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Original Research Article

Isolation and Characterization of *Bacillus thuringinesis* from Soil and Water and Laboratory Testing of Their Insecticidal Activity against *Spodoptera furgiperda* (Lepdoptera: Noctuidae) in Gondar, North Western Ethiopia

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Abstract: Fall armyworm is one of the challenges in the agricultural sector. It is a major insect pest of maize and other crops. Currently, farmers use chemical insecticides to reduce such losses. However, chemical insecticides cause the development of insecticidal resistance in insects, environmental pollution, human health hazards, harm to non-target species, etc. Therefore, agrarian look for safe and effective alternative approaches, biological control to fall armyworm. Bacillus thuringiensis (*Bt*) based biopesticide is a major alternative to solve these problems. This study aimed to isolate and characterize local *Bt* isolates from soil and water samples of different sites in Amhara and Afar Region and laboratory testing of their insecticidal activity against fall armyworm. The data was analyzed using Analysis of Variance. A total of 18 soil and water samples were collected from study sites to isolate *Bt* variety. Morphological and biochemical methods were used to characterize and identify *Bt* isolates. Based on results, a total of 21 *Bt* isolates were recovered from102 bacillus species- *Bt* like a colony and the overall *Bt* index corresponding to the whole sampling areas was 0.2. From total isolates, 7 isolates had a high potential to kill FAW within 72 hrs. W3C and M8E isolate were best as compared to other potential isolates, including reference strains because they were killed after 48 hrs. To conclude Screening of soil and water samples from different sources and habitats may be useful to obtain potential *Bt* isolates with broader host ranges and high potential for insecticidal activity.

Keywords: Bacillus Thuringienesis, Crystal Protein, Insecticidal Activity, Isolates, Spodoptera Furgiperda.

1. INTRODUCTION

1.1. Background of the Study

Agriculture is the backbone of many developing countries. It is not only for surpluses but also it has a substantial contribution to the growing global economy **(**Rameshaiah *et al*., 2015**)**. The global agriculture sector is facing several challenges such as climate change, urbanization, land degradation, evolution and existence of insect pests, diseases, weeds, vertebrate pests and other pests (Siddiqui *et al*., 2015). Of which insect pests are the one and the most important, this cause's considerable crop loss. Several figures suggest that globally about one-sixth of production is lost to insect pests in the field and further losses in storage (Smith, 2015).

In the case of African countries crop losses due to insect pests are estimated at 49% of the expected total crop yield yearly(Ngumbi, 2017). According to the reported data by Loha *et al.* (2018), indicated that Ethiopia, Tanzania, Kenya, Uganda, Malawi and Rwanda loss of 0.9–1.1 billion US$ annually due to reducing small-holder farmer's maize production as a result of poor pest management in the sectors (Loha *et al*., 2018). These indicated that insect pests species are a major role in the economic impact of crop production and among these crop insect pests, fall armyworm is one of the major causes of damage to crops.

The fall armyworm, *Spodopterafurgiperda* (J.E. Smith) (*Lepidoptera: Noctuidae*) (FAW) is innate to America and it is a key pest of maize (*Zea mays L*.) and many other crops throughout the America(Early *et*

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al., 2018). In Brazil, it is also the major pest of maize and decreases maize yields by up to 57%, depending on the crop season and hybrid used (Burtet *et al*., 2017). Recent studies conducted by the Center for Agriculture and Bioscience International (CABI) in 12 maize-producing African countries showed that without proper management, FAW can cause maize yield losses of between 8-21 million tons and monetary losses of up to US$ 6.1 billion, this affecting over 300 million people in Africa, who, directly or indirectly, depend on the crop for food and well-being (Midega *et al*., 2018). According to Kassie *et al*., (2020), FAW poses a significant risk for 9.6 million maize-producing smallholders and causes a loss of more than134,000 tons of maize production in Ethiopia.

The FAW damage about 186 different plant species from 42 families, including maize, a major food staple in sub-Saharan Africa, upon which more than 300 million people depend (Early *et al*., 2018; Abrahams *et al*., 2017; Cock *et al*., 2017). The larvae feed on leaves, stems and reproductive parts of different plant species that include maize, rice, sorghum, sugarcane, cabbage, beet, peanut, soybean, alfalfa, onion, tomato, potato and cotton (CABI, 2016).

Considerable efforts have been made to control the impact of pests in general and fall armyworm in particular such as cultural, chemical and biological methods (Assefa and Ayalew, 2019). The farmers who practice a mechanical method are controlling the pest up to 54%. Control of pests (FAW) is usually achieved through the application of synthetic insecticides (Blanco *et al*., 2014). However, increasing agricultural productivity *via* the use of chemicals resulted in unforeseen problems: increased water use, CO_2emissions and adverse environmental effects, such as extensive land conversion to agriculture and pesticide-driven pollution (Strassburg *et al*., 2014). Besides, the indiscriminate use of synthetic pesticides led to the rapid evolution of pesticide resistance in pests, plus a long list of non-desired detrimental effects, such as the pollution of the environment, human health hazards, harm to non-target species and the rise of secondary pests (Ehler, 2006). Therefore, as a result of the pitfalls of chemical methods agrarian look for safe and effective alternative approaches, biological control to FAW (Aramideh*et al*., 2010).

