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Full Length Research paper

Development of cost-effective media for the culture of *Chilo partellus* larvicide in Kenya

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Stem borers (*Chilo partellus*) are important field insect pests of maize (*Zea mays* L.) and sorghum (*Sorghum bicolor* L.) in Africa. They account for more than 30% yield losses depending on the composition of the pest community. *C. partellus* larvicide like *Bacillus thuringiensis* have been widely and effectively used in *C. partellus* control programs, but the industrial production of theses bacilli is expensive. Here we have attempted to develop three cost-effective media, based on legumes, potato, and whey. Growth and production of the insecticidal proteins from these bacteria were satisfactory; protein concentration yields of 27.60 mg/ml, spore counts of 5.60 × 10^8 CFU/ ml and first-instar *Chilo partellus* larvicidal activity (LC_{50}) of 78 µg/l were obtained with a 72 h culture of this bacterium. Therefore, this investigation suggests that legume, potato and whey-based culture media are more economical and effective for the industrial production of *B. thuringiensis* insecticidal crystal proteins.

Key words: *Bacillus thuringiensis*, larvicidal, *Chilo partellus*, insecticidal crystal proteins, LC_{50}, optical density, sulphate polyacrylamide gel electrophoresis (SDS-PAGE), spore counts.

INTRODUCTION

In Kenya, the spotted stem borers (*Chilo partellus*) destroy an estimated 400,000 metric tons or 13.5% of farmers annual harvest of maize costing about >US$72 million (De Groote, 2002). The management of *C. partellus* has largely been based on chemicals, which are rarely effective particularly due to misuse and resistance development by the pest (Camilla, 2000). In addition, small scale farmers, who form the bulk of the maize producers in Kenya, cannot afford those (Bonhof et al., 2001). Technologies that can reduce yield losses from *C. partellus* damage are necessary to increase maize production to cope with increasing demand for maize in Kenya. Berliner (*Bt*) was thus considered as a possible component in such a pest management system and a series of investigations was undertaken to elucidate its potential for inclusion in such a program (Mugo et al., 2007). In the recent past, it has been the most successful commercial biopesticide with its worldwide application (Pena et al., 2007). When compared with the chemical pesticides, *Bt* has the advantages of being biologically degradable, selectively active on pests and less likely to cause resistance (Lambert and Peferoen, 1992). *Bt* synthesizes an insecticidal cytoplasmic protein inclusion during the stationary phase of its growth cycle (Pena et al., 2007). These crystalline inclusions comprise relatively high quantities of one or more glycoproteins known as delta-endotoxins or cryotoxins (Schnepf et al., 1998). The

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insecticidal activity of *Bt* was attributed largely or completely (depending on the insect) to these parasporal crystals (Bravo et al., 2007). The crystal proteins accumulate in the mother cell and are released upon completion of sporulation (Aronson et al., 1995). Usually *Bt* strains isolated locally are more effective than imported strains due to higher specificity on target host, greater field persistence due to higher adaptation to the natural environment and toxicity at a higher temperature range (Brownbridge, 1991). To derive full benefit from the *Bt* based biopesticides, there is a need for studying influence of carbon-source from cost-effective raw materials on growth, sporulation and crystal protein production by local *Bt* strains that would be used as biopesticides for the indigenous crops.

MATERIALS AND METHODS

Bacteria

Cultures of *B, thuringiensis* subspecies *kenyae (Bt)* were provided by Mr. Richard Rotich, from the Kenya Agricultural Research Insitute (KARI), Nairobi Kenya. Also used in this study were *Bt* strains isolated from soils and termite mounds collected from Kalunya Glade and Lirhanda Hill in Kakamega Forest, Kenya and also from soil samples from Juja, Kenya.

