Fig. 3. PCR analysis for confirmation of sigma factors detected in growth phase.

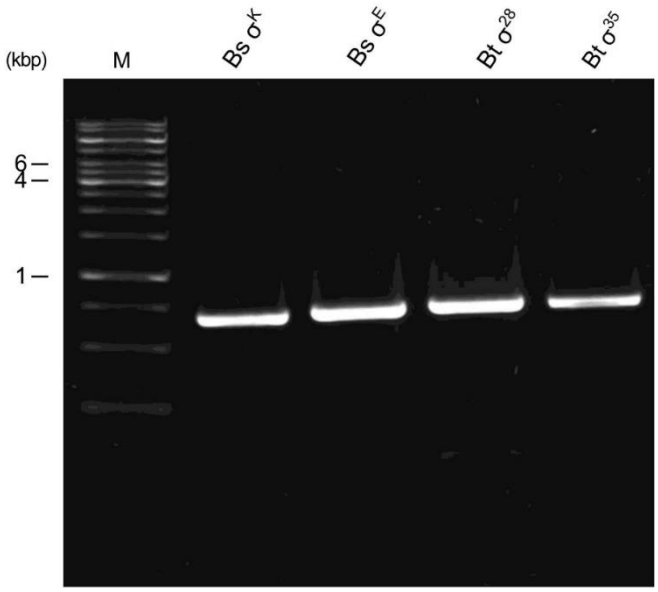

Fig. 4. PCR analysis for confirmation of sigma factors detected in sporulation phase.

Fig. 5. Amino acid sequences alignments of sigma factors detected in growth phase.

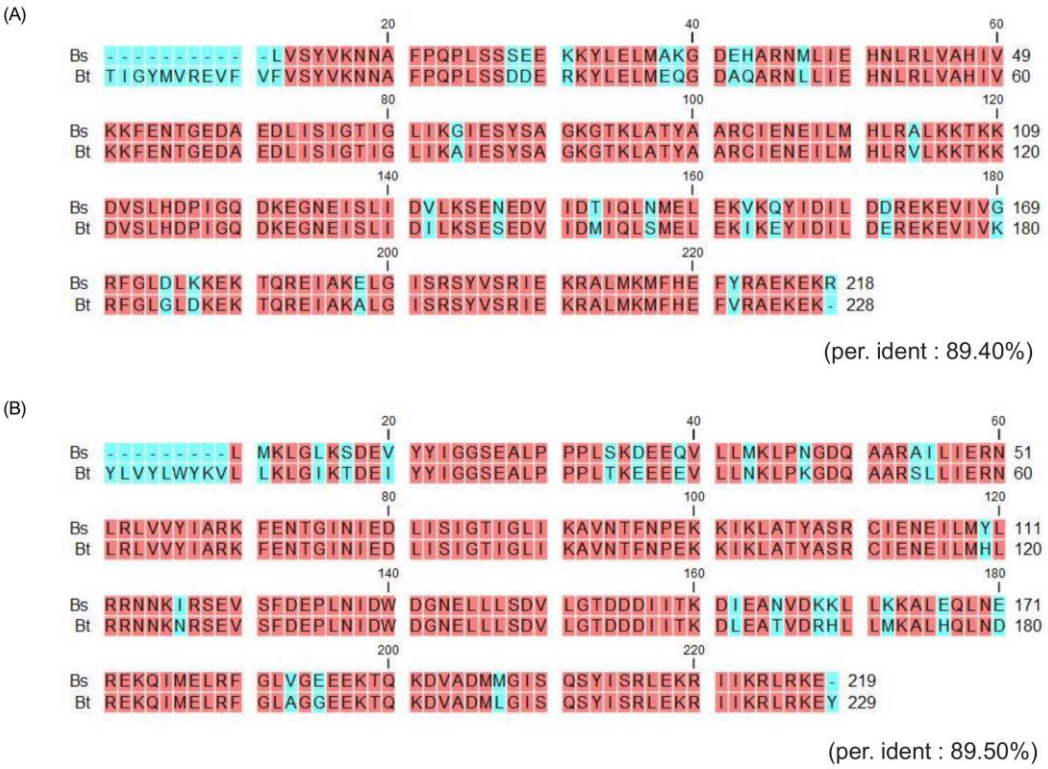

Fig. 6. Amino acid sequences alignments of sigma factors detected in sporulation phase.

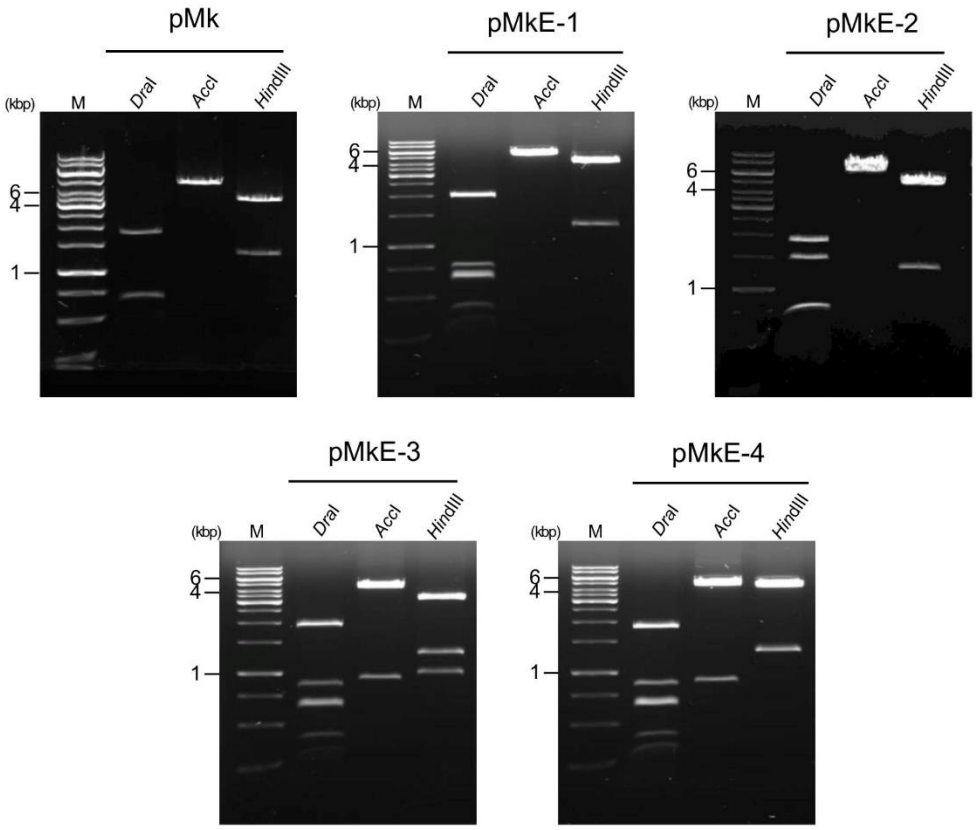

Fig. 7. Restriction endonuclease digestion patterns of the pMkE-X. Lane: M, 1 kbp DNA ladder.

EGFP producing recombinant strains, were constructed by electroporation. The introduction of Bt and Bs strains were analyzed by PCR amplification with specific primers (pHT1k-MCS-F: 5'-ATAGTCCTGTCGGGTTTCGC-3', pHT1k-MCS-R: 5'-GCCTCTTCGCTATTACGCCA-3') (Fig. 8) and electrophoresis (Fig. 9).

3. Expression of EGFP in *Bacillus* strains

For identification of EGFP expression of Bt and Bs/pMkE-X strains, the EGFP expression under the control of the Bt promoters could be observed by confocal laser scanning microscopy due to 720 bp of intact EGFP CDS does exist (Fig. 10). Analysis was conducted twice before and after sporulation. Bt/pMkE-1, 2 strains and Bs/pMkE-1, 2 strains using the sporulation dependent promoter showed higher expression than sporulation phase in the vegetative growth phase. Bt/pMkE-3, 4 and Bs/pMkE-3, 4 showed higher expression in the sporulation dependent phase. The expression of the EGFP in Bs/pMkE-3 strain was the highest expression level and *cry* promoter was selected as the optimized promoter to express Bt toxin in Bs.

