Indian Journal of Exprimental Biology
Vol. 52, June 2014, pp. 637-649

Identification and characterization of the Sudanese *Bacillus thuringiensis* and related bacterial strains for their efficacy against *Helicoverpa armigera* and *Tribolium castaneum*

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Received 1 November 2013; revised 28 January 2014

Forty-four isolates of *Bacillus thuringiensis* like bacteria from various sources in different locations from Sudan were tested for their insecticidal activity. The toxicity of these isolates ranged from 6.6 to 70% to the neonates of cotton bollworm, *Helicoverpa armigera* at 10 ppm concentration. The most effective ones are Kb-29, St-6 and Wh-1 comparable with HD-1. Toxicity of isolates to larvae of the red flour beetle, *Tribolium castaneum* ranged from 20 to 100%. Isolates St-2 and St-23 gave 100% larval mortality within 15 days of exposure and were at par with Ab-8, Ab-12, Kb-26, Kb-30, Om-4, Po-2, Po-5, Po-7, Sa-8 and Wh-5 and were also comparable with *E. coli* clone expressing Cry3 toxin. The most effective five isolates viz., Kb-29, St-2, St-6, St-23 and Wh-1 belonged to *B. thuringiensis*. The St-6 isolate, which also showed high toxicity to *T. castaneum* larvae, had *cry*1 genes along with coleopteran active *cry*28 genes, but not *cry*3 genes. Of the 25 isolates characterized with 16s DNA sequencing, seven belonged to *Paenibacillus* spp., one *Lysinibacillus sphaericus*, one *Bacillus pumilus*, four *Bacillus* spp., and rest 12 belonged to *B. thuringiensis*. Biochemical characterization in each species showed variation. The present study shows potential of some isolates like Kb-29, St-2, St-6, St-23 and Wh-1 as promising bioinsecticides.

Keywords: *Bacillus thuringiensis*, Bioprospecting, Cry toxins, *Helicoverpa armigera*, *Lysinibacillus*, *Paenibacillus*, Sudanese strains, Toxicity, *Tribolium castaneum*

Bacillus thuringiensis Berliner is an ubiquitous soil borne spore forming insect pathogenic bacterium belonging to the group *Bacillus cereus*[1]. It was discovered in 1901 by Shigetane Ishiwata and redescribed from the infected larvae of Mediterranean flour beetle in 1911 by Berliner[2]. The first use of *B. thuringiensis* on the commercial scale was in 1920s for the control of European corn borer in Europe. Since then, *B. thuringiensis* has become a main component of microbial biopesticides as alternative strategies vis-à-vis synthetic insecticides for insect control[3,4]. *B. thuringiensis* produces vegetative insecticidal toxins during vegetative growth and the crystalline (Cry) toxins during sporulation. There is a wide variation within *B. thuringiensis* on the basis of differences in flagellar agglutination and more than 70 serotypes and 92 subspecies are described till date[5,6]. Höfte and Whiteley[7] classified *B. thuringiensis* isolates on the basis of *cry* genes and insecticidal activity, and grouped *cry* genes as lepidopteran specific *cry*1, lepidopteran and dipteran specific *cry*2, coleopteran specific *cry*3 and dipteran specific *cry*4 genes. Currently, Cry toxin proteins are classified on the basis of amino acid identity and homology eliminating need of bioassays, based up on report of the *Bt* delta-endotoxin nomenclature committee in 1993[8]. More than 290 Cry protein holotypes and 500 Cry proteins have been identified on the basis of above criterion till date[9].

B. thuringiensis is highly selective against insect pests due to the specificity of its Cry toxins[10]. Besides, other bacteria like *Bacillus lentimorbus*, *Lysinibacillus sphaericus* and *Paenibacillus papillae* are reported to be pathogenic to insects[6]. The high costs of imported *B. thuringiensis* formulations with uncertain toxicity to the country specific pests necessitates search for locally adapted highly effective strains. Elyass[11] characterized *B. thuringiensis* isolates from various habitats in

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Sudan. Gorashi *et al.*[12] evaluated Sudanese isolates against *Culex quinquefasciatus*. However, very little information is available on their activity against pests of agricultural importance.

Cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) is a very destructive polyphagous pest of agricultural crops. It is distributed in most of Asia, Africa, Australia and the South Mediterranean region[13]. It causes loss of agricultural produce to the extent of $ 5 billion annually[14]. Red flour beetle, *Tribolium castaneum* (Herbst) is a cosmopolitan secondary pest that primarily feeds on the stored cereal grain products resulting in loss of grain weight and contamination with toxic quinones excreted during feeding, thereby affecting nutritional quality[15]. Both insect species are important representatives of Lepidoptera and Coleoptera which have most destructive members affecting field crops and stored grains, respectively.

This communication describes isolation, identification and characterization of Sudanese *B. thuringiensis*-like strains.

Materials and Methods

Sources of the isolates—*B. thuringiensis* like strains were isolated from various sources like infested grains, soil samples and air at localities in Sudan distributed widely between 8 and 22 °N and 31 and 37 °E (Fig.1). The isolation involved sodium acetate selection procedure[16,17]. A sample, (0.5 g) was suspended in 10 mL of nutrient broth supplemented with sodium acetate (0.25 M, *p*H 6.8) in 100 mL

