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Isolation, characterization and screening of *Bacillus thuringiensis* isolates for lepidopteran toxic *cry* genes

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Abstract
A biorational pesticide, *B. thuringiensis* (*Bt*) is a gram positive, spore forming soil bacterium and used as an alternative of chemical insecticides due to the ill effects of synthetic insecticides. *B. thuringiensis* produce crystal proteins during sporulation stage of its growth cycle and it has insecticidal activity against many insect orders *viz*., Lepidoptera, Coleoptera, Diptera, Mallophaga, Hymenoptera and Homoptera. Among these *cry* genes, *cry1, cry2, cry9* genes are having specific insecticidal activity against lepidopteran insects. The aim of the present study is to isolate and characterize *B. thuringiensis* strains based on morphological and biochemical characters and screening of lepidopteran toxic *cry* genes. Nineteen strains were obtained from 50 soil samples. *Bt* index of total *B. thuringiensis* is 0.49%. Three types of crystals *i.e.*, bipyramidal, cuboidal, spherical shape of crystals and spores attached with crystals were present in isolated *B. thuringiensis* strains. PCR analysis with specific primers resulted in the amplification of *cry1* in 7 isolates, *cry2Aa* in 15 isolates and *cry2Ab* in 17 isolates and 4 isolates had combination of three genes *viz., cry1, cry2Aa, cry2Ab*.

Keywords: Native *Bacillus thuringiensis* strains, isolation, characterization, PCR, *cry* genes

1. Introduction
A biorational pesticide, *Bacillus thuringiensis* is a spore forming soil bacterium which is rod shaped, gram positive and facultative aerobic bacterium. During sporulation stage of its growth cycle, it produces proteinaceous parasporal crystals, Cry and Cyt which is encoded by *cry* and *cyt* genes, respectively (Schnepf *et al*., 1998) [31]. Based on amino acid sequence homology, Cry and Cyt proteins are classified into 74 classes of Cry proteins (Cry1 to Cry78) and three classes of Cyt proteins (Cyt1-Cyt3) (https://www.google.com/ Bt+nomenclature&rlz=1C1GGRV). These parasporal crystalline inclusions are having high entomocidal activity against many insect orders *viz*., Lepidoptera, Coleoptera, Diptera, Mallophaga, Hymenoptera and Homoptera (Schnepf *et al.*, 1998; Palma *et al*., 2014) [31, 21]. Among these *cry* genes, *cry1, cry2, cry9* genes are having specific insecticidal activity against lepidopteran insects (Crickmore *et al.*, 1998) [31]. These crystal proteins are the main reason for the development of insect- resistant transgenic plants (Romeis *et al*., 2006) [28]. *B. thuringiensis* has twin advantages i.e. highly specific to targeted pests and it is safe for natural enemies, honeybees and other non-targeted organisms (Vimaladevi *et al.*, 2001; Anitha *et al.*, 2011; Li *et al*., 2012) [2, 16]. It is environmentally safe and biodegradable (Betz *et al*., 2000; Roh *et al.*, 2007; Koch *et al.*, 2015; Raymond and Federici (2017)) [5, 27, 14, 25].
FAO has been reported that, 20 to 40% of yield loss caused by the attack of insect pests and pathogenic organisms (Zhou *et al*., 2001). Most of the damaging pests belong to Lepidoptera (Pimental *et al*., 2009). The present study aims to isolate and characterize *B. thuringiensis* strains based on morphological and biochemical characters and screening of *cry* genes which is showing specific insecticidal activity towards lepidopteran insects by PCR method with specific primers.

2. Materials and Methods
2.1 Soil sample collection and isolation of *B. thuringiensis*
A total of 50 soil samples were collected from different locations of Tirunelveli and Tuticorin Districts. Isolation of *B. thuringiensis* strains was done using sodium acetate selection method as described by Travers *et al.* (1987) [33]. One gram of soil sample was taken in a 250 ml conical flask and it was mixed with 20 ml of LB broth buffered with 0.25 M Sodium acetate. Colonies obtained by sodium acetate selection method are plated in T3 medium and incubated at

30 °C for 48 hrs. Selected *B. thuringiensis* colonies were examined for the presence of crystal proteins using phase contrast microscope. *B. thuringiensis* like colonies were counted for determining *Bt* index (Baig *et al.,* 2010) [3].

