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Genetic improvement of *Bacillus thuringiensis* against the cotton bollworm, *Earias vitella* (Fab.) (Lepidoptera: Noctuidae), to improve the cotton yield in Pakistan

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Abstract

In the present study, *CrylAc* (Crystal protein) gene was cloned under *Cry3A* promoter, which is known to express vegetatively as a sporulation independent promoter, transformed into acrystalliferous strain of *Bacillus thuringiensis*. Its potential was evaluated as a strain to be used for formulation development against the spotted cotton bollworm *Earias vitella* (Fab.) (Lepidoptera: Noctuidae), the major pest of cotton, the important cash crop in Pakistan. The crystal protein produced in a new strain, i.e., *Bt1*, had comparable potency as of crystal protein produced during sporulation. The new strain *Bt1* had the maximum expression of *Cry1Ac* protein during the vegetative stage, i.e., in the first 24 h, thereby reducing the growth period until crystal protein production, from 72 to 24 h. In addition, crystal protein produced during vegetative stage under *Cry3A* promoter remained encapsulated within the cells, which could protect them from UV degradation and was independent of sporulation; therefore; it did not produce spores and thus is not a source to spread *Bt* spores in the environment. It can, therefore, be called as an environmentally friendly pesticide to control cotton pest especially bollworms and ultimately improve the yield of cotton.

Keywords: Genetic improvement, *Bacillus thuringiensis*, *Cry3A* promoter, *Earias vitella*, Biotoxicity assay, Viability assay

Background

Insect pests cause great damage to different cash crops in farming production. Worldwide yield losses vary widely between crops and different geographic areas. Different schemes have been applied to control this agricultural damage, the principal strategy to cut the impairment being the role of chemical insecticides (Federici et al. 2003). The most widely used microbial pesticide worldwide are those based on preparations of the bacterium *Bacillus thuringiensis* (*Bt*). The *Bt* is an aerobic, spore-forming soil bacterium that produces highly specific insecticidal proteins termed as delta endotoxins or *Cry* (crystal) toxin. Delta endotoxins accumulate as crystalline inclusions within the cell during sporulation (Reza et al. 2014). Commercial *Bt* products generally consist of a mixture of spores and crystals, grown in large fermenters and applied as foliar sprays, much like synthetic insecticides. In Pakistan, cotton is susceptible to attack by more than 67 insects, the major lepidopterans bollworms, and they cause heavy damages to cotton yield (FAO 2001).

Biopesticides containing *Bt* are environmentally friendly and effective in a variety of situations. Its products are not as potent or persistent in the field as chemical products. *Bt* products act slowly have a narrow activity spectrum and are not stable in the environment after spraying because they are quickly inactivated by exposure to sun or other environment (Yunus et al. 2011b). Therefore, the duration of pest control has been frequently too short, and its use on many crops is not

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cost effective because too many applications are required to wipe out the pests (Benjamin et al. 2014). The environmental stability of the crystals after spraying is also significant as it defines the duration of pest control and the number of needed applications.

For increasing their efficacy and persistence, *Bt* toxins for field use and C*ry* genes have been cloned and expressed in other microorganisms. A number of different toxin genes, which are effective against lepidopteran, dipteran and coleopteran larvae, from different strains of *Bt* have been cloned and expressed in different host system (Tabashnik et al. 2004; Zhu et al. 2006; Yunus et al. 2011b; Benjamin et al. 2014). Binding of *Bt* toxins to specific sites in the epithelial membrane is a central step in toxin specificity. By using different chimeric genes and using toxins combination, development of resistance in target insects is delayed (Yunus et al. 2011a; Lemes et al. 2017 and Farhat-Touzri et al. 2018).

The expression systems of the *Cry* genes differ and consequently do not compete for rate limiting gene expression vectors. The *CryI* delta endotoxin genes are transcribed from specific sporulation dependent promoters, whereas the *Cry3A* gene is transcribed from a sporulation-independent promoter, which resembles vegetative promoters of other *Cry* toxin genes. This builds it possible to bring about this toxin or other *CryI* toxins at high levels in sporulation-deficient or wild-type backgrounds. Therefore, it may be possible to increase the total quantity of *Cry* toxin produced in a *Bt* strain (Zhu et al. 2006). In the present study, a new recombinant strain *Bt1*, in which the crystal protein *Cry1Ac* produced under C*ry3A* promoter remains encapsulated that provides protective covering of cell membrane around crystal protein to increase stability of toxin in the UV containing environment was developed.