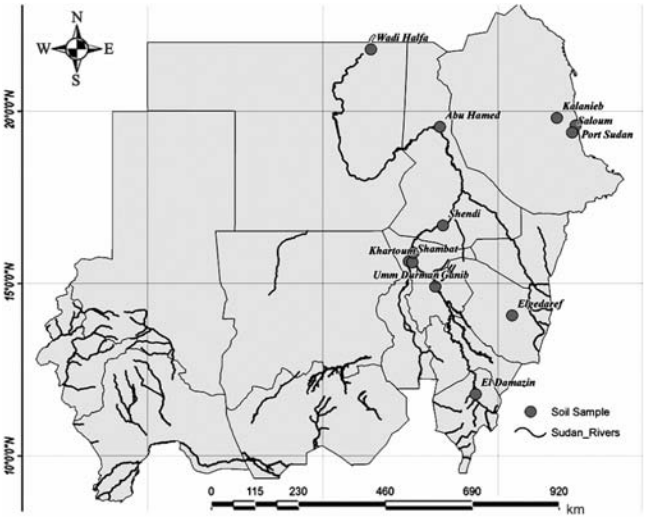

Fig. 1—Map showing locations in Sudan from where soil samples were collected

flask. The mixtures were shaken at 30 ºC and 200 rpm for 4 h. Later, 0.5 mL of supernatant was taken in 10 mL of test tube and was heat-treated for 3 min in water-bath at 80 ºC to remove non-spore-formers. Then, it was diluted 1000-fold and 100 µL each of diluted sample was spread on the nutrient agar plate. The plates were dried and incubated at 30 ºC for 48 h and examined for colony morphology. The samples with *B. thuringiensis* like morphology were selected and stored at 10 ºC. Reference strain, *B. thuringiensis* subsp. *kurstaki* (HD-1) was kindly supplied by Pasteur Institute, Paris, France. BGSC ECE-131 (*E. coli* JM103 recombinant strain with *cry3Aa1* gene from *B. thuringiensis* subsp. *tenebrionis*) and the *Pseudomonas fluorescens* expressing Cry1Ac based formulation MVPII (19.7% toxin) were obtained from Bacillus Genetic Stock Center, Ohio State University, Columbus, USA and Monsanto India Ltd, Bangalore, India, respectively.

Revival of the culture—Nutrient Agar medium was prepared and poured in to Petri plates for solidification. All isolates (44 isolates and one reference strain HD-1) were streaked on the Petri plates and kept at 37 ºC for 24 h. Then Luria Broth test tubes were inoculated by picking single colony from each culture and incubated in an incubator shaker (37 ºC, 200 rpm for 24 h). Glycerol stock (20%) was prepared for each isolate and kept at -80 ºC.

Production of spore-crystal complex—An aliquot (5 mL) of 24 h old pure culture of each isolate was used to inoculate 250 mL of Luria broth in 1 L conical flask for 72 h at 37 ºC with a shaker speed of 200 rpm. After this the broth was centrifuged at 8,000 rpm at 4 ºC for 10 min and the supernatant was discarded and the pellet collected and re-suspended in 5% lactose solution at 1/10th the original volume of the broth (25 mL) and stirred continuously for 30 min, then four volumes (100 mL) of ice-cold acetone were added slowly and stirred for another 30 min. The acetone precipitate powder containing spore-crystal complex was filtered through Whatman filter paper with a vacuum pump and dried overnight in a desiccator, then powdered finely, weighed and stored at 4 ºC till use[18].

Preparation of Cry3 toxin—*E. coli* clone expressing Cry3 (ECE 131) was cultured in Luria Broth containing 50 µg/mL ampicillin for 72 h at 37 ºC with a shaker speed of 150 rpm. It was then harvested by centrifugation at 8,000 rpm at 4 ºC for 10 min. The cells were re-suspended in 50 mM lysis buffer

containing 0.5 mg/mL lysozyme and incubated at 37 ºC with slow shaking for 4 h then centrifuged. The pellet was washed twice with crystal wash and finally with sterile distilled water, and re-suspended in solubilization buffer (50 mM sodium carbonate buffer, 10 mM dithiothreitol, *p*H 10.5) and incubated at 37 ºC for 3 h with slow shaking. This was then centrifuged at 10,000 rpm for 10 min and the supernatant containing the protoxin was treated with 5% trypsin and then quantified[19].

Characterization of *B. thuringiensis* and other bacterial isolates

Insect rearing and bioassays─H. armigera larvae were reared on a chick pea based semi-synthetic diet, at 27 ºC and 60-80% RH as per Gujar *et al*[20]. The adult moths were offered 10% honey solution fortified with multi-vitamin. Adults (about 10 pairs) were kept in plastic jar covered with moist cotton cloth which was replaced daily. The cloth cover containing eggs was kept in a plastic jar lined with a moist filter paper until hatching.

T. castaneum was reared on wheat flour mixed with 5% yeast powder at 27 ºC in glass jars. The adults were separated and were allowed to lay eggs in finely ground wheat flour in Petri plates. These were then separated with sieve to get eggs for bioassays.

*Toxicity against H. armigera─*Each isolate was evaluated against neonate larvae as per bioassay procedure[20]. Stock suspension (10 µL) of spore-crystal complex (acetone powder; 10,000 ppm) was mixed with 10 g diet to get a concentration of 10 ppm. The diet was equally divided into three replicates to which 10 neonates were added per replicate. Diet in the control was mixed with the same volume of sterilized double distilled water. Larval mortality was recorded daily. The data on mortality on the 4th and 7th day of treatment were subjected to analysis of variance using WindoStat 7.9.0.9 programme (Indostat Services, Hyderabad) and the least significant difference was used to compare means. Standard strain HD-1 and MVPII were evaluated at 1 ppm concentration on the mass basis and toxin content, respectively.

*Toxicity against T. castaneum─*All 44 isolates were examined for their toxicity against larvae of *T. castaneum* at a concentration of 100 ppm. Wheat flour was supplemented with yeast at a concentration of 5%. Five grams of this flour were weighed in a Petri plate to which suspension of spore-crystal mixture was added at the specified concentration and mixed thoroughly then divided into three replicates. To each of these replicates were added 10 eggs of the red flour beetle and kept at 27 ºC for one month. The control had the same volume of water in place of the spore-crystal suspension as in treatments. The observations were taken on the number of larvae/pupae/adults surviving at 15 and 30 days of treatment. The mortality data were subjected to analysis of variance using WindoStat programme. *E. coli* clone expressing Cry3Aa1 toxin used as standard was mixed at the concentration of 10 ppm in wheat flour for bioassays.

*Biochemical characterization─*Twenty six isolates including HD-1 and ECE 131 were subjected to biochemical characterization based on sugar fermentation pattern in Brain Heart infusion Broth as per method provided by RAPID HiBacillus™ Identification Kit KB013 (Hi-Media, India). The tests were based on the principle of *p*H change, substrate utilization and other biochemical reactions exhibiting colour change. The whole kit consists of 12 different tests including malonate, Voges Proskauer's, citrate, *o*-nitro phenyl ß-galactose (ONPG), nitrate reduction, catalase, arginine, sucrose, mannitol, glucose, arabinose and trehalose.

*Molecular characterization with 16S rDNA analysis─*Various universal primer combinations designed by Lane *et al.*[21] and Turner *et al.*[22] have been used for PCR amplification of 16S rDNA from members of the domain Bacteria (Table 1). The twenty five isolates were analysed for 16S rDNA sequencing by Saf Labs Private Limited, Navi Mumbai (Assembly programme: Mobyle portal, ABI Sequencer Genetic Analyser 3130). Sequence alignment was performed using the NCBI's (National Centre for Biological Information) Basic Local Alignment Search Tool (BLAST) to compare the sequence data with known sequences submitted on the NCBI database[23]. The sequences were checked by chimera check tool, DECIPHER version 1.1.2 (2012-01-29), trimmed if necessary and then submitted to NCBI for accession numbers[24].