Table 1: Distribution of *B. thuringiensis* in soil samples

Samples	Total Number of colonies	Number of *Bacillus* like isolates	Number of *Bt* isolates	% of Crystalliferous colonies	*Bt* index[1]
KKM 1	57	24	14	58.33	0.58
KKM 2	35	17	9	52.94	0.53
KKM 3	112	53	27	50.94	0.51
KKM 4	36	12	7	58.33	0.58
KKM 5	61	33	12	36.36	0.36
KKM 6	126	18	8	44.44	0.44
KKM 7	114	63	35	55.55	0.55
KKM 8	92	45	25	55.55	0.55
KKM 9	103	20	11	55.00	0.55
KKM 10	118	14	6	42.85	0.42
KKM 11	46	22	9	40.90	0.41
KKM 12	137	52	27	51.92	0.52
KKM 13	115	44	20	45.45	0.45
KKM 14	114	15	5	33.33	0.33
KKM 15	95	35	18	51.42	0.51
KKM 16	38	12	9	75.00	0.75
KKM 17	52	24	11	45.83	0.45
KKM 18	123	44	23	52.27	0.52
KKM 19	117	36	12	33.33	0.33
Total	1691	583	288	49.39	0.49

[1]*Bt* index = The ratio between *B. thuringiensis* colonies and total number of Bacillus like colonies

Table 2: Percentage of *cry* genes in *B. thuringiensis* isolates

cry genes	Number of isolates possessed *cry* genes	*cry* gene % = (No. of isolates possessed *cry* genes/ Total no. of samples)
cry 1	7	36.84
cry 2Aa	15	78.94
cry 2Ab	17	89.47
cry1 & cry2Aa	4	21.05
cry1 & cry 2Ab	6	31.57
cry2Aa & cry2Ab	14	73.68
cry1, cry2Aa, cry2Ab	4	21.05

2.2 Characterization of the *B. thuringiensis* isolates

2.2.1 Morphological characterization

Morphological characters of all the *B. thuringiensis* isolates were observed and compared with the reference strain *Bt* - HD1.

2.2.2 Characterization based on staining

Isolated strains were subjected to Gram staining (Lalitha *et al.*, 2012) [15] and Crystal staining to study the characters of *Bacillus* colonies and for observing crystal proteins (Ammons *et al.,* 2005) [24].

2.3 Biochemical characterization

Further characterization of isolated *B. thuringiensis* strains was done by Biochemical tests include Motility test, Starch hydrolysis test, Methyl Red (MR) test, Voges Proskauer (VP) test and Catalase Test.

2.4 Screening of lepidopteran toxic *cry* genes

2.4.1 DNA extraction from *B. thuringiensis*

Total genomic DNA was extracted from all 20 isolates including reference strain *Bt* -HD1 as described by Wright *et al.* (2017) [36]. Quality and quantity of all isolates including reference strain were deducted by agarose gel electrophoresis as described by Sambrook *et al*. (1989) [30].

2.4.2 PCR Screening

Universal primer and specific primer were used in PCR screening to characterize the isolated *B. thuringiensis* strains. Details about primer sequences and amplicons size were given in Table 3 and PCR screening was done by following the method described by Ramalakshmi *et al*. (2018). PCR amplification of total genomic DNA was carried out in Eppendorf thermal cycler for 30 reaction cycles each. PCR reactions were accomplished by using 25 µl reaction volume containing 30 ng of template DNA of *Bt* mixed with 2.5 µl of 10X PCR buffer (10 mMTris-HCl; pH: 9.0, 50 mM KCl, 1.5 mM MgCl2), 75 µM each of dNTPs, 50 ng each of forward and reverse primers and 1.5 Units of *Taq* DNA polymerase. The condition for the PCRs done with *cry1, cry 2A* and *cry2Ab* primers were as follows: denaturation step of 2 minutes at 94 °C, annealing at 62 °C for 40 seconds, and extension at 72 °C for 1 minute and an extra step of extension at 72 °C for 1 minute. Following amplification, a 15µl sample of each PCR products were resolved by electrophoresis in 2% agarose gel in Tris-borate buffer (45 mM Tris-borate, 1 mM EDTA [pH 8.0]) in that 2 µl of ethidium bromide was added and then electrophoresed at 120 V for 30 to 45 minutes.