Materials and methods

Construction of *Cry1Ac* recombinant strain under *Cry3A* promoter

The wild-type strains of HD-73 subspecies *Kurstaki*, 4AA1 subspecies *tenebrionis*, and acrystalliferous strain 4D22 modified form of HD-73 were procured from BGSC (*Bacillus* Genetic Stock Centre, OH, USA). Total genomic DNA isolated from the HD-73 subspecies *Kurstaki* by Kronstad method (Kronstad 1983) containing the C*ryIAc* gene (Acc # M11068) and 4AA1 subspecies *tenebrionis* strain containing the *Cry3A* promoter (Acc # M30503) were used to amplify the *Cry1Ac* gene and *Cry3A* promoter for cloning into PCR 2.1 TA-cloning vector. The C*ryIAc* construct pAc was PCR amplified from HD-73 genomic DNA with Ac forward and Ac reverse primers, simultaneously incorporating ApaI sites into the both ends of the fragment for cloning into the TA-cloning vector PCR 2.1. The *Cry3A* construct p3A was obtained by using the 3A forward and 3A reverse primers and incorporating the BamHI and ApaI restriction sites into the fragment for cloning into the PCR 2.1 cloning vector (Fig. 1). For comparative study, full-length C*ryIAc* gene was amplified, employing the 1Ac forward and 1Ac reverse primers and cloning into the TA-cloning vector PCR 2.1 exploiting the Sph1 and BamH1 sites in its polylinker region (Table 1). Plasmid DNA from various clones was prepared by the Alkaline Lysis method (Birnboim and Doly 1970).

Polymerase chain reactions (PCR) were performed by a modification of the method by (Saiki et al. 1988). A total volume of 50 μl contained 50–100 ng of DNA template, 0.1 mM dNTPs, 1 pmol/μl of each primer, and 2 units of Taq DNA polymerase in 1xPCR buffer, 2.5–3 mM $MgCl_2$, using a PTC-100 thermocycler (Mj Research Laboratories) according to the expected length of the PCR products. To amplify ~ 0.2 kb DNA fragment from C*ry3A* and 1.8 kb DNA fragment and ~ 3.9 kb full-length gene from the *CryIAc* genomic DNA. The PCR programs were mentioned respectively as follows. For 0.2 kb promoter, the reaction was performed as 94 °C for 1 min followed by 35 cycles, 94 °C for 45 s, 50 °C for 45 s, 72 °C for 1 min, and final extension at 72 °C for 7 min. For 1.8 kb *CryIAc* gene coding region, the reaction was performed at 93 °C for 5 min, followed by 29 cycles, 94 °C for 45 s, 56 °C for 2 min, 72 °C for 2 min, and final extension at 72 °C for 10 min (Fig. 1). Figure 1c depicts the plasmids map for the theoretical sequence in SnapGene (Version 3.0) for *Bt1* in pHB201 and *Bt2* in pHPS9.

Biotixicity assay

Artificial diet containing easily available ingredients was developed to rear the target insect (Rahat 1999). The growth of the insects when reared on artificial diet at (29 ± 2 °C and 65 ± 5% RH) was normal and the larvae completed their development in 17–20 days. The egg hatchability was 100% and the survival rate larvae were 20% (Table 2). *CryIAc* toxin was extracted from *Bt kurstaki* HD-73 using the procedure described by Bietlot (1990). The toxin concentration was determined by the method of (Bradely Bradford 1976). Different concentrations of activated toxin protein ranging from 10 to 300 μg were mixed per gram of diet and air-dried. Two second instar spotted bollworm larvae were placed in each vial containing 2 g of diet. Each assay was performed in triplicate. For a negative control, comparable quantity of the buffer and trypsin was added to the diet, air-dried, and assay established in triplicates. Mortality was monitored till 72 h. Bioassay data were statistically analyzed using Quantal Computer Program at Centre of Excellence in Molecular Biology (CEMB) (Fig. 2).

