CHARACTERISATION OF VARIOUS *BACILLUS THURINGIENSIS* STRAINS HAVING LARVICIDAL ACTIVITY ON *CHILO PARTELLUS* FIRST-INSTAR LARVAE, AFTER CULTURE ON COST-EFFECTIVE MEDIUM, IN KENYA

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Abstract
Bacillus thuringiensis strains are widely used in many larvicidal pest control programs. However, the large-scale production of these biolarvicides is very expensive due to the high cost of the production synthetic medium. In this study, we developed cost-effective media, based on locally available raw materials namely legumes, potato, and whey. The optical density, protein concentration yield, sporulation and *Chilo partellus* larvicidal action were studied by growing bacterial strains in these waste product and in comparison with the conventional medium (NYSM). Protein concentration yield of 27.60 µg/ ml, spore count of 5.60×10^8 and *Chilo partellus* larvicidal activity (LC_{50}) of 78 µg/ l against first-instar larvae were obtained with a 72 h culture of this bacterium. Production of *Bt* Insecticidal Crystal Proteins in NYSM was comparable to the test media and mean values were not significantly different for spore counts: F (media) = 25.19 P>0.01. One-way ANOVA (repeated measures) difference in percentage larval mortalities of *Bacillus thuringiensis* subspecies *kurstaki* (F = 26.88, P>0.05) and *Bt* isolates was not statistically significant. The SDS-PAGE profiles indicated that spore-crystal product from each treatment consisted of proteins with molecular weights of approximately 110-120 kDa and 60-70 kDa. Therefore the investigation suggests that legume, potato and whey-based culture media are more economical for the industrial production of *Bt* Insecticidal Crystal Proteins.

Key words: Bacillus thuringiensis, larvicidal, chilo partellus, insecticidal crystal proteins, LC50, optical density, SDS-PAGE, spore counts

1.0 Introduction

Maize (*Zea mays* L.) and other cereals form a large bulk of staple food in Kenya and in other sub-Saharan African countries. Several strategies have been adopted to control the various diseases transmitted by crop pests. Synthetic insecticides have been effectively used during the past several decades to control many Lepidopteran pests that prey on food crops (Charnley, 1991). But the use of chemical insecticides has become detrimental because of a multiplicity of factors including physiological resistance in the vectors, environmental pollution resulting in bio-amplification of food chain contamination and harmful effects on beneficial insects (Brar*et al*., 2006; Ghribi*et al*., 2007; Prabakaran*et al*., 2008). Hence, there has been an increased interest in recent years in the use of biological vector control agents. The discovery of bacteria like *Bacillus sphaericus* and *Bacillus thuringiensis* which are highly toxic to a variety of pests opened up the possibility of the use of these biolarvicides in pest eradication programs market (Adams *et al*., 1999; Crickmore, 2005). These bacilli have some advantages over conventional insecticides in pest control operations. They are safe for non-target organisms including humans and they are not hazardous to the environment (Alvarez *et al*., 2009). *Bacillus thuringiensis* is a gram-positive aerobic bacterium, producing spores within the sporangium (El-Bendary, 2006; Manzano*et al*., 2009). *Bacillus thuringiensis* synthesizes an insecticidal cytoplasmic protein inclusion during the stationary phase of its growth cycle (Schnepf*et al*., 1998). These crystalline inclusions comprise relatively high quantities of one or more glycoproteins known as delta-endotoxins (Scherrer*et al*., 1973) or Insecticidal Crystal Proteins. In 1989, Hofte and Whiteley, reviewed the known Cry genes and proposed a systematic classification. They distinguished four major classes of delta-endotoxins (Cry1, -2, -3 and 4) and Cytolysins (Cyt) found in the crystals of the mosquitocidal strains, on the basis of their insecticidal activity and molecular properties. The delta-endotoxins belonging to each of these classes were grouped in subclass (A, B, C... and a, b, c...). Generally these proteins are active against insect and invertebrate groups: Cry1 toxic to Lepidopteran, Cry2 toxic to Lepidopteran and Dipteran, Cry3 toxic to Coleopteran, Cry4 and Cry6 toxic to nematodes (Agaisse and Lereclus, 1995; Schnepf*et al*., 1998). *Bacillus thuringiensis* is the most effective microbial control agent active against pests that is available to date (El-Bendary, 2006). The cost to grow and to produce synthetic pesticide formulations through existing fermentation technology is extremely high. Therefore, the use of these pesticides has limitations. To protect the safety of the environment, the use of biopesticides to control crop pests has been emphasized during the last two decades. This warrants developing cheaper media for the culturing of *Bacillus thuringiensis* and thus facilitating the production of biopesticides in a cost-effective manner. Obeta and Okafor (1984) formulated five media (using the seeds of legumes, dried cow blood and mineral salts) and assessed the production of the insecticidal toxins of *Bacillus thuringiensis*serovar. *israelensis* which were effective against Aedes, Anopheles and Culex species of mosquitoes. Similarly, other media containing industrial byproduct, animal parts, fishmeal, soyabean and cornsteep liquor for the production of *Bacillus sphaericus* and *Bacillus thuringiensis*serovar. *israelensis* have also been reported (Sasaki *et al*., 1998; Devi *et al*., 2005; Yezza*et al*., 2006; Chang *et al*., 2008; Ouoba*et al*., 2008). In the present study, we have studied the influence of carbon-source on growth, sporulation and protein production by local *Bacillus thuringiensis*isolates and if the local isolates differ in their delta-endotoxin production and toxicity to *Chilopartellus* larvae.

