academicJournals

Vol. 12(22), pp. 3545-3562, 29 May, 2013
DOI: 10.5897/AJB2013.12347
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Profile of *cry* from native *Bacillus thuringiensis* isolates and expression of *cry*1I

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Accepted 27 May, 2013

The characterization of 255 *Bacillus thuringiensis* isolates of Coorg, Sharavatti and BR hills, containing genes known to be active against coleopteran and lepidopteran insect species was done through PCR amplification using the specific and degenerate primers. The isolates were also tested for their insecticidal activity against *Plutella xylostella.* Among the coleopteran specific genes screened, the most predominant was *cry*1I gene which was present in 18 isolates at a frequency of 7.05%. *cry*1 gene was found to be most abundant (35.39%) among the lepidopteran specific genes. A variant of *cry*1I gene based on amplicon restriction fragment length polymorphism (ARFLP) was cloned into pTZ57R/T and subcloned in an expression vector pQE-30 after amplification of a 2169 bp DNA fragment of *cry*1I gene from *B. thuringiensis* DBT189, the sequence which showed 99% homology with known *cry*1Ia gene from *B. thuringiensis* subsp. *kurstaki*. There were six mismatches between the two amino acid sequences. The *cry*1I- type gene consisted of an open reading frame of 2124 bp that would encode for 720 amino acids. An expected band size of 81 kDa was observed after sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) analysis indicating the expression of *cry*1I gene.

Key words: *Bacillus thuringiensis*, *Plutella xylostella*, ARFLP, cloning, SDS-PAGE.

INTRODUCTION

Chemical insecticides have caused an immense emergence of resistant biotypes no longer controlled by major groups of chemical insecticides. This has led to an increased emphasis in environment friendly insect control strategies to ensure sustainability of the environment (Ozturk et al., 2009). One alternative approach that has received attention is the development of *Bacillus thuringiensis* (Bt) toxins as insecticides. *B. thuringiensis* is a Gram positive, facultative anaerobic, motile bacterium which is entamopathogenic (Schnepf et al., 1998). The Cry protein synthesized during sporulation enables it to be pesticidal to a range of insect species.

The genes encoding for the crystal proteins are named as *cry* genes, and their common characteristic is the expression of the genes during the stationary phase. To date, genes encoding the Cry toxins have been classified into 72 groups divided into class and subclasses according to their amino acid similarity (http://www.lifesci.sussex.ac.uk/home/neil_crickmore/Bttoxin.html). Different Cry proteins have various insecticidal spectra. Wang et al. (2003) reported that Cry1, Cry2 and Cry9 groups exhibit strongest activity to lepidopteran insects; the Cry3, Cry7 and Cry8 groups are most toxic to coleopteran insects whereas Cry4 and Cry11 are most

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Abbreviations: IPTG, Isopropyl-β-D-thiogalactopyranoside; **SDS-PAGE,** sodium dodecyl sulphate polyacrylamide gel electrophoresis; **X-gal,** 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

Table 1. Reference strains used in the study.

Original code	BGSC No.	Genes harboured as per previous studies
B. thuringiensis sub.sp.*kurstaki*	HD1	*cry*1I, *cry*18, *cry*23, *cry*26, *cry*28, *cry*1Aa1, *cry*1Ab2, *cry*1Ac1, *cry*1Ia1, *cry*2Aa1, *cry*2Ab1
B. thuringiensis sub sp. *sotto*	4E2	*cry*14
B. thuringiensis sub sp. *japonensis*	4AT1	*cry*7,8; *cry*9
B. thuringiensis sub sp. *tenebrionis*	4AA1	*cry*3
B. thuringiensis sub sp. *fukukaensis*	4AP1	*cry20*
B. thuringiensis sub sp. *kurstaki*	4D4	*cry*1*, cry*2

toxic to dipteran insects.

The type of the toxin gene and the variety of such genes present in a strain of *B. thuringiensis* determine its level of toxicity and the host range (Hofte et al., 1989). Cloning of such toxin genes has helped in studying specific toxins and isolation of novel Bt strains may lead to the discovery of additional insecticidal proteins with higher toxicity and wider spectrum. Novel toxins are also important for providing alternatives to cope with the emergence of resistant insect populations. This study was focused on characterization of Bt strains which would help in understanding the role of Bt in native environment and the diversity of *cry* genes. An attempt was made to clone a variant of *cry*1I based on ARFLP profile which would be helpful in developing new biopesticides with broader and higher spectrum of toxicity against insect pests belonging to order Coleoptera.

MATERIALS AND METHODS

B. thuringiensis strains, plasmids

About 255 *B. thuringiensis* isolates and reference strains *B. thuringiensis* subsp. *kurstaki* HD1, *B. thuringiensis* sub sp. *japonensis* 4AT1, *B. thuringiensis* sub sp. *tenebrionis* 4AA1, *B. thuringiensis* sub sp. *sotto* 4E2 and *B. thuringiensis* sub sp. *kurstaki* 4D4 (Table 1) were obtained from the culture collection maintained at the Department of Biotechnology, UAS, Dharwad, India. About 113 isolates from Coorg were screened for the presence of lepidopteran and coleopteran specific *cry* genes. All 255 isolates inclusive of isolates from both Coorg and BR Hills were used for screening of coleopteran specific *cry* genes. The T/A cloning vector pTZ 57R/T was obtained from InsTAclone PCR Cloning Kit #K1213, Fermentas and pQE-30 from Qiagen (Cat. No. 32915).

Preparation of *B. thuringiensis* culture for bioassay

Individual native isolates harbouring lepidopteran specific genes were screened by following the leaf dip bioassay method against *Plutella xylostella* along with reference strain *B. thuringiensis* subsp. *kurstaki* (HD1). Individual isolates were streaked on plain Luria agar (Maniatis et al., 1982) plates and overnight incubated at 37°C. Overnight, culture was inoculated in 1 ml Luria in Eppendorf tube and kept for growth (sporulation) under shaking condition at 28°C for 24 h. The culture was re-inoculated in 100 ml modified 'G' medium (MGM) broth (Aronson et al., 1971) in a conical flask and kept for 72 h at 30°C on a shaker at 200 rpm. The culture was serially diluted at 9:1 (900 µl of sterile water and 100 µl of culture) and spread on plane Luria Agar plates for taking colony count before arriving at the concentration of *B. thuringiensis* (1.2×10^6 cfu/ml) to assess its toxicity against test insects.

Dosage = 1.2×10^6 cfu/ml/No. of colonies × 1000

Bioassays

Preliminary leaf dip bioassays for strains containing lepidopteran active *cry* genes was performed against *P. xylostella* as described by Tabashnik and Cushing (1987).

PCR analysis for *cry* gene content

The total DNA was isolated following the method described by Sambrook and Russell (2001). The *cry* specific primers were standardized for annealing temperature by gradient PCR for each set of specific and degenerate primers against total DNA of reference strains respectively. The oligonucleotide sequences of PCR primers and expected size of PCR products of each *cry* gene are shown in Table 2a and b. The reaction mixture varied depending on whether it is a gene specific primer or degenerate primer (10 µl for specific primer and 15 µl for degenerate primer). One of the gene namely, *cry*1I was selected for further cloning and analysis of expression.

Amplification of *cry*1I gene

Gene specific primer which was synthesized at Sigma Aldrich Pvt. Ltd., Bangalore, was used for amplification of *cry*1I gene. The forward and reverse primers used were 5' GGATCCATGAAACTAAAGAATCAAGATAAGC 3' and 3' CTGCAGCATGTTACGCTCAATATGGAGT 5', respectively. PCR was performed with 3U Taq DNA polymerase, 1 mM dNTP, 5 pM primer each, 25 mM $MgCl_2$ in a final volume of 100 µl. Amplification was done in an Eppendoff thermal cycler under the following conditions: 5 min of denaturation at 94°C followed by 35 cycles of amplification with a 1 min denaturation at 94°C, 1 min of annealing at 49.6°C, 2 min of extension at 72°C, final extension step of 45 min at 72°C.

Amplicon restriction fragment length polymorphism (ARFLP)

The novelty of the amplified *cry* genes was done through amplicon restriction fragment length polymorphism (ARFLP) as performed in earlier studies (Kuo et al., 1996) and the amplicon that gave a different ARFLP profile as compared to reference strain *B. thuringiensis* var. *kurstaki* was cloned in pTZ57R/T and later expressed in *Escherichia coli* M15 and *E. coli* SG13009.

Table 2a. List of *cry* primers employed for detection of coleopteran specific *cry* genes in individual isolates.

Gene	Sequence	Size (bp)	Reference
*cry*1I (specific)	FP: ACAATTTACAGCTTATTAAG RP: CTACATGTTACGCTCAATAT	1134	This study
*cry*1I (full length)	FP: GGATCCATGAAACTAAAGAATCAAGATAAGC RP: CTGCAGCATGTTACGCTCAATATGGAGT	2169	This study
*cry*3	FP: CGTTATCGCAGAGATGACATTAAC RP: CATCTGTTGTTTCTGGAGGCAAT	589	This study
*cry*7,8	FP: CCCTTTAGCAAACGATCAAACG RP: ATTGGGCGGTACGTGTCACCTGAC	741	This study
*cry*14	FP: ATAATGCGCGACCTACTGTTGT RP: TGCCGTTATCGCCGTTATT	456	This study
*cry*18	FP: CCGAGGCGATTTGGATAGAT RP: TGCCGGTGTAAACAAAGAAGG	419	This study
*cry*26	FP: CGCGCTGTTCAATTATCAAGTGC RP: ATATGGAAAGAAAAGGCGTGTGGA	362	This study
*cry*28	FP: TACAGTCGCTGTAGTAAGCGCA RP: TGACAGCCAAGTAAATAGCCCTG	862	This study
*cry*34	FP: ATGTCAGCTCGCGAAGTACA RP: TATCTCCTGATCCGCTTTGAG	313	This study
*cry*35	FP: AGTCTTGATGATTCAGGTGTTA RP: CAAGGTACTAATGTCCATCCCATT	479	This study
*cry*36	FP: CTTGTGGATGTGGTTGCCAGCAA RP: CCTCCAAATGTTTGAGCAGCTGTA	1399	This study
*cry*23	FP: CTGTATCGTTCACATGGACGGAA RP: AATGCTTCGCAAGCCTTGTGCA	476	This study
*cry*37	FP: AAGTAGCGACACTGGTTCCCCTA RP: CAAGTCGTACTGTTACACCAGG	140	This study
*cry*55	FP: AGCTCAAACGTTCTAGTCCCAG RP: TTGGATCAGGTGTTTGAGTGC	805	This study

Table 2b. List of *cry* primers employed for detection of lepidopteran specific genes in individual isolates.

