# BIOCONTROL POTENTIAL OF BACILLUS THURINGIENSIS ISOLATED FROM SOIL SAMPLES AGAINST LARVA OF MOSQUITO

**BY**

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**MB/2008/394**

**A RESEARCH PROJECT (MCB 429) SUBMITTED TO THE DEPARTMENT OF MICROBIOLOGY AND BIOTECHNOLOGY FACULTY OF NATURAL SCIENCES, CARITAS UNIVERSITY, AMORJI – NIKE, ENUGU**

**IN PARTIAL FULFILLMENT FOR THE AWARD OF BACHELOR OF SCIENCE (B.Sc.) DEGREE IN MICROBIOLOGY AND BIOTECHNOLOGY**

**AUGUST, 2012.**

# CERTIFICATION PAGE

I certify that this research project was carried out by Onwubiko, Ugochi Caroline (MB/2008/394) in the Department of Microbiology and Biotechnology, Faculty of Natural Sciences, Caritas University, Amorji – Nike, Enugu. The Department recognizes that Onwubiko Ugochi Caroline (MB/2008/394) bears full responsibility of this work.

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# DEDICATION

This work is dedicated to the Almighty God whom, if not for Him, this work would not have been completed, and to my brother; Rev. Fr. Augustus Onwubiko for the unconditional support he provided to drive me to succeed.

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# ABSTRACT

A major challenge for achieving successful mosquito control is overcoming insecticide resistance. *Bacillus thuringiensis* which is one of the most effective biolarvacide for control of species of mosquitoes and monitoring of larval susceptibility is essential to avoid resistance development. Mosquito larvacidal activity of *Bacillus thuringiensis* was assessed by isolating them from ecologically different soil habitats in and around Enugu metropolis. The isolate organisms were confirmed as *Bacillus thuringiensis* based on biochemical characterization and microscopic observation. The larvacidal activity of *Bacillus thuringiensis* isolates was tested against the larval of mosquito by using the standard cup bioassay. The isolates of *Bacillus thuringiensis* showed a significant level of variation in their larvacidal activity.

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## CHAPTER ONE

# INTRODUCTION

*Bacillus thuringrensis* (Bt) is a well known and widely studied bacterium which is known for its use in pest management. Today it is the most successful commercial xenobiotic with its worldwide application when compared with the chemical pesticides; *Bacillus thuringiensis* has the advantages of being biologically degradable, selectively active on pests and less likely to cause resistance. Safety of *Bacillus thuringiensis* formulations for humans, beneficial animals and plants explains the replacement of chemical pesticides in many countries with these environmentally friendly pest control agents.

*Bacillus thuringiensis* was first isolated by the Japanese Scientist Ishiwata (1901) from skilkworm larvae, bombyxmori, exhibiting sotto disease. After 10 years, Berliner (1911) isolated the square gram (+) positive, spore-forming, rod shaped soil bacterium from disease flour moth larvae, Anngasta Kachmiccalla, in the Thuringia region of the Germany and named it as *Bacillus thuringiensis*.

In the early 1930s *Bacillus thuringiensis* was used against Ostrinianubilis, the European corn borer. The first commercial product was available in 1938 in France, with the trade name sporeine (Weiser, 1986). It was *Bacillus thuringiensis* subspecies Kurstaki that was used for the control of the insect

(Lepidopteran) pests in agriculture and forestry (Luthy & Ebersold, 1981). New commercial products arrived in 1980s after the discovering of subspecies *thuringiensis* opened the gate for black fly and mosquito larvae control.

Like all organisms, insect are susceptible to infection by pathogenic microorganisms, many of these infections agents have a narrow host range and therefore, do not cause uncontrolled destruction of beneficial insects and are not toxic to vertebrates. *Bacillus thuringiensis* is a major microorganism, which shows entamopathogenic activity (Glazer & Nikaido, 1995, Schnepf, et al. 1998) which forms parasporal crystals during the stationary phase of its growth cycle.

Most *Bacillus thuringiensis* preparations available on the market contain spores with parasporal inclusion bodies composed of δ – endotoxins. In commercial production, the crystals and spores obtained from fermentation are concentrated and formulated for spray on application according to conventional Agriculture practices (Baum, Kakefuda, & Gawron-Burke, 1996). There are many strains of *Bacillus thuringiensis* having insecticidal activity against insect order (eg Lepidoptera, Diptera, Homoptera, Mollaphage, Coloptera). Only a few of them have been commercially developed.