2.0 Materials and Methods

2.1 Bacteria

The Kenya Agricultural Research Institute (KARI) isolates used in this study were from the *Bacillus thuringiensis* isolate germplasm stored in glycerin. Ten *Bacillus thuringiensis* isolates (no. 12, 14, 20, 21, 30, 37, 46, 47, 53, and 54) were randomly selected from a pool of 68 *Bacillus thuringiensis* isolates and a reference standard *Bacillus thuringiensis* subspecies *kurstaki* isolate also included in the assessment. Also used in this study were *Bacillus thuringiensis*strains 24LBN30°C, 1SKAG37°C, 62LBG37°C, 63KAG37°C, 58SLA25°C and 14SLA30°C isolated previously by Ntabo (2008) from soils and termite mounds collected from Kalunya Glade and Lirhanda Hill in Kakamega Forest and also from soil samples from JKUAT in Juja. The isolates were preserved in the JKUAT GK laboratory at -80°C. The isolates were revived on sterilized 8.0 g/ l nutrient broth (HiMedia Lab, India) and a gelling agent 15.0 g/ l agar (HiMedia Lab, India) media at pH 7.0 and 25°C. The bacterial isolates were then maintained on nutrient agar (HiMedia Lab, India) at 20°C for 72 h and stored at 4°C until used.

2.2 Media Preparation

Table 1: The different media used in this study for enrichment and sporulation of bacteria

Medium no.	Medium composition 10.0 g/ l
1. Basal medium	10.0 g/ l cow blood; 0.02 g/ l $MnCl_2.4H_20$; 0.05 g/ l $MgSO_4.7H_20$; 1.0 g/ l $CaCO_3$.
2.	Poultry litter
3.	10.0 g Legume seeds mixture: groundnut cake (*Arachis hypogea*), cow pea (*Vignaunguiculata*), soya beans (*Glycine soja*), and njugumawe/jugo beans (*Voandzeia subterranean*).
4.	10.0 g Wheat bran/cotton seed
5.	10.0 g Nutrient Yeast Synthetic Medium: 5.0 g glucose, 5.0 g peptone, 3.0 g beef extract, 0.5 g yeast extract.
6.	10.0 g Potato extract
7.	10.0 g Whey Medium

The powders were prepared by sieving and mixed at 30 °C in distilled water for 1 h. The liquids were then decanted and centrifuged (3300 × g for 15 min) and pH adjusted to 7.5 with 1 N NaOH (Lobachemie, India). These extract solution was sterilized at 120 °C and 15 psi for 20 min.

2.3 Growth Conditions

First stage seed culture was prepared by inoculating 10 mL of NYSM broth with one loopfull of *Bt* cells from a slant culture and incubating on an orbital shaker at 37°C, 180 rpm for 6 h. The seed thus prepared was added to 100 mL of various medium in a 500 mL flask at 2% level (v/v) and the flasks were incubated on an orbital shaker at 37°C, 250 rpm for a period of 72 h. This sample was used for assessment of cell mass and larvicidal activity.

2.4 Cell Mass

Each culture was centrifuged at 15,000 x g for 20 min (Prabakaran*et al.*, 2008), supernatant was discarded and the cell pellet was lyophilized. Dry weight was calculated (g/100 mL) and noted in g/ l. The dried samples were used to determine the toxicity against first-instar *Chilopartellus* larvae.