PCR amplification of B. thuringiensis cry genes─ The selected *B. thuringiensis* isolates were grown in Luria Broth at 37 ºC for 24 h and DNA extracted as described by Sambrook *et al*[25]. PCR analysis was performed using the specific *cry1*, *cry2*, *cry3*, and *cry28* primers to detect the respective *cry* genes[26] (Table 2). Each reaction contained 4.3 µL Taq assay

Table 1—Universal Primers for 16S rDNA Analysis

Universal Primer	Sequence	Target group	References
27F:	AGAGTTTGATCMTGGCTCAG	Universal	21
519R:	GWATTACCGCGGCKGCTG	Universal	22
530F:	GCTCTAGAGCTGACTGACTGAGTGCCAGCMGCCGCGG	Universal	21
1100R:	AGGGTTGCGCTCGTTG	Bacteria	22
1114F:	GCAACGAGCGCAACCC	(Bacteria)Universal	21
1492R:	GGTTACCTTGTTACGACTT	Universal	22

Table 2—Characteristics of the primer sets used to identify *cry* genes by PCR analysis.

Primer pair		Tm (°C)	Sequence (5′ to 3′)	Product size (bp)	Reference
Cry1	F	57.0	CATGATTCATGCGGCAGATAAAC	276	26
	R	54.7	TTGTGACACTTCTGCTTCCCATT		
Cry2	F	50.9	GTTATTCTTAATGCAGATGAATGGG	689	37
	R	52.3	CGGATAAAATAATCTGGGAAATAGT		
Cry3	F	55.5	CGTTATCGCAGAGAGATGACATTAAC	701	37
	R	55.7	CATCTGTTGTTTCTGGAGGCAAT		
Cry4	F	51.8	CAAGCCGCAAATCTTGTGGA	797	16
	R	49.7	CGCTACATCTTATGGCTTGT		
Cry28	F	55.7	GTATTGGACCGAGGAGATGAAAGT	589	39
	R	54.4	GTACGGCAAAGCGACAGAACA		

buffer (10X) with $MgCl_2$ (15 mM), 1 µL dNTPs (10 mM), 1 µL of each primer (10 pM), 0.2 µL Taq DNA polymerase (5 U/µL) and 7.5 µL Nuclease free water. Template DNA (10 µL) from the respective samples was added to make the total reaction volume to 25 µL.

The PCR amplification was performed under the following conditions: Initial denaturation at 94 ºC for 3 min, followed by 35 cycles at 94 ºC for 30 sec, 48 ºC for 1 min, 72 ºC for 1.30 min, and final extension step at 72 ºC for 10 min. The conditions used with all primers were similar, except that the annealing temperatures varied according to specificity of *cry* primer[26]. Each experiment was associated with negative (without DNA template) and positive (with a standard template) controls. The reliability of the primers was verified with the following *B. thuringiensis* reference strains: *B. thuringiensis* subsp. *kurstaki* HD-1 for the *cry1* and *cry2*, *E. coli* ECE131 for *cry3Aa1* gene. The PCR products were subjected to electrophoresis (at 80 V for 1 h) on a 1 x Tris-acetate-EDTA (TAE with ethidium bromide) buffer in 1.2% agarose gel. Gels were visualized using Alphaimager™ and analysed with AlphaEase™.

SDS Gel electrophoresis of toxin proteins—*Bt* spore-crystal toxins were characterized using the standard discontinuous buffer system for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as per Laemmli[27]. The spore-crystal mixture samples (10 mg each) of the selected isolates as well as standard ECE-131 were solubilized in 400 µL of solubilization buffer (50 mM sodium carbonate buffer, 10 mM dithiothreitol, pH 10.5) and sonicated twice at output 2 sec pulse, 50% duty cycle, output control setting 8; timer on 2 min (LABSONIC L, Braun Biotech International, Germany). The solubilized samples were incubated at 37 ºC for 3-4 h with slow shaking. After centrifugation at 10,000 rpm for 10 min, supernatant containing solubilized protoxin samples were transferred to new sterilized tubes. Solubilized protoxin was resuspended in equal amount of sample buffer (0.6 mL of 1 M Tris buffer (pH 6.8), 5 mL 50% glycerol, 2 mL 10% SDS, 1 mL 1% bromophenol blue, 50 µL β-mercaptoethanol, 0.9 mL water) and boiled at 95-100 ºC for 5 min. Samples were then loaded and separated by SDS-PAGE using a continuous gel containing a 8% resolving gel and 5% stacking gel. The gel was stained

in staining solution (0.25% Coomassie brilliant blue R250 in methanol: distilled water: acetic acid; 40: 50: 10) for 1-2 h and destained in destaining solution (methanol: distilled water: acetic acid; 40: 50: 10) by washing the stained gel for 3-4 times. BGSC ECE-131 was used as reference for Cry3 toxin. Alphaimager™ Documentation and analysis system was used for gel analysis.

Spore and crystal morphology—Spore-crystal mixture of selected five strains suspended in water was smeared over the glass slide and viewed at 1000x magnification under oil emulsion in phase contrast microscopy. The shape and size of spore and crystal was examined for each isolate and photographed.

Toxin estimation with ELISA—Cry1Ac content in spore-crystal complex of each selected isolates were estimated with their respective ELISA kits (Quan-T ELISA, DesiGen, Jalna, India) as per manufacturer's instructions. Acetone powder (2 mg) of each isolate was mixed in 200 µL of the sample extraction buffer in an eppendorf tube. Then, this suspension was chilled in ice for 10 min, vortexed, and then spinned for 15 min at 8,000 rpm, at 4 ºC. The pellet was discarded and the supernatant was proteolyzed (3.5 µL of trypsin were added to each 100 µL sample). The mixture was then incubated for 30 min at 37 ºC, after that 2.5 µL of 50 mM PMSF was added to each sample and stored at -80 ºC.

Thirty µL of the prepared sample were used per well for quantification of Cry1Ac. Positive and negative controls were prepared and divided into two halves. To each well 150 µL of secondary antibody were added, after which the plates were incubated for 90 min in humid chamber. Then the plates were washed for 5 min two times, followed by addition of the tertiary antibody (250 µL/well), and incubated for 45 min in humid chamber. After this incubation 250 µL of freshly prepared *p*-nitrophenyl phosphate solution (0.1 mg/mL) were added to each well and the plates were incubated for 30 min at room temperature. Then the absorbance of colour formed was read at 405 nm after autozero against control. Then the quantity of toxin produced by each isolate was calculated from the standard curve.

