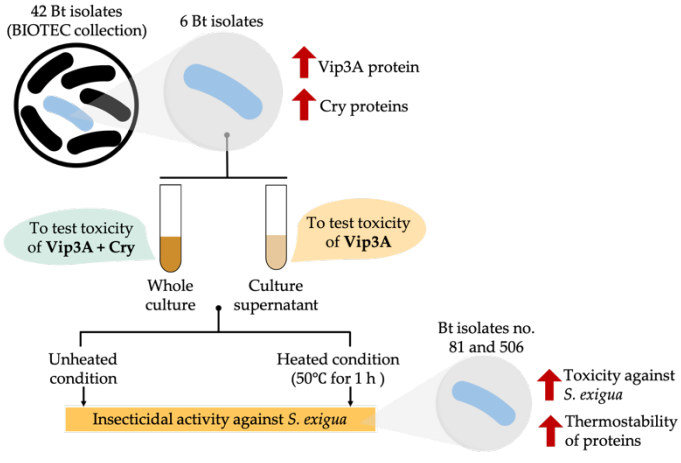
Screening of *Bacillus thuringiensis* isolates for high levels of Vip3A and Cry proteins and high thermostability to control *Spodoptera exigua*

Tharathip Hemthanon[1], Boonhiang Promdonkoy [2] and Panadda Boonserm [1,*]

[1] Institute of Molecular Biosciences, Mahidol University, Salaya, Phutthamonthon, Nakhon Pathom 73170, Thailand; tharathip.hem@student.mahidol.ac.th
[2] National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, 113 Thailand Science Park, Phahonyothin Road, Khlong Neung, Khlomg Luang, Pathum Thani 12120, Thailand; boonhiang@biotec.or.th
* Correspondence: panadda.boo@mahidol.ac.th

Abstract: *Spodoptera exigua* or beet armyworm is one of major agricultural pests that causes serious crops production losses in Thailand. Moreover, *S. exigua* has been reported to develop resistance against chemical pesticides. Nowadays, Cry and Vip3A proteins produced by *Bacillus thuringiensis* or Bt have been used as environmental-friendly biopesticides to control *S. exigua*. The combination of Vip3A and Cry proteins has been found to delay insect resistance development and exhibit synergistic activity against a wide range of insect pests. This study thus aims to screen Bt isolates collected from Thailand for high levels of Vip3A and Cry proteins production and high thermostability to control *S. exigua*. 42 Bt isolates from BIOTEC collection, Thailand were screened by SDS-PAGE analysis to investigate levels and patterns of Vip3A and Cry protein production. Six Bt isolates with high levels of Vip3A protein and different patterns of Cry proteins were selected for insecticidal activity analysis of whole culture extracts containing both Vip3A and Cry proteins and culture supernatant containing secreted Vip3A protein against the second-instar *S. exigua* larvae. Thermostability of proteins was also tested by heating both whole culture and culture supernatant at 50℃ for 1 h before feeding to *S. exigua* larvae. Two Bt isolates (81 and 506) showed 100% larvae mortality 3-4 days after feeding and also retained high larvicidal activity after heating. These two Bt isolates are selected for further studies to improve protein stability and formulation for potential field applications.

Graphical abstract:



Keywords: *Bacillus thuringiensis*; Vip3A protein; Cry protein; *Spodoptera exigua*

1. Introduction

One of major agricultural problems in Thailand is a crop damage from insect pests, especially *Spodoptera exigua* (beet armyworm), resulting in economic losses of crop production. *S. exigua* is a noctuid pest species in order Lepidoptera. During larval stage, they feed on plant leaves, stems and reproductive parts of many plant species, causing serious damages to economically important crops such as asparagus, bean, cabbage, cauliflower, corn, tomato, cotton, and onion (1). As a consequence, farmers extensively use chemical pesticides to control *S. exigua*, leading to toxicity to non-target organisms and natural enemies (2). Moreover, chemical pesticides also affect human health (2) and induce insect resistance development (3, 4). Therefore, using biopesticides is an alternative way to delay insect resistance development and minimize harmful effects to human, animals and environment (5). Bacterial biopesticides, especially those produced by *Bacillus thuringiensis*, are widely used as part of integrated pest management (IPM) programs (5).