Gene	Sequence	Size (bp)	Reference
*cry*1 (degenerate)	FP: AGGCGGTGAATGMBCTGTTTAC RP: CGTTTATCHGCCGCRTGAATC	930	Johnson (2011)
*cry*2	FP: GTTATTCTTAATGCAGATGAATGGG RP:CGGATAAAATAATCTGGGAAATAGT	689-701	Ben dov et al. (1997)

Table 2b. Contd

*cry*8 (degenerate)	FP: GATACRGAAACRTATCCAACGT RP:CATATCTWTRRTTCGGTTGRACTGTA	900	Johnson (2011)
*cry*9 (degenerate)	FP: GGTTCTCAAAGATCCGTGTA RP: MDATYTCTAKRTCTTGACTA	1050	Juarez Perez et al. (1997)
*cry*20	FP: CAATCCCTGGCTTCACTCGT RP: CCGCGGGCATTAGGATT	490	Ejiofar and Johnson (2002)
*cry*1Aa1	FP: GGCAACTATACAGATTATGC RP: TCTAGTGAATCGACTGTACC	635	Designed during the study
*cry*1Ab2	FP: AGGAAGTATTAGGAGTCCAC RP: ATATCTCCTCCTGTAAATCC	639	Designed during the study
*cry*1Ac1	FP: TCCTTAGACATTGATGTAGG RP: TCTGTATTGTTCTCGATCTC	680	Designed during the study
*cry*1Ad1	FP: GTCAGGACATCAAATAACAG RP: ATATCTCCTCCTGTAAATCC	546	Designed during the study
*cry*1Ae1	FP: TAGGTGTATGGGTGATATTC RP: AACTTCTTGTGACACTTCTG	536	Designed during the study
*cry*1Ca1	FP: CCAAACTATGACAATAGGAG RP: CCAAGAAAATACTACACCAG	615	Designed during the study
*cry*1Da1	FP: GTAGCAGACATTTCATTAGG RP: ACATGAATAAGGCTAGTCAG	503	Designed during the study
*cry*1Ea1	FP: ATATAGAAGTAGGGGGACAG RP: TAGCCCTAGTTGATTTGTAG	694	Designed during the study
*cry*1Fa1	FP: GATTTGCTAATACAGACGAC RP: CGTGAACTCACTAAGTGTCC	580	Designed during the study
*cry*1Ia1	FP: AGTACCTAGGGTTGATTTTC RP: TGTACCAGTATTCGTTCTTC	379	Designed during the study
*cry*2Aa1	FP: ATGCGTATAATGTAGTAGCC RP: TATCCTTGTATCTGGAACTG	466	Designed during the study
*cry*2Ab1	FP: ATGTATCTATCTGGTCGTTG RP: ACTCCTTAACCCTAAAGTTG	455	Designed during the study
*cry*2Ac1	FP: AAAGCCTTCTAGTATCTTCC RP: TAGAGGTCTTGCTAAATCTG	521	Designed during the study
*cry*9Aa1	FP: ATCGTAGAGAGTGACATTG RP: TGTTGTCCAGAGATTAGTTC	376	Designed during the study
*cry*9Ca1	FP: GGATCTAAATGCAAGTGTAG RP: ACCATTTACATCGTAGTCAC	697	Designed during the study

N.B: M=(A/C), R=(A/G), W=(A/T), S=(G/C), Y=(C/T), K=(G/T), H=(A/C/T), D=(A/G/T), B=(C/G/T).

Molecular cloning and nucleotide sequencing

PCR amplified products were ligated to the T/A cloning vector pTZ57R/T (Sambrook and Russell, 2001) using the Fermentas DNA ligation kit. The transformed cells were spread on LB agar plates containing X-gal (20 mg/ml), Isopropyl-β-D-thiogalactopyranoside

(IPTG) (24 mg/ml) and ampicillin (100 µg/ml). The plates were then incubated at 37°C for 12 to 16 h and the transformed colonies were further streaked on Luria agar with ampicillin (100 µg/ml). The confirmation for the presence of desired DNA fragment in cloning vector was done by PCR using gene specific primers and by restriction analysis. Nucleotide sequencing was done by using M13 forward and reverse primer at Chromous Biotech Pvt. Ltd., Bangalore. In order to express the *cry*1I gene, the construct containing *cry*1I was inserted into the multiple cloning site of an expression vector pQE30 to generate the recombinant expression construct pAPK3A01. The complete amplified gene was gel purified using the Mini Elute PCR purification kit (Qiagen) according to the manufacturer's instructions. The insert sequence and its reading frame were confirmed by *Bam*HI and *Pst*I digestion. The ligated product was first transferred into *E. coli* JM109 cells for maintenance and then into *E. coli* M15 (pREP4) (Qiagen) and *E. coli* SG13009 (pREP) (Qiagen) for expression analysis.

For confirmation of the clones, the plasmid was isolated by using alkaline lysis protocol of Birmboim and Doly (1979) and restriction analysis was done for the plasmids of selected clones by using *Bam*HI and *Pst*I restriction endonucleases.

Protein analysis and expression studies

For protein analysis, about 5 ml of Luria broth with kanamycin (50 mg/ml) and ampicillin (50 mg/ml) was inoculated with a colony of *E. coli* containing the recombinant construct and incubated at 37°C overnight under shaking conditions. Overnight grown culture was diluted in fresh Luria broth in 1:100 ratio without selection pressure and incubated at 37°C until the culture reached the log phase of growth (A550-0.5 to 1.0) under shaking conditions which will take approximately 3 h. The expression of target protein was induced based on the optimal values of IPTG (1 mM) concentration and it was again incubated for 5 h at 37°C in a shaker. After induction, the protein was extracted and analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). For extraction of proteins, the cell culture was centrifuged at 13,000 rpm for 1 min at room temperature. The pellet was resuspended in 100 µl of $T_{10}E_1$ and 100 µl of 1X SDS gel loading buffer added to it.

The mixture was heated at 90°C on a thermo mixer and centrifuged for 10 min at 4°C. The supernatant was collected in micro centrifuge tubes and protein was quantified by using NanoDrop. The protein preparations were analysed by SDS-PAGE as described by Sambrook et al. (2001).

Statistical analysis

For all investigated parameters, the analysis of variance (ANOVA) was performed using the MSTATC software. The measurements of treatments were compared using Duncan's multiple range tests at the 0.01 significance level.

RESULTS AND DISCUSSION

Bioassay for insecticidal activity

At 24 h, the mean percent mortality ranged between 0 to 43.33% mortality. The highest mortality was recorded in DBT153 (43.33%) followed by DBT2564 (33.33%), while in reference strain HD1, the mortality was 46.66% (Table 3 and Plate 1). The cumulative mean percent mortality after 48 h exposure ranged between 0 to 53.33%. The highest mortality was recorded in native isolate DBT153 (53.33%), followed by DBT2564 (43.33%) and DBT112 (36.66%) compared to reference strain *B. thuringiensis* subsp *kurstaki* (HD1) which showed mortality of 73.33%. At 72 h exposure, the cumulative mortality of third instar larvae of *P. xylostella* ranged from 0 to 100%. The reference strain HD1 exhibited 100% mortality, whereas DBT153 exhibited 83.33% mortality. The mortality range of isolates was less as compared to previous reports.

Higher toxicity of native isolates than reference strain (HD1) against *P. xylostella* was reported earlier by Shilpa (2005) and Marutesh (2007).

cry profiling of native isolates

The melting temperatures were standardized for each primer set. The specific amplicons obtained for some of the genes is shown in Plate 2. Most of the isolates showed amplification of at least one *cry* gene. PCR can be used for prediction of toxicity of isolates (Salehi et al., 2008). Among the coleopteran specific *cry* genes, the most predominant was *cry*1I gene which was present in 18 native isolates at a frequency of 7.05%. *cry*7,8 and *cry*3 were present in almost equal frequency of 6.66 and 6.27%, respectively (Figure 1). Similar results were reported by Nazarian et al. (2009) which was the first exploration in which *B. thuringiensis* isolates were screened almost for all coleopteran-active *cry* genes (19 *cry* genes) and *cry*1I was found to be in 48.5% frequency. *cry*1I and *cry*7,8 were found to be predominant in a previous study (Mahadeva et al., 2011). About 13 isolates amplified for *cry*14. *cry*18 was present in six isolates. *cry*26 and *cry*36 were present in an equal frequency of 5.49%. About 4.13% of the isolates contained *cry*34 and *cry*35. *cry*28 was present in four isolates. Only one isolate amplified for *cry*55 and *cry*23. None of the isolates amplified for *cry*37. Among the reference strains HD1 amplified for *cry*1I, *cry*18, *cry*26, *cry*28, *cry*23, *cry*1, *cry*1Aa1, *cry*1Ab2, *cry*1Ac1, *cry*1Ia1, *cry*2Aa1 and *cry*2Ab1; 4AA1 amplified for *cry*3; 4AT1 amplified for *cry*7,8; *cry*8 and *cry*9; 4E2 amplified for *cry*14.

Many of the isolates harboured more than one *cry* genes. DBT178 harboured *cry*28, *cry*34 and *cry*35 whereas *cry*34, *cry*35 and *cry*36 were present in DBT202. DBT211 contained *cry*1I, *cry*34 and *cry*35. DBT340 had *cry*3, *cry*7,8; *cry*26 and *cry*36. *cry*3, *cry*26 and *cry*36 were present in DBT344. Only one isolate amplified for *cry*55 namely, DBT333 (Table 4). It was also found that the toxicity correlated with the number of *cry* genes in an individual isolate namely, DBT112 harboured 5 *cry* genes (*cry*2, *cry*1Aa1, *cry*1Ac1, *cry*1Ae1, *cry*2Ab1) and showed 76.66% mortality. Similarly, DBT111 harboured three *cry* genes (*cry*2Ab1, *cry*1I, *cry*26) and showed 60% mortality after 72 h of treatment. Among the genes screened which are specific for lepidopteran pests, *cry*1 gene was found to be most abundant (35.39%) followed by *cry*2 (33.62%) (Figure 2). High frequencies of *cry*1 and *cry*2 genes

Table 3. Efficacy of native isolates of *Bacillus thuringiensis* against DBM [*Plutella xylostella* (L.)].