Results

Production of spore crystal complex—The spore-crystal complex produced by each isolate differed in quantity (Table 3). As many as 10 isolates produced more than 1 g of powder from 250 mL culture over 72 h. The high production from 250 mL culture each was recorded for isolate Kb-26 (1.22 g) and Gf-18 (1.21 g) and least for isolate Ab31 (0.35 g). The yield ranged from 0.14 to 0.48% for 72 h of incubation.

Toxicity against cotton bollworm—The isolates differed greatly in their toxicity to the larvae of *H. armigera* wherein mortality ranged from 6.6% (St-5) to 70% (Wh-1) as compared to 0% in control on the 4th day of treatment. In contrast, MVPII showed 90% mortality at 1 ppm of Cry1Ac. Only three effective isolates viz., Kb-29, St-6 and Wh-1 showed more than 50% mortality and were better than HD-1 at 1 ppm on the 4th day of treatment. Additional isolates Gg-2, St-2 and St-14 also showed higher than 50% mortality on the 7th day of treatment (Table 4).

Toxicity against the red flour beetle—Mortality of larvae of *T. castaneum* exposed to the different isolates ranged from 20% (Wh-4) to 100% (St-2, St-23) at 100 ppm, as compared to only 16.6% in the untreated control, and 33.3% in the reference strain, ECE 131. More than 70% of the isolates resulted in mean mortality more than 50% after 15 days. After 30 days only nine isolates Wh-1, Wh-4, Gz-6, Ab-1, Dn-1, Po-1, Po-41, Sd-2 and Om-6 showed less than 50% mortality. Bt isolates St-2 and St -23 showing 100% mortality were at par with Ab-8, Ab-12, Kb-26, Kb-30, Om-4, Po-1, Po-5, Po-7, Sa-8 and Wh-5 (Table 4).

Biochemical analysis—Twenty four isolates (100%) showed positive reaction for nitrate reduction, catalase production and arginine presence; and negative reaction for malonate and Voges Proskauer tests. However, HD-1 showed negative test for arginine. All isolates except for St-22 isolate were found positive for glucose; All isolates except Po-1, Po-2, St-22 were positive for sucrose. All isolates except Po-2 and St-22 were positive for trehalose. All isolates except Ab-8, Ab-12, Po-1, Sa-8, Wh-5 showed negative reaction for citrate utilization. All isolates except Fh-6, Kb-26, Kh-3, Po-1, Po-7, Sa-8 and HD-1 showed positive test for ONPG. The isolates within a species like *B. thuringiensis* showed differential responses to biochemical tests (Table 5).

16S rDNA gene analysis—Twenty five isolates submitted to NCBI were identified as *Bacillus.* sp. (Ab-8, Kb-30, Po-2, Po-7), *B. pumilus* (Sh-13), *B. thuringiensis* (Ab-12, Fh-6, Kb-29, Om-5, Po-5,

Table 3—Production of spore-crystal complex of each isolate

Serial No.	Isolate code	Location/source	Amount (g/250 mL broth)
1.	Ab-1	Abu Hamad/ cultivated soil	0.7795
2.	Ab-3	Abu Hamad/ cultivated soil	0.8439
3.	Ab-7	Abu Hamad/ cultivated soil	1.0824
4.	Ab-8	Abu Hamad/ cultivated soil	1.0073
5.	Ab-12	Abu Hamad/ cultivated soil	0.6968
6.	Ab-31	Abu Hamad/ cultivated soil	0.3467
7.	Dn-1	El damazin-Blue Nile/ cultivated soil	0.7939
8.	Dn-4	El damazin-Blue Nile/ cultivated soil	0.7445
9.	Fh-6	Elfaki Hashim/ cultivated soil	0.6618
10.	Gf-18	Elgedaref/ cultivated soil	1.2058
11.	Gg-1	Ganib farm/ bare soil	0.6831
12.	Gg-2	Ganib farm/ bare soil	0.8385
13.	Gz-6	Gezira/ Blue Nile bank	0.9628
14.	Kb-26	Kalanieb/ cultivated soil	1.2164
15.	Kb-29	Kalanieb/ cultivated soil	0.7572
16.	Kb-30	Kalanieb/ cultivated soil	0.7512
17.	Kh-3	Khartoum/ river Nile bank	1.0316
18.	Lb-1	Air/ indoor	0.8969
19.	Om-4	Omdurman- Khartoum/ cultivated soil	0.8188
20.	Om-5	Omdurman- Khartoum/ bare land	0.6485
21.	Om-6	Omdurman- Khartoum/ cultivated soil	0.8311
22.	Po-1	Port Sudan/ bare land	0.8208
23.	Po-2	Port Sudan/ bare land	1.0576
24.	Po-5	Port Sudan/ bare land	0.9462
25.	Po-7	Port Sudan/ bare land	0.9721
26.	Po-41	Port Sudan/ bare land	0.6961
27.	Po-42	Port Sudan/ bare land	0.7585
28.	Sa-2	Saloum/ cultivated soil	0.8612
29.	Sa-8	Saloum/ cultivated soil	0.8162
30.	Sa-49	Saloum/ cultivated soil	1.0938
31.	Sd-2	Shendi/ cultivated soil	0.7871
32.	Sd-3	Shendi/ cultivated soil	0.9360
33.	Sh-13	Shambat-Nr Khartoum/ cultivated soil	0.8855
34.	Sh-14	Shambat-Nr Khartoum/ cultivated soil	1.0391
35.	St-2	Stored infested lentil	0.8398
36.	St-5	Stored infested wheat	0.8805
37.	St-6	Stored infested lentil	0.8561
38.	St-13	Stored infested wheat	0.9802
39.	St-14	Stored infested wheat	1.1062
40.	St-22	Stored infested wheat	0.7872
41.	St-23	Stored infested wheat	0.7209
42.	Wh-1	Wadi Halfa/ bare land	0.7859
43.	Wh-4	Wadi Halfa/ bare land	1.0776
44.	Wh-5	Wadi Halfa/ bare land	0.8843

Table 4—Toxicity of *B. thuringiensis* isolates at 10 ppm to the neonates of *H. armigera* and at 100 ppm to the neonates of *T. castaneum*