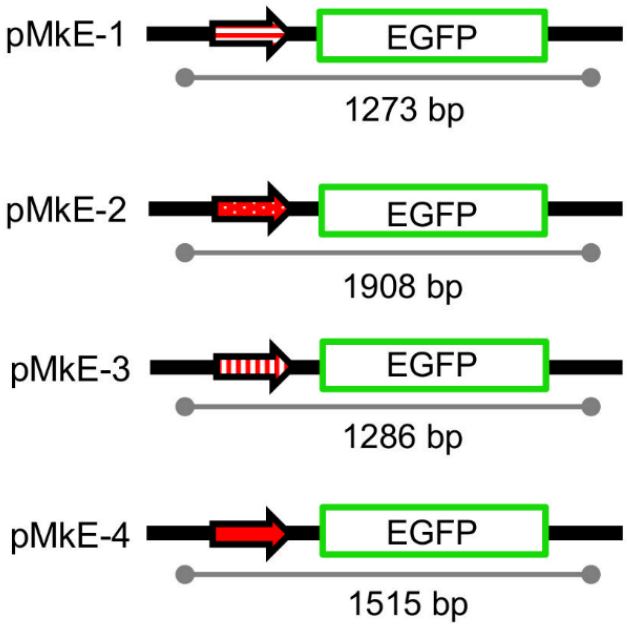

Fig. 8. Target size of pMkE-X PCR amplification using pHT1k-MCS-F and pHT1K-MCS-R.

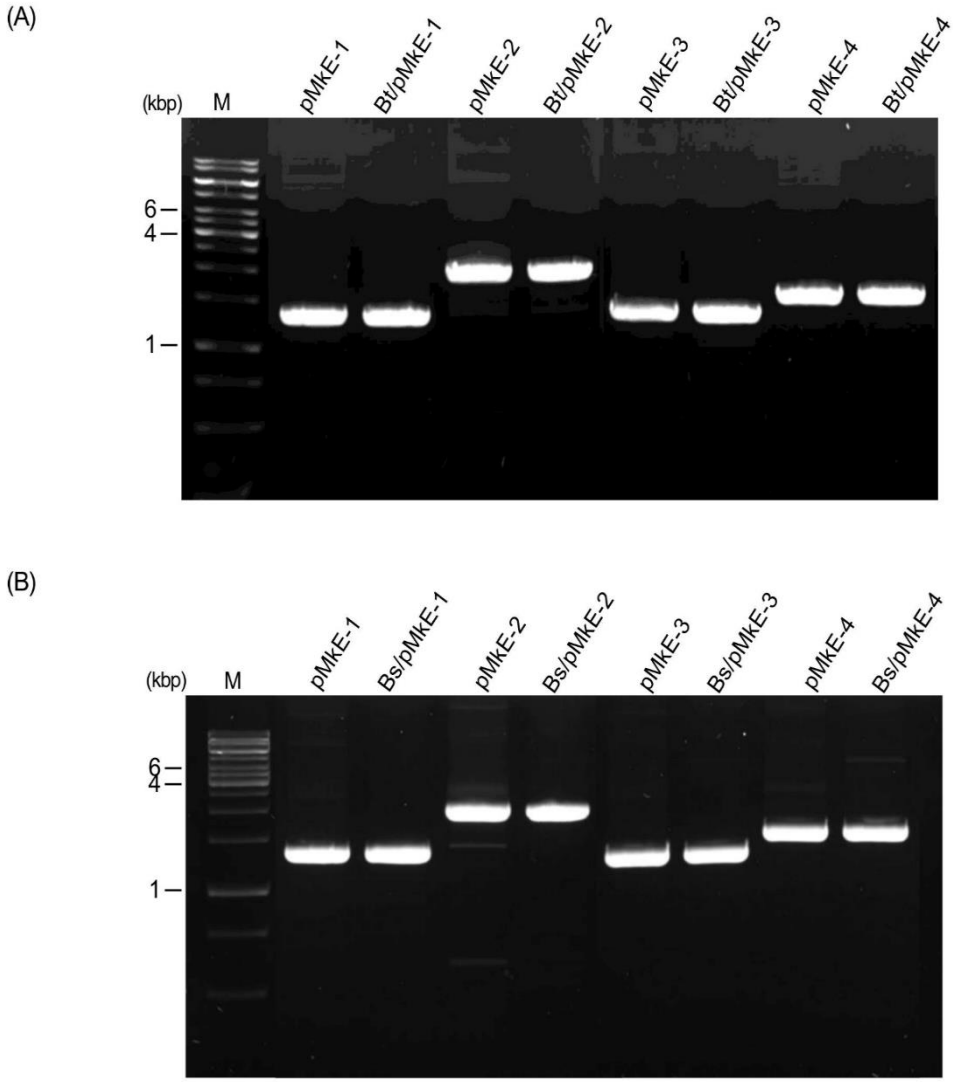

Fig. 9. Confirmation of recombinant *Bacillus* strains introducing pMkE-X. (A) The result of PCR analysis to identify Bt transformants. Lane: M, 1 kbp DNA ladder. (B) The result of PCR analysis to identify Bs transformants. Lane: M, 1 kbp DNA ladder.

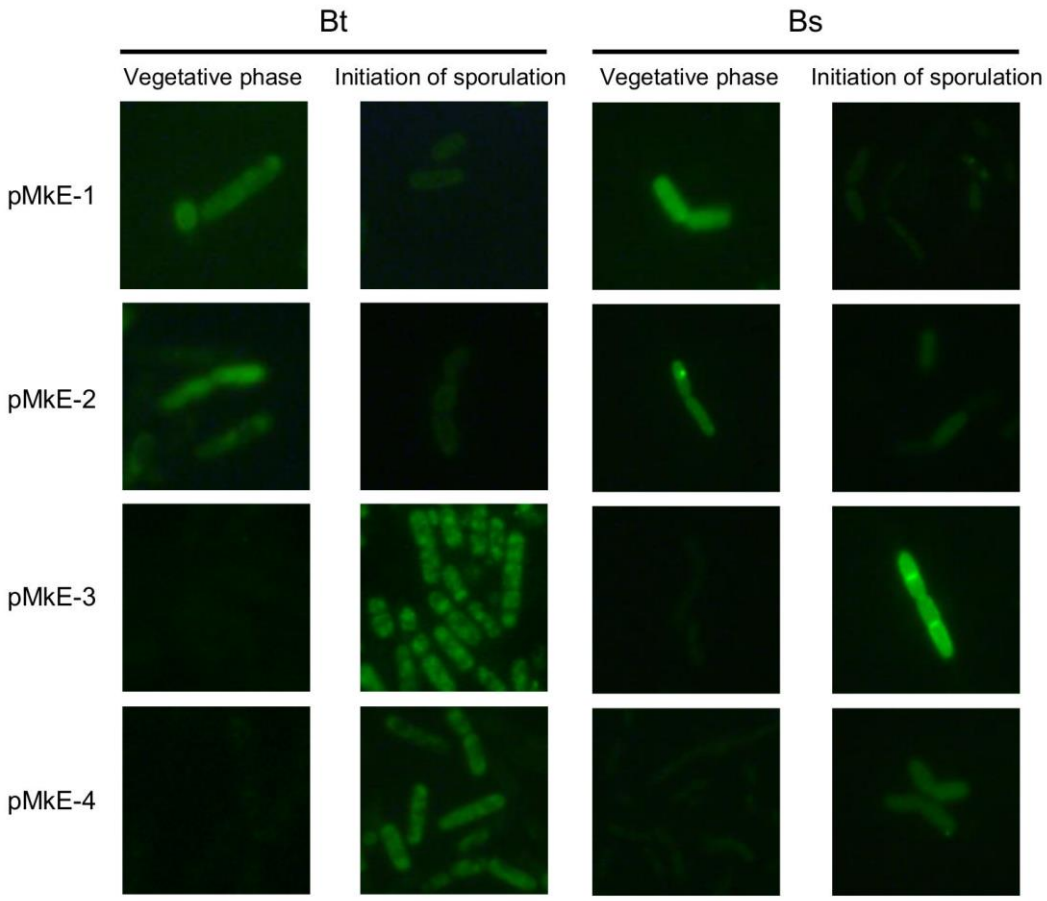

Fig. 10. EGFP expression in Bt/pMkE-X and Bs/pMkE-X strains by confocal laser scanning microscopy.

DISCUSSION

Bacillus are gram-positive soil bacterium, and a number of *Bacillus* isolates of the bacterium are commercially produced due to their biocontrol abilities. In addition, the regulation of sporulation genes in the *Bacillus* group may share common features (Adams et al., 1991). In this work, similarities of sigma factors between Bt 4Q7 and Bs were analyzed. The σ^H of Bs and Bt showed 82.09% similarity, and σ^A showed 90.28% similarity. Also, the Bs σ^K with σ^{28} of Bt showed 89.40% similarity and Bs σ^E with σ^{35} of Bt showed 89.50% similarity. All amino acid sequences showed high similarities, and this result suggested that sigma factors between Bt and Bs might be complemented. Therefore, the promoter associated with these sigma factors might be expressed even though the origins of promoter genes are different.

The pMkE-series EGFP expressing vectors under the control of four types Bt promoter were constructed and introduced in the *B. subtilis* subsp. *subtilis* (KACC 17109) strain. All recombinant Bt, and Bs strains expressed EGFP in the appropriate timing that promoters work. The expression of the EGFP in Bs/pMKE-3 strain was the highest expression level in Bs, and *cry* promoter was selected as the optimized promoter to express Bt toxin in Bs. In this experiment, the cause of the difference in expression amount within Bs was not accurately identified. Therefore, in further study, the difference in the expression amount of quantitative EGFP such as qPCR will be confirmed, and the experiment to confirm the difference in expression for Cry proteins instead of EGFP is necessary.