*Bacillus thuringiensis* insecticides are divided into three groups, group one has been used for the control of lepidopterans. These groups of insecticides are formulated with *Bacillus thuringiensis* Subspecies. Kurstaki, group two contains thesandiego and tenebrionis strains of *Bacillus thuringiensis*and has been applied for the control of certain celopterans and their larvae. Group three contains the Israelensis strains of *Bacillus thuringiensis* which has been used to control black flies and mosquitoes.

## CRYSTAL COMPOSITION AND MORPHOLOGY

The existence of parasporal inclusions in Bt was first noted I 1915 (Berliner 1915) but their protein composition was not delineated until the 1950s (Angus 1954). Hannay (1953) detected the crystalline fine structure that is a property of most of the parasporal inclusion. *Bacillus thuringiensis* subspecies can synthesize more than one inclusion, which may contain different ICPs. ICPs have been called data endotoxins; however since the term endotoxin usually refers to toxin associated with the other membranes of gram-negative bacteria, comprising a core lipopoly saccharide. Depending on their ICP composition, the crystals have various forms (bipyramidal, cuboidal, flat rhomboid, or a composition with two or more crystal types. A partial correlation between crystal morphology, ICP composition, and bioactivity against target insects has been established (Bulla et al.1977). Hofte and Whitely, 1989, Lynch and Baumman, 1985).

## GENERAL CHARACTERISTICS OF *BACILLUS THURINGLENSIS*

*Bacillus thuringiensis* is a member of the genes *Bacillus* and like the other members of the taxon, has the ability to form endospores that are resistant to inactivation by heat, desiccation and organic solvent. The spore formation of the organism varies from terminal to subterminal in sporangia that are not swollen, therefore, *Bacillus thuringiensis resembles* other members of Bacillus species in morphology and shape (Stahly, Andrews, & Yousten, 1991). The organism is gram-positive and facultitative anaerobes. The shape of the cells of the organism is rod. The size when grown in standard liquid media varies 3 – 5um.

The most distinguishing features of *Bacillus thuringiensis* from other closely related Bacillus species. (eg *Bacillus anthracis*, *Bacillus. cereus*) is the presence of the parasporal crystal body that is near to the spore outside the exosporangium during the endospore formation, which is shown in figure 1:1 (Andrews, Bibilops, & Bulla, 1985; Andrews, Faust, Wabiko, Raymond, & Bulla, 1987; Bulla, Faust, Andrews, & Goodma, 1995). *Bacillus thuringiensis* is an insecticide producing variant of *Bacillus cereus* (Gordon, Haynes, & Pang, 1973) several Bt species also produce *Bacillus cereus* type

enterotooxin (Carlson, & Kolsto, 1993) plasmids coding for the insecticidal toxin of *Bacillus thuringiensis* have been transferred into B. cereus to make it a crystal producing variant of *Bacillus thuringiensis*(Gonzalez, Brown, Carlton, 1982) molecular methods including genomic restriction digestion analysis and 16 rRNA sequence comparison support that *Bacillus thuringiensis*, *Bacillus anthracis* and *Bacillus cereus* are closely relocated species and they should be considered as a single species (Carlson, Caugant, & Kolstra, 1994; Ash , Farrow, Dorsch, Stackebrandt, & Collins. 1991; Helgason et al.2000).

**CLASSIFICATION OF *BACILLUS THURINGIENSIS* SUBSPECIES**

The classification of *Bacillus thuringiensis* based on the serological analysis of the flagella antigens was introduced in the early 1960s (de Barjac & Bonnefoi, 1962). This classification by serotype has been supplemented by morphological and biochemical criteria (de Barjac, 1981). Clutill (1977), explains that only 13 *Bacillus thuringiensis* subspecies were toxic to lepidopteran Larva only. And apparently Nematode (Narva *et; al.*, 1991) enlarged the host range and markedly increased the number of subspecies up to the end of 1998, over 67 subspecies based on flagella H – Serovars had been identified.

**ECOLOGY AND PREVALENCE OF *BACILLUS THURINGRENSIS***

Although our knowledge about *Bacillus thuringiensis* occurs naturally and it can also be added to an ecosystem artificially to control pest, prevalence of *Bacillus thuringiensis* in nature can be said as “natural” and can be isolated when there is no previous record of application of the organism for pest control.

The *Bacillus thuringiensis* which belong to artificial habitat areas are sprayed based insecticides (mixture of spores and crystal). (Stahly et al. 1991). Thus, it is obvious that *Bacillus thuringiensis* is widespread in nature. However, the normal habitat of the organism is soil. The organism grows naturally as asaprophyle, feeding on dead. Organic matter, therefore, the spores of Bacillus *thuringiensis* persist in soil and its vegetative growth occurs when there is nutrient available. Moreover *Bacillus thuringiensis* has recently been isolated from marine environments (Maeda et al. 2000) and from soil of Antarctica also (Foresty & Logan 2000).