2.5 Spore-delta Endotoxin Recovery

The spore-delta-endotoxin complexes in the final whole culture were recovered as bacterial powders at the end of fermentation by the acetone precipitation method of Wessel and Flugge, 1984.

2.6 Crystal Protein Staining

A bacterial smear was prepared; air dried and heat fixed. It was placed inside a staining tray, covered with blotting paper and the paper saturated with basic fuchsin. The slide was heat fixed and examined under oil immersion objective (Lillie, 1977).

2.7 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 (Hoben and Somasegaran, 1982).

2.8 Protein Extraction and Concentrations

The protein extraction concentrations of the samples were determined by Bradford method (Bradford, 1976; Armelle, 1991).

2.9 SDS-PAGE

A protein molecular weight marker was from the Protein mixture, Amersham Biosciences (UK). The proteins were separated by using a vertical polyacrylamide gel apparatus. Electrophoresis was run at 20 mA at the beginning

when the samples reached to the separating gel, then the current was increased to 40 mA and the electrophoresis was continued until the samples reached to the end of the gel (Laemmli 1970).

2.10 Toxicity Test

100 mg of potato: legumes: whey powder mixed in ration 1:1:1 was suspended 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. 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

2.11 Data Analysis

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.

3.0 Results

3.1 Occurrence of Bacilli

The isolates were processed through gram's staining. All the isolates were identified as bacilli (Figure 1), spore staining of the colonies resulted in gram positive (+) rods andthere was formation of one or more parasporal crystalline bodies adjacent to the spore.

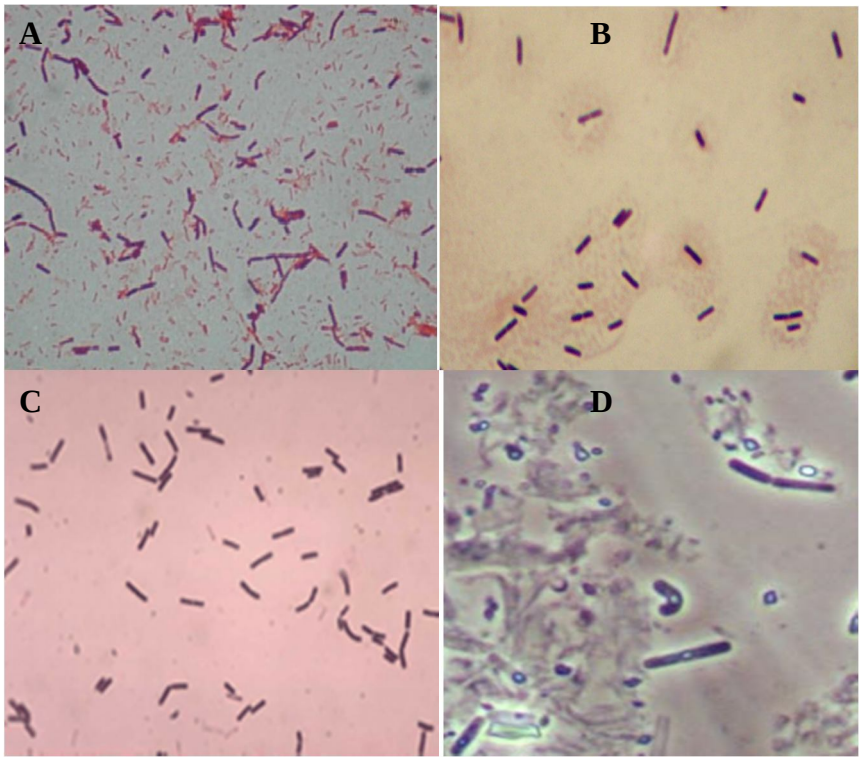

Figure 1: Photomicrographs of Bacillus thuringiensisproduced from Gram stain (A, B and C) and (D) produced from legume test culture medium

Table 2 shows the effect of temperature on the growth of different *Bacillus thuringiensis* isolates. The OD 600nm of culture was taken as indicators of bacterial growth. For all the isolates maximum growth was recorded at 37°C temperature.