Table 3: Primer sequences and amplicons size

S. No	Primer sequences	cry genes	Product size (bp)	References
1.	FP: 5'CATGATTCATGCGGCAGATAAAC3' RP: 5'TTGTGACACTTCTGCTTCCCATT	cry1	277	Ben-dov *et al*. (1997) [4]
2.	FP: 5'GTTATTCTTAATGCAGATGAATGGG3' RP: 5'CGGATAAAATAATCTGGGAAATAGT3'	cry2A	700	Ben-dov *et al*. (1997) [4]
3.	FP: GTTATTCTtAATGCAGATGAATGGG RP: TGGCGTTAACAATGGGGGGAGAAAT	cry2Ab	546	Ben-dov *et al*. (1997) [4]

3. Results and Discussion

3.1 Isolation of *B. thuringiensis* strains

Totally 50 soil samples were collected from various regions of Tirunelveli and Tuticorin district. Nineteen strains were isolated by following the sodium acetate selection methodology given by Travers *et al*. (1987) [33]. Based on the crystal protein presence 288 colonies out of 583 colonies and 19 isolates were identified as *B. thuringiensis* (Table 4). Recovery of *B. thuringiensis* from native soil samples was highest in isolate KKM 16 (0.75%) followed by isolate KKM 1 and KKM 4 (0.58%) and lowest in isolates KKM 14 and KKM 19 (0.33%) and similar results also reported by Lone *et al*, (2017) [18]. The estimated average *Bt* index of native soil sample was 0.49%.

3.2 Morphological characterization

A total of 583 colonies were isolated based on the morphological characters like colony color, colony shape, colony margin, colony elevation, and colony surface and subcultured in T3 medium for single colony isolation (Figure 1). Most of the isolates were showed creamy white to off white colonies with regular or irregular shape, entire or errose or undulated margin, slightly raised elevation and glistening surface. Kavitha *et al.* (2011) [26] and Rampersad *et al.* (2005) [24] also observed creamy white to off white colonies with smooth edges and flat to slightly raised elevation in their studies. Similar colonial morphology results were also reported by El-kersh *et al.* (2012).

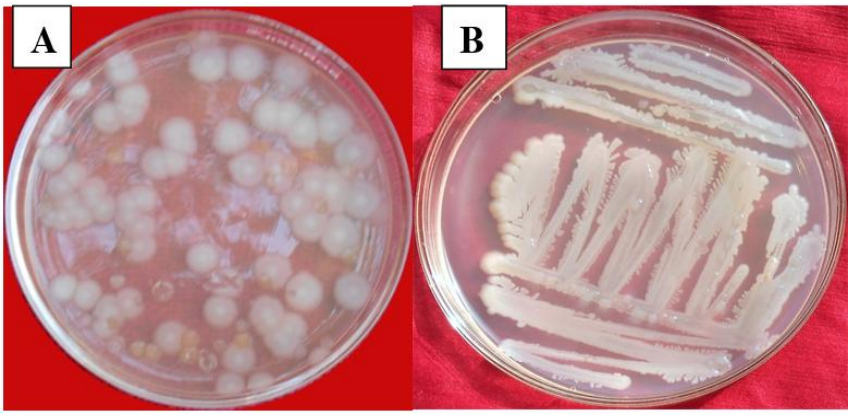


Fig 1: Colony morphology of *B. thuringiensis* strain (A - Creamy white, fried egg like Colonies indicating a *Bacillus* colony; B - Single colony isolation)

3.3. Characterization based on staining

3.3.1 Gram staining

Gram staining method was used to distinguish whether it was a gram positive or gram negative bacteria. Rod shaped and violet color cells indicated the presence of gram positive. *Bacillus* species (Figure 2) and Das *et al*. (2015) [7] also reported similar results.