However the true role of the bacteria is not clear. Although it produces parasporal crystal inclusions that are toxic to many orders of insects, some species of *Bacillus thuringiensis* from diverse environments show no insecticidal activity. The insecticidal activities of Bacillus thuringiensis are rare in nature. For example, Iriarte et al.(2000) reported that there is no relationship between mosquito breeding sites and pathogenic action level of *Bacillus thuringiensis* in the surveyed aquatic habitats. While another study

suggested that habitat with a high density of insect were originated by the pathogenic action of this bacterium (Itoqou Apoyolo et al.1995).

**OTHER PATHOGENIC FACTORS OF *BACILLUS THURINGIENSIS***

At the period of the active growth cycle, the strains of Bacillus thuringiensis produce extracellular compounds; this compound might yield to virulence. These extracellular compounds include proteases, chitinases phospholipases, and vegetative conseticidal protein (Zhang et al. 1993; Sohneff et al. 1998).

*Bacillus thuringrensis* also produces antibiotics compounds having antifungal activity (stab et al. 1994). However the crystal toxins are more effective then these extracellular compounds and allow the development of the bacteria in dead insect larvae.

*Bacillus thuringiensis* strains also produce a protease, which is called inhibitor. This protein attacks and selectively destroys cecropiris and attacisis which are antibacterial proteins in insects, as a result of this, the defence response of the insect collapses. This protease activity is specific, it attacks an

open hydrophobic region near C – terminus of the cecropin and it does not attack the globular proteins (Duthambar & Steiner, 1984).

Other important insecticidal proteins which are unrelated to crustal proteins are vegetative insecticidal protein. These proteins are produce by some strains of *Bacillus thuringiensis* during vegetative growth.

**MORPHOLOGICAL PROPERTIES OF *BACILLUS THURINGIENSIS***

Colony forms can help to distinguish *Bacillus thuringiensis* colonies from other *Bacillus* species. The organism forms white, rough colonies, which spread out and can expand over the plate very quickly. *Bacillus thuringiensis* strains have unswallon and ellipsoidal spores that lie in the subterminal position. The presence of parasporal crystals that are adjacent to the spore in another cell is the best criteria to distinguish *Bacillus thuringiensis* from other closely related *Bacillus* species. The size number, of parasporal inclusion and morphology may vary among *Bacillus thuringiensis* strains. However, four distinct crystal morphologies are apparently the typical bipyramidal crystal, related to crystal proteins (Aronson et al. 1976). Cuboidal usually associated with bipyramidal crystal (Ohba&Aizawi 1986), amorphous and composite crystals related to cry4 and cry proteins (federicet al. 1990), and flat, square crystal related to cry3 proteins (Hernstadet al. 1986, Lopezmeza & Ibarra, 1996

The classification was based in part on the possession of parasporal bodies. Bernard et al.(1997) isolated 5303 *Bacillus thuringiensis* from 80 different countries and 2793 of them were classified according to their crystal shape.

*Bacillus thuringiensis* vary's based on geographical or environmental location. Each habitat may contain novel *Bacillus thuringiensis* isolated that have more toxic effects on target insects. Intensive screening programs have been identified *Bacillus thuringiensis* strain from soil, plant surfaces and stored product dust samples. Therefore many strain collections have been described in the literature, such as Assian (Chak et al. 1994, Ben – Dov et al. 1997, 1999) and Maxican (Bravo et al. 1998).

Therefore the aim of this study is to isolate *Bacillus thuringiensis* from soil sample and to isolate *Bacillus thuringiensis* against larva of mosquito or to determine *Bacillus thuringiensis* against larva of mosquito.

# CHAPTER TWO

# LITERATURE REVIEW

Commercial *Bacillus thuringiensis* (Bt) products are microbial pest control agent (MPCAs) containing specific insecticidal crystalline proteins (ICPs) and most often living spores as well as formulating agent. They processed fermentation products. (Baumann et al., 1993; and Hansen et al. 1998).