Table 2: Effect of temperature on the growth of Bacillus thuringiensis, as depicted by the optical density of the bacterial culture at 600 $_{nm}$, 10 h after inoculation at 37 ℃

Isolate	**Incubation temperature**			
	20 ℃	30 ℃	37 ℃	40 ℃
KARI 46	0.55 ±0.12	0.60 ±0.15	0.72 ±0.22	0.55 ±0.17
KARI 37	0.60 ±0.12	0.63 ±0.15	0.64 ±0.22	0.46 ±0.17
62LBG37°C	0.26 ±0.12	0.44 ±0.15	0.81 ±0.22	0.62 ±0.17
KARI 30	0.40 ±0.12	0.49 ±0.15	0.55 ±0.22	0.42 ±0.17
KARI 20	0.43 ±0.12	0.51 ±0.15	0.60 ±0.22	0.45 ±0.17
KARI 21	0.10 ±0.12	0.36 ±0.15	0.77 ±0.22	0.32 ±0.17
KARI 47	0.26 ±0.12	0.32 ±0.15	0.93 ±0.22	0.45 ±0.17
63KAG37°C	0.37 ±0.12	0.41 ±0.15	1.25 ±0.22	0.39 ±0.17
14SLA30°C	0.24 ±0.12	0.39 ±0.15	1.03 ±0.22	0.51 ±0.17
KARI 53	0.29 ±0.12	0.36 ±0.15	0.69 ±0.22	0.42 ±0.17
24LBN30°C	0.33 ±0.12	0.41 ±0.15	1.00 ±0.22	0.35 ±0.17
1SKAG37°C	0.19 ±0.12	0.46 ±0.15	1.19 ±0.22	0.41 ±0.17
58SLA25°C	0.39 ±0.12	0.51 ±0.15	0.89 ±0.22	0.39 ±0.17
KARI 54	0.35 ±0.12	0.43 ±0.15	0.69 ±0.22	0.37 ±0.17
KARI 12	0.21 ±0.12	0.58 ±0.15	0.96 ±0.22	0.51 ±0.17
KARI 14	0.34 ±0.12	0.64 ±0.15	0.88 ±0.22	0.44 ±0.17

Maximum growth was observed between pH 6.5-7.5 for all the *Bt* isolates (Table 3).

Table 3: Effect of pH on the growth of Bacillus thuringiensis, as depicted by the optical density of the bacterial culture at 600 $_{nm}$, 10 h after inoculation at pH 7

Isolates	**pH**						
	4	5	6	7	8	9	10
KARI 46	0.17±0.11	0.25±0.10	0.47±0.16	0.78±0.13	0.53±0.12	0.41±0.11	0.18±0.18
KARI 37	0.13±0.11	0.27±0.10	0.41±0.16	0.65±0.13	0.49±0.12	0.35±0.11	0.15±0.18
62LBG37°C	0.22±0.11	0.35±0.10	0.46±0.16	0.76±0.13	0.54±0.12	0.32±0.11	0.16±0.18
KARI 30	0.15±0.11	0.38±0.10	0.45±0.16	0.64±0.13	0.53±0.12	0.35±0.11	0.13±0.18
KARI 20	0.23±0.11	0.36±0.10	0.47±0.16	0.89±0.13	0.57±0.12	0.31±0.11	0.09±0.18
KARI 21	0.26±0.11	0.31±0.10	0.43±0.16	1.00±0.13	0.49±0.12	0.37±0.11	0.15±0.18
KARI 47	0.16±0.11	0.33±0.10	0.41±0.16	0.83±0.13	0.55±0.12	0.33±0.11	0.16±0.18
63KAG37°C	0.22±0.11	0.29±0.10	0.40±0.16	0.72±0.13	0.51±0.12	0.29±0.11	0.15±0.18
14SLA30°C	0.14±0.11	0.25±0.10	0.42±0.16	0.67±0.13	0.49±0.12	0.30±0.11	0.22±0.18
KARI 53	0.19±0.11	0.27±0.10	0.46±0.16	0.73±0.13	0.41±0.12	0.35±0.11	0.19±0.18
24LBN30°C	0.11±0.11	0.26±0.10	0.42±0.16	0.68±0.13	0.43±0.12	0.31±0.11	0.16±0.18
1SKAG37°C	0.23±0.11	0.29±0.10	0.45±0.16	0.69±0.13	0.52±0.12	0.36±0.11	0.12±0.18
58SLA25°C	0.19±0.11	0.34±0.10	0.46±0.16	0.75±0.13	0.58±0.12	0.35±0.11	0.15±0.18
KARI 54	0.11±0.11	0.27±0.10	0.41±0.16	0.64±0.13	0.45±0.12	0.34±0.11	0.13±0.18
KARI 12	0.17±0.11	0.33±0.10	0.42±0.16	0.61±0.13	0.46±0.12	0.29±0.11	0.14±0.18
KARI 14	0.18±0.11	0.31±0.10	0.45±0.16	0.63±0.13	0.45±0.12	0.38±0.11	0.15±0.18