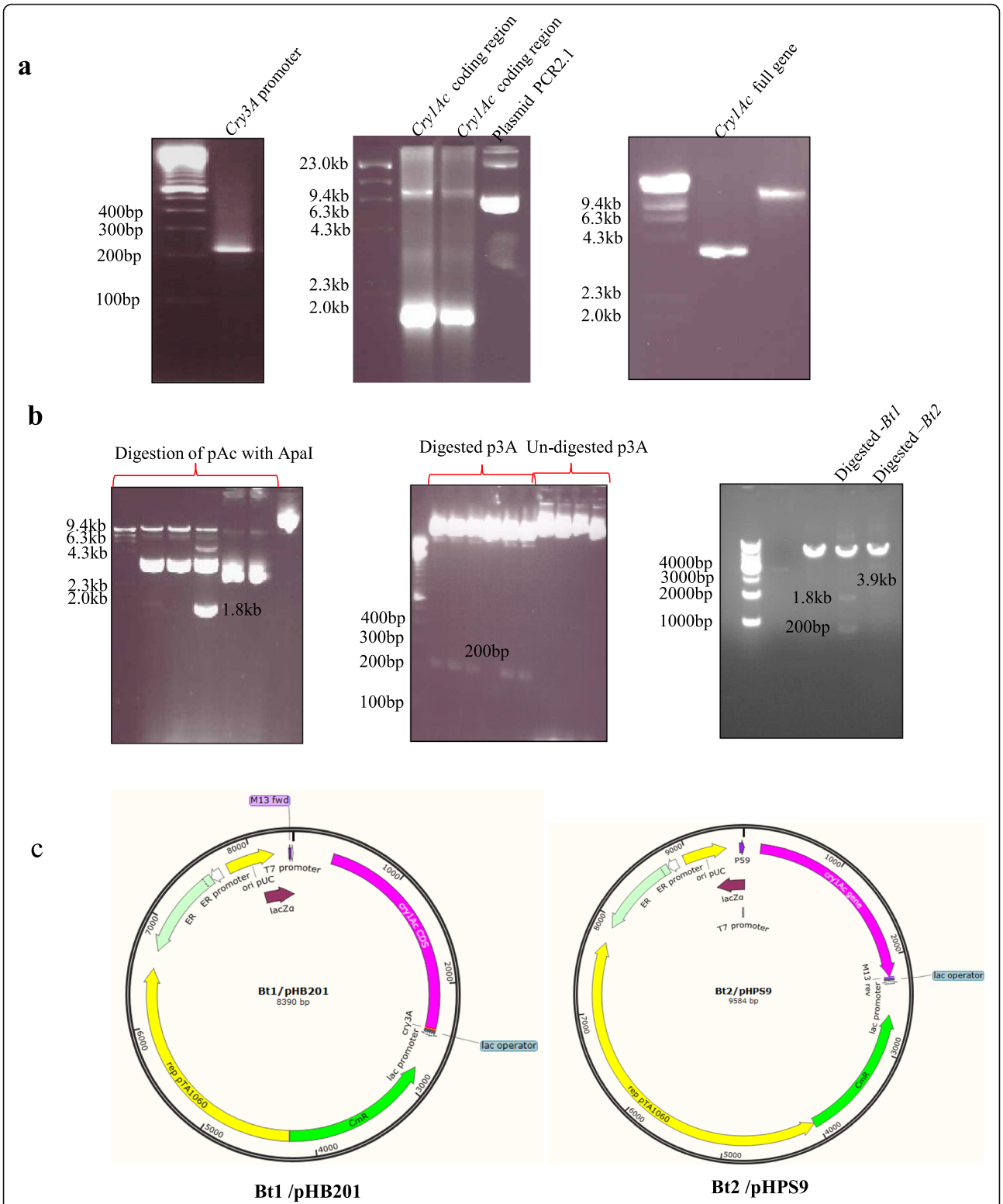

Fig. 1 Cloning of *Cry1Ac* coding region under *Cry3A* promoter in pHB201 shuttle vector (*Bt1)* and full-length *Cry1Ac* gene in pHSP9 shuttle vector (*Bt2)*. **a** Gel electrophoresis of the PCR amplified products for *Cry3A* promoter 200 bp, *Cry1Ac* coding region 1.8 kb, and *Cry1Ac* 3.9 kb full-length gene. **b** Restriction digestion of positive clones with ApaI and EcoRI enzymes of expression cassette in shuttle vector pHB201 (*Bt1*) and full-length *Cry1Ac* gene in shuttle vector pHPS9 (*Bt2*) release fragments of specific sizes. **c** Plasmids map predict the theoretical sequence of recombinant strains *Bt1* and *Bt2* in SnapGene