S/N	Isolate	Mean% mortality at different intervals after treatment			S/N	Isolate	Mean% mortality at different intervals after treatment		
		Diamondback moth					Diamond back moth		
		24 HAT	48 HAT	72 HAT			24 HAT	48 HAT	72 HAT
Coorg Isolate									
1	DBT100	3.33 (6.14)^{fg}	6.66 (12.28)^{ijk}	6.66 12.28)st	58	DBT 157	10 (18.42)^{def}	16.6 (23.84)^{efghi}	30 (33.19)^{lmno}
2	DBT101	16.66 (23.84)^{cde}	16.6 (23.84)^{efghi}	26.66 30.98)^{mnop}	59	DBT 158	3.33 (6.14)^{fg}	6.66 (12.28)^{ijk}	6.66 (12.28)st
3	DBT102	6.66 (12.28)^{efg}	6.66 (12.38)^{ijk}	10 (18.42)^{rs}	60	DBT 159	3.33 (6.14)^{fg}	10 (18.42)^{ghij}	10 (18.42)^{rs}
4	DBT 103	13.33 (21.13)^{cde}	13.33 (21.13)^{fghi}	23.33 (28.76)^{nopq}	61	DBT 160	13.33 (21.13)^{cde}	16.6 (23.84)^{efghi}	20 (26.55)^{opq}
5	DBT 104	0 (0.00)^{g}	0 (0.00)^{k}	0 (0.00)^{u}	62	DBT161	23.33 (28.76)^{bcd}	30 (33.19)^{cdef}	36.66 (37.20)^{jklm}
6	DBT 105	0 (0.00)^{g}	0 (0.00)^{k}	0 (0.00)^{u}	63	DBT 162	23.33 (28.76)^{bcd}	30 (33.19)^{cdef}	50 (44.98)^{ghij}
7	DBT 106	16.66 (23.84)^{cde}	30 (33.19)^{cdef}	40 (39.21)^{jkl}	64	DBT 163	13.33 (21.13)^{cde}	16.6 (23.84)^{efghi}	20 (26.55)^{opq}
8	DBT 107	3.33 (6.14)^{fg}	6.66 (12.28)^{ijk}	10 (18.42)^{rs}	65	DBT 164	16.66 (23.84)^{cde}	23.3 (28.76)^{cdefg}	26.66 (30.98)^{mnop}
9	DBT 108	6.66 (12.28)^{efg}	6.66 (12.28)^{ijk}	6.66 (12.28)st	66	DBT 165	10 (18.42)^{def}	16.6 (23.84)^{efghi}	20 (26.55)^{opq}
10	DBT 109	23.33 (28.76)^{bcd}	26.6 (30.98)^{cdefg}	46.66 (43.05)^{hij}	67	DBT 166	16.66 (23.84)^{cde}	23.3 (28.76)^{cdefg}	30 (33.19)^{lmno}
11	DBT 110	10 (18.42)^{def}	20 (26.55)^{defgh}	30 (33.19)^{lmno}	68	DBT 167	6.66 (12.28)^{efg}	10 (18.42)^{ghij}	10 (18.42)^{rs}
12	DBT 111	23.33 (28.76)^{bcd}	30 (33.19)^{cdefg}	60 (50.74)^{defg}	69	DBT 168	0 (0.00)^{g}	0 (0.00)^{k}	0 (0.00)^{u}
13	DBT 112	30 (33.19)^{abc}	36.66 (37.20)^{bcd}	76.66 (61.19)^{bc}	70	DBT 169	13.33 (21.13)^{cde}	16.6 (23.84)^{efghi}	20 (26.55)^{opq}
14	DBT 113	6.66 (12.28)^{efg}	16.6 (23.84)^{efghi}	30 (33.19)^{lmno}	71	DBT 170	16.66 (23.84)^{cde}	26.6 (30.9)^{cdefg}	40 (39.21)^{jkl}
15	DBT 114	13.33 (21.13)^{cde}	23.3 (28.76)^{cdefg}	40 (39.21)^{jkl}	72	DBT 171	26.6 (30.98)^{abcd}	36.66 (37.20)^{bcd}	46.66 (43.05)^{hij}
16	DBT 115	16.66 (23.84)^{cde}	26.6 (30.98)^{cdefg}	50 (44.98)^{ghij}	73	DBT 172	23.3 (28.76)^{bcd}	26.66(30.98)^{cdefg}	60 (50.74)^{defg}
17	DBT 116	6.66 (12.28)^{efg}	6.66 (12.28)^{ijk}	6.66 (12.28)st	74	DBT 173	20 (26.55)^{bcd}	23.3 (28.76)^{cdefg}	23.33 (28.76)^{nopq}
18	DBT 117	0 (0.00)^{g}	0 (0.00)^{k}	0 (0.00)^{u}	75	DBT 174	13.33 (21.13)^{cde}	26.6 (30.9)^{cdefg}	33.33 (35.20)^{klmn}
19	DBT 118	26.66(30.98)^{abcd}	30 (33.19)^{cdefg}	70 (56.76)^{cd}	76	DBT 175	13.33 (21.13)^{cde}	16.6 (23.84)^{efghi}	16.66 (23.84)^{pqr}
20	DBT 119	13.33 (21.13)^{cde}	20 (26.55)^{defgh}	26.66 (30.98)^{mnop}	77	DBT 176	16.66 (23.84)^{cde}	26.6 (30.9)^{cdefg}	40 (39.21)^{jkl}
21	DBT 120	13.33 (21.13)^{cde}	13.33 (21.13)^{fghi}	16.66 (23.84)^{pqr}	78	DBT 177	13.33 (21.13)^{cde}	20 (26.55)^{defgh}	20 (26.55)^{opq}
22	DBT 121	10 (18.42)^{def}	10 (18.42)^{ghij}	10 (18.42)^{rs}	79	DBT 178	13.33 (21.13)^{cde}	26.6 (30.9)^{cdefg}	33.33 (35.20)^{klmn}
23	DBT 122	20 (26.55)^{cde}	20 (26.55)^{defgh}	20 (26.55)^{opq}	80	DBT 179	10 (18.42)^{def}	13.33 (21.13)^{fghi}	13.33 (21.13)^{qr}
24	DBT 123	10 (18.42)^{def}	13.33 (21.13)^{fghi}	13.33 (21.13)^{qr}	81	DBT 180	26.6 (30.98)^{abcd}	30 (33.19)^{cdef}	66.66 (54.76)^{cde}
25	DBT 124	3.33 (6.14)^{fg}	6.66 (12.28)^{ijk}	6.66 (12.28)st	82	DBT 181	13.33 (21.13)^{cde}	20 (26.55)^{defgh}	26.66 (30.98)^{mnop}
26	DBT 125	6.66 (12.28)^{efg}	6.66 (12.28)^{ijk}	6.66 (12.28)st	83	DBT 182	16.66 (23.84)^{cde}	23.3 (28.76)^{cdefg}	23.33 (28.76)^{nopq}
27	DBT 126	6.66 (12.28)^{efg}	10 (18.42)^{ghij}	10 (18.42)^{rs}	84	DBT 183	23.3 (28.76)^{bcd}	26.6 (30.98)^{cdefg}	33.33 (35.20)^{klmn}
28	DBT 127	0 (0.00)^{g}	0 (0.00)^{k}	0 (0.00)^{u}	85	DBT 184	10 (18.42)^{def}	13.33 (21.13)^{fghi}	20 (26.55)^{opq}
29	DBT 128	13.33 (21.13)^{cde}	13.33 (21.13)^{fghi}	20 (26.55)^{opq}	86	DBT 185	6.66 (12.28)^{efg}	13.33 (21.13)^{fghi}	23.33 (28.76)^{nopq}
30	DBT 129	16.66 (23.84)^{cde}	23.3 (28.76)^{cdefg}	36.66 (37.20)^{jklm}	87	DBT 186	13.33 (21.13)^{cde}	23.3 (28.76)^{cdefg}	46.66 (43.05)^{hij}
31	DBT 130	13.33 (21.13)^{cde}	20 (26.55)^{defgh}	20 (26.55)^{opq}	88	DBT 187	16.66 (23.84)^{cde}	26.6 (30.98)^{cdefg}	53.33 (46.90)^{fghi}
32	DBT 131	3.33 (6.14)^{fg}	6.66 (12.28)^{ijk}	10 (18.42)^{rs}	89	DBT 188	13.33 (21.13)^{cde}	16.6 (23.84)^{efghi}	23.33 (28.76)^{nopq}
33	DBT 132	16.66 (23.84)^{cde}	16.6 (23.84)^{efghi}	23.33 (28.76)^{nopq}	90	DBT 189	3.33 (6.14)^{fg}	6.66 (12.28)^{ijk}	10 (18.42)^{rs}

Table 3. Contd

34	DBT 133	0 (0.00)^{g}	0 (0.00)^{k}	0 (0.00)^{u}	91	DBT 190	20 (26.55)^{cde}	26.6 (30.98)^{cdefg}	50 (44.98)^{ghij}
35	DBT 134	13.33 (21.13)^{cde}	13.33 (21.13)^{fghi}	23.33 (28.76)^{nopq}	92	DBT 191	23.3 (28.76)^{bcd}	26.6 (30.9)^{cdefg}	36.66 (37.20)^{jklm}
36	DBT 135	0 (0.00)^{fg}	0 (0.00)^{k}	0 (0.00)^{u}	93	DBT 192	10 (18.42)^{def}	16.6 (23.84)^{efghi}	30 (33.19)^{lmno}
37	DBT 136	6.66 (12.28)^{efg}	6.66 (12.28)^{ijk}	10 (18.42)^{rs}	94	DBT 193	6.66 (12.28)^{efg}	13.33 (21.13)^{fghi}	13.33 (21.13)^{qr}
38	DBT 137	6.66 (12.28)^{efg}	13.33 (21.13)^{fghi}	16.66 (23.84)^{pqr}	95	DBT 194	6.66 (12.28)^{efg}	16.6 (23.84)^{efghi}	20 (26.55)^{opq}
39	DBT 138	3.33 (6.14)^{fg}	3.33 (6.14)^{jk}	3.33 (6.14)^{tu}	96	DBT 195	6.66 (12.28)^{efg}	10 (18.42)^{ghij}	13.33 (21.13)^{qr}
40	DBT 139	16.66 (23.84)^{cde}	20 (26.55)^{defgh}	30 (33.19)^{lmno}	97	DBT 196	16.66 (23.84)^{cde}	26.6 (30.9)^{cdefg}	40 (39.21)^{jkl}
41	DBT 140	0 (0.00)^{g}	0 (0.00)^{k}	0 (0.00)^{u}	98	DBT 197	6.66 (12.28)^{efg}	6.66 (12.28)^{ijk}	13.33 (21.13)^{qr}
42	DBT 141	13.33 (21.13)^{cde}	16.6 (23.84)^{efghi}	20 (26.55)^{opq}	99	DBT 198	16.66 (23.84)^{cde}	20 (26.55)^{defgh}	23.33 (28.76)^{nopq}
43	DBT 142	3.33 (6.14)^{fg}	6.66 (12.28)^{ijk}	10 (18.42)^{rs}	100	DBT 199	23.33 (28.76)^{bcd}	30 (33.19)^{cdef}	46.66 (43.05)^{hij}
44	DBT 143	6.66 (12.28)^{efg}	10 (18.42)^{ghij}	10 (18.42)^{rs}	101	DBT 200	13.33 (21.13)^{cde}	26.6 (30.9)^{cdefg}	40 (39.21)^{jkl}
45	DBT 144	16.66 (23.84)^{cde}	20 (26.55)^{defgh}	30 (33.19)^{lmno}	102	DBT 201	16.66 (23.84)^{cde}	20 (26.55)^{defgh}	26.66 (30.98)^{mnop}
46	DBT 145	0 (0.00)^{g}	0 (0.00)^{k}	0 (0.00)^{u}	103	DBT 202	6.66 (12.28)^{efg}	13.33 (21.13)^{fghi}	16.66 (23.84)^{pqr}
47	DBT 146	6.66 (12.28)^{efg}	6.66 (12.28)^{ijk}	10 (18.42)^{rs}	104	DBT 203	0 (0.00)^{g}	6.66 (12.28)^{ijk}	10 (18.42)^{rs}
48	DBT 147	16.66 (23.84)^{cde}	20 (26.55)^{defgh}	26.66 (30.98)^{mnop}	105	DBT 204	0 (0.00)^{g}	0 (0.00)^{k}	0 (0.00)u
49	DBT 148	10 (18.42)^{def}	13.33 (21.13)^{fghi}	16.66 (23.84)^{pqr}	106	DBT 205	13.33 (21.13)^{cde}	16.6 (23.84)^{efghi}	23.33 (28.76)^{nopq}
50	DBT 149	0 (0.00)^{g}	0 (0.00)^{k}	0 (0.00)^{u}	107	DBT 206	13.33 (21.13)^{cde}	26.6 (30.9)^{cdefg}	43.33 (41.13)^{ijk}
51	DBT 150	0 (0.00)^{g}	0 (0.00)^{k}	0 (0.00)^{u}	108	DBT 207	23.33 (28.76)^{bcd}	30(33.19)^{cdef}	53.33 (46.90)^{fghi}
52	DBT 151	16.66 (23.84)^{cde}	26.6 (30.9)^{cdefg}	36.66 (37.20)^{jklm}	109	DBT 208	13.33 (21.13)^{cde}	20 (26.55)^{defgh}	33.33 (35.20)^{klmn}
53	DBT 152	10 (18.42)^{def}	16.6 (23.84)^{efghi}	20 (26.55)^{opq}	110	DBT 209	3.33 (6.14)^{fg}	10 (18.42)^{ghij}	13.33 (21.13)^{qr}
54	DBT 153	43.33 (41.13)^{ab}	53.33 (46.90)^{b}	83.33 (66.11)^{b}	111	DBT 210	0 (0.00)^{g}	3.33 (6.14)^{jk}	10 (18.42)^{rs}
55	DBT 154	3.33 (6.14)^{fg}	6.66 (12.28)^{ijk}	10 (18.42)^{rs}	112	DBT 211	3.33 (6.14)^{fg}	10 (14.99)^{hij}	13.33 (21.13)^{qr}
56	DBT 155	20 (26.55)^{cde}	26.6 (30.9)^{cdefg}	40 (39.21)^{jkl}	113	DBT 212	13.33 (21.13)^{cde}	16.6 (23.84)^{efghi}	30 (33.19)^{lmno}
57	DBT 156	3.33 (6.14)^{fg}	6.66 (12.28)^{ijk}	6.66 (12.28)st					
114	DBT 2514	10 (18.42)^{def}	13.33 (21.13)^{fghi}	20 (26.55)^{opq}	157	DBT 2557	20 (26.55)c^{de}	23.3 (28.76)c^{defg}	33.33 (35.20)^{klmn}
115	DBT 2515	13.33 (21.13)^{cde}	20 (26.55)^{defgh}	30 (33.19)^{lmno}	158	DBT 2558	0 (0.00)^{g}	0 (0.00)^{k}	0 (0.00)^{u}
116	DBT 2516	3.33 (6.14)^{fg}	3.33 (6.14)^{jk}	10 (18.42)^{rs}	159	DBT 2559	6.66 (12.28)^{efg}	13.33 (21.13)^{fghi}	23.33 (28.76)^{nopq}
117	DBT 2517	0 (0.00)^{g}	3.33 (6.14)^{jk}	10 (18.42)^{rs}	160	DBT 2560	0 (0.00)^{g}	0 (0.00)^{k}	0 (0.00)u
118	DBT 2518	13.33 (21.13)^{cde}	16.6 (23.84)^{efghi}	26.6 (30.98)^{mnop}	161	DBT 2561	3.33 (6.14)^{fg}	3.33 (6.14)^{jk}	10 (18.42)^{rs}
119	DBT 2519	26.66 (30.98)^{abcd}	30 (33.19)^{cdef}	43.33 (41.13)^{ijk}	162	DBT 2562	13.3 (21.13)^{cde}	20 (26.55)^{defgh}	66.66 (54.76)^{cde}
120	DBT 2520	3.33 (6.14)^{fg}	6.66 (12.28)^{ijk}	10 (18.42)^{rs}	163	DBT 2563	3.33 (6.14)^{fg}	3.33 (6.14)^{jk}	10 (18.42)^{rs}
121	DBT 2521	3.33 (6.14)^{fg}	6.66 (12.28)^{ijk}	20 (26.55)^{opq}	164	DBT 2564	33.33(35.20)^{abc}	43.336 (41.13)^{bc}	76.66 (61.19)^{bc}
122	DBT 2522	16.6 (23.84)^{cde}	23.3 (28.76)^{cdefg}	33.33 (35.20)^{klmn}	165	DBT 2565	0 (0.00)^{g}	0 (0.00)^{k}	3.33 (6.14)^{t}u
123	DBT 2523	3.33 (6.14)^{fg}	3.33 (6.14)^{jk}	10 (18.42)^{rs}	166	DBT 2566	6.66 (12.28)^{efg}	10 (18.42)^{ghij}	16.66 (23.84)^{pqr}
124	DBT 2524	6.66 (12.28)^{efg}	6.66 (12.28)^{ijk}	20 (26.55)^{opq}	167	DBT 2567	0 (0.00)^{g}	0 (0.00)^{k}	0 (0.00)u
125	DBT 2525	6.66 (12.28)^{efg}	16.6 (23.84)^{efghi}	26.66 (30.98)^{mnop}	168	DBT 2568	6.66 (12.28)^{efg}	10 (18.42)^{ghij}	16.66 (23.84)^{pqr}
126	DBT 2526	0 (0.00)^{g}	3.33 (6.14)^{jk}	10 (18.42)^{rs}	169	DBT 2569	0 (0.00)^{g}	0 (0.00)^{k}	0 (0.00)u