Chapter 2.

Construction and Characterization of the Dual-functional *Bacillus subtilis* strain

INTRODUCTION

Insects of the lepidopteran pests which are *Spodoptera frugiperda*, *S. exigua* (Lepidoptera: Noctuidae), and *Plutella xylostella* are an important polyphagous lepidopteran pest that causing significant economic damage by infesting various crop plants a wide host range of more than 80 plant species (Adamczyk Jr et al., 1998; Chandrasena et al., 2018; Furlong et al., 2013; Moulton et al., 2000; Peterson & Higley, 2000). For controlling these problems, synthetic chemical insecticides were used (Gutiérrez-Moreno et al., 2019; Young, 1979). In addition, the plant pathogenic fungi result in considerable losses to crop yields. However, the extensive use of chemical pesticides leads to an accumulation of pesticide residues in agricultural fields. It brings out severe environmental problems including insecticide-resistant pests.

For this reason, there have been many attempts to replace chemical pesticides with microbial biopesticides. Bt have been broadly used in agriculture for the control of pests. Biological controls using microorganism agents have been used in fungicides. Bs have been widely used for biopesticides because of these secondary metabolites like surfactin and fengycin.

In the previous study, to improve insecticidal activities, the recombinant Bt plasmid pMBD-ABC was constructed which is simultaneously encoding *cry1Ac*, *cry1Be* and *cry1C* genes (Park, 2022). In this study, the pMBD-ABC was introduced into a Bs strain, which has high antifungal activities against *Sclerotinia sclerotiorum*, *Botrytis cinerea* and *Colletotrichum acutatum*.

MATERIALS AND METHODS

1. Bacterial strains, plasmids, and media

The *Escherichia coli* TOP 10 strain (Thermo Fisher Scientific, Waltham, MA, USA) was used for amplification of the plasmid DNA, pMBD-ABC which is encoded *cry1Ac*, *cry1Be*, and *cry1C* (Park, 2022). The pMBD-ABC contains both *E. coli* and Bt origins of replication and erythromycin and kanamycin resistance genes (Fig. 11). Each *cry* genes were derived from other Bt strains (Table 4). Bt 4Q7/pMBD-ABC were grown at 30℃ with vigorous shaking of 220 rpm in LB medium used as a control group. The GYS medium was used to express of crystal proteins. *Bacillus subtilis* subsp. *subtilis* (KACC 17109) strain were grown at 30℃ with vigorous shaking of 220 rpm in LB medium. GYS medium was used for expression cry toxins.

2. Genetic manipulation of Bs

The genomic DNA of Bs (KACC 17109) was prepared using a TaKaRa MiniBEST Bacteria Genomic DNA Extraction Kit Ver. 3.0 (Takara Co., Tokyo, Japan) according to the manufacture's protocols. Plasmid DNA of *Bacillus* transformants were isolated using alkaline lysis method after lysozyme treatment.

3. Morphological observation and protein analysis

Bacillus were primarily cultured in 20 ml of LB medium with shaking at 30°C

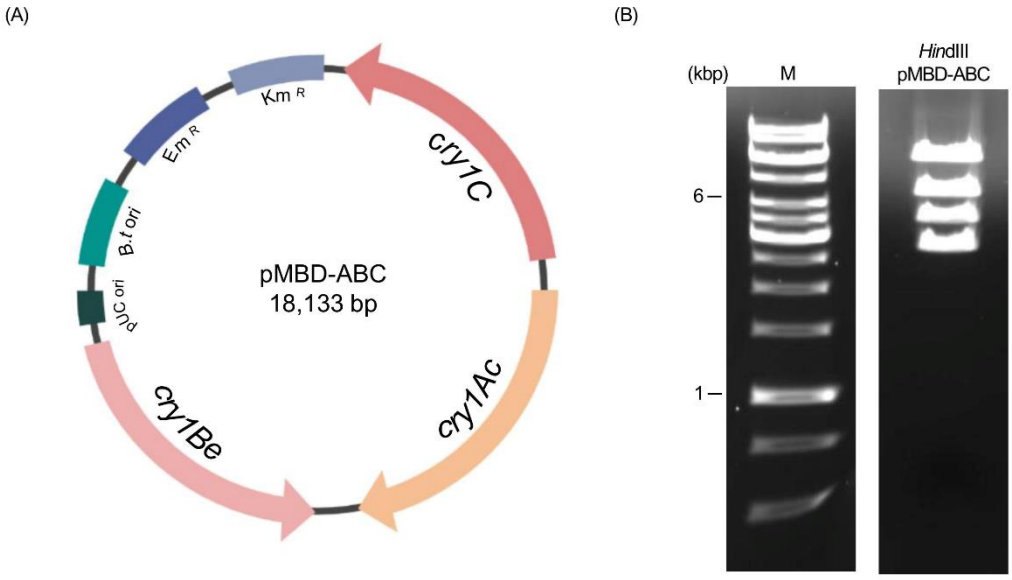

Fig. 11. Plasmid map of insecticidal protein producing vector pMBD-ABC. Km^R, kanamycin resistant gene; Em^R, erythromycin resistant gene; pUCori, *E. coli* replication origin, Btori, Bt origin (A). Restriction endonuclease digestion patterns of the cry toxins producing basis vector (B), pMBD-ABC. Lane: M, 1 kbp DNA ladder.

Table 4. Origins of *cry* genes in pMBD-ABC.

***cry* genes**	**Targeted insect pests (common names)**	**Insect order**
cry1A(*c*)	Tobacco budworm, cabbage lopper, cotton bollworm	Lepidoptera
cry1B	Cabbage worm	Lepidoptera
cry1C	Cotton leaf worm, Beet armyworm, fall armyworm	Lepidoptera

(Bt) and 37°C (Bs) overnight, and then 200 μl of the primary culture was inoculated into 20 ml of GYS at 30°C and 37℃ until autolysis. Spore-crystal mixtures were harvested by centrifugation at 10,000 rpm for 10 min, washed 2 times in washing solution (1 M NaCl with 0.01% Tween 20) and resuspended in distilled water. The morphology of parasporal inclusions were observed using scanning electron microscopy (SEM) (Carl Zeiss, SUPRA 55VP, Oberkochen, Germany), and transmission electron microscopy (TEM) (FEI, Talos L120C, Hillsboro, OR, USA).

For the SEM examination, the washed Bt samples were primary fixed using Karnovsky's fixative (Morris, 1966) then post fixed in ethanol-propylene oxide and 1% OsO_4. After dehydration using ethanol, hexamethyldisilazane (HMDS) specimen drying was performed to prepare SEM sample. After that, it is sputter-coated with gold in an IB-5 ion coater (Hitachi, Tokyo, Japan) for 5 min and then examined. For TEM samples preparation, after performing a series of procedures such as fixation, dehydration, replacement, and soaking, embedding the samples with 100% Spurr's resin was performed. After sectioning, and staining, the samples were observed as previously described (Wallis & Griffin, 1973). To analyze insecticidal protein expression, the insecticidal proteins from *Bacillus* transformants were harvested and analyzed by 12% SDS–PAGE (Laemmli, 1970).

4. Insects

The *S. exigua*, *P. xylostella* and *S. frugiperda* used in this experiment, adapted to breeding on an artificial diet. *S. exigua* were obtained from the Rural Development Administration (Jeonju, Jeollabuk-do, Republic of Korea). The artificial diet of *S. exigua*

was comprised of 10% wheat germ, 10% kidney bean powder, 3% whole milk powder, 6% yeast, 1.5% ascorbic acid, 0.2% sorbic acid, 1.5% vitamin mixture, 0.45% Methyl 4-hydroxybenzoate (MPH), 0.75% salt mixture, and 27% agar. The *S. frugiperda* were obtained from Jeon-Buk National University (Jeonju, Jeollabuk-do, Republic of Korea) and the artificial diet of *S. frugiperda* was comprised 12.5% wheat germ, 12.5% kidney bean powder, 7% yeast, 1.5% ascorbic acid, 0.2% sorbic acid, 0.5% Methyl 4-hydroxybenzoate (MPH), and 3% agar. The *P. xylostella* were adapted to breeding on rapeseed (Brassica napus). The larvae were bred in the growth chamber at 70% RH and 25℃ (Li et al., 2002).

5. Bioassay

Sporulating Bt cells were prepared following a previous study to determine the insecticidal activity of Cry toxins produced by Bt strains (Park, 2022). Thirty 3rd instar larvae of *P. xylostella* and the same number of 1st instar larvae of *S. exigua* and *S. frugiperda* were fed on 2 cm × 2 cm Chinese cabbage leaves dipped in serially diluted spore-parasporal inclusion suspensions. In each assay, larval mortality was recorded at 5 days after treatment. All tests were performed with spore-parasporal inclusion suspensions and repeated three times.