Isolate code	Mean corrected mortality (%)			
	H. armigera		*T. castaneum*	
	4 days	7 days	15 days	30 days
Wh-1	70 (56.78) ^{ab}	70 (56.78)^{bc}	36.66 (29.15)	36.66 (29.15)
St-6	56.6 (48.84) ^{bc}	63.3 (52.77)^{bcd}	76.66 (58.15)^{cde}	76.66 (58.15)^{cdef}
Kb-29	53.3 (46.92) ^{bcd}	53.3 (46.92)^{cdef}	66.66 (50.80)^{cde}	66.66 (50.80)^{f}
Gg-2	46.6 (42.99) ^{cde}	53.3 (46.92)^{cdef}	66.66 (50.80)^{cde}	66.66 (50.80)^{f}
St-22	46.6 (42.99) ^{cde}	50.0 (45.00)^{defg}	46.66 (36.31)	50.0 (38.64)
St-14	40.0 (38.85) ^{cdef}	53.3 (46.92)^{cdef}	76.66 (58.15)^{cde}	100 (85.94)^{a}
Sh-14	40.0 (39.14) ^{cdef}	50.0 (44.91) ^{defg}	36.66 (27.38)	76.66 (58.15)^{cdef}
Ab-12	40.0 (39.6) ^{cdef}	43.3 (41.07) ^{efg}	93.33 (75.13)^{abcd}	93.33 (75.13)^{abcd}
Kh-3	40 (39.14) ^{cdef}	43.3 (41.15) ^{efg}	43.33 (34.37)	50.00 (39.00)
Sd-3	36.6 (36.93) ^{cdef}	36.6 (36.93) ^{fg}	73.33 (55.82)^{cde}	76.66 (58.84)^{cdef}
Om-5	33.3 (36.00) ^{def}	33.3 (36.00) ^{g}	90.00 (72.11)^{abc}	93.33 (75.13)^{abc}
St-2	33.3 (34.92) ^{def}	53.3 (46.92)^{cdef}	100.0 (85.94)^{a}	100.0 (85.94)^{a}
Po-7	33.3 (35.21) ^{def}	46.6 (43.07)^{defg}	90.00 (72.11)^{abcd}	90.00 (72.11)^{abcde}
Gf-18	33.3 (35.00) ^{def}	33.3 (35.00) ^{g}	80.00 (64.97)^{bcde}	80.00 (64.97)^{bcdef}
Sa-49	30.0 (29.34) ^{ef}	40.0 (38.06) ^{efg}	83.33 (63.68) ^{bcde}	86.66 (66.71)^{abcdef}
Po-5	30.0 (32.21) ^{ef}	43.3 (41.07) ^{efg}	96.66 (80.54)^{ab}	96.66 (80.54)^{ab}
Ab-3	26.6 (30.78) ^{ef}	43.3 (41.07) ^{efg}	83.33 (64.19)^{bcde}	83.33 (64.19)^{bcdef}
Fh-6	26.6 (30.78) ^{ef}	40.0 (39.14) ^{efg}	43.33 (31.10)	53.33 (34.37)
Ab-31	26.6 (30.99) ^{ef}	26.6 (30.99)	60.00 (46.06)	63.33 (48.49)
Om-4	26.6 (30.99) ^{ef}	33.3 (35.21) ^{g}	88.66 (69.09) ^{abcde}	90.00 (72.11) ^{abcde}
Ab-7	26.6 (30.99) ^{ef}	26.6 (30.99)	80.00 (61.17)^{bcde}	83.33 (64.19) ^{bcdef}
St-13	23.3 (28.78) ^{f}	23.3 (28.28)	56.66 (43.74)	63.33 (48.49)
Kb-30	23.3 (28.78) ^{f}	30.0 (32.71)	90.00 (72.11) ^{abcd}	90.00 (72.11) ^{abcde}
Om-6	23.3 (28.78) ^{f}	36.6 (37.14)^{efg}	33.33 (25.07)	43.33 (32.20)
St-23	23.3 (28.78) ^{f}	43.3 (41.15) ^{efg}	100.0 (85.94) ^{a}	100.0 (85.94) ^{a}
Sd-2	23.3 (28.78) ^{f}	33.3 (34.92) ^{g}	40 (31.58)	46.66 (36.21)
Sa-2	23.3 (28.78) ^{f}	40.0 (39.14) ^{efg}	80.00 (64.06)^{bcde}	80.00 (64.06)^{bcdef}
Sh-13	16.6 (23.85)	40.0 (38.85) ^{efg}	63.33 (48.49)	63.33 (48.49)
Po-41	23.3 (28.78) ^{f}	33.3 (35.21) ^{g}	26.66 (19.86)	30.00 (22.65)
Wh-4	23.3 (28.28) ^{f}	30.0 (33.00)	20.00 (15.84)	20.00 (15.84)
Gg-1	23.3 (28.28) ^{f}	26.6 (30.99)	73.33 (55.82)^{cde}	73.33 (55.82) ^{def}
Dn-4	26.6 (30.29)	20.0 (26.07)	66.66 (50.80) ^{cde}	76.66 (58.15) ^{cdef}
Lb-1	20.0 (26.07)	20.0 (26.07)	60.00 (46.16)	60.00 (46.16)
Ab-8	20.0 (23.49)	23.3 (25.49)	86.66 (69.09) ^{abcde}	90.00 (72.11) ^{abcde}
Kb-26	16.6 (23.36)	16.6 (23.36)	86.66 (69.09) ^{abcde}	90.00 (72.11) ^{abcde}
Gz-6	16.6 (23.85)	20.0 (26.07)	36.66 (27.08)	36.66 (27.08)
Ab-1	16.6 (23.85)	16.6 (23.36)	40.00 (29.87)	43.33 (32.30)
Wh-5	16.6 (23.85)	16.6 (23.85)	86.66 (69.60) ^{abcde}	86.66 (69.60) ^{abcdef}
Sa-8	13.3 (19.06)	23.3 (28.07)	86.66 (69.09) ^{abcde}	86.66 (69.09) ^{abcdef}
Dn-1	13.3 (18.56)	13.3 (18.56)	23.33 (15.55)	23.33 (15.55)
Po-42	13.3 (21.14)	13.3 (21.14)	50.00 (38.62)	60.00 (46.48)
Po-1	10.0 (16.35)	13,3 (19.06)	46.66 (36.80)	46.66 (36.80)
St-5	06.6 (13.64)	06.6 (13.64)	83.33 (63.68) ^{bcde}	86.66 (69.09)^{abcdef}
Po-2	06.6 (13.64)	13,3 (19.06)	86.66 (66.71)^{abcde}	86.66 (69.09)^{abcdef}
HD-1	46.6 (41.15) ^{cde}	56.6 (48.84)^{bcde}	----------	----------
MVP11	90 (71.56) ^{a}	100 (85.94)^{a}	----------	----------
**E. coli* expressing *cry*3 (ECE-131)	----------	----------	33.33 (23.07)	40.00 (29.41)
control	00.0 (4.05)	06.6 (11.55)	16.66	16.66