B. thuringiensis or Bt is a Gram-positive, rod-shaped, and spore-forming bacterium (6). During the sporulation phase, Bt can produce crystal (Cry) proteins which are classified into 73 groups (Cry1 to Cry73) based on their amino acid sequence homology. These Cry proteins exert insecticidal activity against several insect orders such as Lepidoptera, Coleoptera, Diptera, Hemiptera, and Hymenoptera (6, 7). When larvae ingest Cry proteins, originally produced as a protoxin form, protoxins are solubilized under midgut alkaline condition and activated by gut proteases. The activated toxin subsequently binds to a specific receptor on midgut cell membrane, leading to pore formation and larvae death (6). During the vegetative growth phase, Bt can produce and secret vegetative insecticidal proteins (Vip) into culture supernatant. Vip proteins are classified into four families (Vip1, Vip2, Vip3, and Vip4). Among Vip proteins, Vip3 protein shows insecticidal activity against a wide range of lepidoptera pests (6). Similarly, Cry1 and Cry2 proteins also show insecticidal activity against lepidoptera species (8), especially Cry1C, Cry1D, Cry1F and Cry2A proteins (4). However, long-term usage of Cry proteins as biopesticides can induce insect resistance development via genetic modification of receptor on insect's midgut cell membrane as has been reported in Cry1Ca-resistant *S. exigua* (9-11). Vip3A protein, however, can be used to control insect pests that are resistant to Cry proteins due to the receptors recognized by Vip3A are different from those of Cry proteins (12, 13). In addition, the combination of Cry and Vip3A proteins showed synergistic activity against a board-range of insect pests and delay insect resistance development (8, 14).

To improve insecticidal activity of biopesticides and prevent insect resistance development, combination of both Cry and Vip3A proteins seems to be a promising strategy for insect pest control. However, the use of Vip3A in pest control is limited by its short shelf life when stored at high temperatures (15). In this study, we aim to screen Bt isolates from BIOTEC collection that produce high levels of Vip3A and Cry proteins with high thermostability for control of *S. exigua*.

2. Materials and Methods

2.1 Bacterial strains and *Spodoptera exigua* larvae

All 42 *B. thuringiensis* (Bt) isolates were collected from several parts of Thailand and stored at Biocontrol Technology Research Team, National Center for Genetic Engineering and Biotechnology, Thailand. *S. exigua* larvae were obtained from BIOTEC Nuclear Polyhedrosis Virus Pilot Plant for Insect Pest Control at Thailand Science Park, Thailand.

2.2 Screening of *B. thuringiensis* isolates for Cry protein production

Single colony of 42 Bt isolates was streaked on sporulation media (2xSG agar) and incubated at 30℃ for 4 days. Spores of Bt were resuspended in 3 ml sterile distilled water and analyzed by using SDS-PAGE to monitor protein profiles.

2.3 Screening of *B. thuringiensis* isolates for producing high levels of Vip3A proteins

Single colony of 42 Bt isolates was inoculated in 2 ml Luria-Bertani (LB) broth and incubated at 37℃ with shaking at 200 rpm for 16-18 h. Then 60 µl overnight cultures were inoculated in 3 ml Terrific broth (TB) and incubated at 30℃ with shaking at 200 rpm for 4 days. The culture supernatant and cell pellet were collected by centrifugation at 8,500 rpm, 4℃ for 10 min. The cell pellet was resuspended in 200 µl sterile distilled water. The whole culture, culture supernatant and cell pellet were analyzed by SDS-PAGE to monitor Vip3A protein production.

2.4 Screening of *B. thuringiensis* isolates with high thermostability and high insecticidal activity against *S. exigua*

Bt isolates with high levels of Vip3A protein production were tested insecticidal activity in two sets including whole culture extracts containing both Vip3A and Cry proteins and culture supernatant containing secreted Vip3A protein against second-instar larvae of *S. exigua*. For unheated condition, 20 µl of whole culture and culture supernatant were overlaid on a semi-artificial diet surface and allowed to dry before adding a second-instar larva in each well. For heated condition, 20 µl of whole culture and culture supernatant were heated at 50℃ for 1 h before overlaid on a semi-artificial diet surface (12 larvae were tested for each sample). Plates were incubated at room temperature and numbers of dead larvae were measured daily for 7 days.