6. Plant pathogenic fungi

To examine *in vitro* antifungal activity, the following fifteen phytopathogenic fungi were prepared: *C. jacksonii* causing dollar spot, *R. solani* AG-4 causing damping off, two types of *R. solani* AG 2-2(IV) large patch and brown patch, *F. graminearum* causing head

blight, *F. asiaticum* causing head blight, *F. oxysporum* f. sp. *cucumerinum* causing fusarium wilt, *F. oxysporum* f. sp. *lycopersici* causing fusarium wilt, *G. graminis* causing root rot, *P. aphanidermatum* causing damping off, *P. ultimum* causing damping off and root rot, *B. cinerea* causing gray mold, *F. verticillioides* causing corn ear and stalk rot, *C. coccodes* causing black dot and *P. infestans* causing late blight.

7. *In vitro* dual-culture analysis

Using *in vitro* dual-culture analysis, *Bacillus* strains were subjected to in vitro antifungal activity assay against the above fifteen phytopathogenic fungi. Potato dextrose agar (PDA) medium was used as basal medium. A plug (0.5 cm in diameter) containing mycelium was taken from 7-day-old target fungi and placed at the centre of PDA dual plates. Single *Bacillus* colonies were streaked at a distance of about 3 cm from the fungus. After 3-7 days, the inhibition zone between bacterial colony and fungal pathogen was investigated (Lim et al., 2017).

8. Minimum inhibitory concentrations (MICs) test

In primary experiment, the tryptic soy broth (TSB) medium (1.7% tryptone, 0.3% soytone, 0.25% glucose, 0.5% sodium chloride, 0.25% Dipotassium phosphate, pH 7.3 ± 0.2) was used as the optimal medium for the production of antifungal substances. *Bacillus* strains were cultured in TSB medium at 37°C and 150 rpm for 3 days (Fig. 12). Culture fermentations were harvest after 3 days and tested for in vitro activity against nine plant fungi using a modified version of the antimicrobial bioassay of Nair et al. (Nair et al.,

1992). The fifteen fungal microorganisms were used to determine the minimum inhibitory concentrations (MICs).

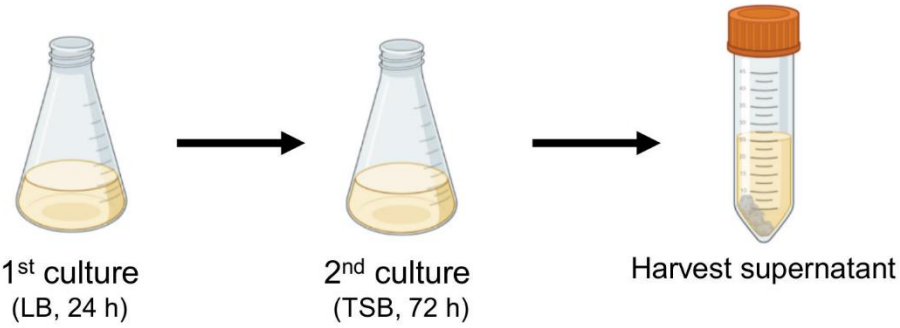

Fig. 12. Schematic diagram of harvesting *Bacillus* strains culture fermentations.

TSB medium was used as the basal medium for *Bacillus* strains, and fungi were incubated in potato dextrose broth (PDB). After 7 days culture, Fungi culture medium were filtered and cell pellets were harvested and then washed 2 times in DDW. And then pellets were resuspended in PDB containing 0.1% (v/v) by homogenizing. These fungal suspensions were diluted to 1% concentration in PDB (Fig. 13). The 1% fungal suspension was transferred to individual wells of a 96-well microplate. *Bacillus* culture fermentations were added to the wells at final concentrations of 0.15625% to 10%. Each diluted fermentation was pipetted into wells of a 96 well micro plate (Cell Wells; Corning Glass Works, Corning, NY) to determine the MICs against the fungal species. Control wells containing fungal suspension without fermentation treatment and pure PDB medium were prepared. Three replicates of each concentration for each fungus were incubated at 25℃ for the rest of the test fungi (Fig. 14). The lowest concentrations of the culture fermentation that completely inhibited mycelial growth were defined as the MICs (Park et al., 2005).

9. Statistical analysis

Statistical analysis was performed by one-way ANOVA using SPSS Statistics version 24 (SPSS Inc., IL, USA). Lethal concentration 50 was calculated through Probit analysis. Multiple comparisons of the mean values were conducted by post 56 hoc test using Scheffé's method. P values less than 0.05 were considered statistically significant.

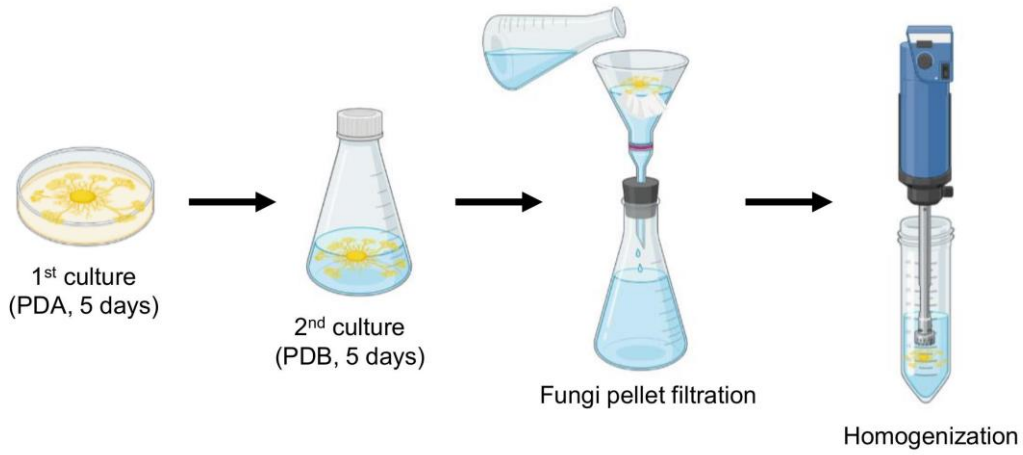

Fig. 13. Schematic diagram of preparing 1% fungal suspensions.

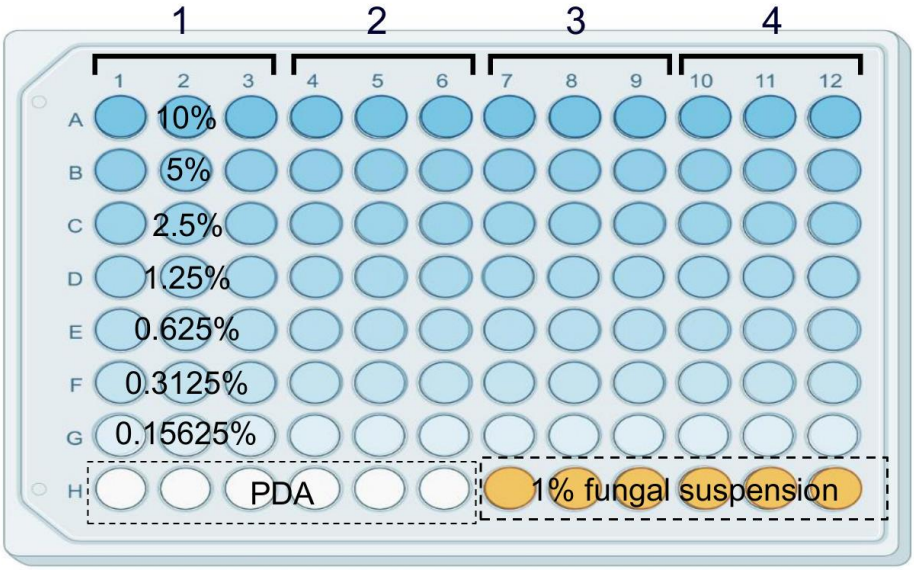

Fig. 14. Schematic diagram of MIC tests treated in 96-well plates.

RESULTS

1. Generation of Bs-Z2 crystal toxin producing strain

To confirm the recombinant Bs strain, the PCR analysis with specific primers about *cry1Ac*, *cry1Be*, and *cry1C* was performed. The size of the amplification was about 4 kbp. The result of PCR analysis showed that Bs/pMBD-ABC was constructed (Fig. 15). This strain has been deposited in the KACC database under identification number KACC 92465 P and named to Bs-Z2 strain. Also, to confirm the transcription of *cry* genes, RT-PCR was conducted for three *cry* genes encoded in the pMBD-ABC (Fig. 16).