*Bacillus thuringiensis* is a facultative anaerobic motile, gram – positive, spore forming bacterium. The formation of parasporal crystals adjacent to the endospore during sporulation stage three to four distinguished Bt from other Bacillus species. *Bacillus thuringiensis* like other Bacillus species, has been classified on the basis of its cellular, cultural, biochemical and genetic characteristics (Baumannet al. 1984; Barkley 1986; Slepecky; 1992; Hansenet al. (1998). In 1958, Heimpel and Augus1958, introduce a

classification scheme to identify these crstalliferous bacteria base on their morphological and biochemical characteristics but that specific biochemical characteristics do not always refer to a specific serotype (Helyasonet al. 1998; Hansen et al.1998).

## MODE OF ACTION ON TARGET ORGANISM

The sporulation bacillus thuringiensis with insecticidal crystalline proteins or spore – ICP complexes must be ingested by a susceptible insect larva. The efficacy of the ICP depends on the solubilizaiton, in the midgut (Heimpel&Anges 1993) resulting in the conversion of the protoxin to the biologically active toxin by proteolytic enzymes, specific membrane receptor binding by the C-termminal domain of the active toxin, and pore formation by the N-terminal domain with subsequent lysis of the epithelia cell. Proliferation of the vegetative cells into the haemocoel may result in a septicuemia contributing to the cause of death.

Receptor binding by the ICP is the major determinant of host specificity by the different *Bacillus thuringiensis* (ICPs) insecticidal crystalline proteins (Barkley et al. 1996). The mode of action of *Bacillus thuringiensis*has been reviewed by Schneptet al.(1998) and can be summarized in the following stages:

1. Ingestion of sporulation Bt and ICP by an insect harva.

2. Solubilization of the crustalline ICP in the midgut.
3. Activation of the ICP by protease.
4. Binding of the activated ICP to specific receptors in the midgut cell membrane.
5. Insection of the toxin in the cell membrane and formation, followed by destruction of the epithelia cells (Cooksey 1971 &Smodley 1996).
6. Subsequent *Bacillus thuringiensis* spore germination and septicaemia may enhance motality.

The specific bioactivity of Bt is dominated by the ICPs that are encoded by the cry gene and are active against subsceptible species (Feitelson 1993; Zukowski 1995) the ICP must be effective against the target organism (Visseret al.1993).

*Bacillus theringiensis* is effective against the early stages of mosquito larvae and does not affect mosquito to eggs, mature larvae, pupae or adults. (Murray & Daniels, 2007). Mosquito larvae must eat the Bt formulated product containing dormant spores. Crystals which are known as insecticidal crystals proteins (ICPs) or delta – endotoxinal produced during *Bacillus thuringiensis* sporulation. The mosquito larvae stop feeding and die when these proteins are converted into toxins that work by damaging the gut wall of mosquitoes. (Lacey, & Merrit, 2003).

## MECHANISM OF ACTION OF *BACILLUS THURINGENSIS* FORMULATION

The ICP spore complexes of *Bacillus thuringiensis* are ingested by susceptible insect larva. In the midgut the parasporal crystalline ICP is dissociated to the protoxinform, and the protoxin is then activated to a holotoxin by gut proteases (Warrenet al. 1984; Jaquetet al. 1987; Aronson et al. 1991; Honee 1993). Shortly the gut becomes paralysed and the larun cease to feed.

The ICP structure and function have been reviewed in detail by Schneptet al.(1998) binding of the ICP to pultative receptors is a major determinant of ICPs specificity and the formation of pores in midgut epithelia cell is a major mechanisms of toxicity (Van Frankenhuzen, 1993).

Binding to specific receptors has been demonstrated to be closely related to the insecticidal spectrum of the ICPs (Denolfet al. 1997). Van Rieet al. (1998) found the effinity of these toxins similar for the tobacco budworm (Heliothisvirescen) and tomato hornworm (manducasexta) brush bord or membrane vesicles, but the number of binding sites differed and reflected varied bioactivity. However, the toxin affinity for binding sites does not appear constant for all insects.

## GENERAL APPLICATION OF *BACILLUS THURINGIENSIS*

Application Agricultural and Forest: Commercial use of Bt on Agricultural and forest crops dates back nearly 30 years, when it became available in France – use of B+ has increased greatly in recent years and the number of companies with a commercial interest in Bt products has increased from four in 1980 to at least 18 (Van Frankenhuyzen, 1993), several commercial *Bacillus thuringiensis*products have been supplied to crops using conventional spraying technology. Various formulation have been used on major crops such as cotton, maize, soybeans, potatoes, tomatoes, various crops trees and stored grains (Carlton et al. 1990).