3. Results

3.1. Screening of B. thuringiensis isolates for producing high levels of Vip3A and Cry proteins

3.1.1. Protein profiles of Cry protein production

Protein profiles of spores extracted from 42 Bt isolates are divided into 6 patterns based on protein bands observed by SDS_PAGE (Figure 1). Pattern 1 consists of 29 Bt isolates (98, 115, 187, 233, 234, 256, 487, 495, 507, 527, 534, 557, 559, 562, 563, 568, 572, 580, 583, 584, 585, 586, 595, 597, 605, 608, 611, 612, and 613). Pattern 2 consists of 6 Bt isolates (64, 104, 501, 506, 520, and 565) and pattern 3 consists of 4 Bt isolates (2, 5, 498, and 556). All Bt isolates in patterns 1, 2, and 3 showed protein bands with molecular mass around 20 kDa. A protein band of around 27 kDa was detected in only pattern 1 while a major protein band at around 22 kDa was observed in both patterns 2 and 3. However, an extra band of protein at 30 kDa was observed in pattern 2. Patterns 4, 5, and 6 are protein profiles of Bt isolates 81, 228, and 609, respectively that did not show those major protein bands as observed from the previous three patterns. Bt isolates 81 and 228 showed a major protein band of around 65 kDa while a protein band of around 47 kDa was present in only Bt isolate 228. Bt isolate 609 showed an extra protein band with a mass around 43 kDa that is different from other patterns. For screening of Cry protein production, both Cry1 and Cry2 proteins, which are highly active against lepidoptera larvae (8), are targets for selection. The protoxin forms of Cry1 and Cry2 proteins are around 130-140 and 65-70 kDa, respectively (16) while their activated forms have molecular sizes around 60 and 50 kDa, respectively (6, 17). Five patterns, except pattern 4, have a protein band with molecular mass around 70 kDa which is predicted to be a protoxin form of Cry2 protein. Five patterns, except pattern 3 showed a band with molecular mass around 60 kDa which seems to be activated form of Cry1 protein.

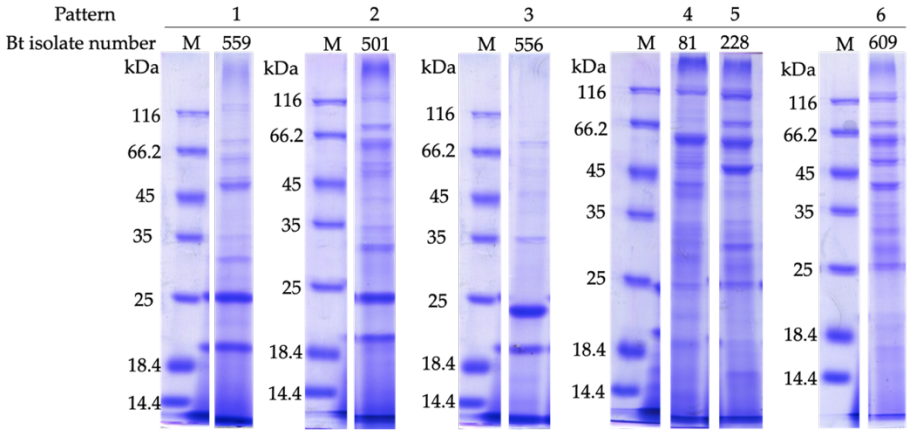


Figure 1. 12% SDS-PAGE analysis of spores of 42 Bt isolates are divided into 6 patterns. Protein profiles of Bt isolates 559, 501, 556, 81, 228, and 609 which are representatives of patterns 1-6, respectively. Lane M represents protein molecular weight marker.

3.1.2. Vip3A protein production

The whole culture, culture supernatant and cell pellet from each of 42 Bt isolates were analyzed by SDS-PAGE. A protein band of Vip3A with a mass of 88 kDa is expected to be found in both whole culture and culture supernatant (15). Six Bt isolates (81, 228, 501, 506, 559, and 609) showed intense protein bands at around 80 kDa in both whole culture and culture supernatant (Figure 2), suggesting the high levels of Vip3A protein production in these isolates.