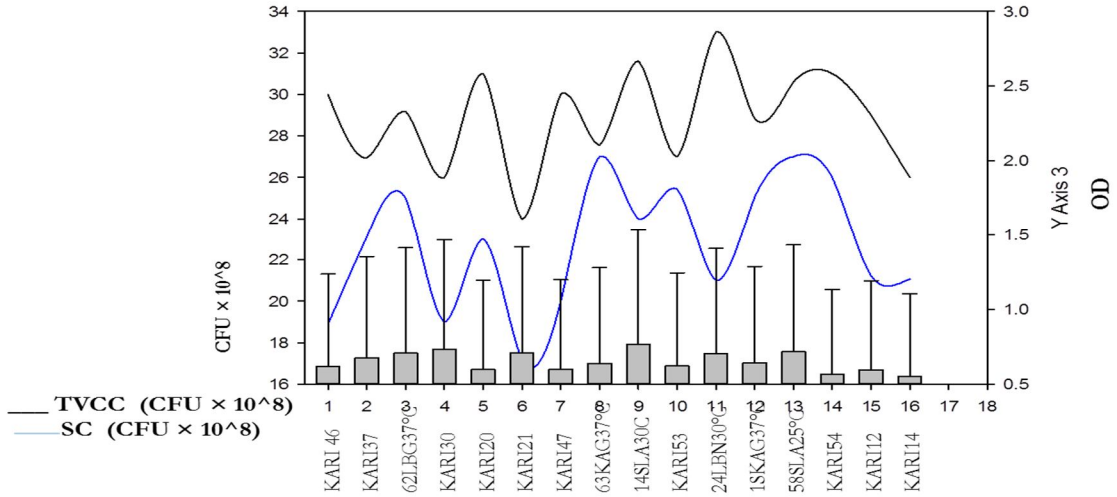

Figure 2: The means for optical density and total viable cell count/spore count of Btisolatesafter 72 h fermentation in six different media: NYSM[a], Legume[b], Potato[bc], Whey[cd], Wheat/ Cotton[de], Poultry[e]. Mean values for media with same letters are not significant

Bacterial isolates cultured in NYSM medium recorded spore counts of 3.87-4.14 × 10^8 CFU/ mL and protein concentrations of 28.75 µg/ ml while legume recorded4.89-5.60 × 10^8 CFU/ mL, 27.60µg/ ml, potato 2.45-3.25 × 10^8 CFU/ ml, wheat/ cotton 0.83-1.34 × 10^7 CFU/ ml , whey 1.72-2.44 × 10^7 CFU/ ml and poultry litter medium 0.64-0.83 × 10^7 CFU/ ml.The overall growth and production of *Bt*Insecticidal Crystal Proteins in NYSM was comparable to the test media, mean values were not significantly different for optical density: F (media)=12.23, F(time)=4.28; P>0.01total viable cell count: F (media)=55.29, P>0.01spore count: F (media)=25.19 P>0.01.