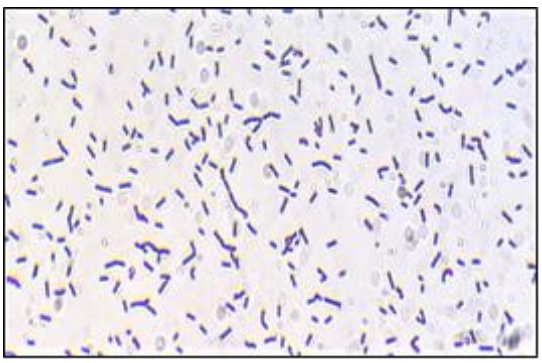


Fig 2: Gram positive *Bacillus* spp

3.3.2 Crystal staining

All isolates were subjected to coomassie brilliant blue staining for further characterization based on crystal morphology. All colonies were examined under light microscope and 288 colonies out of 583 colonies showed the presence of crystal proteins (Figure 3). Nineteen isolated strains possessed three types of crystals *i.e.*, bipyramidal, cuboidal, spherical shape of crystals and spores attached with crystals. Among three types of crystal, cuboidal shape of crystal showed highest frequency (42.36%) followed by bipyramidal crystal (27.43%) and lowest frequency was observed in spherical shape of crystal (10.41%) (Table 4). The present findings are in accordance with the findings of (Ramalakshmi *et al.*, 2010) [23], (Reyaz *et al.*, 2017) [29]. Ammouneh *et al.* (2011) [1] and Kaviyapriya *et al.* (2019) [12] observed bipyramidal and cuboidal parasporal bodies in their studies.

Table 4: Types of crystals present in *B. thuringiensis* isolates

S. No	Crystal morphology	Bt isolates	
		Number	%
1.	Bipyramidal	79	27.43
2.	Cuboidal	122	42.36
3.	Spherical	30	10.41
4.	Spores attached with crystals	57	19.79
Total		288	

3.4 Biochemical characterization

All strains showed positive results to motility test, methyl Red (MR) test, and catalase test. All isolates were negative for voges proskauer (VP) test and isolates KKM 3, KKM 4, KKM 8, KKM 11, KKM 13, KKM 15, KKM16, KKM17 and KKM18 showed negative reaction to starch hydrolysis test. These findings are similar with the findings of Das *et al*. (2015) [7]. El- kersh *et al.* (2016) [10] also reported that isolated *B. thuringiensis* strains showed 90% motile activities.

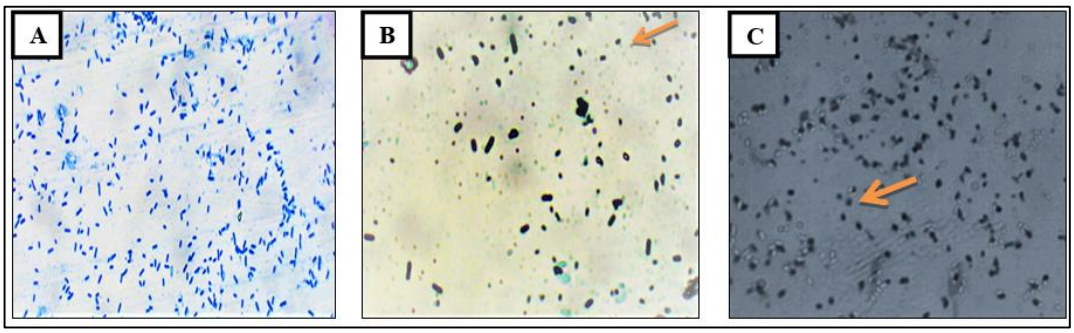


Fig 3: Crystal morphology of *B. thuringiensis* strains A) Vegetative cells B) Cuboidal shape of crystal C) Bipyramidal shape of crystal