Values in parentheses are arcsine transformed means. Means are results of three replicates;
* *E. coli cry*3 clone examined at 10 ppm

Table 5—Biochemical characterization of some important isolates

Isolate Annotation	Citrate	ONPG	Arginine	Sucrose	Mannitol	Glucose	Arabinose	Trehalose
Ab-8 *Bacillus* sp. (KF305085)	+	+	+	+	-	+	+	+
Ab-12 *Bt* (KF305086)	+	+	+	+	+	+	+	+
Fh-6 *Bt* (KC201678)	-	-	+	+	-	+	+	+
Gg-A *Pp* (KC107790)	-	+	+	+	-	+	+	+
Kb-26 *Paenibacillus* sp.(KF30508)	-	-	+	+	-	+	-	+
Kb-29 *Bt* (KC201674)	-	+	+	+	-	+	+	+
Kb-30 *Bacillus* sp. (KF305084)	-	+	-	+	+	+	+	+
Kh-3 *Paenibacillus* sp. (JX674042)	-	-	+	+	+	+	+	+
Om-5 *Bt* (JX660701)	-	+	+	+	-	+	+	+
Po-1 *Paenibacillus* sp. (JX841102)	+	-	+	-	-	+	+	+
Po-2 *Bacillus* sp. (KF305081)	-	+	+	-	+	+	-	-
Po-5 *Bt* (JX391979)	-	+	+	+	-	+	+	+
Po-7 *Bacillus* sp.(KF305082)	-	-	+	+	+	+	+	+
Po-42 *Pp* (JX841103)	-	+	+	+	-	+	+	+
aS-8 *Bt* (KF305080)	+	-	+	+	+	+	+	+
Sh-13 *Bp* (JX857699)	-	+	+	+	-	+	-	+
Sh-14 *Pp* (KC201676)	-	+	+	+	-	+	+	+
St-2 *Bt* (KC201677)	-	+	+	+	-	+	+	+
St-6 *Bt* (JX841104)	-	+	+	+	-	+	+	+
St-14 *Ls (*KC107791)	-	+	+	+	-	+	+	+
St-22 *Bt* (KC201675)	-	+	+	-	-	-	+	-
St-23 *Bt* (JX674040)	-	+	+	+	-	+	+	+
Wh-1 *Bt* (KC107789)	-	+	+	+	-	+	+	+
Wh-5 *Bt* (JX674041)	+	+	+	+	-	+	+	+
ECE- 131	+	-	+	-	+	+	+	+
HD-1	-	-	-	+	+	+	-	+

All isolates were positive for catalase and nitrate reduction, and negative for malonate and Voges Proskauer; *Bp*, *Bacillus pumilus*; *Bt*, *Bacillus thuringiensis*; *Ls*, *Lysinibacillus sphaericus*; *Pp*, *Paenibacillus popillae*,.

Sa-8, St-2, St-6, St-22, St-23, Wh-1, Wh-5), *Paenibacillus popilliae* (Gg-2, Om-4, Po-42, Sh-14), *Paenibacillus sp.* (Kb-26, Kh-3, Po-1), and *Lysinibacillus sphaericus* (St-14) (Table 6).

Identification of cry genes in Bacillus isolates—Of the most effective five strains, St-6 showed the presence of *cry1* gene with PCR amplification product of 362 and presence of *cry28* with a prominent band of 400 bp instead of expected band size of 466 bp (Fig. 2a,d). The expected size of PCR product of *cry1*, *cry2, cry3* and *cry28* genes are 276, 689-701, 589 and 466 bp, respectively. We identified strain Kb-29 which produced unexpected 342 bp product when assayed with *cry1* gene specific primer, suggesting that it may harbor an unique *cry1* gene. The *cry1* primer produced amplicon of 276 bp product as expected which was also seen in both HD-1 and HD-73. No *cry1* gene was detected in St-2, St-23 and Wh-1 (Fig. 2).

The *cry2* genes were found in standard strains, HD-1 and *E. coli* ECE-126 with amplicons of 716 and 683 bp, respectively. None of selected isolates showed presence of *cry2* (Fig. 2b). However, Ab-12 (*B. thuringiensis*) and Kh-3 (*Paenibacillus* spp.) isolates showed presence of *cry2* genes as these produced amplicons of 690 and 685 bp, respectively.

Only *E. coli* ECE-131 strain showed presence of *cry3Aa1* gene by producing 589 bp amplicon (Fig. 2c). None of isolates showed presence of *cry3* gene.

Characterization of toxin proteins by SDS-PAGE—The protein profile analysis from selected best isolates revealed that St-23 and St-6 isolates showed major protein bands around 130, 63-75, 48 and 20-25 kDa, respectively (Fig. 3). These proteins may belong to Cry1 and Cry2 protoxin and their activation products. The protein of 29 kDa may correspond to the cytolytic (Cyt) toxin. HD-1, Om-4, Fh-6 and Kb-29 isolates showed 63-75 and 20-25 kDa, while Sh-13, St-22, Wh-5, St-14, Gg-2 and Wh-1 showed proteins

Table 6—GenBank accession numbers of 16S rDNA sequences

Isolate annotation	Scientific name	BankIt No.	Accession No.
Ab-8	*Bacillus* sp.	1642373	KF305085
Ab-12	*Bacillus thuringiensis*	1642374	KF305086
Fh-6	*Bacillus thuringiensis*	1584597	KC201678
Gg-2	*Paenibacillus popilliae*	1578072	KC107790
Kb-26	*Paenibacillus* sp.	1642371	KF305083
Kb-29	*Bacillus thuringiensis*	1584578	KC201674
Kb-30	*Bacillus sp.*	1642372	KF305084
Kh-3	*Paenibacillus* sp.	1566459	JX674042
Om-4	*Paenibacillus popilliae*	1566570	JX841101
Om-5	*Bacillus thuringiensis*	1565217	JX660701
Po-1	*Paenibacillus* sp.	1567373	JX841102
Po-2	*Bacillus* sp.	1642358	KF305081
Po-5	*Bacillus thuringiensis*	1550883	JX391979
Po-7	*Bacillus* sp.	1642370	KF305082
Po-42	*Paenibacillus popilliae*	1567382	JX841103
Sa-8	*Bacillus thuringiensis*	1642354	KF305080
Sh-13	*Bacillus pumilus*	1569168	JX857699
Sh-14	*Paenibacillus popilliae*	1584588	KC201676
St-2	*Bacillus thuringiensis*	1584596	KC201677
St-6	*Bacillus thuringiensis*	1567633	JX841104
St-14	*Lysinibacillus sphaericus*	1578077	KC107791
St-22	*Bacillus thuringiensis*	1584584	KC201675
St-23	*Bacillus thuringiensis*	1566428	JX674040
Wh-1	*Bacillus thuringiensis*	1578062	KC107789
Wh-5	*Bacillus thuringiensis*	1566435	JX674041