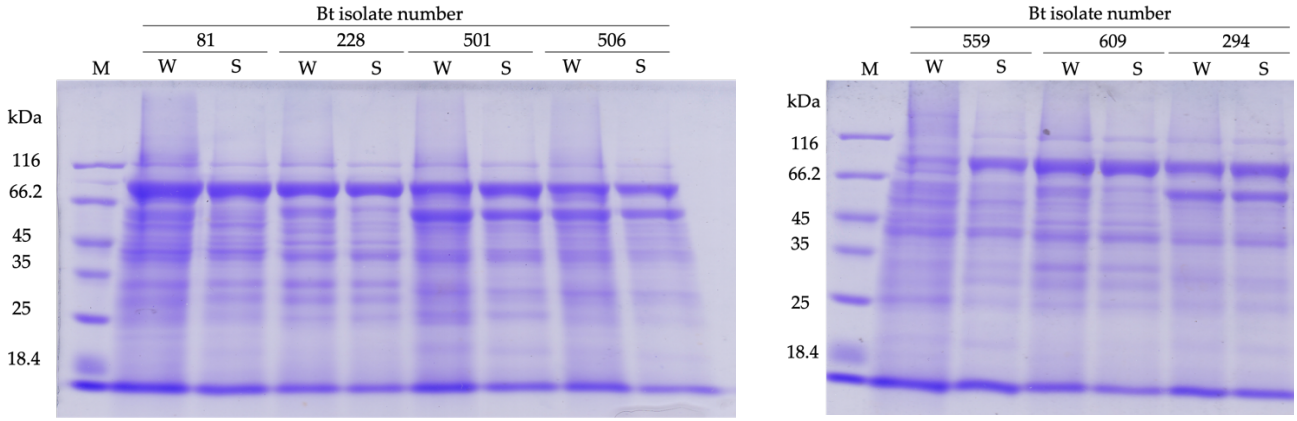


Figure 2. 12% SDS-PAGE analysis of whole culture (W) and culture supernatant (S): (**a**) Bt isolates 81, 228, 501, and 506; (**b**) Bt isolates 559, 609, and 294. Lane M represents unstained protein molecular weight marker.

3.2. Screening of B. thuringiensis isolates with high insecticidal activity against S. exigua and high thermostability

The combination of Vip3A and Cry proteins from whole culture extracts and Vip3A protein secreted into culture supernatant were biologically assayed on *S. exigua* larvae (Table 1). The whole culture, and culture supernatant of Bt isolates 81, 501 and 294 (as a positive control) showed 100% larval mortality after three days post feeding for unheated condition. While Bt isolates 228, 506 and 609 showed 100% larval mortality after three and four days post feeding. The whole cultures of all Bt isolates showed higher toxicity than that of their culture supernatant. For heated samples to test thermostability of Vip3A protein (Table 2), Bt isolates 506 and 294 (positive control) retained insecticidal activity with 100% larval mortality for both whole culture and culture supernatant. The culture supernatant of Bt isolates 81 and 501 showed 100% after four days post feeding. Taken together

from these results, whole cultures of Bt isolates 81 and 506 showed higher toxicity than that of their culture supernatant and also retained insecticidal activity after heated when compared with other Bt isolates.

Table 1. The percentage of *S. exigua* larvae mortality after treated with whole culture and culture supernatant of Bt isolates 81, 228, 501, 506, 559, 609, and 294 under unheated condition.

Bt isolate number		Larval mortality (%)						
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
81	Whole culture	8.33	83.33	100				
	Culture supernatant	8.33	50	100				
228	Whole culture	0	33.33	100				
	Culture supernatant	0	8.33	58.33	100			
501	Whole culture	0	66.67	100				
	Culture supernatant	0	50	100				
506	Whole culture	8.33	75	100				
	Culture supernatant	0	8.33	58.33	100			
559	Whole culture	0	41.67	75	100			
	Culture supernatant	16.67	33.33	66.67	83.33	91.67	91.67	91.67
609	Whole culture	0	8.33	91.67	100			
	Culture supernatant	0	16.67	66.67	100			
294	Whole culture	0	91.67	100				
	Culture supernatant	0	75	100				
TB		0	0	0	0	0	0	8.33

Table 2. The percentage of *S. exigua* larvae mortality after treated with whole culture and culture supernatant of Bt isolates 81, 228, 501, 506, 559, 609, and 294 under heated condition.

Bt isolate number		Larval mortality (%)						
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
81	Whole culture	0	25	100				
	Culture supernatant	0	8.33	75	100			
228	Whole culture	0	8.33	83.33	100			
	Culture supernatant	0	0	58.33	100			
501	Whole culture	0	91.67	100				
	Culture supernatant	8.33	25	91.67	100			
506	Whole culture	8.33	50	100				
	Culture supernatant	8.33	50	100				
559	Whole culture	0	25	75	83.33	91.67	91.67	91.67
	Culture supernatant	8.33	16.67	75	100			
609	Whole culture	0	16.67	83.33	100			
	Culture supernatant	0	16.67	66.67	100			
294	Whole culture	16.67	91.67	100				
	Culture supernatant	0	66.67	100				
TB		0	0	0	0	0	0	8.33