Bacterial culture media

The conventional laboratory culture broth Nutrient Yeast Extract Medium (NYSM), used as reference medium in the present study was prepared by mixing glucose (5 g), peptone (5 g), NaCl (5 g), beef extract (3 g), yeast extract (0.5 g), mineral solutions (10.0 g/l cow blood; 0.02 g/l $MnCl_2.4H_20$ (s. d. fine-chem ltd); 0.05 g/l $MgSO_4.7H_20$ (Lab Tech Chemicals); and 1.0 g/l $CaCO_3$ (Sigma-Aldrich, Germany) (10 ml) in an appropriate volume (1 L) of double distilled water (pH 7.5). Legumes, potato and whey-based culture media was collected from farms and brought to the laboratory, washed in tap water, air-dried and stored at room temperature. A known quantity of these dried substrates (10 g/l) was boiled in ordinary tap water for 15 min. After cooling, the extracts were filtered and the pH of the filtrate was adjusted (pH 7.5). 1 L volume of the extract medium was dispensed in each of the three conical flasks (2 L capacities each) for culturing *Bt*. Similarly, flasks were kept for conventional medium (NYSM) also. All the culture media were autoclaved (at 120 °C/20 lb/in^2/20 min).

Bacterial growth

A small amount of *Bt* was inoculated separately in 2 ml each NYSM medium and allowed to grow for 12 h at 37°C as pre-cultures (50 µl each) were inoculated into sterile culture media. The cultures were allowed to grow under constant agitation (120 rev/min) at 37°C in an orbital shaker. Culture samples (2 ml) were drawn from each culture medium at 6 h intervals from 0 to 72 h. The pH and culture turbidity were measured using a digital pH meter and UV-VIS spectrophotometer. These were also examined microscopically for the presence of spore-crystal mixtures (Maniatis et al., 1982).

Total viable cell count and spore count

Total viable cell and spore counts were determined in the final whole culture by the pour plate method. Serial decimal dilutions of the final whole culture were made in sterile 1% peptone water (Oxoid), and 0.5 ml of each dilution in triplicate was added to a Petri dish, followed by the addition of 10 ml of plate count agar (Oxoid) at 45°C. The culture and agar were mixed thoroughly and allowed to set. Plates were incubated at 32°C for 24 to 48 h. Plates with 30 to 300 colonies were counted with a colony counter (Gallenkamp Ltd.). For spore counts, cultures were pasteurized at 65°C for 20 min before serial dilutions were made (Hoben and Somasegaran, 1982).

Spore-crystal toxin recovery from culture media

As soon as the cultures were fully sporulated, the spore-crystal toxin complex was recovered by centrifugation (10,000 *g*/30 min/4°C) using super speed centrifuge and the spore-crystal free supernatants were discarded. The spore-crystal mixtures were thoroughly washed three times each with 0.1 M NaCl and sterile double distilled water (10,000 *g*/30 min/4°C). Finally, these were washed with protease inhibitor, phenyl methyl sulphonyl fluoride (PMSF, 1mM, Sigma), re-suspended in the required volume of double distilled water and stored at -20°C, until further use, for biochemical studies and toxicity bioassays (Wessel and Flugge, 1984).

Protein estimation

A small volume of the stored spore/crystal sample was centrifuged (10,000 *g*/15 min/4°C) and the pellets were solubilized in solubilization buffer (50 mM $NaHCO_3$, 10 mM dithiothreitol, pH 10) and incubated for 2 to 3 h, at 25 to 30°C. After centrifugation and extraction (same rpm as above), the pure solubilized protein (from *Bt*) was quantified for protein estimation (Bradford, 1976) with bovine serum albumin (BSA, Sigma) as standard.

SDS-PAGE

A total of 5 µg protein equivalent samples from *Bt* spores-crystals (NYSM and the test media) was mixed with an equal volume of sample loading buffer and boiled for 5 min and separated on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) unit, according to Laemmli (1970). The protein bands were stained with Coommasie Brilliant Blue R-250 and visualized.