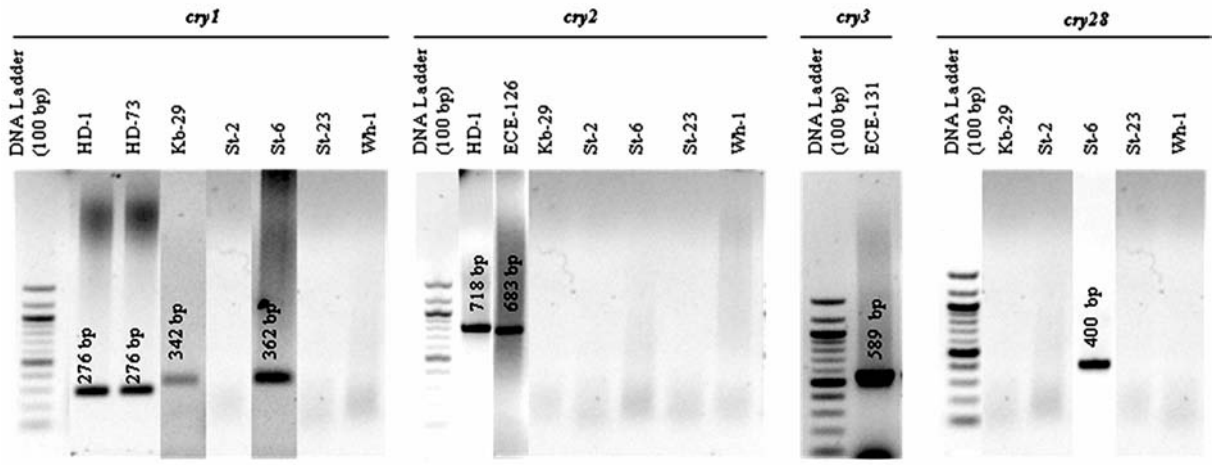

Fig. 2—(a,b,c,d). *cry1, cry2, cry3* and *cry28* genes screening by PCR analysis of local *Bacillus thuringiensis* isolates.

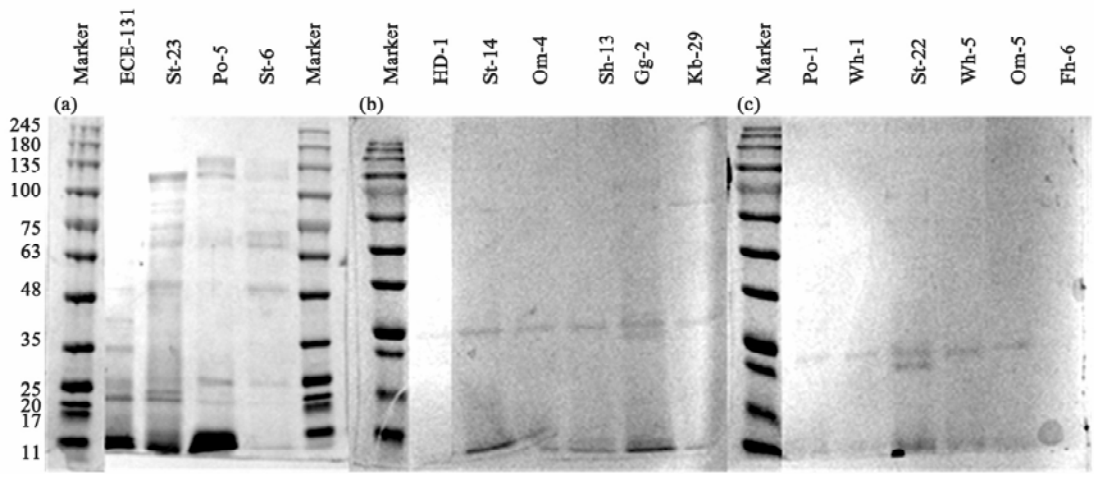

Fig. 3─(a,b,c). SDS-PAGE profile of selected *Bacillus thuringiensis* native strains.