Table 3. Contd

127	DBT 2527	6.66 (12.28)^{efg}	6.66 (12.28)^{ijk}	20 (36.55)^{opq}	170	DBT 2570	6.66 (12.28)^{efg}	16.6 (23.84)^{efghi}	30 (33.19)^{lmno}
128	DBT 2528	13.33 (21.13)^{cde}	16.6 (23.84)^{efghi}	26.66 (30.98)^{mnop}	171	DBT 2571	0 (0.00)^{g}	3.33 (6.14)^{jk}	6.66 (12.28)st
129	DBT 2529	0 (0.00)^{g}	3.33 (6.14)^{jk}	10 (18.42)^{rs}	172	DBT 2572	16.6 (23.84)^{cde}	23.3 (28.76)^{cdefg}	33.33 (35.2)^{klmn}
130	DBT 2530	3.33 (6.14)^{fg}	6.66 (12.28)^{ijk}	10 (18.42)^{rs}	173	DBT 2573	16.6(23.84)^{cde}	23.3 (28.76)^{cdefg}	40 (39.21)^{jkl}
131	DBT 2531	6.66 (12.28)^{fg}	6.66 (12.28)^{ijk}	20 (26.55)^{opq}	174	DBT 2574	13.3(21.13)^{cde}	26.6 (30.9)^{cdefg}	33.33 (35.20)^{klmn}
132	DBT 2532	0 (0.00)^{g}	3.33 (6.14)^{jk}	10 (18.42)^{rs}	175	DBT 2575	0 (0.00)^{g}	0 (0.00)^{k}	0 (0.00)u
133	DBT 2533	10 (18.42)^{def}	10 (18.42)^{ghij}	26.66 (30.98)^{mnop}	176	DBT 2576	0 (0.00)^{g}	0 (0.00)^{k}	0 (0.00)u
134	DBT 2534	0 (0.00)^{g}	0 (0.00)^{k}	0 (0.00)u	177	DBT 2577	6.66 (12.28)^{efg}	13.3 (21.13)^{fghi}	20 (26.55)^{opq}
135	DBT 2535	3.33 (6.14)^{fg}	6.66 (12.28)^{ijk}	10 (18.42)^{rs}	170	DBT 2570	6.66 (12.28)^{efg}	16.6 (23.84)^{efghi}	30 (33.19)^{lmno}
136	DBT 2536	6.66 (12.28)^{efg}	6.66 (12.28)^{ijk}	13.33 (21.13)^{qr}	171	DBT 2571	0 (0.00)^{g}	3.33 (6.14)^{jk}	6.66 (12.28)st
137	DBT 2537	3.33 (6.14)^{fg}	3.33 (6.14)^{jk}	6.66 (12.28)st	172	DBT 2572	16.6 (23.84)^{cde}	23.3 (28.76)^{cdefg}	33.33 (35.2)^{klmn}
138	DBT 2538	0 (0.00)^{g}	3.33 (6.14)^{jk}	10 (18.42)^{rs}	173	DBT 2573	16.6(23.84)^{cde}	23.3 (28.76)^{cdefg}	40 (39.21)^{jkl}
139	DBT 2539	6.66 (12.28)^{efg}	10 (18.42)^{ghij}	16.66 (23.84)^{pqr}	174	DBT 2574	13.3(21.13)^{cde}	26.6 (30.9)^{cdefg}	33.33 (35.20)^{klmn}
140	DBT 2540	3.33 (6.14)^{fg}	3.33 (6.14)^{jk}	10 (18.42)^{rs}	175	DBT 2575	0 (0.00)^{g}	0 (0.00)^{k}	0 (0.00)u
141	DBT 2541	0 (0.00)^{g}	3.33 (6.14)^{jk}	10 (18.42)^{rs}	176	DBT 2576	0 (0.00)^{g}	0 (0.00)^{k}	0 (0.00)u
142	DBT 2542	13.33 (21.13)^{cde}	16.6 (23.84)^{efghi}	26.66 (30.98)^{mnop}	177	DBT 2577	6.66 (12.28)^{efg}	13.3 (21.13)^{fghi}	20 (26.55)^{opq}
143	DBT 2543	3.33 (6.14)^{fg}	3.33 (6.14)^{jk}	6.66 (12.28)st	178	DBT 2578	3.33 (6.14)^{fg}	13.3 (21.13)^{fghi}	23.33 (28.76) ^{nopq}
144	DBT 2544	33.33(35.20)^{cde}	23.3 (28.76)^{cdefg}	63.33 (52.75)^{def}	179	DBT 2579	3.33 (6.14)^{fg}	3.33 (6.14)^{jk}	6.66 (12.28)st
145	DBT 2545	13.33 (21.13)^{cde}	20 (26.55)^{defgh}	30 (33.19)^{lmno}	180	DBT 2580	3.33 (6.14)^{fg}	10 (18.42)^{ghij}	16.66 (23.84)^{pqr}
135	DBT 2535	3.33 (6.14)^{fg}	6.66 (12.28)^{ijk}	10 (18.42)^{rs}	181	DBT 2581	6.66 (12.28)^{efg}	10 (18.42)^{ghij}	20 (26.55)^{opq}
136	DBT 2536	6.66 (12.28)^{efg}	6.66 (12.28)^{ijk}	13.33 (21.13)^{qr}	182	DBT 2582	6.66 (12.28)^{efg}	13.33 (21.13)^{fghi}	13.33 (21.13)^{qr}
137	DBT 2537	3.33 (6.14)^{fg}	3.33 (6.14)^{jk}	6.66 (12.28)st	183	DBT 2583	0 (0.00)^{g}	0 (0.00)^{k}	0 (0.00)u
138	DBT 2538	0 (0.00)^{g}	3.33 (6.14)^{jk}	10 (18.42)^{rs}	184	DBT 2584	13.33 (21.13)^{cde}	23.3 (28.76)^{cdefg}	33.33 (35.20)^{klmn}
139	DBT 2539	6.66 (12.28)^{efg}	10 (18.42)^{ghij}	16.66 (23.84)^{pqr}	185	DBT 2585	6.66 (12.28)^{efg}	13.33 (21.13)^{fghi}	26.66 (30.98)^{mnop}
140	DBT 2540	3.33 (6.14)^{fg}	3.33 (6.14)^{jk}	10 (18.42)^{rs}	186	DBT 2586	3.33 (6.14)^{fg}	6.66 (12.28)^{ijk}	10 (18.42)^{rs}
141	DBT 2541	0 (0.00)^{g}	3.33 (6.14)^{jk}	10 (18.42)^{rs}	187	DBT 2587	3.33 (6.14)^{fg}	3.33 (6.14)^{jk}	6.66 (12.28)st
142	DBT 2542	13.33 (21.13)^{cde}	16.6 (23.84)^{efghi}	26.66 (30.98)^{mnop}	188	DBT 2588	3.33 (6.14)^{fg}	10 (18.42)^{ghij}	20 (26.55)^{opq}
143	DBT 2543	3.33 (6.14)^{fg}	3.33 (6.14)^{jk}	6.66 (12.28)st	189	DBT 2589	16.6 (23.84)^{cde}	23.33(28.76)^{cdefg}	56.66 (48.82)^{efgh}
144	DBT 2544	33.33(35.20)^{cde}	23.3 (28.76)^{cdefg}	63.33 (52.75)^{def}	190	DBT 2590	3.33 (6.14)^{fg}	6.66 (12.28)^{ijk}	13.33 (21.13)^{qr}
145	DBT 2545	13.33 (21.13)^{cde}	20 (26.55)^{defgh}	30 (33.19)^{lmno}	191	DBT 2591	0 (0.00)^{g}	3.33 (6.14)^{jk}	10 (18.42)^{rs}
146	DBT 2546	26.66 (30.98)^{abcd}	33.33 (35.20)^{cde}	43.33 (41.13)^{ijk}	192	DBT 2592	0 (0.00)^{g}	0 (0.00)^{k}	0 (0.00)u
147	DBT 2547	3.33 (6.14)^{fg}	6.66 (12.28)^{ijk}	20 (26.55)^{opq}	193	DBT 2593	10 (18.42)^{def}	16.6 (23.84)^{efghi}	23.33 (28.76)^{nopq}
148	DBT 2548	6.66 (12.28)^{efg}	10 (18.42)^{ghij}	16.66 (23.84)^{pqr}	178	DBT 2578	3.33 (6.14)^{fg}	13.3 (21.13)^{fghi}	23.33 (28.76) ^{nopq}
149	DBT 2549	3.33 (6.14)^{fg}	6.66 (12.28)^{ijk}	20 (26.55)^{opq}	179	DBT 2579	3.33 (6.14)^{fg}	3.33 (6.14)^{jk}	6.66 (12.28)st
150	DBT 2550	3.33 (6.14)^{fg}	3.33 (6.14)^{jk}	13.33 (21.13)^{qr}	180	DBT 2580	3.33 (6.14)^{fg}	10 (18.42)^{ghij}	16.66 (23.84)^{pqr}
143	DBT 2543	3.33 (6.14)^{fg}	3.33 (6.14)^{jk}	6.66 (12.28)st	181	DBT 2581	6.66 (12.28)^{efg}	10 (18.42)^{ghij}	20 (26.55)^{opq}
144	DBT 2544	33.33(35.20)^{cde}	23.3 (28.76)^{cdefg}	63.33 (52.75)^{def}	182	DBT 2582	6.66 (12.28)^{efg}	13.33 (21.13)^{fghi}	13.33 (21.13)^{qr}