Toxicity studies

Powders of *Bt* produced from the three cost-effective media and control were assayed against laboratory-reared first instar larvae of *C. partellus*. A standard primary powder of *Bt* subspecies *kurstaki* (*Btk*) was included in the assay for comparison. 100 mg of NYSM, potato, legumes, and whey powder was suspended respectively in 1,000 ml of distilled water containing 1% (vol/vol) Tween 80. Serial dilutions of this suspension were made in distilled water. 15 larvae were added to 150 ml of each dilution in 250 ml white plastic cups. Three cups were used per dilution. Controls consisted of three cups each containing 150 ml of distilled water and 15 larvae for each powder assayed. Larval food was provided by adding a small

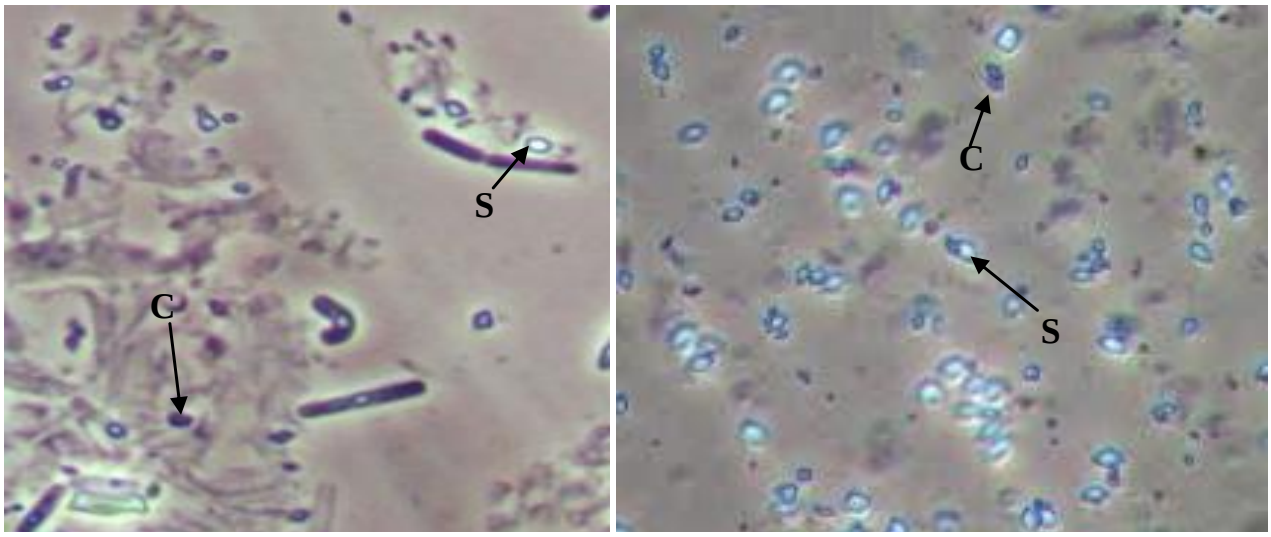

Figure 1. Photomicrograph of *Bt* spore-crystal mixture produced from potato, legume and whey formulated media. S, spores; C, Crystal (×1000).

portion of finely ground oaf flakes (Quaker) mixed with dried yeast powder. Each experiment was incubated at 20 ± 5°C for 48 h, and each assay repeated three times. Observations were made at 6 h for paralysis and knockdown effects. Mortality counts were made at 24 h and 48 h. A larva was presumed dead if it did not move when touched with a blunt needle.

Statistical studies

A one-way ANOVA test was used to compare mean maximum spore count among media and pairwise comparison of the media was done using the Duncan's multiple comparison test based on least significance difference. Probit analysis for calculation of LC_{50} values was carried out using the statistical software SPSS 18.0 for windows.

RESULTS

During activation and multiplication of the isolates, the growth of the local *Bt* and *Btk* isolates occurred between 24 to 72 h producing smooth creamish-white colonies which were rough edged and slightly raised from the nutrient agar. Under the culture conditions described, lysis of the *Bt* cells was complete after 72 h and most of the released protein crystals settled at the bottom of the flask. Almost complete separation of the endotoxin protein crystals from the spores and cell debris was achieved by decantation of the frothy spent culture and high speed centrifugation at 10,000 rpm where the crystals formed a white pellet at the bottom of the tube leaving the spores and cell debris in the supernatant fraction. Serial washing, decanting and centrifugation rid the crystals of spent culture components, spores and cell debris. Microscopy revealed a high concentration of the crystalline inclusions in the pellets obtained (Figure 1).