In addition, the crystal morphology of Bs-Z2 was analyzed by scanning electron microscopy (SEM), and transmission electron microscopy (TEM) (Fig. 17). The parasporal inclusion proteins of Bs-Z2 was analyzed by SDS-PAGE (Fig. 18).

2. Insecticidal activities of Bs-Z2

To evaluate the insecticidal activities of the Bs-Z2 against 1st instar larvae of *S. exigua* and *S. frugiperda*, and 3rd instar larvae of *P. xylostella*. Compared to the original strain of Bt 4Q7/pMBD-ABC, the insecticidal activity of Bs-Z2 strain against *Spodoptera* spp. was less than Bt (Table 5, 6). However, the insecticidal activity against *P. xylostella* was similar to both strains (Table 7). All larvae were blackened to death, therefore the results were demonstrated the cry toxins were activated in insect guts (Fig. 19, 20, and 21).

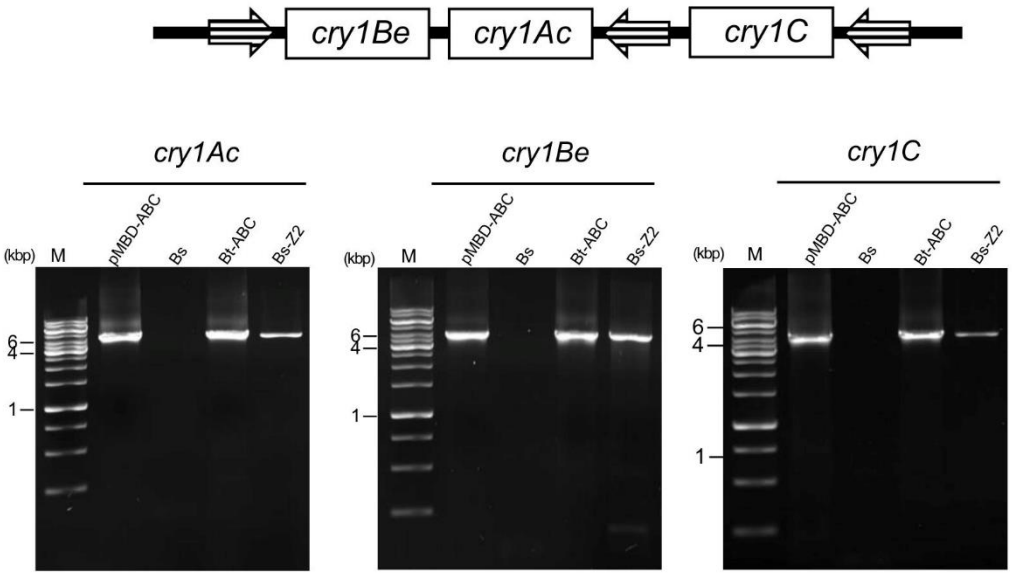

Fig. 15. Confirmation of the introducing pMBD-ABC in Bs by PCR. Lane: M, 1 kbp DNA ladder.

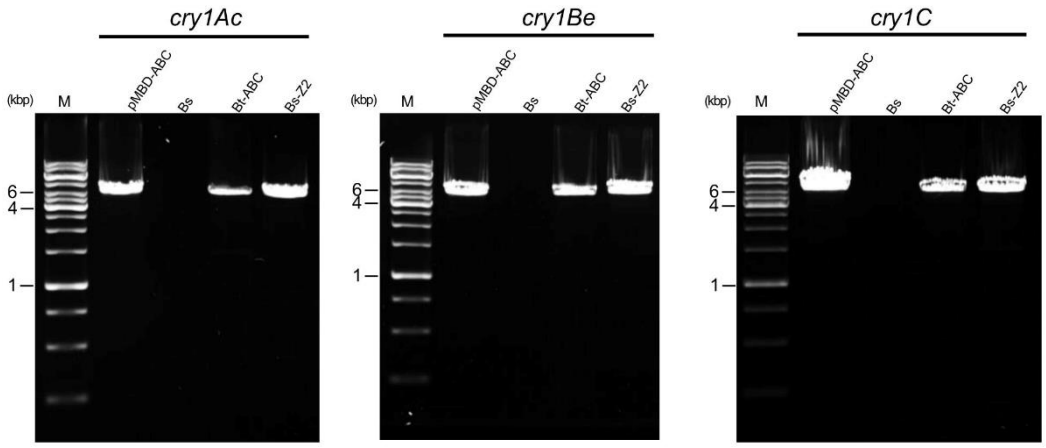

Fig. 16. Confirmation of transcription of *cry* genes by RT-PCR. Lane: M, 1 kbp DNA ladder.

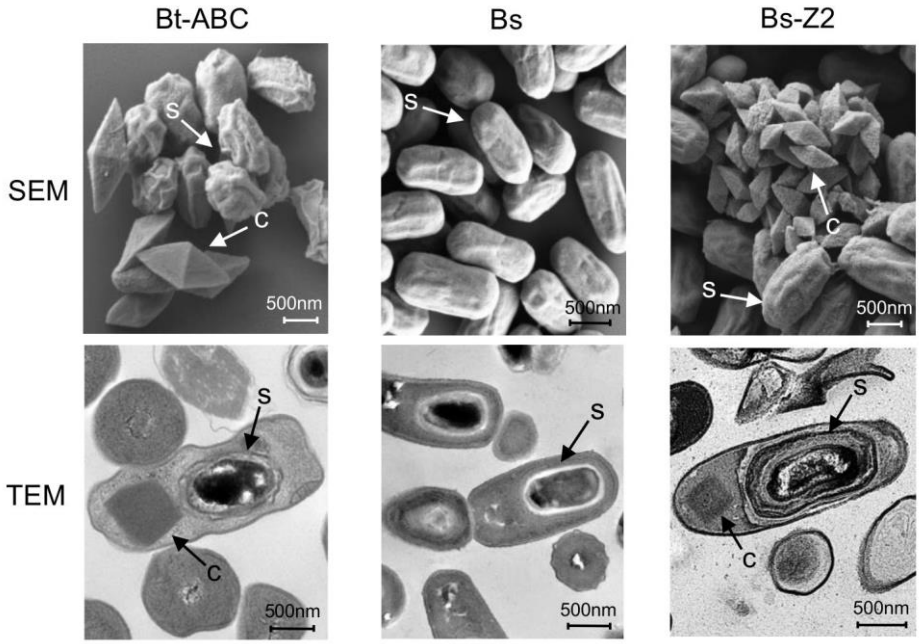

Fig. 17. Parasporal inclusions and spores of Bt-ABC and Bs-Z2. Parasporal inclusion (C) and spores (S) of *Bacillus* strains observed by scanning electron microscopy, and transmission electron microscopy.

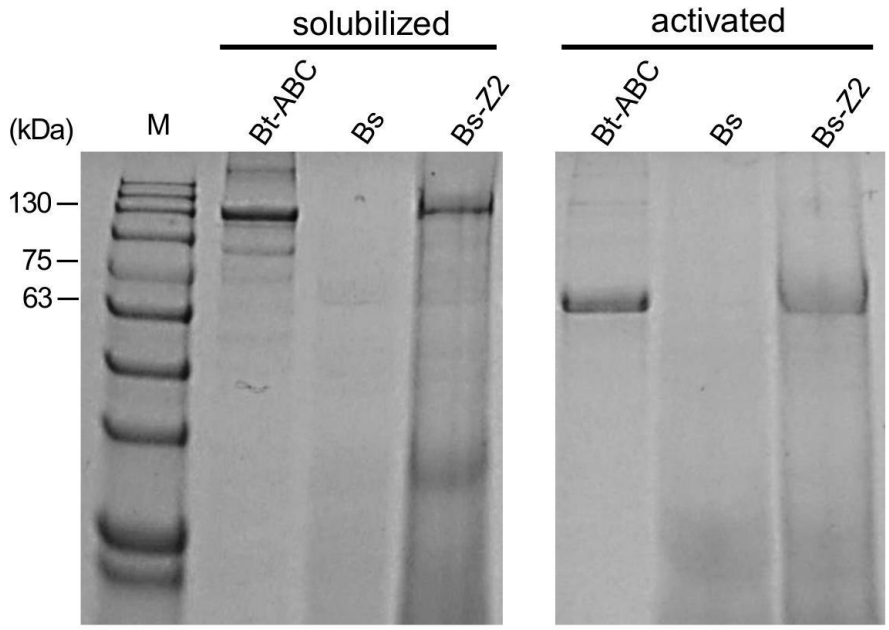

Fig. 18. SDS-PAGE analysis of insecticidal proteins of Bt-ABC and Bs-Z2 recombinant strain. Insecticidal crystal proteins produced by *Bacillus* recombinant strains were harvested and analyzed on 12% of SDS-PAGE gel. Lane: M, Protein molecular weight marker.

Table 5. Insecticidal activities of Bs-Z2 against *S. exigua*.