Table 3. Contd

145	DBT 2545	13.33 (21.13)^{cde}	20 (26.55)^{defgh}	30 (33.19)^{lmno}	183	DBT 2583	0 (0.00)^{g}	0 (0.00)^{k}	0 (0.00)u
146	DBT 2546	26.66 (30.98)^{abcd}	33.33 (35.20)^{cde}	43.33 (41.13)^{ijk}	184	DBT 2584	13.33 (21.13)^{cde}	23.3 (28.76)^{cdefg}	33.33 (35.20)^{klmn}
147	DBT 2547	3.33 (6.14)^{fg}	6.66 (12.28)^{ijk}	20 (26.55)^{opq}	185	DBT 2585	6.66 (12.28)^{efg}	13.33 (21.13)^{fghi}	26.66 (30.98)^{mnop}
148	DBT 2548	6.66 (12.28)^{efg}	10 (18.42)^{ghij}	16.66 (23.84)^{pqr}	186	DBT 2586	3.33 (6.14)^{fg}	6.66 (12.28)^{ijk}	10 (18.42)^{rs}
149	DBT 2549	3.33 (6.14)^{fg}	6.66 (12.28)^{ijk}	20 (26.55)^{opq}	187	DBT 2587	3.33 (6.14)^{fg}	3.33 (6.14)^{jk}	6.66 (12.28)st
150	DBT 2550	3.33 (6.14)^{fg}	3.33 (6.14)^{jk}	13.33 (21.13)^{qr}	188	DBT 2588	3.33 (6.14)^{fg}	10 (18.42)^{ghij}	20 (26.55)^{opq}
151	DBT 2551	0 (0.00)^{g}	3.33 (6.14)^{jk}	10 (18.42)^{rs}	189	DBT 2589	16.6 (23.84)^{cde}	23.33(28.76)^{cdefg}	56.66 (48.82)^{efgh}
152	DBT 2552	6.66 (12.28)^{efg}	6.66 (12.28)^{ijk}	10 (18.42)^{rs}	190	DBT 2590	3.33 (6.14)^{fg}	6.66 (12.28)^{ijk}	13.33 (21.13)^{qr}
153	DBT 2553	3.33 (6.14)^{fg}	6.66 (12.28)^{ijk}	16.66 (23.84)^{pqr}	191	DBT 2591	0 (0.00)^{g}	3.33 (6.14)^{jk}	10 (18.42)^{rs}
154	DBT 2554	0 (0.00)^{g}	0 (0.00)^{k}	0 (0.00)u	192	DBT 2592	0 (0.00)^{g}	0 (0.00)^{k}	0 (0.00)^{u}
155	DBT 2555	3.33 (6.14)^{fg}	6.66 (12.28)^{ijk}	13.33 (21.13)^{qr}	193	DBT 2593	10 (18.42)^{def}	16.6 (23.84)^{efghi}	23.33 (28.76)^{nopq}
156	DBT 2556	26.6 (30.9)^{abcd}	26.6 (30.98)^{cdefg}	43.33 (41.13)^{ijk}					
157	DBT 2557	20 (26.55)^{cde}	23.3 (28.76)^{cdefg}	33.33 (35.20)^{klmn}	HD1	46.66 (43.05)^{a}	73.33 (58.98)^{a}	93.33 (77.67)^{a}	HD1
158	DBT 2558	0 (0.00)^{g}	0 (0.00)^{k}	0 (0.00)u		**24 h**	**48 h**	**72 h**	
159	DBT 2559	6.66 (12.28)^{efg}	13.33 (21.13)^{fghi}	23.33 (28.76)^{nopq}	SE M±	3.99	3.64	2.24	SEM±
155	DBT 2555	3.33 (6.14)^{fg}	6.66 (12.28)^{ijk}	13.33 (21.13)^{qr}	CD (1%)	14.62	13.33	8.22	CD (1%)
156	DBT 2556	26.6 (30.9)^{abcd}	26.6 (30.98)^{cdefg}	43.33 (41.13)^{ijk}	CV (%)	47.93	33.69	15.31	CV (%)

HAT – Hours after treatment; HD1 – *B. thuringiensis* sub.sp.*kurstaki*- Reference strain. Figure in paranthesis are arc sine transformed values.

were found in the *B. thuringiensis* collection which was similar to that described in other reports (Bendov et al., 1997; Bravo et al., 1998; Kim, 2001; Wang et al., 2003), whereas, *cry*9 gene displayed the lowest frequency. The study on the diversity of *cry* gene combinations in Thailand revealed that *cry*1 and *cry*2 genes often appeared together, which is similar to the observations from Israel (Ben-Dov et al., 1997) and China (Kim, 2001; Wang et al., 2003). *cry*1Ad1 and *cry*1Ca1 was present only in two isolates. None of the isolates amplified for *cry*1Da1. 23.89% of the isolates amplified for *cry*2Ab1.

About 13 isolates amplified for *cry*1Ia1 and 12 isolates for *cry*1Aa1. *cry*2Aa1 and *cry*1Ac1 was present in 19 isolates. Six isolates amplified for *cry*2Ac1 and three isolates for *cry*9Aa1; *cry*1Ae1 was found in 17 isolates. *cry*9Ca1 was present in 4.42% of the isolates. About five isolates contained *cry*1Ab2 (Table 5 and Figure 3). It has been reported that most of the commercial *Bt* formulations used for the control of Lepidopteran pests contain toxins of Cry1A family, especially Cry1Aa, Cry1Ab and Cry1Ac proteins (Hofte and Whiteley, 1989). The diversity of *cry* gene profiles suggest that there could be different strains of *Bt* which could be toxic to insects belonging to order Lepidoptera and Coleoptera. There may be more than one *cry* gene in a given isolate. Martinez and Caballero, (2002) found as many as eight *cry* genes in one isolate.

Analysis by amplicon fragment length polymorphism (ARFLP)

The *cry*1I amplicons of DBT189 and DBT212 was restricted with restriction endonucleases *Eco*RI, *Nhe*I and *Xba*I. The pattern of restriction was similar to that of the reference strain HD1. But when the PCR amplicon of DBT189 was restricted with *Hind*III, there were differences in restriction fragments in relation to the reference strain HD1. There was one *Hind*III site in the isolate DBT189 giving rise to 1584 and 585 bp bands as compared to the reference strain HD1 which gave restricted fragments of 1300, 585 and 284 bp indicating the presence of two *Hind*III sites in HD1 (Plate 3). PCR-RFLP is the first method specifically designed to detect novel *cry* genes (Kuo and Chak, 1996). It was also used by Wang et al. (2003) to detect new *cry* genes.

Cloning and nucleotide sequencing

*cry*1I gene fragments of about 2.1 kb amplified by PCR from the genomic DNA of Bt isolate DBT189 was cloned into a cloning vector pTZ57R/T and this

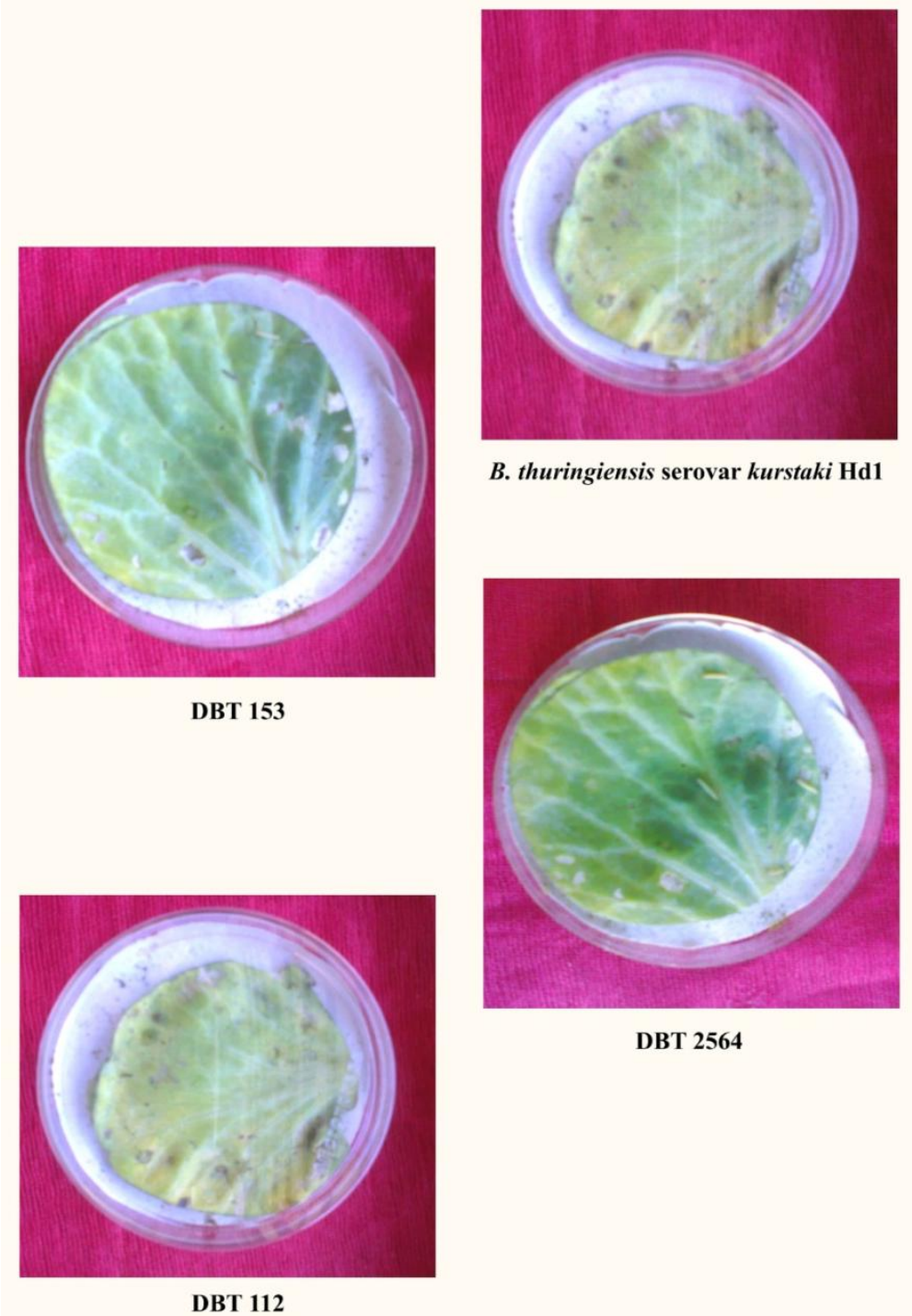
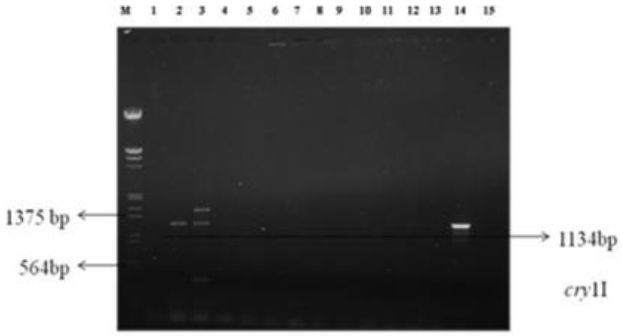