Table 1 Forward and reverse primers of *Cry1Ac* genes and *Cry 3A* promoter region

Gene/Promoter	Primers
Primers for full length *Cry1Ac* gene	Forward
	5′ AT**GCATGC**GTGAATTGCAGGTAAATGGTTC 3′ Sph1
	Reverse
	5′ CG**GGATCC**TTACTATTCCTCCATAAGGAGTAAT 3′ BamH1
Primers for coding region of *Cry1Ac* gene	1Ac Forward
	5′ TTT**GGGCCC**ATGGATAACAATCCGA 3′ Apa1
	1Ac Reverse
	5′ TTT**GGGCCC**TTCAGCCTCGAGTG 3′ Apa1
Primers for *Cry3A* promoter region	Pro Forward
	5′ CG**GGATCC**GGACTATTATAATCAT 3′ BamH1
	Pro Reverse
	5′ TTT**GGGCCC**TTTTCTTCCTCCC3′ Apa1

Cell viability assay

For the laboratory biotoxicity assay, dry and wet formulations were developed for recombinant strains of *Bt1* and *Bt2*, and spore viability assay was performed for both formulations. Cell viability assay for recombinant *Bt* strain *Bt1* was performed with acetone. For this purpose, *Bt1* was grown in 500 ml LB-Chloramphenicol (10 μg/g) at 37°C for 24 h, followed by harvesting cells at 7000 RPM at 4°C for 10 min, and cells were washed twice by resuspending the cell pellet in acetone and centrifugation afterward. One gram of pellet was resuspended in 9 ml of autoclaved 0.89% saline solution and vortexes thorough. This dilution was used as a stock to prepare six further serial dilutions. From each of first six serial dilutions, 100 μl was spread on LB-chloramphenicol plates in triplicates for each dilution. Positive control contained cells washed with autoclaved saline instead of acetone. Plates were incubated at 30°C overnight. The positive control plates showed growth in all plates whereas treated pellet with acetone showed very few colonies in first three dilutions, and there was no growth in plates for further three dilutions. When bioassays were performed for both types of pellets against the target pest, similar larval mortality was observed in control cells as well as in treating cells (Fig. 2).

Table 2 Life cycle of spotted bollworm (*Earias vitella*) on locally developed synthetic diet for laboratory bioassay

Stage of larvae	Days and percentage
Egg stage	
Egg hatching time	6–7 days
Egg hatching	90%
Larval stage	
Survival rate of neonatal larvae	80%
Survival rate of second and four instar larvae	98%
Time period (neonatal to four instar)	18 days
Pupal stage	
Pupation after four instar	95%
Time period (pupa to moth)	5–9 days
Adult stage (moth)	
Moth emergence rate	80%
Longevity	5–8 days

Leaf bioassay for *Bt1* formulation

Infectivity test was conducted to check the fate of *Bt1* formulation in the laboratory against *E. vitella*. These treatment contained ten replicates. In the petri plates, to retain moisture on the round filter paper ½ ml, water was spreaded. Top tender undamaged leaves of about 4 cm in length were plucked from CEMB cotton field. Leaves were washed by sterilized distilled water to remove dust particles. The formulation was weighed as 1.25 g and mixed in 500 ml sterilized distilled water. Ten leaves were sprayed by a prepared solution using a hand sprayer, then air-dried the leave; for control, simple sterilized distilled water was used. Second instar larvae of *E. vitella* were kept on leave for 72 h. Mortality was recorded, after 72 h.

Results and discussion

Resistance development is the major hurdle for *Bt* biopesticide. The present study addresses such problem and developed an improved recombinant strain *Bt1* in which crystal protein was protected from UV degradation and avoided the spreading of spores in the environment. For this purpose, the expression of an active toxin fragment of *CryIAc* gene was studied under the *Cry3A* promoter. It has been well documented that unlike *Cry1Ac*, *Cry3A*