In the main naturally occurring Bt strain have been used but transgenic microorganism expressing and by genetic manipulation and in some cases, these have reached the commercial market. These modified organisms have been developed in order to increase host range, prolong field activity or improve delivery of toxin to target organisms, for examples, the coleopteran active crygene has been transferred to a hepidopteran – active Bt (Cartonet al. 1990). A plasmid bearing an ICP gene has been transferred from *Bacillus thuringiensis* to a non-pathogenic leaf colonizing isolate of pseudomonas fluorescens, fixation of the transgenic cells produces ICP contained within a membrane which prolongs persistence (Gelenter, 1990). The delivery of ICP effectiveness has been shown in European corn borer, feeding within plant stems (Beach 1990). Improvement in performance arising from such

modification are such that transgenic organisms and their products are likely to be used much more widely in the future.

# CHAPTER THREE

# MATERIAL AND METHOD

## SOIL SAMPLE COLLECTION

Six samples including soil, dead insects, dry leaf residues animal feces and stored product dust sample were collected from Emene, Keyetta and Atisan market, where there is no previous record of application of *Bacillus thuringiensis* based insecticides, the collected samples are summarized. The soil samples were taken two to five (2 – 5cm) below the surface, after scraping of the surface material with sterile spatula. Finally collected samples were stored in sterile plastic bag at $40^{o}$C.

Soil sample were collected from Emene about two in number, then two dead insects where collected from federal housing and (2) different or the same dry leaf where collected from Atisan market for the experiments.

## ISOLATION OF *BACILLUS THURINGIENSIS*

According to Edwards D.L. and G. Soares (1988), sodium acetate, heat treatment method was applied to isolate *Bacillus thuringiensis*from environment samples.

Approximately, 0.25g at each sample were suspended in 15 x 160mm test tubes containing 10ml Nutrient broth with concentrations of sodium acetate 0.12m and 0.25m (pH. 6.8). The samples were also suspended in nutrient broth without sodium acetate as negative control. Next suspensions were vortexed vigorously and incubated overnight at 37$^{o}$C in a shaking water bath. Afterwards, the samples were pasteurize for 5 minute at 80$^{o}$C in order to kill vegetative bacterial cells, and to eliminate non-sporeforming bacterial cells, following heat treatment, the samples were placed on nutrient agar plates which were incubated overnight at 35$^{o}$C. Finally bacterial colonies were separated by their colony morphology. The colonies which showed *Bacillus thuringiensis*like colony morphology were rough, while and spread out over the plate. These colonies were subcultured on nutrient agar plates and incubated for 48hrs at 30$^{o}$C to check the position of the spore in the bacterial

cell by light microscopy. Because of this reason, simple staining and endospore method were used or carried out.

## ISOLATION OF *BACILLUS THURINGIENSIS* FROM SOIL

Ten soil samples were collected from rhizosphere of Alfa alfa plant from four different locations in Emene market in Enugu state. Soil samples (Ig) were added to L – broth medium (20ml) supplemented with sodium acetate (0.25m, pH 6.8) in a conical flask (125ml). The mixture was shaken at 1,000 xg for 4 hrs. at 30$^{o}$C. Sample of about 0.5ml in 10ml test tube were heated for 5 minutes in a water bath at 80$^{o}$C to kill vegetative cells and nonsporeforming bacteria, and used to inculate L-agar medium plates, without adding sodium acetate to the medium. Colonies formed after overnight growth at 30$^{O}$C were transferred onto TCHA medium (tryptone, 5 g/litre, cascinhydroly – sate, 2g/litre; $K_2H_p0_4$, 12.5mm, $mgS0_4$, 12.5mM, $MnS0_4$, 0.05mM, $ZnS0_4$, 1.2mM, $Fe_2$ $(S0_4)3$. 1.2mM, $H_2S0_4$, 0.5%, $Cacl_2$, 25mM and Nutrient broth 15gm (litre) complemented with 0.3% glucose. Culture were allowed to grow and sporalated for 40hrs. at 30$^{O}$C then examined by light microscope based on colony morphology and crystal shape. (Braun, 2000).

## SIMPLE STAINING

Simple staining means that one dye and one step procedure was used to stain microbial cells. Spores of Bacillus species do not stain, and they may be seen

as unstained bodies within bacterial cells stained with methylene blue. Smears of Bacillus isolates were prepared and they were fixed by heat. The bacterial smears were then plodded with methylene blue. Staining lasted for 5min. Finally distaining was performed by washing under the tap water and stained bacterial colonies were observed under oil – immersion objective. In addition endospore staining with malachite green was performed for a better observation of Bacillus spores. This staining procedure involved primary staining with malachite green for 5 min and steam heat to drive the stain into spores. This stain was retained by endospores but washed out of the rest cells with water. Cells were then counterstained with the red dye safranin. The spores appeared green and cells appeared red after staining by this procedure.