The highest optical densities were obtained at 37°C from isolates: 62LBG37°C, *Bt* 20, 63KAG37°C, 1SKAG37°C, *Bt* 47, *Bt* 12 and *Bt* 54 respectively. The results of optical density show that pH 7 was the most suitable pH for the maximum growth of 62LBG37C, *Bt* 21, *Bt* 47, and *Bt* 52 while pH 7.5 was suitable for 14SLA30°C and pH 5.5 was suitable for *Bt* 20.

The mass of the resulting pellets ranged from 0.460 g for *Bt* 30 to 0.225 g for *Bt* 20 (mean = 0.314 g ±0.084 g) (Table 1). The protein mass of the pellets was significantly different among the isolates ($t_{16.616,\ 19,\ 0.000}$). Spore counts in the pellets ranged from 4.89×10^8 to 5.60×10^8 (mean = 5.23 ±1.53) (Table 1). There was significant difference ($t_{16.014,\ 19,\ 0.000}$) on the percent protein content in the pellets. Isolates *Bt* 30 and *Bt* 47 recorded higher contents while 24LBN30°C, 63KAG37°C, and *Bt* 20 had low protein content. The protein yield in the nutrient broth ranged from 2.22 to 4.60 mg/ml of broth (mean = 3.112 mg ± 0.938 mg) and was significantly different ($t_{23.328,\ 19,\ 0.000}$) across the different *Bt* isolates.

When compared to high molecular weight standards in SDS-PAGE analysis, the solution and pellet of the dissolved spore-crystal product from each treatment had proteins with molecular weights of approximately 110 to 120 and 60 to 70 kDa (Figures 2 and 3). The major polypeptides present in the spore-crystal complex of *Bt* produced from Legume medium (1SKAG37°C and 24LBN30°C), NYSM medium (58SLA25°C and *Bt* 20), potato medium (58SLA25°C and *Bt* 30) and whey medium

Table 1. Masses of pellets of *Bt* isolates and their protein quantities.

***Bt* isolate number**	**Media**	**Mean absorbance (OD 600)**	**Spore count (CFU/ml)**	**Protein in pellet (mg/ml)**
46	Legume	1.47	3.80×10^8	4.18
	Potato	1.80	5.10×10^8	2.22
	Whey	1.00	2.50×10^8	3.25
37	Legume	1.48	3.80×10^8	4.01
	Potato	1.92	5.00×10^8	3.22
	Whey	1.07	2.90×10^8	3.68
62LBG37C	Legume	1.45	2.60×10^8	4.51
	Potato	1.82	4.10×10^8	3.09
	Whey	1.21	2.40×10^8	3.78
30	Legume	1.40	3.40×10^8	3.87
	Potato	1.92	5.30×10^8	3.02
	Whey	1.33	1.40×10^8	4.15
20	Legume	1.44	3.40×10^8	4.12
	Potato	1.84	4.10×10^8	3.00
	Whey	0.71	2.00×10^8	3.45
21	Legume	1.35	3.50×10^8	3.69
	Potato	1.91	3.30×10^8	3.07
	Whey	1.30	1.30×10^8	2.68
47	Legume	1.33	4.00×10^8	4.60
	Potato	1.95	3.70×10^8	2.71
	Whey	0.51	1.00×10^7	3.72
63KAG37C	Legume	1.29	2.40×10^8	4.12
	Potato	1.90	3.70×10^8	3.04
	Whey	1.09	2.60×10^8	4.02
14SLA30C	Legume	1.33	3.80×10^8	3.92
	Potato	1.99	4.40×10^8	3.14
	Whey	0.99	1.00×10^7	3.91
53	Legume	1.14	3.50×10^8	4.04
	Potato	1.64	4.90×10^8	3.02
	Whey	1.21	3.00×10^8	3.34
24LBN30C	Legume	1.04	2.70×10^8	4.12
	Potato	2.39	4.70×10^8	3.08
	Whey	0.94	2.20×10^8	3.85
1SKAG37C	Legume	1.05	3.00×10^8	4.43
	Potato	1.66	4.70×10^8	3.32
	Whey	0.72	2.00×10^8	3.00
58SLA25C	Legume	1.38	3.00×10^8	4.63
	Potato	1.93	4.80×10^8	3.11
	Whey	1.27	2.50×10^8	4.45
54	Legume	1.35	3.40×10^8	4.52
	Potato	1.79	5.40×10^8	3.05
	Whey	0.61	2.20×10^8	3.73
12	Legume	1.45	3.30×10^8	4.17
	Potato	1.90	5.30×10^8	3.09
	Whey	1.13	1.90×10^7	3.71
14	Legume	1.44	3.20×10^8	4.08
	Potato	1.92	5.60×10^8	3.12
	Whey	0.52	2.70×10^8	3.09