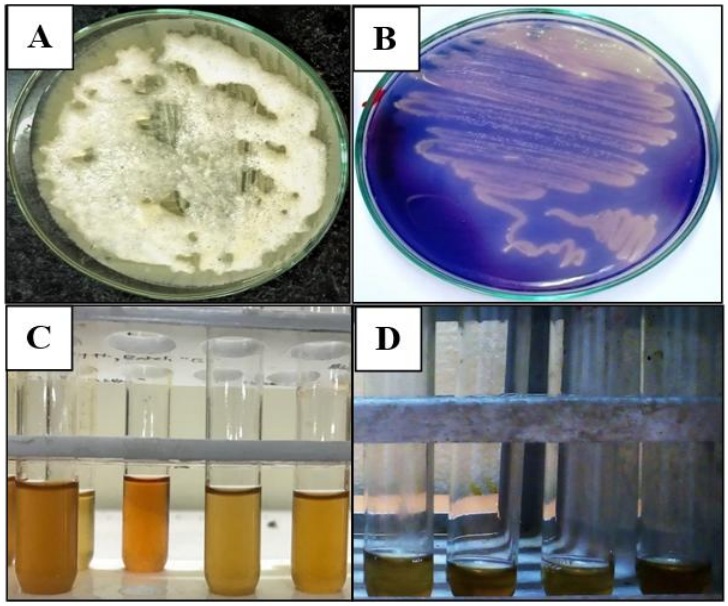


Fig 4: A) Catalase test B) Starch hydrolysis C) Methyl red test D) Voges proskauer test

Table 5: Biochemical characters of *B. thuringiensis* strains

S. No	Isolates	Gram staining	Motility test	Methyl Red (MR) test	Voges Proskauer (VP) test	Starch hydrolysis test
1.	KKM 1	Gram positive	Motile	Positive	Negative	Positive
2.	KKM 2	Gram positive	Motile	Positive	Negative	Positive
3.	KKM 3	Gram positive	Motile	Positive	Negative	Negative
4.	KKM 4	Gram positive	Motile	Positive	Negative	Negative
5.	KKM 5	Gram positive	Motile	Positive	Negative	Positive
6.	KKM 6	Gram positive	Motile	Positive	Negative	Positive
7.	KKM 7	Gram positive	Motile	Positive	Negative	Positive
8.	KKM 8	Gram positive	Motile	Positive	Negative	Negative
9.	KKM 9	Gram positive	Motile	Positive	Negative	Positive
10.	KKM 10	Gram positive	Motile	Positive	Negative	Positive
11.	KKM 11	Gram positive	Motile	Positive	Negative	Negative
12.	KKM 12	Gram positive	Motile	Positive	Negative	Positive
13.	KKM 13	Gram positive	Motile	Positive	Negative	Negative
14.	KKM 14	Gram positive	Motile	Positive	Negative	Positive
15.	KKM 15	Gram positive	Motile	Positive	Negative	Negative
16.	KKM 16	Gram positive	Motile	Positive	Negative	Negative
17.	KKM 17	Gram positive	Motile	Positive	Negative	Negative
18.	KKM 18	Gram positive	Motile	Positive	Negative	Negative
19.	KKM 19	Gram positive	Motile	Positive	Negative	Positive