Isolates having ellipsoidal and subterminal spores in unswollen bacterial cells were identified as *Bacillus thuringiensis*

**BIOCHEMICAL IDENTIFICATION**

When the isolates were placed on the dots, they were allowed to sporulate and were determined to have the *Bacillus thuringiensis* biochemical profile. Putatice *Bacillus thuringiensis*colonies were screened by light microscopy for crystal morphology. Subdivisions within the Bt species by biochemical type were also made at this time. According to Thomas E.O. and Ohba 1986, fourteen biochemical tests were performed to identify isolates. For this study,

only the results of the following four the most relevant biochemical tests were presented: glucose and sucrose and catalase production. This enabled us to divide the *Bacillus thuringiensis*isolates in these groups was parallel to the distribution obtained by additional tests.

"The + sign indicates a positive reaction, ie utilization of lactose, acid production from glucose and sucrose, and production of catalase.

## MATERIALS AND METHOD OF *BACILLUS THERINGIENSIS* AGAINST MOSQUITO LAVA

**Insects:** Three species of mosquito were used as target insects,culexquinquefusciatus say Aedesacqypti L, and Anopheles (Pallas). The mosquitos were maintained in insectary with the methods described by Weiser (1991).

**Bioassay:** In primary screening, all of the *Bacillus thuringiensis*isolates selected were tested against secondary stage larva of mosquito. Twenty five larvae were transferred into each test tubes (7 x 9) cm with 50ml sterile distilled water. The stock suspension of cultures of *Bacillus thuringiensis*from broth slants was diluted to $10^{-1}$, $10^{-2}$ and $10^{-3}$ in sterile water and 0.5ml of each dilution was added to each of the three test tube. The test tube were kept at $30^{O}$C. Larval mortality was stored 48hrs. After treatment, those isolates that caused mortality the same were kept for further

experiment. After the promising isolate were found, further screening was carried out for the *Bacillus thuringiensis* against mosquito larva.

Based on the result further screening, the fermentation product were diluted Cwofold to get a series dilutions of shaking flask cultures. The larvae of mosquito were treated and ($LC_{50}$) lethal concentration values of spores and crystal complex were determined by using the method described above (Luo *et al.*, 1998) for each isolate.

**CATALASE TEST**

With the aid of a wooden applicator stick or wire loop, small amount of a well isolated 24hrs colony of an organism was put into the test tube four to five drops of 3% hydrogen perioxide ($H_2O_2$) were added into the test tube, then the test tube was placed against a dark background and immediate bubble formation was observed. Immediate effectiveness (bubble formation) indicates positive catalase while no bubble formation indicates negative catalase.

**OXIDASE ENZYME ACTIVITY**

Using aspectic techniques, TrypticSog (is) agar plates were inoculated with the inoculums. The inoculated plates were incubated at $37^{O}C$ for 24 – 28hrs then 2 – 3 drops of P – aminodimethylamine presence or absence of colour

change was observed within 10 – 30 sec. after adding the reagent presence of colour change from pink to purple indicates positive while absence of colour change indicates negative.

**SUGAR TEST**

Each test of the isolates was tested for its ability to ferment a given sugar with the production of acid and gas or acid only. Most bacteria especially gram negative bacteria utilize different sugar as source of carbon and energy with the production of either acid or gas. This test is used as an acid in their different.

The growth medium, peptone water was prepared in a conical flask and the indicators, bromocre sol purple was added. The mixture was dispensed into test tubes containing Durham's tube. The tubes with their content were sterilized by autoclaving at 121$^{o}$C for 15 minutes. 1% solution of the sugar was prepared and sterilized separately at 115$^{o}$C for 110 minutes. This was then aseptically dispensed in 5ml adequate volume into the tube containing the peptone water and indicator. The tubes were incubated at 37$^{o}$C. Acid and gas production or acid only were observed after about 24hrs of incubation.

Acid production was indicated by the change of the medium from light green to yellow colour while gas production was indicated by the presence of gas in Durham's tubes. The control tubes were not incubated.

**Methyl Red Test**

Tubes of buffered glucose – peptone broth were lightly inoculated at 37$^{o}$C for not less than 48hrs. About 5 drops was added into 5ml of the culture. The production of a bright red colour immediately on the addition of the reagent showed a positive test.

**Indole Test**

Tubes of peptone water were inoculated with young culture of isolates. The tubes were incubated at 37$^{o}$C for 48hrs. About 4 drops of kollac reagent were added into 1ml of each of the culture tubes. Positive test was indicated by a red colour occurs immediately at upper part of the test tube.