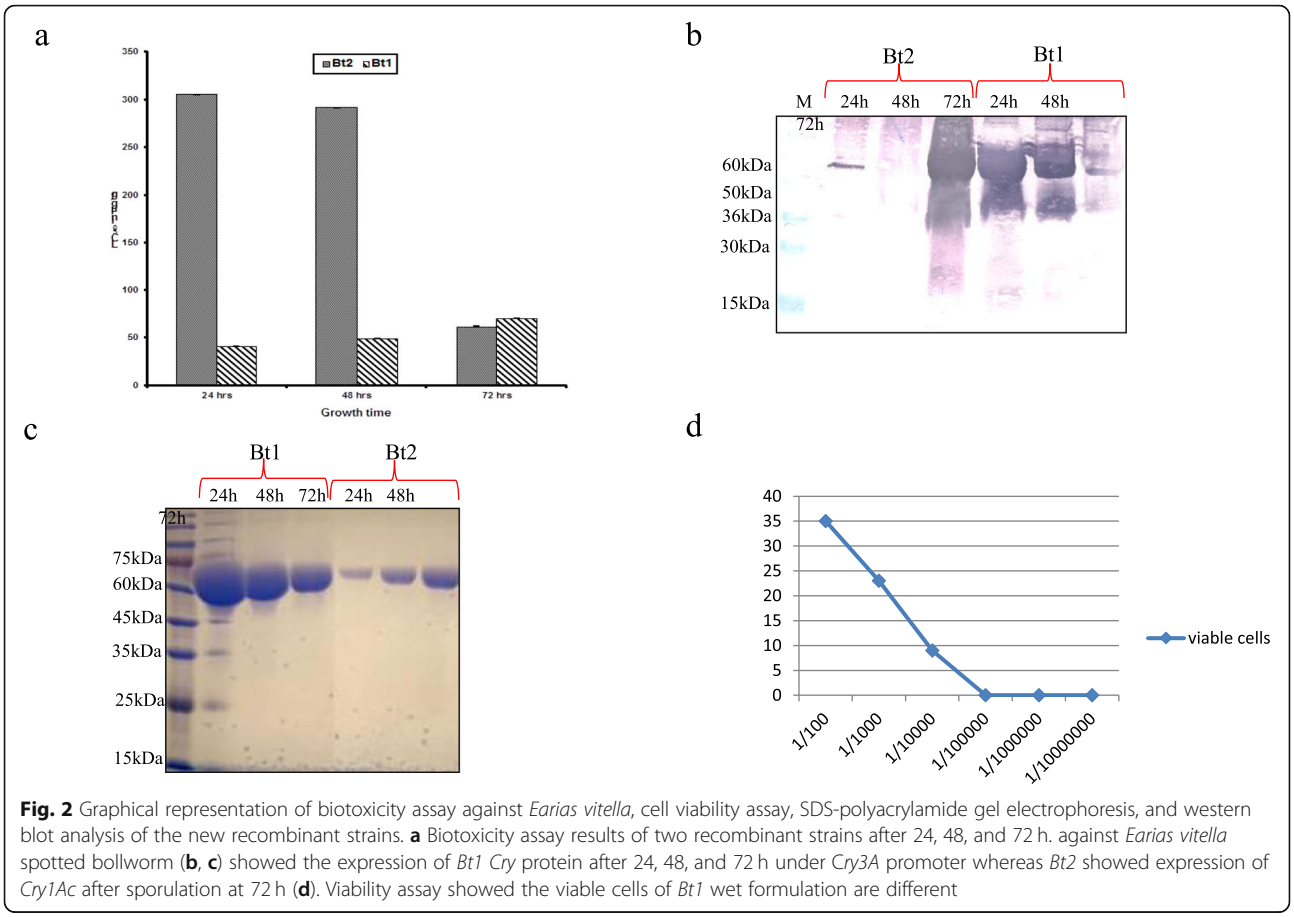

Fig. 2 Graphical representation of biotoxicity assay against *Earias vitella*, cell viability assay, SDS-polyacrylamide gel electrophoresis, and western blot analysis of the new recombinant strains. **a** Biotoxicity assay results of two recombinant strains after 24, 48, and 72 h. against *Earias vitella* spotted bollworm (**b**, **c**) showed the expression of *Bt1 Cry* protein after 24, 48, and 72 h under *Cry3A* promoter whereas *Bt2* showed expression of *Cry1Ac* after sporulation at 72 h (**d**). Viability assay showed the viable cells of *Bt1* wet formulation are different

expression is independent of the genes involved in the initiation of sporulation and sporulation sigma factors σE and σK. The *Cry3A* gene expression is activated at the onset of stationary phase from a promoter resembling those recognized by the RNA polymerase containing primary sigma factor σA (Agaisse and Lereclus 1995). Therefore, transcription at the *Cry3A* promoter is initiated earlier than the promoters of other *Cry* genes that are transcribed by RNA polymerase containing sporulation-specific sigma factors, σE and σK. The gene expression under *Cry3A* promoter can make it possible to produce this toxin at high levels before the onset of sporulation in *Bt.*