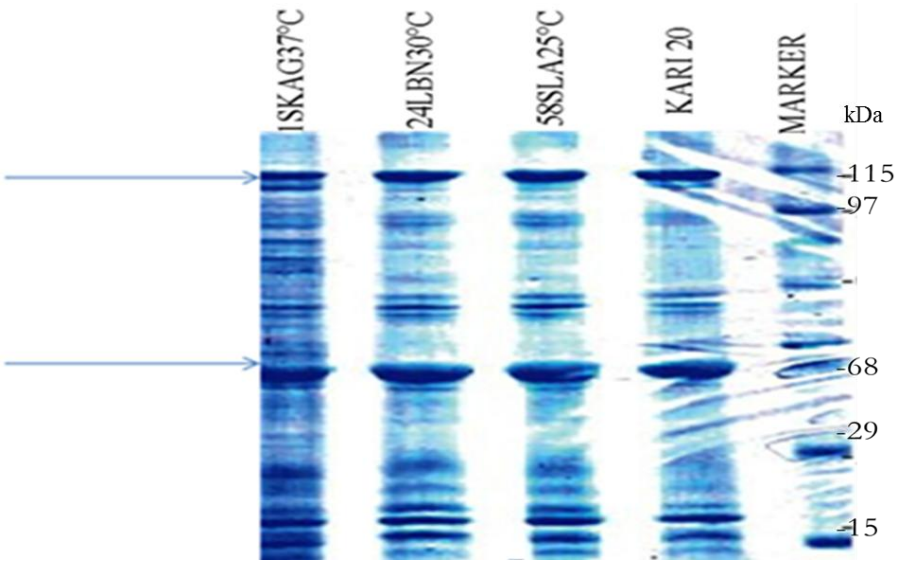

Figure 2. The protein bands (arrows) of delta-endotoxin and spore mixture of *Bt* isolates during its fermentation from Legume medium (1SKAG37°C and 24LBN30°C) and NYSM medium (58SLA25°C and *Bt* 20) as determined by SDS-PAGE.