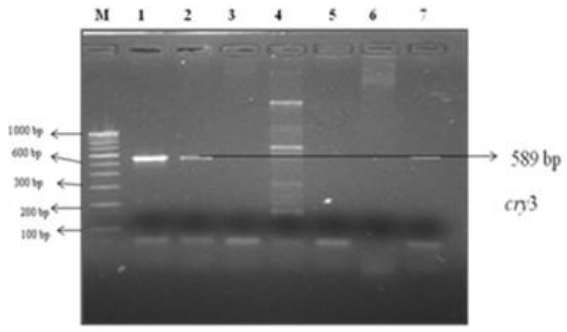
Plate 1. Bioassay of native *Bt* isolates against diamond back moth (*Plutella xylostella*).

construct was transferred into *E. coli* DH5α and transformants were confirmed by restriction analysis using *Bam*HI and *Pst*I endonucleases separately giving rise to linear fragment of 5.05 kb including both vector and insert. This construct was named as pAPK101. A computer based homology search program of NCBI revealed that the gene is a novel *cry*1I-type gene. The construct pAPK101 containing full length *cry*1I was sequenced through primer walking employing M13 primers. The available sequence information from cloned fragments was analysed using BLAST algorithm available at http://www.ncbi.nlm.nih.gov. Multiple alignment of amino



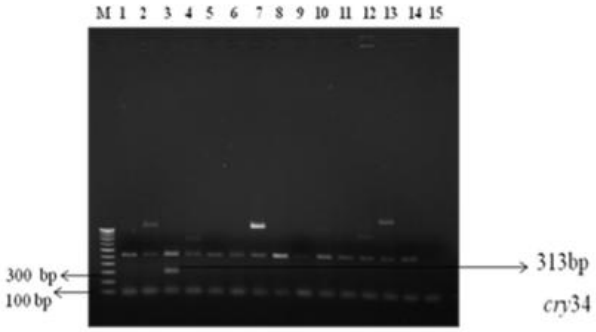
A

M: λ DNA *Eco*RI/*Hind*III digest
Lane 2, 3 and 14 showing amplicon of 1134 bp for *cry*1I gene from DBT183, DBT212 and HD1 respectively



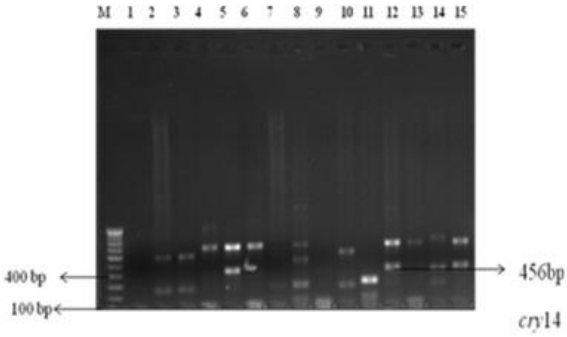
B

M: 100 bp DNA ladder
Lane 1, 2 and 7 showing amplicon of 589 bp for *cry*3 gene from DBT141, DBT142 and 4AA1 respectively



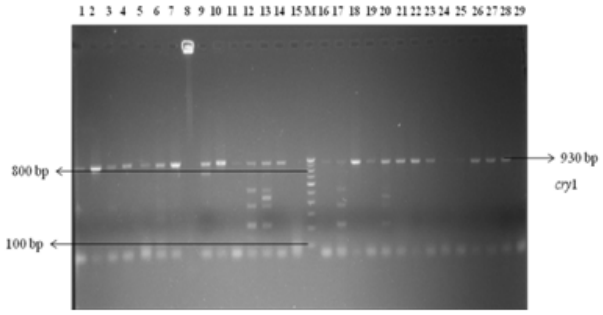
C

Marker: 100 bp DNA ladder
Lane3 showing amplicon of 313 bp for *cry*34 gene from DBT128

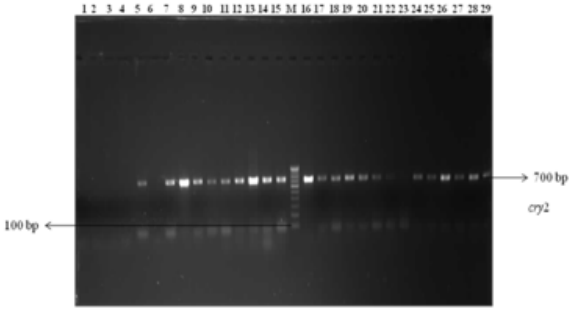


D

M: 100 bp DNA ladder
Lane 5 , 12, 14 and 15 showing amplicon of 456 bp for *cry*14 gene from DBT105, DBT136, DBT143 and DBT140 respectively



E



F

M: 100 bp DNA ladder
Lane 1, 2, 3, 4, 5, 6, 7, 9, 10, 11, 12, 13, 14, 16, 17, 18, 19, 20, 21, 22,23,25,26,27,28 showing amplicon of 930 bp for *cry*1 from DBT120, DBT125, DBT128, DBT123, DBT 126, DBT140, DBT146, DBT172, DBT159, DBT155, DBT160, DBT195, DBT162, DBT165, DBT170, DBT157, DBT178, DBT185, DBT174, DBT150, DBT105, DBT210, DBT211 and DBT212 respectively

M: 100 bp DNA ladder
Lane 5, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 20, 21,22,24,25,26,27,28,29 showing amplicon of 700 bp for *cry*2 from DBT148, DBT152, DBT192, DBT206, DBT153, DBT173, DBT181, DBT182, DBT184, DBT189, DBT190, DBT193, DBT196, DBT197, DBT201, DBT202, DBT209, DBT 164, DBT178, DBT198, DBT123, DBT158 and DBT14 respectively

Plate 2. Screening of native Bt isolates harbouring coleopteran and lepidopteran specific *cry* genes.

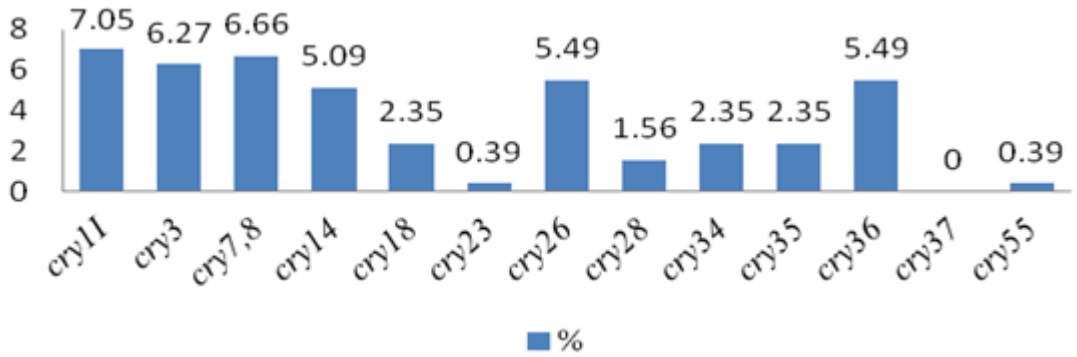


Figure 1. Distribution of coleopteran active *cry*-type genes in 255 *B. thuringiensis* isolates identified by PCR analysis with specific primers.

Table 4. Native *Bacillus thuringiensis* isolates showing the presence of *cry* genes toxic to Coleopterans in Coorg and BR Hills isolates.

***cry* gene**	**Isolate**	**Number**
*cry*1I	DBT105, DBT107, DBT139, DBT167, DBT182, DBT183, DBT189, DBT210, DBT211, DBT212, DBT111, DBT 136, DBT200, DBT265, DBT268, DBT321 DBT352, DBT369	17
*cry*3	DBT141, DBT142, DBT199, DBT200, DBT258, DBT311, DBT312, DBT340, DBT343, DBT344, DBT347, DBT360, DBT367, DBT368, DBT369, DBT370	16
*cry*7,8	DBT 104,DBT173, DBT 181, DBT 191, DBT310, DBT321, DBT340, DBT345, DBT356, DBT358, DBT368, DBT243, DBT246, DBT265, DBT268, DBT291, DBT235	17
*cry*14	DBT105, DBT136, DBT140, DBT143, DBT161, DBT174, DBT183, DBT210, DBT245, DBT249, DBT250, DBT317, DBT346	13
*cry*18	DBT190, DBT258, DBT264, DBT273, DBT317, DBT346	6
*cry*23	DBT 171	1
*cry*26	DBT111, DBT118, DBT131, DBT142, DBT171, DBT245,DBT247,DBT333, DBT340, DBT343, DBT344, DBT345, DBT347, DBT354	14
*cry*28	DBT158, DBT178, DBT248, DBT258	4
*cry*34	DBT128, DBT178, DBT201, DBT202, DBT211, DBT235	6
*cry*35	DBT128, DBT178, DBT201, DBT202, DBT211, DBT235	6
*cry*36	DBT197, DBT202, DBT242, DBT254, DBT259, DBT264, DBT312, DBT340, DBT343, DBT344, DBT347, DBT352, DBT360, DBT367	14
*cry*55	DBT333	1
*cry*37	-	-

acid sequence of pAPK101 showed 99% homology to that of published *cry*1I sequence. It encoded a protein consisting of 720 amino acids. The sequence was subjected to further analysis in BT1 software of GENETOOL for finding the restriction sites and NCBI open reading frame search database for finding the ORF. The sequence analysis revealed the same results as that observed in the ARFLP pattern (Table 6).