The *CryIAc* active toxin fragment from *Bt kurstaki* HD-73 from BGSC (*Bacillus* Genetic Stock Centre) was cloned under the *Cry3A* promoter sequences obtained from *Bt* strain *tenebrionis* 4AA1 (BGSC). Expression studies of *Cry1Ac* from strains *Bt1* revealed that only 24 h of growth were required to obtain maximum levels of toxin under the vegetative *Cry3A* promoter in comparison to the *Bt2* strain, where the expression of *Cry1Ac* gene reached its maximum level after 72 h, from its native, sporulation-dependent promoter. The expression of *Cry1Ac* in *Bt2* strain was comparable to the expression of *Cry1Ac* in *Bt kurstaki* strain HD-73 (Figs. 1 and 2). Several studies have been conducted so far indicating that the *Cry3A* promoter was able to enhance the expression of other *Cry* genes and significantly increased the production of those *Cry* proteins (Kronstad et al. 1983;Cannon 1995; Park et al. 1998). Recently, Chen et al. (2005) reported that both *Cry3A* promoter and promoter/Shine-Dalgarno sequence combinations were able to enhance synthesis of Vip184 and to change its expression time course in the different *Bt* strains. Another survey suggested that the amount of Vip3Aa7 protein produced under the control of *Cry3A* promoter was two to three folds more than that of vip3Aa7 native promoter. Chaoyin et al. (2007) demonstrated recently that the yields of *Cry1Ac* protein obtained from *Cry3A* promoter were higher than that produced from *Cry1Ac* promoter. In comparison to the expression of full-length *Cry1Ac* protein, the expression of an active toxin fragment of *Cry1Ac* from *Cry3A* promoter was found. Interestingly, the absence of C-terminal sequences of *Cry1Ac* required for the packaging and crystal formation does not seem to interfere the stability or efficacy of the toxin. Wet and dry sprayable formulations of *Bt1* and *Bt2* recombinant strains were successfully developed. The field bioactivity assays against *E. vitella* showed comparable efficacies of both formulations. Comparative

laboratory assays of *Cry1Ac* proteins produced from *Bt1* and *Bt2*, after 24, 48, and 72 h respectively, for the maximum toxin yield, against *E. vitella* demonstrated slightly lower LC50 values (40.5 ± 0.35 μg/g) for *Bt1* than in *Bt2* (61 ± 0.71 μg/g) suggesting *Bt1* to be more effective (Fig. 2a). The biotoxicity assays showed that after killing the vegetative cells in formulation, there was no effect on protein activity and percentage of insect mortality, which was the same for both viable and non-viable cells (Fig. 2d).

Conclusion

The new strain *Bt1* had the maximum expression of *Cry1Ac* protein during the vegetative stage, i.e., in first 24 h reducing the growth period until the maximum toxin yield, from 72 to 24 h, and thereby significantly reducing the production cost. In addition, the crystal protein produced during vegetative stage under *Cry3A* promoter remains encapsulated that provides a protective covering of cell membrane around crystal protein to increase stability of toxin in the UV containing environment. Most importantly, this approach provides a mean to produce spore-free, environment friendly formulation as sporulation ensues well after 24 h. Preliminary data showed that the *Bt1* formulation was as stable as *Bt2* with reference to formulation development and storage in dried form; however, more studies are required to estimate their comparative stability against UV under field environment.

Abbreviations

Bt: *Bacillus thurigiensis*; *Bt1*: Recombinant strain of *Cry1Ac* under *Cry3A* promoter in pHB201; *Bt2*: Recombinant strain of full-length *Cry1Ac* gene in pHPS9; Cry: Crystal protein; Cry: Crystal protein gene; PCR: Polymerase chain reaction

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Author's contributions

FY and RM designed the work. FY did the experiments and analyzed the data. FY, GR, and HZ were involved in the MS preparation and revised the paper. All authors read and approved the final manuscript.

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Availability of data and materials

NA

Ethics approval and consent to participate

NA

Consent for publication

This is to state that this work is the author's PhD dissertation work so I give permission for the publication.

Competing interests

The authors declare that they have no competing interests.

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