## CHAPTER FOUR

## RESULT OF SAMPLE COLLECTION AND ISOLATION

Six samples comprising soil. Soil from marshy places, dead insects, dry lead residues, animal feces and stored product dust samples, were collected from Emene, Keyetta and Atisan Market.

Six strains of spore – forming bacteria in was tested in two sodium acetate concentration (09.12m, 0.2m) in order to determine their ability to germinate

in acetate buffered medium. They report of germination of *Bacillus thuringiensis*strains was usually inhibited by 0.26m sodium acetate and concentration, 0.12 while other *Bacillus thuringiensis*(non – acetated isolates) strains could germinate in high sodium acetate concentration. In this study two different acetate concentration also Nutrient broth without sodium acetate (negative control) wave used to increase the efficiency of the isolation rate of *Bacillus thuringiensis*strains from environmental samples and to compare sodium acetate selection results/heat treatment and negative control with heat treatment gave similar result. The isolate was place on nutrient agar for the examination of morphological result which are listed in the table below.

**COLONY MORPHOLOGY OF *BACILLUS ISOLATES*.**

**Table 1**

**Sample A**

| **Colony Code** | **Morphology** | **No. of Isolate** | **% of Total Isolate** |
|---|---|---|---|
| A – 0.12 | Creamy, rough, slightly raise spread over the plate. | 2 | 25% |

| | | | |
|---|---|---|---|
| A – 0.25 | Creamy, opaque, big, serrated | 3 | 37% |
| A – NSA | Creamy, spread over the plate | 3 | 37% |

**Sample B**

| **Colony Code** | **Morphology** | **No. of Isolate** | **% of Total Isolate** |
|---|---|---|---|
| B – 0.12 | Dull opaque, flat smooth edge, big. | 4 | 36% |
| B – 0.25 | Creamy, rough edge spread over the plate glistering. | 3 | 27% |

| | | | |
|---|---|---|---|
| B – NSA | Creamy, flatting spread over the plate, rough edge. | 4 | 36% |

**Sample C**

| Colony Code | Morphology | No. of Isolate | % of Total Isolate |
|---|---|---|---|
| C – 0.12 | Creamy, tiny, glistering, rough edges | 4 | 31% |
| C – 0.25 | Creamy, flat, round spread over the plate | 4 | 31% |
| C – NSA | Creamy, round, flat glistering | 5 | 38% |

**BIOCHEMICAL TEST**

The isolate were tested for biochemical test which are catalase, oxidase, sugar, methyl red, indole and pore test and results of these test are listed below.

**Table 4.2**

| Isolate code | Gram stain | Oxidase | Catalase | Sugar | | | Methyl red | Indole | Spore test |
|---|---|---|---|---|---|---|---|---|---|
| | | | | L | S | G | | | |
| $A_1$ | +ve rods | + | + | + | + | + | + | + | + |
| 0.12 | | | | | | | | | |
| $A_2$ | +ve rods | + | + | + | + | + | + | + | + |
| $B_1$ | +ve rods | + | + | + | + | + | + | + | + |
| $C_1$ | +ve rods | + | + | + | + | + | + | + | + |
| $A_1$ | +ve rods | + | + | + | + | + | + | + | + |
| 0.25 | | | | | | | | | |
| $A_2$ | +ve rods | + | + | + | + | + | + | + | + |
| $B_1$ | +ve rods | + | + | + | + | + | + | + | + |
| $C_1$ | +ve rods | + | + | + | + | + | + | + | + |

**BIOASSSAY**

The bioassay is done using serial dilation method, using different concentration of *Bacillus thuringiensis*against mosquito larvae. Therefore the

different concentrations are $10^{-7}$, $10^{-6}$, $10^{-5}$, $10^{-4}$, $10^{-5}$, $10^{-2}$ and $10^{-1}$ the result is list below.

**Table 4.3**

| | $X_1$ | $X_2$ | $X_3$ |
|---|---|---|---|
| $A_1$ 0.12 | + | + | + |
| $A_1$ 0.25 | + | + | + |
| $A_2$ 0.25 | + | + | + |
| $B_1$ 0.25 | + | + | + |
| $C_1$ 0.12 | + | + | + |
| $C_1$ 0.25 | + | + | + |

## CHAPTER FIVE

## DISCUSSION

In this study *Bacillus thuringiensis* was examined in different habitats like soil, animal dung, birds dropping and grain dust, where no *Bacillus thuringiensis*have been applied before. Although *Bacillus thuringiensis* was found in all the samples but its occurrence in soil samples was found to be relatively high compared to other types of samples. The isolation of *Bacillus thuringiensis* from soil sample is variably successful with the rates ranging from 3.85% and from 22 – 50% (Martin & Travers 1989).