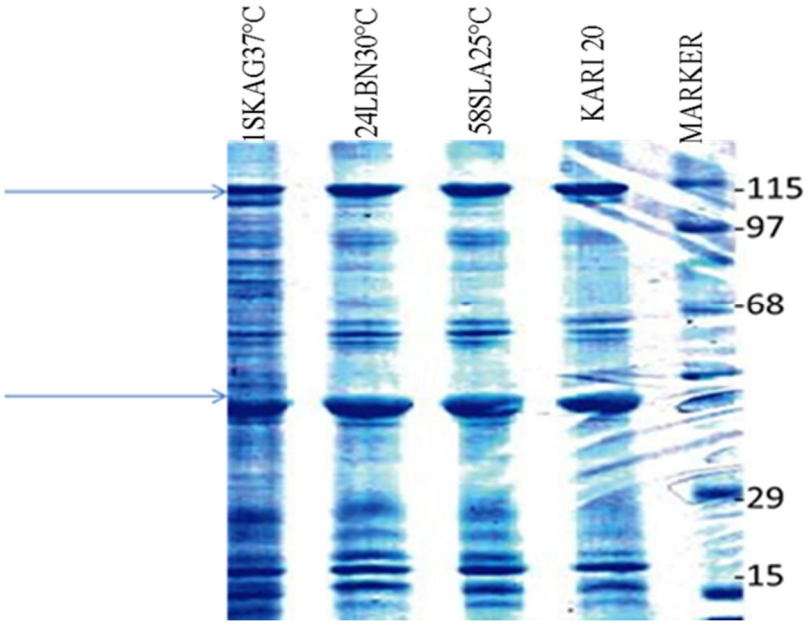

Figure 3: Protein bands of delta-endotoxin of Btisolates: Legume (1SKAG37°C and 24LBN30°C), NYSM (58SLA25°C and KARI 20 as determined by SDS-PAGE

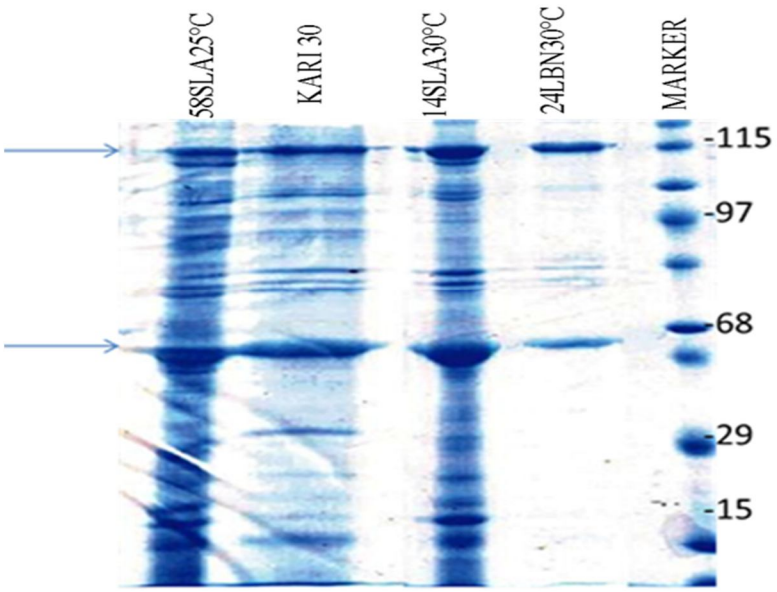

Figure 4: Protein bands of delta-endotoxin of Btisolates: potato (58SLA25°C and KARI 30), whey (14SLA30°C and 24LBN30°C) as determined by SDS-PAGE

Spore-crystal product from each treatment produced proteins with molecular weights of approximately 110-120 kDa and 60-70 kDa.*Bt* production of crystal toxin was linked to sporulation as parasporal crystals appear near spore, time of their appearance when viewed by phase contrast closely coincides with spore formation. The clear and conspicuous protein bandsprotein profiles as indicators of BT Insecticidal Crystal Proteins were correspondingly related to their larvicidal activity (Figures 3 and 4).

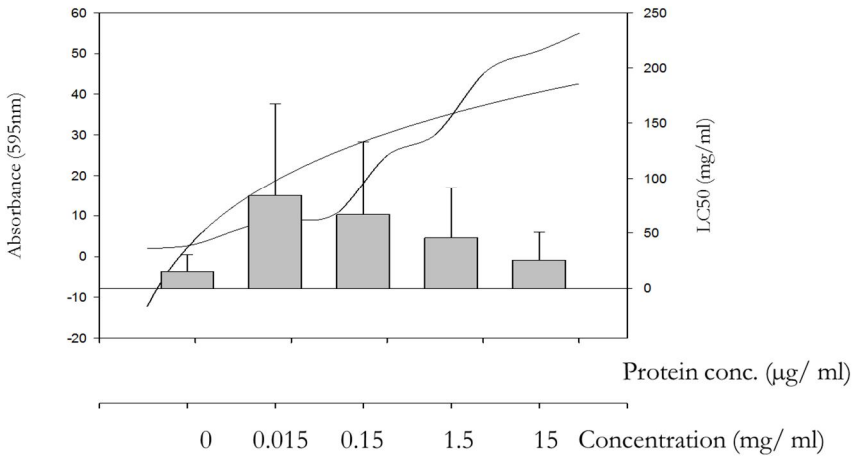

Figure 5: Percent mortality of neonate C. partellus larvae on treatment with five toxin concentrations of, the standard, Bacillus thuringiensis subspecies kurstaki(y-axis right) and the mean protein concentrations of Bacillus thuringiensis isolatesdetermined from thestandard curve of Bt proteins using BSA protein standard (y-axis left)

Five concentrations were used toidentify the right concentration treatment for screening the *Bt* isolates. 0.015 mg/ ml conc. recorded highest LC50 (78 µg/ l) on larvae of 1st instar *C. partellus* larvae (Figure 5).