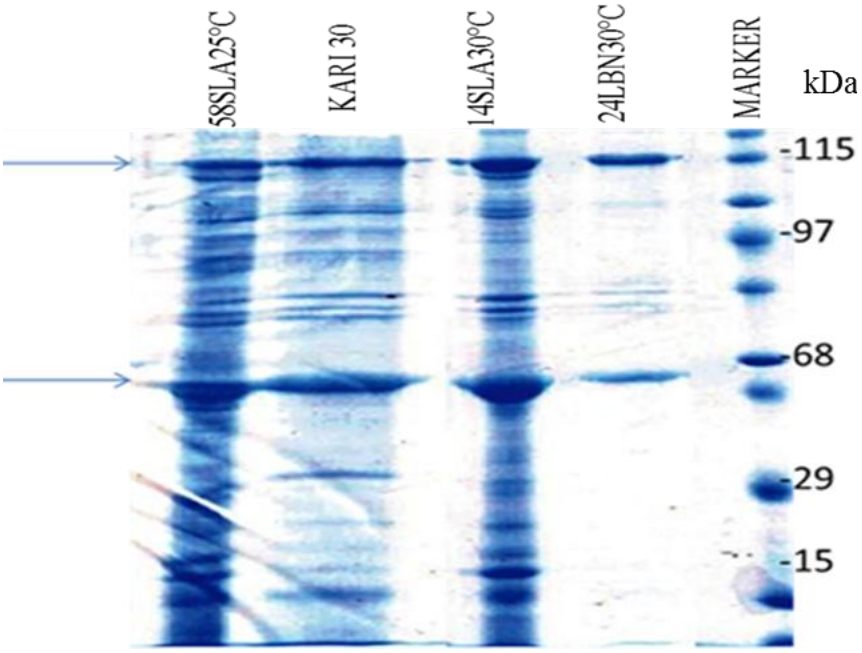

Figure 3. The protein bands (arrows) of delta-endotoxin and spore mixture of *Bt* isolates during its fermentation

(14SLA30°C and 24LBN30°C) were clear and conspicuous. The protein profiles as indicator of *Bt.*

Crystal toxins were correspondingly related to their larvicidal activity.

The neonate larvae started feeding immediately after introduction into the Petri dish and preferred the underside of the leaf. Their feeding intensity slowed down with time and some larvae moved away from the meal

Table 2. Percent mortality of neonate *C. partellus* larvae on treatment with five toxin concentrations of, the standard, *Bacillus thuringiensis* subspecies *kurstaki* (*Btk*).

Concentration (mg/ ml)	Mean larval mortality at 48 (%)	Intercept	Slope (±SE)	LC_{50} (mg/ml)	x^2 (df)
0	10	1.2	0.8 ±0.33	0.001	5.462
0.015	78	2	1.1 ±0.25	0.011	
0.15	72	1.8	1.2 ±0.19	0.008	
1.5	49	1.7	1 ±0.21	0.006	
15	20	1.4	0.9 ±0.27	0.003	

after 24 h. Most larvae in the treatment especially with higher delta-endotoxin concentrations stopped feeding after 48 h, appeared weak and stunted in growth compared to the control upon when death was also observed. For instance, with the 0.015 mg/ml endotoxin treatment, 78% of the larvae were found to be dead from the diet after 48 h. After 48 h, only 10% of larvae were dead with the 0 mg/ml endotoxin treatment, 72% were dead with the 0.15 mg/ml endotoxin treatment, 49% dead with the 1.5 mg/ml endotoxin treatment and 20% of the larvae were dead with the 15 mg/ml endotoxin treatment. Leaf damage was observed to be less on inoculated leaf disks compared to that in the control. Upon death, the larvae appeared dark and shrunk compared to the control. A dead larva was washed, ground and aseptically inoculated onto nutrient agar plate, creamish growth was observed around the larvae which confirmed that the larval mortality was due to ingestion of *Bt* endotoxins. In the set of starved larvae, mortality was 100% in 144 h (Table 2).

A 10% larval mortality on the control treatment was observed after 48 h while 30% recorded after 48 h (Table 2). The cause of this mortality was probably due to drastic changed of weather and laboratory conditions from source of larvae to bioassay laboratory resulting in weak neonates, dehydration or infection; in the subsequent bioassays, eggs were sourced in the yellow state and allowed to acclimatize to the bioassay lab conditions before hatching and maize leaves were also thoroughly washed with distilled water, the filter paper in the Petri dish wetted daily with distilled water and the larvae placed back on the leaf if they had moved far away and got trapped under the filter paper which greatly reduced control larvae mortality. The LC_{50} value estimate for reference isolate *Btk* was 0.011 mg/ml after 48 h (regression coefficient = 0.031005, 95% confidence limit, SE = 0.13747).