During sporulation endorspare formation which matured after 24 hours and started to released in the medium after 24 hours. Then for 48 hours many growth of endospore matured and were release into the medium.

## CONCLUSION

*Bacillus thuringiensis*is a ubiquitous gram positive and sporeforming bacterium, the organism produce interacellular crystalline proteins which are topic to insects during the stationary phase of its growth cycle because of its insectividal activity *Bacillus thuringiensis* has been used for a long time as a bioplesticide. However it is still necessary to search for more toxin to control insects orders which have the ability to develop resistance against such pesticide and to also provide alternative for chemical insecticide. Mosquito – toxin stains of *Bacillus thuringiensis* have been reported from different

continent and their source include soil, plant, insect, animal feces and water, *Bacillus thuringiensis* belonged to other subspecies/serotype, within the subspecies there strain which exhibited different level of toxicity against the target organisms. Some were very high, while some are high, some moderate, others poor and non toxic, and certain subspecies showed dual toxicity to mosquito larvae.

A classical features of all the mosquitocidal*Bacillus thuringiensis* strain is that they all posses a large transferable plasmid which is responsible for the toxicity and it carries cry genes that code for cry toxins. Loss of plasmid from *Bacillus thuringiensis* strain is likely to make them non pathogen or non toxin.

**RECOMMENDATIONS**

I recommended that *Bacillus thuringiensis* should be used in our environment including drinking water reservoirs, for control of mosquito, black fly and nuisance insect larvae.*Bacillus thuringiensis* product should contain insecticide crystalline protein and free from other microorganism and biological active metabolites. Also good industrial large scale practice standard should be employed for the production of *Bacillus thuringiensis* products. Therefore more research on the fate of *Bacillus thuringiensis*spare and ICPs in the environment is needed, this should cover the natural

occurrence of *Bacillus thuringiensis* and be in food and its relationship to exposure to *Bacillus thuringiensis*from its pesticide use.

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# APPENDIX 1

The media that were used

## NUTRIENT BROTH PREPARATION

Weigh 13g of powder, dispense in 1 litre of deionized $H_{20}$. Allow to soak for 10 minutes swirl to mix, then dispense into final containers sterilize by autoclaving at 121% for 15 minutes.

**Composition**

| | |
|---|---|
| Beaf extract | 1.0g |
| Yeast extract | 2.0g |
| Peptone | 5.0g |
| Sodium chloride | 5.0g |

**PEPTOME WATER**

**Preparation**

Dispense 15g in 1 litre of deionised the water, soak for 10 minutes, swirl to mix, then dispense into final container, sterilize by autoclaving for 15 minutes at 121%. Carbohydrate and a pH indicator can be added for studying fermentation reaction.

**Composition**

| | |
|---|---|
| Peptone | 5.0g |
| Trypeptone | 5.0g |
| Sodium chloride | 5.0g |

**NUTRIENT AGAR (NA)**

This media was used for the enumeration of bacterial cells and also to maintain pure culture. Nutrient agar is a general medium, it was therefore used on the assumption as many organisms as possible will grow on it.

**Composition**

The medium is composed of the following

| | |
|---|---|
| Distilled water | 1000ml |
| Yeast extract | 200g |

| | |
|---|---|
| Lab. Lem Co Powder | 1g |
| Sodium chloride | 5.0g |
| Agar No3 | 15g |
| pH | 7.4 |

**PREPARATION**

The medium was prepare as directed by the manufacturer 25g of powdered nutrient agar (oxide) was suspended in 600ml of distilled water and made to dissolve by heating. This was sterilized by autoclaving at $121^{O}C$ for 15 minutes. The medium was allowed to cool for about $45^{O}C$ and then poured into sterile petri dishes, the medium was allowed to solidify and therefore inoculated with the samples.

# APPENDIX 2

## LIST OF ABBREVIATIONS AND ACRONYMS

| **Abbreviated Terms** | **Full Term** |
|---|---|
| *B.t* | *Bacillus thuringiensis* |
| ICP | Insecticidal crystalline Protein |
| *B. anthracis* | *Bacillus anthracis* |
| B. Cereus | Bacillus Cereeus |
| *B. thuringiensis* | *Bacillus thuringiensis* |
| MPCAs | Microbial Pest Control/Agents |
| $LC_{50}$ | Lathel Concentration |