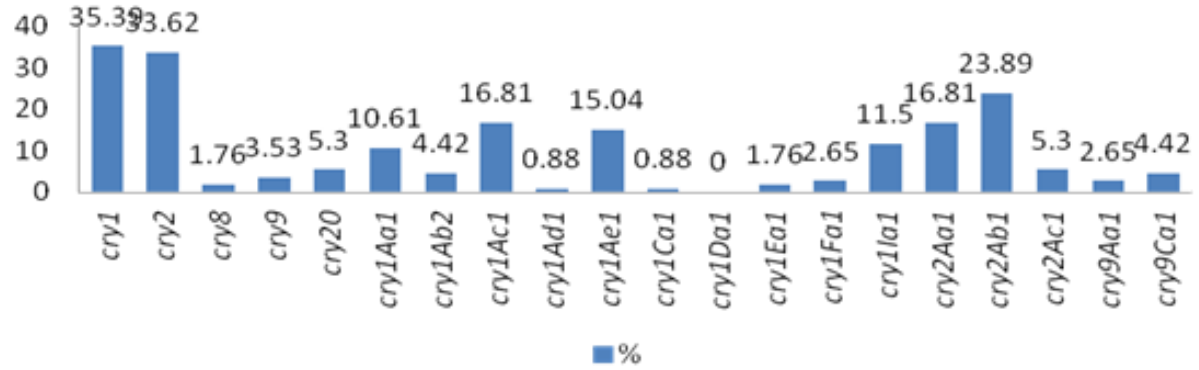


Figure 2. Distribution of Lepidopteran active *cry*-type genes in 113 *B. thuringiensis* isolates identified by PCR analysis with specific and degenerate primers.

Table 5. Native *Bacillus thuringiensis* isolates showing the presence of *cry* genes toxic to Lepidopterans in Coorg isolates.

***cry* gene**	**Isolate**	**Number**
*cry*1	DBT120, DBT125, DBT128, DBT123, DBT 126, DBT140, DBT146, DBT172, DBT159, DBT155, DBT160, DBT195, DBT162, DBT165, DBT170, DBT157, DBT178, DBT185, DBT174, DBT150, DBT105, DBT210, DBT211, DBT212, DBT135, DBT139, DBT107, DBT167, DBT149, DBT133, DBT156, DBT138, DBT143, DBT115, DBT144, DBT129, DBT137, DBT154, DBT203, DBT183	40
*cry*2	DBT148, DBT152, DBT192, DBT206, DBT153, DBT173, DBT181, DBT182, DBT184, DBT189, DBT190, DBT193, DBT196, DBT197, DBT201, DBT202, DBT209, DBT 164, DBT178, DBT198, DBT123, DBT158, DBT147, DBT151, DBT197, DBT194, DBT204, DBT186, DBT199, DBT177, DBT188, DBT180, DBT169, DBT109, DBT100, DBT106, DBT116, DBT112	38
*cry*8	DBT156, DBT166	2
*cry*9	DBT179, DBT186, DBT188, DBT192	4
*cry*20	DBT134, DBT145, DBT178, DBT179, DBT180, DBT207	6
*cry*1Aa1	DBT192, DBT173, DBT182, DBT184, DBT190, DBT197, DBT179, DBT204, DBT112, DBT104, DBT117, DBT121	12
*cry*1Ab2	DBT116, DBT172, DBT195, DBT185, DBT107	5
*cry*1Ac1	DBT182, DBT190, DBT178, DBT123, DBT147, DBT186, DBT177, DBT169, DBT113, DBT109, DBT100, DBT106, DBT116, DBT124, DBT110, DBT112, DBT102, DBT104, DBT125	19
*cry*1Ad1	DBT126	1
*cry*1Ae1	DBT112, DBT102, DBT104, DBT120, DBT125, DBT128, DBT126, DBT175, DBT146, DBT172, DBT159, DBT155, DBT160, DBT161, DBT162, DBT170, DBT185	17
*cry*1Ca1	DBT146	1
*cry*1Da1	-	-
*cry*1Ea1	DBT122, DBT185	2
*cry*1Fa1	DBT142, DBT171, DBT195	3
*cry*1Ia1	DBT194, DBT204, DBT186, DBT199, DBT177, DBT188, DBT191, DBT113, DBT106, DBT117, DBT183, DBT105, DBT211	13
*cry*2Aa1	DBT207, DBT166, DBT148, DBT176, DBT192, DBT153, DBT182, DBT184, DBT193, DBT197, DBT205, DBT208, DBT209, DBT164, DBT178, DBT158, DBT147, DBT113, DBT109	19
*cry*2Ab1	DBT148, DBT152, DBT192, DBT206, DBT153, DBT181, DBT182, DBT184, DBT189, DBT193, DBT196, DBT203, DBT178, DBT123, DBT158, DBT147, DBT197, DBT186, DBT177, DBT191, DBT100, DBT116, DBT124, DBT112, DBT102, DBT104, DBT118, DBT111	27

Table 5. Contd.

*cry*2Ac1	DBT150, DBT155, DBT193, DBT197, DBT210, DBT211	6
*cry*9Aa1	DBT121, DBT126, DBT133	3
*cry*9Ca1	DBT125, DBT129, DBT147, DBT168, DBT171	5

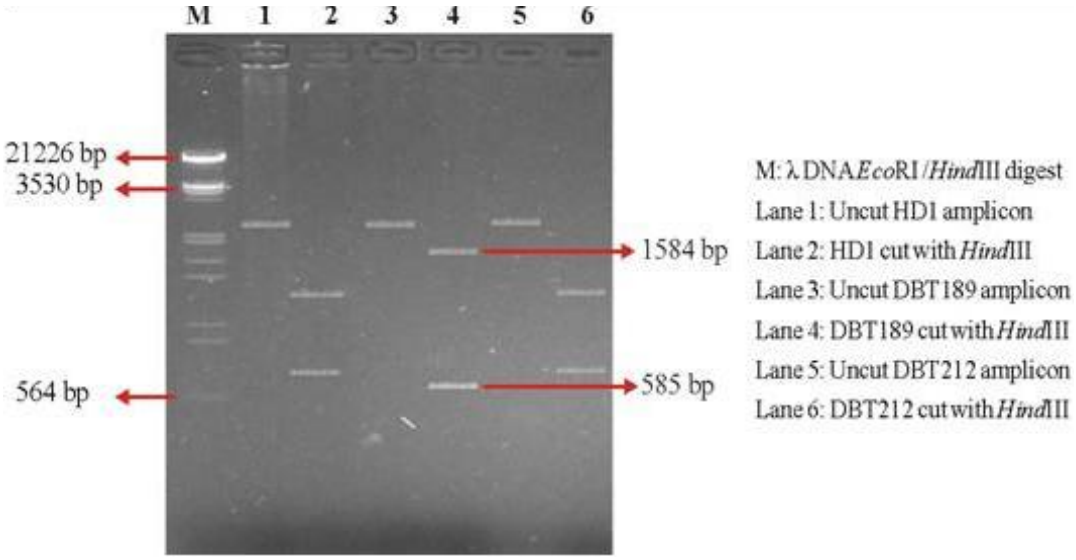


Plate 3. ARFLP pattern. M: λ DNA digested with *Eco*RI and *Hind*III; Lane 1: PCR product of HD1 undigested; Lane 2: PCR product of HD1 digested with *Hind*III; Lane 3: PCR product of DBT189 undigested, Lane 4: PCR product of DBT189 digested with *Hind*III; Lane 5: PCR product of DBT212 undigested, Lane 6: PCR product of DBT212 digested with *Hind*III.

The conserved domains of the cloned *cry*1I sequence were analysed using NCBI CDD search database. The toxic domain of *cry*1I ranged between 60 to 640 amino acids and contained N, M and C super family domains of endotoxins which include most of the N-terminal region which indicates its toxic potential. The alignment of Cry1I (pAPK101) and reference strain *B. thuringiensis* sub sp. *kurstaki* (Accession No. AJ315121.1) revealed changes in six amino acids at the position 15, 217, 426, 657, 711 and 712 amino acid residues. Different Cry proteins vary in toxicity against one insect species, while different insect species vary in susceptibility to a particular Cry protein. This variability may be due to significant differences in the amino acid sequence between proteins (Barboza et al., 1998) but occasionally the toxicity of a particular Cry protein may vary considerably owing to minor differences in sequences. Minor differences from a holotype sequence are frequently found in nature and are considered to be 'natural variants'; however, no relationship has been established between this variability and adaptability. Most of these differences normally have no effect on toxicity, except when they occur in particular regions of the molecule. Because the toxic effect of Cry1 proteins is restricted to the N-terminal half of the molecule (δ endotoxin), any difference in activity may be attributed to the amino acid differences in this moiety.

Multiple alignment of amino acid of pAPK101 with reference AJ315121.1 revealed six amino acid substitutions: N for X (position 15 bp), G for X (position 217 bp), K for E (position 426 bp), Q for R (position 657), N for K (position 711) and E for Q (position 712). This resulted in substitution of amino acids: K (lysine) to E (glutamic acid), Q (glutamine) to R (arginine), N (asparagine) to K (phenylalanine) and E (glutamic acid) to Q (glutamine) (Figure 3). The N-terminal region (from M1-Q10) consists of positively charged amino acids which may function as a signal peptide. According to the results described by Kostichka et al. (1996), the analysis of the deduced *cry1I* protein sequence of the T01328 isolate also revealed the presence of a N-terminal sequence that functions as a signal peptide.

Expression studies

The gene of interest was cloned and expressed in pQE30 giving rise to a recombinant vector of 5630 bp. The confirmation of the cloned gene was on the basis of release of insert of 2169 bp along with vector and insert 5630 bp (Plate 4). SDS-PAGE analysis revealed that a 81 kDa protein was produced in *E. coli* induced with IPTG (Plate 5). Similar protein with a molecular weight of appro-

Figure 3. Alignment of amino acid of Cry1I (pAPK101) with AJ315121.1.

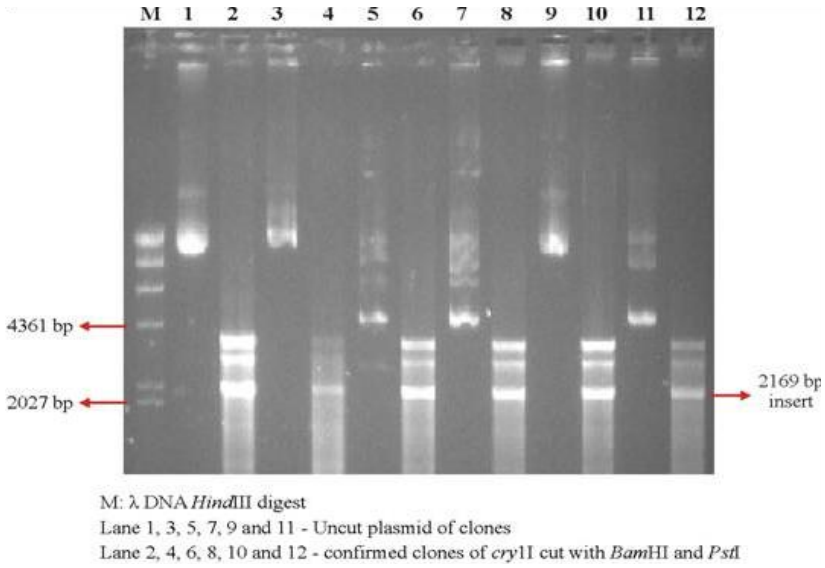


Plate 4. Restriction confirmation of *cry*1I clones in *E.coli* M15 and SG13009. M: λ DNA HindIII digest; Lanes 1, 3, 5, 7, 9: uncut plasmid; Lanes 2, 4, 6, 8, 10, 12: confirmed clones of *cry*1I cut with *Bam*HI and *Pst*I.