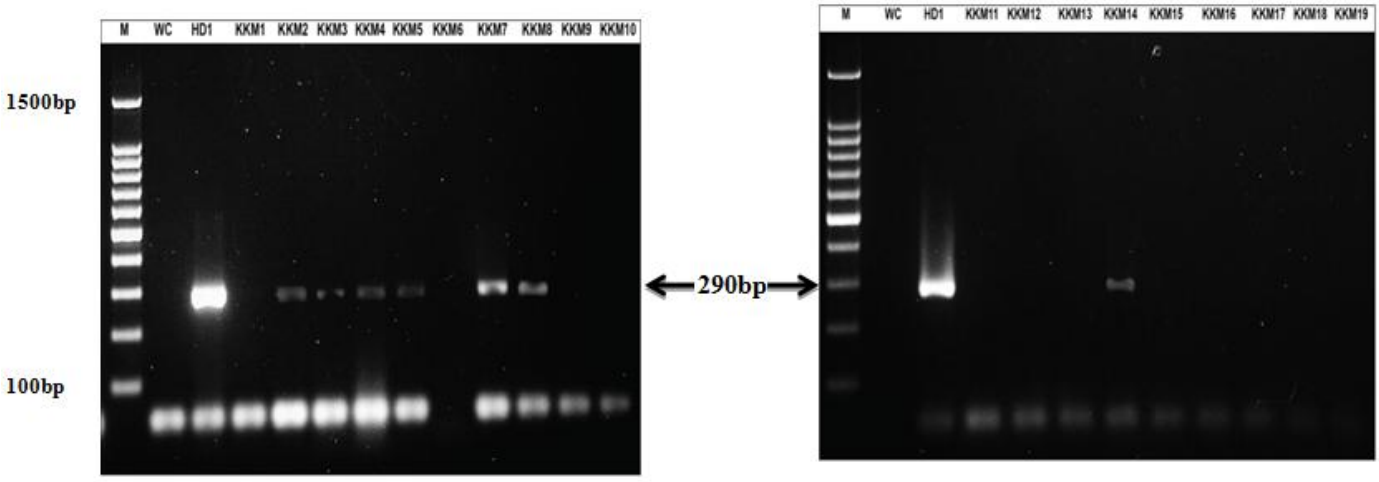
3.5. PCR screening

PCR analysis was performed with three specific primers *i.e.*, *cry1*, *cry2Aa*, *cry2Ab* to screen lepidopteran toxic *cry* genes. Three primers were successfully amplified in the isolates with specific product sizes of 277, 700, and 546 respectively (Figure 4). *cry1* gene was present in 7 isolates with the frequency of

36.84% and 15 native isolates were amplified for *cry2Aa* gene which showed 78.94% frequency. About 17 isolates possessed *cry2Ab* gene which showed high frequency (89.47%) compared to others. These findings are similar to the findings of (Reyaz *et al.*, 2017) [29] and they reported that *cry1, cry2Aa, cry2Ab* genes were amplified in six strains. Djenane *et al.* (2017) [9] also found similar results in PCR amplification and reported that 50% of the isolates harbored *cry1*, *cry2*, or *cry9* genes, and 69.3% contained a *vip3* gene. Wang *et al.* (2003) [35] also found that 70% of the isolates harbouring *cry2* genes.

In this study *cry1* & *cry2Aa* combination was existed in 4 isolates which showed 21.05% frequency and these results were related with Ben-Dov *et al.* (1997) [4], Bravo *et al.* (1998) [6], Kim (2000) [13], Zhang *et al.* (2000) [11], Wang *et al.* (2003) [35], Thammasittirong and Attathom (2008) [32]. Combination of *cry1 & cry 2Ab* gene was present in 6 isolates with frequency of 31.57% whereas 15 isolates had combination of *cry2Aa & cry2Ab* genes with the frequency of 73.68%. The results are agreement with the findings of Liang *et al.* (2011) [17] they revealed that the combination of *cry2Aa/cry2Ab* genes were the most frequently observed in their studies. Combination of *cry1, cry2Aa, cry2Ab* genes were observed in 4 isolates *viz.,* KKM 2, KKM 4, KKM 7, KKM 14 and 21.05% of frequency was observed in this combination. Naqvi *et al.* (2017) [20] also showed the results of triple gene *viz., cry1Ac*, *cry2Ab*, and *EPSPS* combination. Disribution of *B. thuringiensis* and frequency of *cry* genes were represented in Table 1 and 2, respectively. *cry2Ab* gene was abundant in the native isolates compared to other two genes. Mendoza *et al.* (2012) [19] also reported that cry2 gene was abundant in the isolated strains. Reyaz *et al.* (2017) [29] also reported that 90% isolates harbouring *cry2Ab* gene. Lone *et al.* (2017) [18] also found different combinations of *cry* genes in their studies. Reference strain *Bt*- HD1 was amplified for three genes *viz., cry1, cry2Aa, cry2Ab.*