Among the different *Bt* treatments, only isolates 24LBN30°C, 63KAG37°C and *Bt* 20 recorded mortality of 10% at 48 h of observation (Table 3). However, while *Btk* increased mortality towards the end of the observation, *Bt* 30, *Bt* 47 and *Bt* 54 stabilized at 60% from 48 h. Although, isolate 58SLA25°C recorded the first mortality of 40% at 24 h, it is the only isolate that recorded 73% mortality in the observation period, at 48 h. The *Btk* recorded 83% mortality at 48 h. No mortality was observed with 14SLA30°C, ISKAG37°C, *Bt* 21, *Bt* 12 and the control throughout the experiment period. Calculations of LT_{50} shows that *Btk* was the most toxic, causing 50% mortality after only 24 h, followed by 58SLA25°C at 48 h and *Bt* 30 at 48 h. One way ANOVA (repeated measures) revealed that the difference in percent larval mortalities of the standard isolate *Btk* and the *Bt* isolates was statistically significant ($p<0.05$) except the isolates with less than 20% mortality; *Bt* 37 ($F_{(1.0,5.0)} = 6.4$, $p>0.05$) and *Bt* 20 ($F_{(1.0,5.0)} = 0.122$, $p>0.05$).

DISCUSSION

The growth of the Kenyan isolates on nutrient agar and nutrient broth was similar to that reported by Wamaitha (2006) which shows the viability of local (Kenyan) *Bt* isolates. The protein purity values recorded by this study were highly variable probably due to the large diversity of isolates that may have differences in optimum growth conditions and a variety of insecticidal crystal protein produced (Aronson et al., 1995) which suggests that the culturing and extraction method may need to be optimized for each isolate in order to obtain equally high delta-endotoxin yields of high purity. The procedure used by this study has been reported to be well suited for large scale production of endotoxin extracts for pesticidal application (Osir and Vundla, 1999). The mean protein yield from the pellets varied from 20 to 30% similar to what is reported by Lereclus et al. (1993) although using different protocol. Higher protein values may have resulted from using lower centrifugation speeds and prior decantation of froth containing cell debris and spores effectively resulting in a lighter pellet.

Two commonly used *Bt* strains in commercial formulations for control of lepidopterans are *Bt kurstaki* and *Bt aizawai*, with the letter strains showing better larval control in situations where *Bt kurstaki* is becoming

Table 3. Percent cumulative mortality of *C. partellus* first-instar larvae exposed to 0.015 mg/ ml endotoxins from *Bt* isolates.

*Bt i*solate	Mean larval mortality at 48 h (%)	Intercept	Slope (±SE)	LC_{50} (mg/ml)	x^2 (df)
Bt kurstaki	83	1.9	1.1 ±0.33	0.011	11.425 (2)
58SLA25°C	73	1.8	1.1 ±0.33	0.009	
30	60	1.8	1 ±0.25	0.008	
47	60	1.8	1 ±0.25	0.008	
54	30	1.5	0.8 ±0.19	0.005	
62LBG37°C	30	1.5	0.8 ±0.19	0.005	
46	30	1.5	0.8 ±0.19	0.005	
14	30	1.5	0.8 ±0.19	0.005	
53	30	1.5	0.8 ±0.19	0.005	
37	20	1.3	0.6 ±0.21	0.003	
24LBN30°C	10	1.1	0.5 ±0.27	0.0001	
63KAG37°C	10	1.1	0.5 ±0.27	0.0001	
20	10	1.1	0.5 ±0.27	0.0001	
14SLA30°C	0				
ISKAG37°C	0				
21	0				
12	0				

less effective due to resistance development of the pests like the diamond black moth (Schnepf et al., 1998; Polanczyk et al*.,* 2000). The findings of this study closely resemble those of Wang'ondu (2001) with the more toxic isolates (*Bt* 44 and *Bt* 48) being obtained from the lowest LT_{50} values. Significant correlations between endotoxin yield and toxicity among the different isolates illustrates that different *Bt* isolates produce different delta-endotoxins which may differ in toxicity against different target pests (Uribe et al., 2003).

RECOMMENDATIONS

These results form a basis for further investigation of the local *Bt* isolates showing efficacy against *C. partellus* such as determination of the Cry proteins therein and how temperature and pH would affect their toxicity. It is also recommended that the toxicity of these isolates be investigated against other local lepidopteran pests in order to determine their target range.

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