Strains	Observation time (days)	N	Slope (± SE)	LC_{50} (µg/mL)	95% Fiducial limits	
					Lower	Upper
Bt-ABC	4	30	0.856 ± 0.134	0.380	0.125	0.727
Bs-Z2	4	30	0.866 ± 0.124	0.770	0.408	1.316

Table 6. Insecticidal activities of Bs-Z2 against *S. frugiperda.*

Strains	Observation time (days)	N	Slope (± SE)	LC_{50} (µg/mL)	95% Fiducial limits	
					Lower	Upper
Bt-ABC	4	90	0.964 ± 0.167	0.451	0.028	1.188
Bs-Z2	4	90	1.197 ± 0.158	1.604	0.707	2.931

Table 7. Insecticidal activities of Bs-Z2 against *P. xylostella.*

Strains	Observation time (days)	N	Slope (± SE)	LC_{50} (µg/mL)	95% Fiducial limits	
					Lower	Upper
Bt-ABC	4	90	2.385 ± 0.382	0.121	0.070	0.175
Bs-Z2	4	90	1.959 ± 0.339	0.113	0.050	0.190

Fig. 19. Morphological abnormalities of dead *S. exigua* larvae after treatment.

Fig. 20. Morphological abnormalities of dead *S. frugiperda* larvae after treatment.

Fig. 21. Morphological abnormalities of dead *P. xylostella* larvae after treatment.

3. *In vitro* antifungal activities

In vitro dual-culture analysis is widely used to evaluate antifungal activities of microorganisms. Considerable inhibition of fungal growth was observed against *C. jacksonii*, *F. asiaticum*, and *R. solani* AG-4 Large patch (Fig. 22). Also, the MIC test was conducted, and Bt 4Q7, Bt/pMBD-ABC, and Bs KACC 17109 strains were used as control. Because of MIC test to confirm antifungal activity on plant pathogenic fungi of Bs-Z2 strain, Bs-Z2 strain showed the strongest antifungal activity with 0.3125% MIC value for *C. jacksonii*, and also showed strong activities with 1.25% MIC value for *F. asiaticum* and 2.5% MIC value for *R. solani* AG-4 Large patch (Table 8). Therefore, the results showed that Bs-Z2 strain has antifungal activity against the three plant pathogenic fungi described above.

In addition, Bs-Z2 strain showed higher antibacterial activity than that of Bt strain used as negative control, and the same MIC value compared to wild type Bs strain (KACC 17109) before plasmid introduction. This result means that the Bs-Z2 strain was maintained at the same level as that of wild-type Bs (KACC 17109).

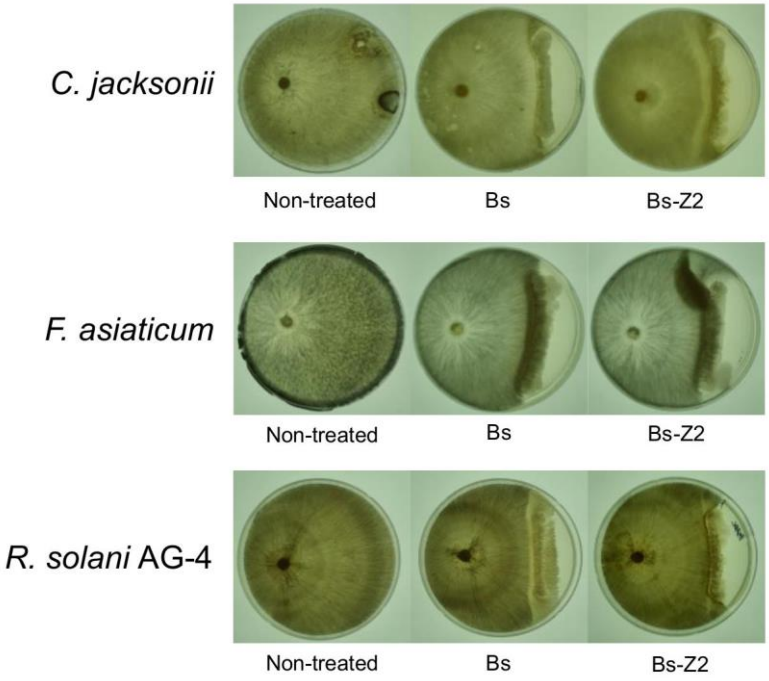

Fig. 22. *In vitro* dual-culture analysis against Bs recombinant strain.

Table 8. Minimum inhibitory concentration (MIC) values of the culture filtrate of Bs-Z2 against three phytopathogenic fungi.

No.	Phytopathogenic fungi	MIC value (%)	
1	*Clarireedia jacksonii*	Bt	2.5
		Bt-ABC	2.5
		Bs	0.31
		Bs-Z2	0.31
2	*Fusarium asiaticum*	Bt	-
		Bt-ABC	-
		Bs	1.25
		Bs-Z2	1.25
3	*Rhizoctonia solani* AG-4	Bt	10
		Bt-ABC	10
		Bs	2.5
		Bs-Z2	2.5

DISCUSSION

Bs strains have been studied because of its rapid growth, low nutritional requirements, safety to humans and animals, and their antibacterial activities. There have been many reports about their secondary metabolites used in agriculture. A Bs strain has been shown to have inhibitory against the *Fusarium* head blight disease by *Fusarium graminearum* (Wang et al., 2007). Moreover, an antifungal chitinase gene cloned from Bs has been shown to exhibit inhibitory activity against *R. solani*, a phytopathogen that causes dampling-off in radish seedlings (Yang et al., 2009). The lipopeptides from Bs, like surfactin and fengycin were able to inhibit the growth of phytopathogenic fungi. Another strain in *Bacillus* genus, Bt strains produce Cry proteins showed insecticidal activities against Lepidopteran (Cry1 of 130-140 kDa) larvae. For this reason, the biopesticides based on the *Bacillus* are one of the most effective biological products in agriculture. Nonetheless, there are few reports of *Bacillus* strains with both antibacterial and insecticidal activities. Therefore, this study aimed to generate improved *Bacillus* strain which have a dual-functional activity against phytopathogens and insect pests co-harmful to one crop.

To construct improved *Bacillus* strain, pMBD-ABC cloned *cry1Ac, cry1Be,* and *cry1C* genes was introduced into Bs and then named Bs-Z2. Cry proteins produced by Bs-Z2 showed high level of insecticidal activities against *P. xylostella* and *Spodoptera* spp. respectively. However, the insecticidal activity for *Spodoptera* spp. was slightly less compared to Bt/pMBD-ABC (Table 5, 6). There are several examples of the expression in Bt c*ry* genes in *Bacillus* species, including *B. megaterium* (Bora et al., 1994), *B. cereus*

(Moar et al., 1994), *B. licheniformis*, and *B. subtilis* (Theoduloz et al., 2003). Similar to the results seen in this study, the expression of Cry proteins in these *Bacillus* species was frequently less than the expression level in Bt. Importantly, the recombinant Bs-Z2 strain retained its antifungal activity after transformation. This result suggests that the gene(s) controlling the antifungal activity may be located on the Bs chromosome. Of note, an antifungal factor, a Bs chitinase, has been cloned from chromosomal DNA (Yang et al., 2009). Although Bt toxins were produced within the Bs due to similar sigma factors, the size and activity of toxin made by Bs appeared to be slightly lower than that produced by Bt (Fig. 17).

In conclusion, these results demonstrate that a recombinant Bs-Z2 strain could be used to control harmful insect pests and plant fungal diseases simultaneously in one crop. Field trials using this recombinant strain as a dual control agent are now being performed by this laboratory. Moreover, since this study confirmed that Bs can express Bt toxin under the control of Bt promoter, in further study, it might help generate improved strains with higher insecticidal activity by introducing pMBD-ABC to other Bs strains, which have insecticidal activity originally (Mnif et al., 2013).

LITERATURE CITED

Adamczyk Jr, J., Holloway, J., Church, G., Leonard, B., & Graves, J. (1998). Larval survival and development of the fall armyworm (Lepidoptera: Noctuidae) on normal and transgenic cotton expressing the *Bacillus thuringiensis* CryIA (c) δ-endotoxin. Journal of Economic Entomology, ***91*(2)**, 539-545.

Adams, L. F., Brown, K. L., & Whiteley, H. R. (1991). Molecular cloning and characterization of two genes encoding sigma factors that direct transcription from a *Bacillus thuringiensis* crystal protein gene promoter. J Bacteriol, **173(12)**, 3846-3854.

Afsharmanesh, H., Perez-Garcia, A., Zeriouh, H., Ahmadzadeh, M., & Romero, D. (2018). Aflatoxin degradation by *Bacillus subtilis* UTB1 is based on production of an oxidoreductase involved in bacilysin biosynthesis. Food Control, **94**, 48-55.

Agaisse, H., & Lereclus, D. (1995). How does *Bacillus thuringiensis* produce so much insecticidal crystal protein? Journal of Bacteriology, **177(21)**, 6027-6032.