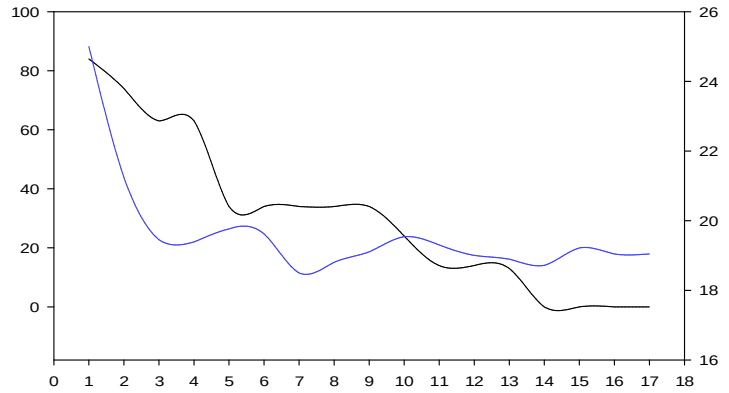

Figure 6: Mean percent cumulative mortality of C. partellus first-instar larvae exposed to 0.015mg/ ml endotoxins from Btisolates (y-axis left) and protein concentrations of Btisolatesafter 72 h fermentation in six different media (y-axis right)

The highest toxicity was recorded by 58SLA25°C (F=21.04), that is, 73 % mortality at 48 h. No mortality was observed for 14SLA30°C (F=4.00), 1SKAG37°C (F=1.92), KARI 21 (F=2.29), KARI 12 (4.00), and the control (F=2.74). One-way ANOVA (repeated measures) difference in percentage larval mortalities of *Bacillus thuringiensis* subspecies *kurstaki* (F=26.88,P>0.05) and *Bt*isolates was not statistically significant (Fig. 6).

4.0 Discussion

37 °C and pH 6.5-7.5 (tables 2 and 3) were optimum for the maximum BT growth, this compares to pH 7.0 – 9.4, 30 °C *Bacillus thuringiensis* subspecies *kurstaki*spore production in broiler litter extracts (Adams *et al*., 1999). Legume spore counts of 4.89-5.60 × 10^8(Fig. 2) compares to 0.85-5.08 × 10^9obtained from similar medium by Obeta and Okafor, 1984.For test media and NYSM; lag phaseconsisted of 1 h after which there was rapid multiplication of bacterial cells and spore maturation till 48 h. The culture density reached a plateau (range 1–1.5) and cell lysiscaused the release of endotoxins into the medium reaching a peak at 72 h. It is not necessarily important to end up with high spore counts because high spore counts do not always mean high toxicity (Fig. 2). Major polypeptides yielded crystal proteins 110-120 kDa and 60-70 kDa(Figs. 3 and 4) comparable to 135 kDa produced by *Bacillus thuringiensiss* ubspecies *kurstaki* (Adams *et al*., 1999).Maximum toxicity (LC50 83 µg/ l) was observed in the conventional medium and low cost medium (LC50 73 µg/ l) after 72h (Figures 5 and 6). The efficacy of *Bt* Insecticidal Crystal Proteins produced was highly effective against *C. partellus* first-instar larvae as determined from LC_{50}values.

5.0 Conclusion

There was good growth in all the media. *Bt* delta-endotoxins produced from test media are equally efficient as that of the standard, *Bacillus thuringiensis* subspecies *kurstaki* for *C. partellus* control. Legumes/potato/whey, are cheap/cost-effective for the industrial production of *C. partellus* pathogenic *Bt*, especially in developing countries, towards the pest control program.

6.0 Recommendations

Developing countries should search for other low cost media supplements to reduce costs for development of pesticides to counter the ever increasing insect pests. The toxicity of these isolates be investigated against other local Lepidopteran pests in order to determine their target range.

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