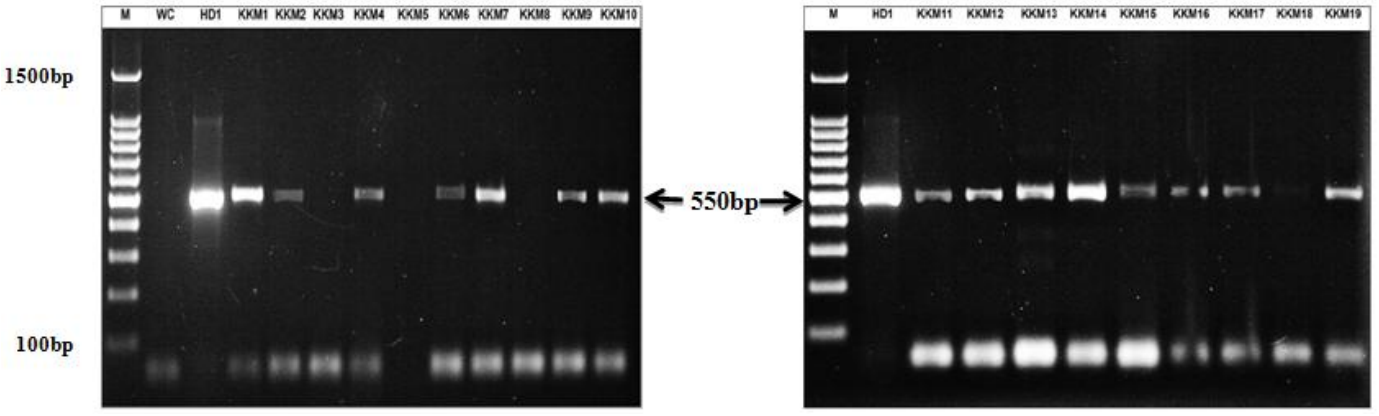
cry1 genes



M: 100bp marker; **WC:** negative control; **HD1:** Positive control; **KKM 1 to 10:** Isolated *Bt* strains

M: 100bp marker; **WC:** negative control; **HD1:** Positive control; **KKM 11 to 19:** Isolated Bt strains

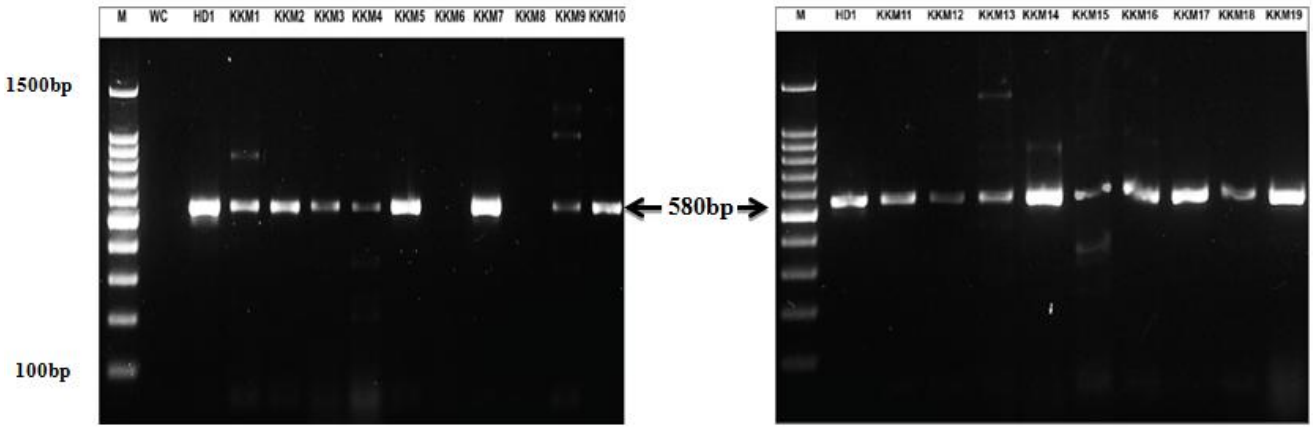
***cry2Aa* genes**



M: 100bp marker; **WC:** negative control; **HD1:** Positive control; **KKM 1 to 10:** Isolated *Bt* strains

M: 100bp marker; **HD1:** Positive control; **KKM 11 to 19:** Isolated *Bt* strains

***Cry2Ab* genes**



M: 100bp marker; **WC:** Negative control; **HD1:** Positive control; **KKM 1 to 10:** Isolated *Bt* strains

M: 100bp marker; **HD1:** Positive control; **KKM 11 to 19:** Isolated *Bt* strains

Fig 5: Amplification of lepidopteran toxic *cry* genes

4. Conclusion

Novel native strains are needed to combat the problems faced by the insect resistance. In this present study, nineteen native *B. thuringiensis* strains were isolated from soil. All isolates were showed the presence of either single or combination of lepidopteran toxic cry genes viz., cry1, cry2Aa, cry2Ab.

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