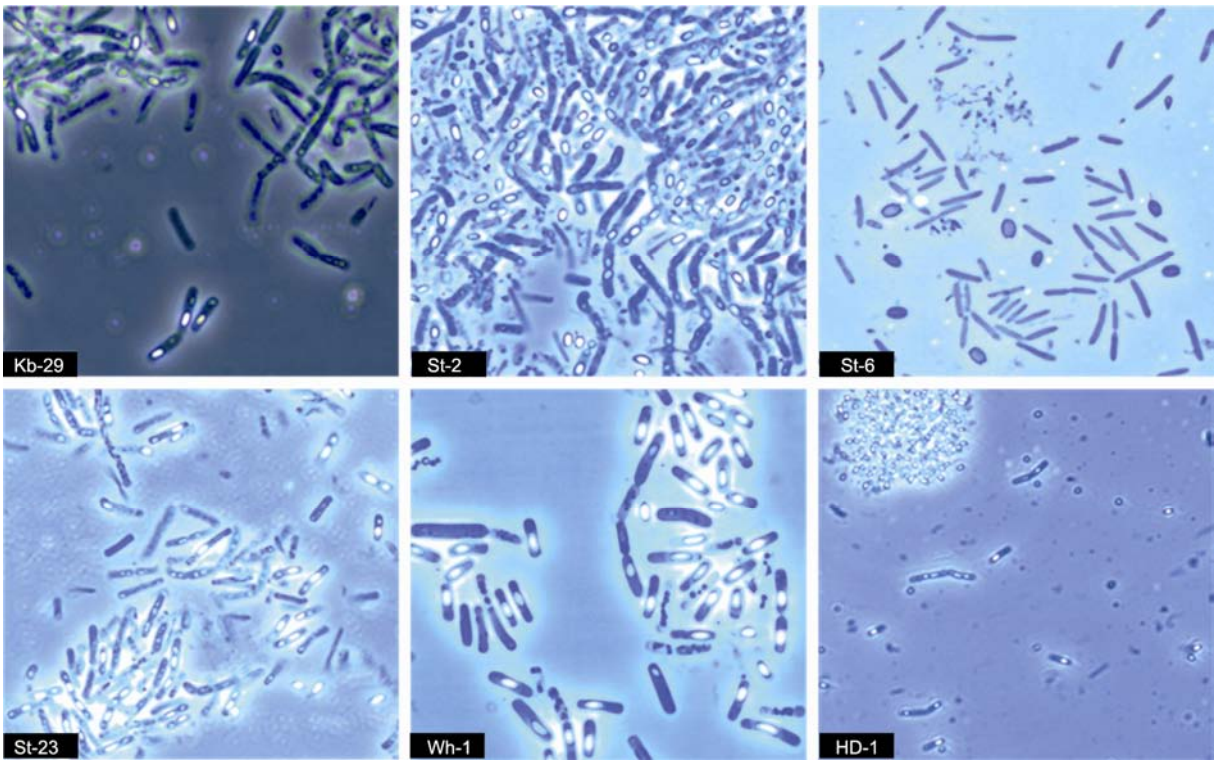

Fig. 4—Phase contrast microscopy of Kb-29, St-2, St-6, St-23, Wh-1 with standard HD-1. Magnification 1000x.

between 20 and 25 kDa. *E. coli* ECE131 specific for Cry3 toxin clone produced 19, 25, 32, 42, 67 kDa proteins while the above isolates produced 19 and 25 kDa proteins.

Morphology of selected B. thuringiensis isolates by microscopy─Morphological studies of the selected five isolates were performed by phase contrast microscopy. The results revealed that standard strain HD-1 produced bipyramidal shaped crystals. Kb-29, St-2, St-23, Wh-1 produced spherical shaped crystals whereas St-6 isolate produced very small size crystals (Fig. 4). All isolates produced ellipsoidal shaped spores except St-6.

Cry1Ac toxin estimation with ELISA─Of the five selected isolates, St-6 spore-crystal complex contained 4.64 ppm Cry1Ac. The lateral flow strips showed presence only of Cry1Ac.

Discussion

Bacterial isolates from Sudan differed a lot in their biochemical and molecular characteristics. These included *Bacillus* sp., *B. thuringiensis*, *Paenibacillus* sp. and *L. sphaericus*, although *B. thuringiensis*-like bacteria dominated (48%) in the collection. Using the biochemical characterization as criterion for *B. thuringiensis* isolates originally developed by Martin *et al.*[28], all isolates showed positive tests for catalase and nitrate reduction; and negative tests for malonate and Voges Proskauer, latter for 2-3 butanediol production from glucose. However, these tests were not specific. Thus, some isolates, despite being identified with 16S rDNA sequences, differed to some extent, owing to their being different strains/serotypes. Keshavarzi[29] isolated and differentiated *B. thuringiensis* strains collected from various locations in Iran, solely on the basis of biochemical traits and reported predominance of *B. thuringiensis* var. *kurstaki*. Haggag and Abou-Yousef[30] reported diversity of nine *B. thuringiensis* isolates from Egypt. El-Kersh *et al.*[31] found differences in 64 *B. thuringiensis* isolates from Saudi Arabia characterized with 16S rRNA sequencing and biochemically. Similar studies on the diversity of *B. thuringiensis* were reported in India[32-35], Iran[36] and Israel[37]. The production kinetics in terms of spore-crystal complex showed the differences amongst strains which could be genetic in nature. The high production of spore-crystal complex could be important in economics of commercialization.

Besides, the amount of Cry toxins and relative proportion of effective toxins in the spore-crystal complex will decide the efficacy. It is well known that strain that produces only one Cry toxin is less toxic than strain producing a mixture of toxins[38]. The *cry* genes were found to vary in different isolates. In some cases, amplicons of unexpected sizes were produced, suggesting differences in *cry* genes. St-6 strain which was effective against lepidopteran and coleopteran insects showed presence of respective *cry1* and *cry28* genes[39]. Wojciechowska *et al.*[40] observed a novel protein band with 125 kDa for *cry28* in *Bacillus thuringiensis* ssp. *finitimus*.

Of the five selected isolates, only two isolates Kb29 and St-6 showed presence of *cry1* gene while St-6 also showed *cry28*, whereas both had lepidopteran as well as coleopteran toxicity. Variations in *cry* gene contents and distribution are likely associated with differences in the biological, geographical and ecological properties of the collection areas[41]. Patel *et al.*[42] reported diversity of *cry* genes in strains collected from different regions of the country. Of all *cry* genes studied, *cry1* gene was the most abundant; with no major difference in distribution and diversity of *cry* genes in agricultural and non-agricultural samples except the absence of *cry3* and *cry13* genes in non-agricultural soil samples.

Interestingly, this study also showed that many isolates were highly toxic to Coleoptera than to Lepidoptera which is due to high concentration used in bioassays for *T. castaneum*. Pereyra-Alferez *et al.*[43] also reported effective strain C-9 against *Oryzaephilus surinamensis* and *T. castaneum* in wheat flour bioassays. *E. coli cry3* clone resulted in less than 50% mortality of larvae of the red flour beetle at 10 ppm concentration as against 22% mortality of Andean weevil when treated with Cry3Aa[41].

Analysis of SDS-PAGE further revealed that these strains produced a range of proteins of different molecular weights between 20 and 130 kDa depending up on the strain. These proteins could be Cry toxins/protoxins and their products. St-6 which showed presence of *cry* genes also expressed protein bands corresponding to *cry1* and *cry28* genes.

This study confirms the presence of many different *B. thuringiensis*-like subspecies and strains. Such a rich collection especially from the localities unexplored until recent past also opens up new vista for search of highly effective strains for pest management. The present study shortlisted some of the isolates (Kb-29, St-2, St-6, St-23 and Wh-1) which could be a starting point for developing them as bioinsecticides. Further studies are needed on fermentation and formulation of these isolates, and testing them under field conditions in a suitable agroecosystem.

Aknowledgement

N.E.G. is grateful to the Indian government for the award of CV Raman fellowship for African researchers for a period of 6 months in 2012 and to ENRRI, Khartoum for study leave to avail the fellowship. We thank two anonymous reviewers for their very useful comments. We are grateful to Director, Indian Agricultural Research Institute, New Delhi for providing infrastructure and facilities for carrying out these studies.

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