4. Discussion

In this study, 42 Bt isolates collected from various locations in Thailand were screened for high expression levels of both Cry and Vip3A proteins for further development as biopesticides for control of *Spodoptera* spp. Through observations of protein profiles by SDS_PAGE analysis, Bt isolates 81, 228, 501, 506, 559 and 609 were selected

according to their high production of both Cry and Vip3A proteins with molecular masses ranging from 50-140 kDa for Cry proteins and 80 kDa for Vip3A protein. However, the protein identity produced by these Bt isolates still needs to be confirmed by mass spectrometry. Subsequently, the selected Bt isolates were tested insecticidal activity against *S. exigua* larvae. Among these Bt isolates' samples, whole culture extracts of Bt isolates 81 and 506 showed highest toxicity. Whereas, their culture supernatant are less toxic to *S. exigua* larvae than the whole culture extracts. This result could be explained that the whole culture contains both Vip3A and Cry proteins that may work together to kill *S. exigua* larvae while culture supernatant contains only secreted Vip3A protein, hence exhibiting less toxicity than when it is mixed with Cry proteins. A combination of Vip3A and Cry proteins has been shown to confer synergistic activity by enhancing pore-forming activity on midgut cell membrane (18). Similarly, different Cry proteins also show synergistic activity by forming oligomer complexes of different Cry molecules that promote better receptor binding ability as compared with that from a single group of Cry molecules (19). The synergism activity of Vip3A and Cry1Ia proteins against *Spodoptera* spp. has been reported by Bergamasco et al., 2012 (19). In another study, the combination of Vip3Aa29 and Cyt2Aa3 proteins also showed synergistic activity against *S. exigua* (20).

Although Vip3A protein is promising to be applied together with Cry proteins for pest control, the use of Vip3A is limited by its short shelf life when stored at high temperatures due to its production as a soluble form with protease susceptibility compared to insoluble form of Cry proteins (15). Hence in this study, the thermostability of both whole culture and culture supernatant of Bt isolates 81 and 506 was investigated by heating them at 50℃ for 1 h before feeding to *S. exigua* larvae. We found that both Bt isolates 81 and 506 retained high larvicidal activity after being heated. Previous study of Soonsanga et al.,2018 reported that a key amino acid residue at tyrosine-776 of Vip3Aa64 from Bt isolate 294 is responsible for thermostability of this protein upon high temperature storage (15). It thus remains to be investigated whether amino acid sequences of Vip3A proteins from Bt isolates 81 and 506 are homologous or distinct from that of Vip3Aa64. In addition, these two Bt isolates are selected for further studies to improve protein stability and formulation for field applications.

5. Conclusions

In this study, 6 out of 42 Bt isolates (81, 228, 501, 506, 556 and 609) with high levels of Vip3A and Cry protein production were selected based on SDS-PAGE analysis. Among those selected isolates, 2 Bt isolates (81 and 506) showed high insecticidal activity against *S. exigua* larvae and retained high toxicity after heating at 50℃, supporting their potentials to be developed as biopesticides to control *Spodoptera* spp. in the fields.

Author Contributions: Conceptualization, B.P. and P.B.; methodology, T.H.; writing—original draft preparation, T.H.; writing—review and editing, P.B. and B.P.; funding acquisition, B.P. and P.B.; All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Agricultural Research Development Agency (ARDA) and Mahidol University, grant number PRP6305031920 (to P.B. and B.P.) and the Development and Promotion of Science and Technology Talents Project (DPST) to (T.H.).