Arima, K., Kakinuma, A., & Tamura, G. (1968). Surfactin, a crystalline peptidelipid surfactant produced by *Bacillus subtilis*: Isolation, characterization and its inhibition of fibrin clot formation. Biochemical and biophysical research communications, **31(3)**, 488-494.

Asaka, O., & Shoda, M. (1996). Biocontrol of *Rhizoctonia solani* Damping-Off of Tomato with *Bacillus subtilis* RB14. Appl Environ Microbiol, **62(11)**, 4081-4085.

Baum, J. A., & Malvar, T. (1995). Regulation of insecticidal crystal protein production in *Bacillus thuringiensis*. Molecular microbiology, **18(1)**, 1-12.

Bennallack, P. R., Burt, S. R., Heder, M. J., Robison, R. A., & Griffitts, J. S. (2014). Characterization of a novel plasmid-borne thiopeptide gene cluster in *Staphylococcus epidermidis* strain 115. J Bacteriol, **196(24)**, 4344-4350.

BESSON, F., & Michel, G. (1987). Isolation and characterization of new iturins: iturin D and iturin E. The Journal of antibiotics, **40(4)**, 437-442.

Bora, R. S., Murty, M., Shenbagarathai, R., & Sekar, V. (1994). Introduction of a lepidopteran-specific insecticidal crystal protein gene of *Bacillus thuringiensis* subsp. *kurstaki* by conjugal transfer into a *Bacillus megaterium* strain that persists in the cotton phyllosphere. Applied and Environmental Microbiology, **60(1)**, 214-222.

Bravo, A., Agaisse, H., Salamitou, S., & Lereclus, D. (1996). Analysis of *cryIAa* expression in sigE and sigK mutants of *Bacillus thuringiensis*. Molecular and General Genetics MGG, **250(6)**, 734-741.

Cawoy, H., Bettiol, W., Fickers, P., & Ongena, M. (2011). *Bacillus*-based biological control of plant diseases. Pesticides in the modern world-pesticides use and management, 273-302.

Chandrasena, D. I., Signorini, A. M., Abratti, G., Storer, N. P., Olaciregui, M. L., Alves, A. P., & Pilcher, C. D. (2018). Characterization of field-evolved resistance to *Bacillus thuringiensis*-derived Cry1F δ-endotoxin in *Spodoptera frugiperda* populations from Argentina. Pest management science, **74(3)**, 746-754.

Corvey, C., Stein, T., Düsterhus, S., Karas, M., & Entian, K.-D. (2003). Activation of subtilin precursors by *Bacillus subtilis* extracellular serine proteases subtilisin (AprE), WprA, and Vpr. Biochemical and biophysical research communications, **304(1)**, 48-54.

Crickmore, N., Zeigler, D. R., Feitelson, J., Schnepf, E., Van Rie, J., Lereclus, D., Baum, J., & Dean, D. H. (1998). Revision of the Nomenclature for the *Bacillus thuringiensis* Pesticidal Crystal Proteins. Microbiology and Molecular Biology Reviews, **62(3)**, 807-813.

Donovan, W. P., Engleman, J. T., Donovan, J. C., Baum, J. A., Bunkers, G. J., Chi, D. J., Clinton, W. P., English, L., Heck, G. R., Ilagan, O. M., Krasomil-Osterfeld, K. C., Pitkin, J. W., Roberts, J. K., & Walters, M. R. (2006). Discovery and characterization of Sip1A: A novel secreted protein from *Bacillus thuringiensis* with activity against coleopteran larvae. Appl Microbiol Biotechnol, **72(4)**, 713-719.

Dunlap, C. A., Bowman, M. J., & Rooney, A. P. (2019). Iturinic Lipopeptide Diversity in the *Bacillus subtilis* Species Group – Important Antifungals for Plant Disease Biocontrol Applications [Original Research]. Frontiers in Microbiology, **10**.

Earl, A. M., Losick, R., & Kolter, R. (2008). Ecology and genomics of *Bacillus subtilis*. Trends in microbiology, **16(6)**, 269-275.

Estruch, J. J., Warren, G. W., Mullins, M. A., Nye, G. J., Craig, J. A., & Koziel, M. G. (1996). Vip3A, a novel *Bacillus thuringiensis* vegetative insecticidal protein with a wide spectrum of activities against lepidopteran insects. Proc Natl Acad Sci U S A,

93(11), 5389-5394.

Federici, B. A., Park, H.-W., & Bideshi, D. K. (2010). Overview of the basic biology of *Bacillus thuringiensis* with emphasis on genetic engineering of bacterial larvicides for mosquito control. Open Toxinology Journal, **3(2)**, 83-100.

Furlong, M. J., Wright, D. J., & Dosdall, L. M. (2013). Diamondback moth ecology and management: problems, progress, and prospects. Annual review of entomology, **58**, 517-541.

Gutiérrez-Moreno, R., Mota-Sanchez, D., Blanco, C. A., Whalon, M. E., Terán-Santofimio, H., Rodriguez-Maciel, J. C., & DiFonzo, C. (2019). Field-evolved resistance of the fall armyworm (Lepidoptera: Noctuidae) to synthetic insecticides in Puerto Rico and Mexico. Journal of Economic Entomology, **112(2)**, 792-802.

Höfte, H., & Whiteley, H. (1989). Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiological reviews, **53(2)**, 242-255.

Jiang, C., Shi, J., Liu, Y., & Zhu, C. (2014). Inhibition of *Aspergillus carbonarius* and fungal contamination in table grapes using *Bacillus subtilis*. Food Control, **35(1)**, 41-48.

Jurat-Fuentes, J. L., & Crickmore, N. (2017). Specificity determinants for Cry insecticidal proteins: Insights from their mode of action. Journal of invertebrate pathology, **142**, 5-10.

Kaleh, A. M., Singh, P., Mazumdar, P., Chua, K. O., & Harikrishna, J. A. (2022).

Halotolerant rhizobacteria isolated from a mangrove forest alleviate saline stress in Musa acuminata cv. Berangan. Microbiological Research, **265**, 127176.

Kim, P.-I., Ryu, J.-W., Kim, Y.-H., & Chi, Y.-T. (2010). Production of biosurfactant lipopeptides iturin A, fengycin, and surfactin A from *Bacillus subtilis* CMB32 for control of Colletotrichum gloeosporioides. Journal of microbiology and biotechnology, **20(1)**, 138-145.

Laemmli, U. K. (1970). Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature, **227(5259)**, 680-685.

Lambert, B., & Peferoen, M. (1992). Insecticidal promise of *Bacillus thuringiensis*. BioScience, **42(2)**, 112-122.

Lee, N.-K., Kim, W.-S., & Paik, H.-D. (2019). *Bacillus* strains as human probiotics: characterization, safety, microbiome, and probiotic carrier. Food science and biotechnology, **28**, 1297-1305.

Li, G., Pang, Y., Chen, Q., Su, Z., & Wen, X. (2002). Studies on the Artificial Diet for Beet Armyworm, *Spodoptera exigua*. Chinese Journal of Biological Control, **18(3)**, 132-134.

Lim, S. M., Yoon, M.-Y., Choi, G. J., Choi, Y. H., Jang, K. S., Shin, T. S., Park, H. W., Yu, N. H., Kim, Y. H., & Kim, J.-C. (2017). Diffusible and volatile antifungal compounds produced by an antagonistic *Bacillus velezensis* G341 against various phytopathogenic fungi. The plant pathology journal, **33(5)**, 488.

Maget-Dana, R., & Peypoux, F. (1994). Iturins, a special class of pore-forming lipopeptides: biological and physicochemical properties. Toxicology, **87(1-3)**, 151-174.

Meena, K. R., & Kanwar, S. S. (2015). Lipopeptides as the antifungal and antibacterial agents: applications in food safety and therapeutics. BioMed research international, 2015.

Mnif, I., Elleuch, M., Chaabouni, S. E., & Ghribi, D. (2013). *Bacillus subtilis* SPB1 biosurfactant: Production optimization and insecticidal activity against the carob moth Ectomyelois ceratoniae. Crop Protection, **50**, 66-72.

Moar, W. J., Trumble, J., Hice, R. H., & Backman, P. A. (1994). Insecticidal activity of the CryIIA protein from the NRD-12 isolate of *Bacillus thuringiensis* subsp. *kurstaki* expressed in *Escherichia coli* and *Bacillus thuringiensis* and in a leaf-colonizing strain of *Bacillus cereus*. Applied and Environmental Microbiology, **60(3)**, 896-902.

Morris, L. J. (1966). Separations of lipids by silver ion chromatography. Journal of Lipid Research, **7(6)**, 717-732.

Moulton, J. K., Pepper, D. A., & Dennehy, T. J. (2000). Beet armyworm (*Spodoptera exigua*) resistance to spinosad. Pest Management Science: formerly Pesticide Science, **56(10)**, 842-848.