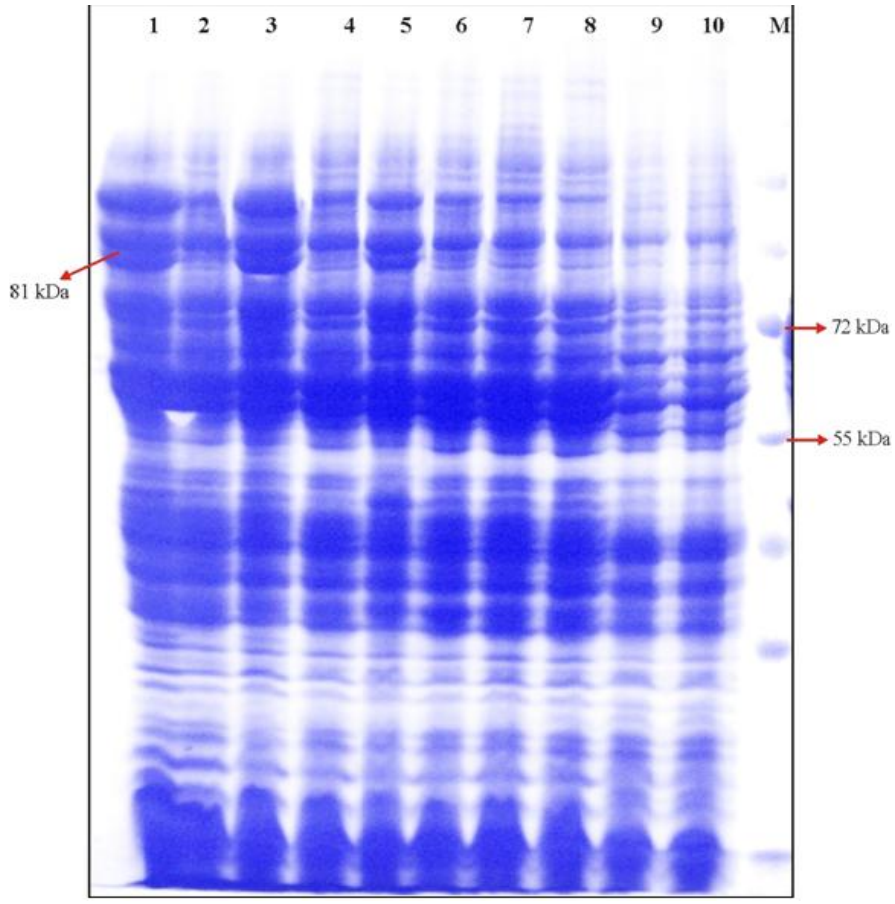


Plate 5. Detection of the recombinant protein in a 10% SDS gel. Lanes 1, 3 and 5: Proteins from induced clones; Lanes 2, 4 and 6: Proteins from uninduced clones; Lane 7: Protein from induced host *E. coli* M15 (pREP4); Lane 8: Protein from uninduced host *E. coli* M15 (pREP4); Lanes 9 and10: Proteins from the *E. coli* cell extract with the empty vector; M: Prestained Protein Ladder marker SM0671.

Table 6. Amplicon restriction fragment length polymorphism of full length *cry*1I.

Enzyme	Restriction fragment size (bp)		
	HD1	DBT189	DBT212
*Hind*III	1300, 585 and 284	1584, 585	1300, 585 and 284
*Eco*RI	1450, 719	1450, 719	1450, 719
*Nhe*I	1700, 469	1700, 469	1700, 469
*Xba*I	1125, 1044	1125, 1044	1125, 1044

ximately 81 kDa was observed in a study conducted by Bergamsco et al. (2011). Cry1I toxins are of special interest since they present toxicity against insects of the Lepidoptera and Coleoptera orders. Other proteins, as Cry1B, Cry1C and Cry2A have also been found to exhibit action against more than one order (Zhong et al., 2000; Widner and Whiteley, 1990). Cry-type proteins (for example, Cry1A, Cry1A, Cry2A, Cry3A and Cry9C-type) have been widely applied in transgenic plants, but the problem of narrow insecticidal spectrum and insect resistance have recently been observed due to lengthy use of high concentrations of a single Bt toxin (Romeis et al., 2006). Most of the toxins cloned consist of lepidopteran active proteins till now, thus, search for more *Bt* strains harbouring coleopteran specific genes is important.

The *cry*1I protein may prove to be an alternative to combat the insect resistance problem and so it will be worthwhile to fully elucidate its insecticidal potential. Further studies are in progress to ascertain the nature of the protein and its novelty and toxicity tests are being carried out to widen the understanding of its effects on different insect orders.

ACKNOWLEDGEMENT

This study was supported by the Department of Biotechnology, Government of India, New Delhi.

REFERENCES

Aronson AI, Angelo N, Holt SC (1971). Regulation of extracellular protease production in Bacillus cereus T: characterization of mutants producing altered amount of protease. J. Bacteriol. 106:1016-1025.

Barboza-Corona JE, Loapez-Meza JE, Ibarra JE (1998). Cloning ande xpression of the *cry*1Ea4 gene of *Bacillus thuringiensis* and the comparative toxicity of its gene product. World J. Microbiol. Biotechnol. 14:437-441.

Bendov E, Zaritsky A, Dahan E, Barak Z, Sinai R, Manasherob R, Khamraev A, Troitskaya E, Dubitsky A, Berezina N, Margalith Y (1997). Extended screening by PCR for seven *cry* group genes from field collected strains of *Bacillus thuringiensis*. Appl. Environ. Microbiol. 63(12):4883-4890.

Bergamasco VB, Goncalves JF, Polanczk RA, Desiderio JA, Lemos MV (2011). Expression of a new *Bacillus thuringiensis cry*1Ia gene in *Escherichia coli* with strong activity against cotton pests. Australian J. Bas. Appl. Sci. 5(12) : 526-533.

Birmboim HC, Doly J (1979). A rapid alkaline lysis procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7(6):1515-1523.

Bravo A, Sarabio S, Lopez L, Ontiveros H, Abarca C, Oritz A, Oritz M, Lina L, Villalobos FJ, Pena G, Munez-Valdez M, Soberon M, Uintero R (1998). Characterization of *cry* genes in a Mexican *Bacillus thuringiensis* strain collection. Appl. Environ. Microbiol. 64(12):4965-4972.

Ejiofar AO, Johnson T (2002). Physiological and molecular detection of crystalliferous *Bacillus thuringiensis* strains from habitats in the South Central United States. J. Ind. Microbiol. Biot. 28(5) : 284-290.

Hofte H, Whiteley HR (1989). Insecticidal crystal protein of *Bacillus thuringiensis*. Microbiol. Rev. 53(2): 242-255.

http://www.lifesci.sussex.ac.uk/home/neil_crickmore/Bttoxin.html

Johnson LW (2011). Analysis of *cry* contents in native *Bacillus thuringiensis* isolates and cloning of *cry* gene. M. Sc. Thesis, Uni. Agric. Sci., Dharwad (India).

Juarez-Perez VM, Ferrandis MD, Frutox R (1997). PCR based approach for detection of novel *Bacillus thuringiensis cry* genes. Appl. Environ. Microbiol. 63(8):2997-3002.

Kim HS, Li MS (2001). Molecular Cloning of a new crystal protein gene *cry*1Af1 of *Bacillus thuringiensis* NT0423 from Korean Sericultural Farms, Curr. Microbiol. 43(6):408-413.

Kuo WS, Chak KF (1996). Identification of novel *cry*-type genes from *Bacillus thuringiensis* strains on the basis of restriction fragment length polymorphism of the PCR-amplified DNA. Appl. Environ. Microbiol. 62(4):1369–1377.

Mahadeva Swamy HM, Asokan R, Nagesha SN, Arora DK, Birah A, Mahmood R (2011). Cloning, characterization and diversity of insecticidal crystal protein genes of *Bacillus thuringiensis* native isolates from soils of Andaman and Nicobar Islands, Curr. Microbiol. 63(5):420–425.

Maniatis T, Fritsch EF, Sambrook J (1982). Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring, Harbor, New York.

Martinez C, Caballero P (2002). Contents of *cry* genes and insecticidal toxicity of *Bacillus thuringiensis* strains from terrestrial and aquatic habitats. J. Appl. Microbiol. 92(4): 745-752.

Marutesh SA (2007). Molecular characterization and efficacy of native isolates of *Bacillus thuringiensis* (Berliner) against cruciform pests with special reference of DBM. M.Sc. Thesis, Uni. Agric. Sci., Dharwad (India).

Nazarian A, Jahangiri R, Jouzani GS, Seifinejad A, Soheilivand S, Bagheri O, Keshavarzi M, Alamisaeid K (2009). Coleopteran-specific and putative novel *cry* genes in Iranian native *Bacillus thuringiensis* collection. J. Invert. Pathol. 102(2):101–109.

Ozturk F, Acik L, Ayyaz A, Bozdogan B, Suludere Z (2009). Turkish Isolation and Characterization of Native *Bacillus thuringiensis* Strains from Soil and Testing the Bioactivity of Isolates Against *Ephestia Kuehniella* Zeller (Lepidoptera:Pyralidae) Larvae. J. Biochem. 33(4): 202-208.

Romeis J, Meissle M, Bigler F (2006). Transgenic crops expressing *Bacillus thuringiensis* toxins and biological control. Nat. Biotechnol. 24(1):63–71.

Salehi JG, Seifinejad A, Saeedizadeh A, Nazarian A, Yousefloo M, Soheilivand S, Mousivand M, Jahangiri R, Yazdani M, Amiri RM, Akbari S (2008). Molecular detection of nematicidal crystalliferous *Bacillus thuringiensis* strains of Iran and evaluation of their toxicity on free-living and plant-parasitic nematodes. Can. J. Microbiol. 54(10): 812-822.

Sambrook J, R ussel DW (2001). Molecular Cloning: A laboratory

manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
Schnepf E, Crickmore N, Vanrie J, Lereclus D, Baum J, Feitelson J, Zeigler D R, Dean DH (1998). *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. 62: 775-806.
Shilpa HT (2005). Evaluation of native *Bacillus thuringiensis* isolates against *H. armigera* and *P. xylostella*. M.Sc. Thesis, Uni. Agric. Sci., Dharwad (India).
Tabashnik BE, Cushing NL (1987). Leaf residue Vs topical bioassays for assessing insecticide resistance in the diamond back moth, *Plutella xylostella* L. FAO Pl. Prot. Bull. 35:11-14.
Wang JA, Boets J, Re V, Ren G (2003). Characterization of *cry*1, *cry*2 and *cry*9 genes in *Bacillus thuringiensis* isolates from China. J. Invert. Pathol. 82(1): 63-71.
Widner WR, Whiteley HR (1990). Location of the dipteran specificity region in a lepidopteran-dipteran crystal protein from *Bacillus thuringiensis*. J. Bacteriol. 172: 2826–2832.
Zhong C, Ellar DJ, Bishop A, Johnson C, Lin S, Hart ER (2000). Characterization of *Bacillus thuringiensis* delta-endotoxin which is toxic to insects in three orders. J. Invert. Pathol. 76(2): 131-139.