Acknowledgments: We thank Biocontrol Technology Research Team of BIOTEC for kindly providing Bt isolates and BIOTEC Nuclear Polyhedrosis Virus Pilot Plant for Insect Pest Control for supplying *S. exigua* larvae. Special thanks are given to Ms. Monrudee Srisaidap and Ms. Chutchanun Trakulnalueamsai for laboratory technical supports.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Capinera JL. Beet Armyworm, *Spodoptera exigua* (Hübner) (Insecta: Lepidoptera: Noctuidae). UF/IFAS Extension. 2020.
2. Aktar MW, Sengupta D, Chowdhury A. Impact of pesticides use in agriculture: their benefits and hazards. *Interdiscip Toxicol*. 2009;2(1):1-12.
3. Che W, Shi T, Wu Y, Yang Y. Insecticide resistance status of field populations of *Spodoptera exigua* (Lepidoptera: Noctuidae) from China. *J Econ Entomol*. 2013;106(4):1855-62.
4. Hernandez-Martinez P, Ferre J, Escriche B. Broad-spectrum cross-resistance in *Spodoptera exigua* from selection with a marginally toxic Cry protein. *Pest Manag Sci*. 2009;65(6):645-50.
5. Usta C. Microorganisms in Biological Pest Control — A Review (Bacterial toxin application and effect of environmental factors). *Current Progress in Biological Research* 2013.
6. Palma L, Munoz D, Berry C, Murillo J, Caballero P. *Bacillus thuringiensis* toxins: an overview of their biocidal activity. *Toxins (Basel)*. 2014;6(12):3296-325.
7. Nimsanor S, Chimpalee S, Rungrod A, Trakulnaleamsai C, Jongsareejit B, Promdonkoy B. Cloning and characterization of vegetative insecticidal protein (Vip3A) from Thai isolated *Bacillus thuringiensis*. E-Journal, *Science and Technology Silpakorn University*. 2016;3:9.
8. Figueiredo CS, Zara FJ, Desidério JA. Effect of the Cry1, Cry2 and Vip3 protein combinations on the control of *Anticarsia gemmatalis* (Erebidae) and *Chrysodeixis includens* (Noctuidae) Lepidoptera. *International Journal of Pest Management*. 2020:1-9.
9. Herrero S, Gechev T, Bakker PL, Moar WJ, de Maagd RA. *Bacillus thuringiensis* Cry1Ca-resistant *Spodoptera exigua* lacks expression of one of four Aminopeptidase N genes. *BMC Genomics*. 2005;6:96.
10. Soberón M, Monnerat R, Bravo A. Mode of action of Cry toxins from *Bacillus thuringiensis* and resistance mechanisms. *Microbial Toxins*. 2016. p. 1-13.
11. Hernandez-Martinez P, Navarro-Cerrillo G, Caccia S, de Maagd RA, Moar WJ, Ferre J. Constitutive activation of the midgut response to *Bacillus thuringiensis* in Bt-resistant *Spodoptera exigua*. *PLoS One*. 2010;5(9).
12. Bel Y, Banyuls N, Chakroun M, Escriche B, Ferre J. Insights into the structure of the Vip3Aa insecticidal protein by protease digestion analysis. *Toxins (Basel)*. 2017;9(4).
13. Shao E, Zhang A, Yan Y, Wang Y, Jia X, Sha L. Oligomer formation and insecticidal activity of *Bacillus thuringiensis* Vip3Aa toxin. *Toxins (Basel)*. 2020;12(4).
14. Chakrabarty S, Jin M, Wu C, Chakraborty P, Xiao Y. *Bacillus thuringiensis* vegetative insecticidal protein family Vip3A and mode of action against pest Lepidoptera. *Pest Manag Sci*. 2020;76(5):1612-7.
15. Soonsanga S, Rungrod A, Audtho M, Promdonkoy B. Tyrosine-776 of Vip3Aa64 from *Bacillus thuringiensis* is important for retained larvicidal activity during high-temperature storage. *Curr Microbiol*. 2019;76(1):15-21.
16. Ruiz de Escudero I, Estela A, Porcar M, Martinez C, Oguiza JA, Escriche B. Molecular and insecticidal characterization of a Cry1I protein toxic to insects of the families Noctuidae, Tortricidae, Plutellidae, and Chrysomelidae. *Appl Environ Microbiol*. 2006;72(7):4796-804.
17. Liu S, Wang S, Wu S, Wu Y, Yang Y. Proteolysis activation of Cry1Ac and Cry2Ab protoxins by larval midgut juice proteases from *Helicoverpa armigera*. *PLoS One*. 2020;15(1):e0228159.
18. Lemes AR, Davolos CC, Legori PC, Fernandes OA, Ferre J, Lemos MV. Synergism and antagonism between *Bacillus thuringiensis* Vip3A and Cry1 proteins in *Heliothis virescens, Diatraea saccharalis* and *Spodoptera frugiperda*. *PLoS One*. 2014;9(9):e107196.
19. Bergamasco VB, Mendes DR, Fernandes OA, Desiderio JA, Lemos MV. *Bacillus thuringiensis* Cry1Ia10 and Vip3Aa protein interactions and their toxicity in *Spodoptera* spp. (Lepidoptera). *J Invertebr Pathol*. 2013;112(2):152-8.
20. Yu X, Liu T, Sun Z, Guan P, Zhu J, Wang S. Co-expression and synergism analysis of Vip3Aa29 and Cyt2Aa3 insecticidal proteins from *Bacillus thuringiensis*. *Curr Microbiol*. 2012;64(4):326-31.