Nair, M. G., Mishra, S. K., Putnam, A. R., & Pandey, R. C. (1992). Antifungal anthracycline antibiotics, spartanamicins A and B from *Micromonospora* spp. J Antibiot (Tokyo), **45(11)**, 1738-1745.

Palanisamy, P. (2008). Biosurfactant mediated synthesis of NiO nanorods. Materials Letters, **62(4-5)**, 743-746.

Pardo-López, L., Soberón, M., & Bravo, A. (2013). *Bacillus thuringiensis* insecticidal three-domain Cry toxins: mode of action, insect resistance and consequences for crop protection. FEMS Microbiol Rev, **37(1)**, 3-22.

Park, J.-H., Choi, G. J., Jang, K. S., Lim, H. K., Kim, H. T., Cho, K. Y., & Kim, J.-C. (2005). Antifungal activity against plant pathogenic fungi of chaetoviridins isolated from *Chaetomium globosum*. FEMS Microbiology Letters, **252(2)**, 309-313.

Park, M. G. (2022). Synthetic biological approaches to develop improved Bacillus thuringiensis insecticides. 개량된 Bacillus thuringiensis 살충제를 개발하기 위한 합성생물학적 접근법.

Peterson, R. K., & Higley, L. G. (2000). Biotic stress and yield loss. CRC Press.

Priest, F. G. (1993). Systematics and ecology of *Bacillus*. *Bacillus subtilis* and other gram-positive bacteria: Biochemistry, physiology, and molecular genetics, 1-16.

Schneider, J., Taraz, K., Budzikiewicz, H., Deleu, M., Thonart, P., & Jacques, P. (1999). The structure of two fengycins from *Bacillus subtilis* S499. Zeitschrift für Naturforschung C, **54(11)**, 859-866.

Strange, R. N., & Scott, P. R. (2005). Plant disease: a threat to global food security. Annual review of phytopathology, **43(1)**, 83-116.

Su, Y., Liu, C., Fang, H., & Zhang, D. (2020). *Bacillus subtilis*: a universal cell factory

for industry, agriculture, biomaterials and medicine. Microbial Cell Factories, **19(1)**, 173.

Tang, Q., Bie, X., Lu, Z., Lv, F., Tao, Y., & Qu, X. (2014). Effects of fengycin from *Bacillus subtilis* fmbJ on apoptosis and necrosis in *Rhizopus stolonifer*. Journal of Microbiology, **52(8)**, 675-680.

Thakore, Y. (2006). The biopesticide market for global agricultural use. Industrial Biotechnology, **2(3)**, 194-208.

Theoduloz, C., Vega, A., Salazar, M., Gonzalez, E., & Meza-Basso, L. (2003). Expression of a *Bacillus thuringiensis* δ-endotoxin *cry1Ab* gene in *Bacillus subtilis* and *Bacillus licheniformis* strains that naturally colonize the phylloplane of tomato plants (*Lycopersicon esculentum*, Mills). Journal of Applied Microbiology, **94(3)**, 375-381.

Vanittanakom, N., Loeffler, W., Koch, U., & Jung, G. (1986). Fengycin--a novel antifungal lipopeptide antibiotic produced by *Bacillus subtilis* F-29-3. J Antibiot (Tokyo), **39(7)**, 888-901.

Wallis, M. A., & Griffin, R. L. (1973). A routine method for embedding animal tissues in Spurr resin for electron microscopy. J Clin Pathol, **26(1)**, 77-78.

Wang, J., Liu, J., Chen, H., & Yao, J. (2007). Characterization of *Fusarium graminearum* inhibitory lipopeptide from *Bacillus subtilis* IB. Applied Microbiology and Biotechnology, **76(4)**, 889-894.

Westers, L., Westers, H., & Quax, W. J. (2004). *Bacillus subtilis* as cell factory for pharmaceutical proteins: a biotechnological approach to optimize the host organism. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, **1694(1-3)**, 299-310.

Wong, H. C., Schnepf, H. E., & Whiteley, H. R. (1983). Transcriptional and translational start sites for the *Bacillus thuringiensis* crystal protein gene. Journal of Biological Chemistry, **258(3)**, 1960-1967.

Yang, C.-Y., Ho, Y.-C., Pang, J.-C., Huang, S.-S., & Tschen, J. S.-M. (2009). Cloning and expression of an antifungal chitinase gene of a novel *Bacillus subtilis* isolate from Taiwan potato field. Bioresource Technology, **100(3)**, 1454-1458.

Young, J. R. (1979). Fall armyworm: control with insecticides. Florida Entomologist, 130-133.

Zweers, J. C., Barák, I., Becher, D., Driessen, A. J., Hecker, M., Kontinen, V. P., Saller, M. J., Vavrová, L. u., & van Dijl, J. M. (2008). Towards the development of *Bacillus subtilis* as a cell factory for membrane proteins and protein complexes. Microbial Cell Factories, **7**, 1-20.

살균 활성과 살충 활성을 동시에 가지는 재조합 *Bacillus subtilis* 균주의 제작 및 특성 구명

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초록

식물병과 해충은 세계 농업 식량 손실의 주요 원인으로 손꼽히며, 전체 생산량 손실의 약 20%를 차지하고 있다. 이러한 농업 피해를 최소화하기 위해, 현대 농업에서는 살충제, 살균제, 제초제 등의 다양한 화학 농약들을 사용하고 있다. 그러나 화학 농약은 환경 독성, 잔류 독성, 해충 저항성 등의 문제를 야기시켰으며, 화학 농약의 오남용을 줄이고자 새로운 친환경 농약들이 개발되었다. 친환경 농작물에 대한 수요와 화학농약에 의한 환경 오염 문제가 대두되면서 이러한 친환경 농약의 수요는 날로 증가하고 있다. 그 중 생물 농약은 자연계에 존재하는 생물체 및 그로부터 유래한 소재를 이용하여 농작물 생산 및 보존에 피해를 미치는 병원균, 해충 및 잡초 등을 방제하는 작물보호제로 현대에는 식물이나 미생물 등을 이용한 새로운 친환경 생물 농약 연구가

활발히 진행되고 있다.

Bacillus 속에 속하는 균주들은 대표적인 미생물 농약으로 이용되고 있다. *Bacillus* 속은 호기성 그람 양성의 간균으로 토양이나 연안, 심지어 해양에서 발견될 만큼 다양한 환경에 널리 분포한다. *Bacillus* 균주들은 내생포자(endospore)를 형성하며 다양한 활성을 지닌 2차 대사산물을 분비하고, 종에 따라서는 특정 곤충 목에 대한 살충활성을 지니기도 한다.

Bacillus 속의 대표 종인 *Bacillus subtilis* (Bs)는 다양한 이차 대사산물과 효소를 생산해내는 것으로 알려져 있으며, 이로 인해 산업적으로 다방면에서 활용되고 있다. 농업에서는 이러한 이차 대사산물의 항균활성을 이용한 살균제가 주로 개발되고 있다. 또 다른 종인 *Bacillus thuringiensis* (Bt)는 곤충 병원성 세균으로 곤충 병원성 단백질인 δ-endotoxin을 생산하여 이를 이용한 살충제 연구가 활발히 이루어지고 있다.

이처럼 미생물을 이용한 다양한 친환경 제제들을 생산해내기 위해서는, 개량된 미생물 균주의 확보가 매우 중요하다. 따라서 본 연구에서는 개량된 *Bacillus* 균주를 확보하기 위하여, 살균활성과 살충활성을 동시에 가지는 균주를 개발하고자 하였다. 식물 병원성 곰팡이에 대한 항균활성을 지닌다고 알려진 Bs (KACC 17109) 균주에 Bt의 Cry toxin 유전자(*cry1Ab, cry1Be,* 그리고 *cry1C*)를 도입하여 새로운 균주를 제작하고자 하였으며 해당 균주를 Bs-Z2 (KACC 92465P)로 명명하였다. 본 연구에서는 또한 개량 균주인 Bs-Z2 균주의 살균활성과 살충활성을 평가하였다. 그 결과, *C. jacksonii, F. asiaticum,* 그리고 *R. solani*

AG-4 Large patch에 대한 살균활성을 보였으며, 나비목 해충인 *Spodoptera frugiperda*, *S. exigua*, 그리고 *Plutella xylostella*에 대한 강한 살충 활성 효과를 보였다.

이상의 결과를 종합하면, Bs-Z2 균주가 식물 병원성 곰팡이와 나비목 해충에 대해 유의미한 방제능을 가짐을 확인할 수 있었으며, 이는 향후 식물병과 해충을 동시에 방제할 수 있는 복합 기능성 미생물 제제를 제작하는데 있어 유용할 수 있을 것으로 기대된다.

검색어: 미생물농약, *Bacillus*, 살충활성, 살균활성, δ-endotoxin

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