Among these biological control, bacteria based biopesticides are the most widely applicable and are cheaper than the other modes of pest biocontrol. Several species of bacteria can infect different varieties of insects, but those bacteria belonging to the genus *Bacillus* are most widely applicable as biopesticides (Sharma and Sharma, 2011). Interestingly, *Bacillus thuringiensis* (*Bt*) is a Gram-positive, saprophytic, aerobic and spore-forming soil bacterium that was first isolated from diseased larvae of *Bombyxmori*(an economically important insect, called the silkworm) in Japan (Ishiwata, 1901). *Bacillus thuringiensis* (*Bt*) has been used successfully as a biological insecticide over the last 60 years and constitutes 90% of all commercial bio-insecticides, due to its high specificity, safety and effectiveness in the control of a wide spectrum of human disease vectors and agriculture-pests including fall armyworms (Naster *et al*., 2002).

Search for new *Bt* strains may lead to the discovery of new insecticidal proteins with higher toxicity which will be important for providing alternatives to cope up with the emergence of resistant insect populations. *Bacillus thuringienesis* strains isolated locally are usually more effective than imported strains due to higher specificity on the target host, greater field persistence due to higher adaptation to the natural environment and toxicity at a higher temperature range (Brownbridge, 1989). Therefore, the purpose of this study is to isolate and characterize *Bt* from soil and water to evaluate their insecticidal activity against fall armyworm (*Spodopterafurgiperda* (*Lepidoptera: Noctuidae*)) in North Gondar and Dubeti (Afar), Ethiopia.

2. MATERIALS AND METHODS

2.1. Study Period and Design

CRD design was used to conduct this study. The insecticidal efficiency of the 7 potential isolates was tested and the tests were carried out in triplicate. A treatment with no bacteria was prepared as the negative control. This study was conducted from October 2019 to September 2020 in the Cellular and Microbial and Molecular biology Laboratory, Institute of Biotechnology, University of Gondar, Gondar.

2.2. Sample Type, Sampling Technique and Sample Collection

From the study areas, soil samples weighed 10 grams each were collected by scraping the soil surface 4-10cm deep, using a sterile spatula and polyethylene tube (Zeleke W. Tenssay *et al*., 2009). The above study areas were purposively selected and a total of 15 soil samples (3 soil samples from each sites) were particularly taken from forest areas. Similarly, 20ml of water samples were collected from the forest area of Ras Dejen (Ras dashen) at three different sites. All of the collected samples were transported to the Institute of Biotechnology, a microbiology laboratory under sterile conditions for further analysis.

2.3. Processing of Soil Sample

From the collected soil samples one gram was taken distinctly and dissolved in 9 ml of sterile normal saline solution (0.85%: w/v) and homogenized vigorously with a vortex mixer for 2 min. The sample solutions were heat-shocked at 80ºC for 10 min, in a water bath to destroy non-spore formers and vegetative *Bacillus* cells. Then homogenized and heat-treated soil solutions were serially diluted in sterile normal saline, to prepare 10^{-1} – 10^{-5} dilutions. Then 100 µl volume

sofserially diluted soil samples were taken and plated on nutrient agar medium and incubated aerobically at 37ºC for 24 hrs (Chilcott and Wigley, 1993).

2.4. Isolation and Identification of *Bt* Isolates

After 24 hrs of incubation, the bacterial isolates were obtained and characterized using various morphological (Microscopic and colony morphology) and biochemical tests as described by Fawole and Oso (2001). A single individual colony from 10^{-3} - 10^{-5} agar culture was picked and transferred to the Tryptoya Soya Broth (TSB) consequently grown for 24hrs at 37°C. The singularity of colonies was checked through sub-culturing TSB grown cultures on nutrient agar for 24 hrs at 37°C.

2.4.1 Microscopic Observation of Morphological Features of Spores and Parasporal Bodies

Bacterial colonies were picked and transferred into LB broth, subculture on nutrient agar and incubated it for 24 hrs. From the sub-cultured colonies, smears were prepared and a Gram staining test was done following a method initially described by Chilcott and Wigley (1988). Spores and parasporal bodies were examined after incubation of LB broth media at 30ºC for 72 hrs and stained according to Ammons *et al.*, (2002).

2.4.2 Biochemical Typing

Twenty-one *Bt* isolates which were Gram-positive and had a parasporal body were selected and subjected to consecutive biochemical tests including; Catalase test, Oxidase test, Starch hydrolysis, TSI, Methyl red, Indole test, Citrate utilization, Urease test, Mannitol salt agar tests were conducted to know further nature of isolates(Rajeswari and Bhuvaneswari, 2016).

2.4.3 Hemolytic Activity

Isolates were refreshed by sub-culturing on a nutrient agar plate and incubated overnight at 27°C. Fresh cultures were inoculated onto blood agar plates containing 2% (v/v) sheep erythrocytes at 27°C for 24 hrs. The sheep erythrocytes were kindly provided from college of veterinary medicine farm at Gondar University. The formations of the zone of hemolysis surrounding colonies were examined at the end of incubation (Ichikawa *et al*., 2008).

2.4.4 Motility Testing

Isolates were inoculated onto the middle of the tubes from top to bottom by using an inoculation loop on a modified motility agar medium containing (1% (w/v) tryptone, 0.5% (w/v) NaCl and 0.3% (w/v) agar) with phenol red as a color indicator and incubated at 30°C overnight. The isolates that correspond positive or negative to the motility test were recorded in contrast to a reference strain *Bti* and *Btk* were used as a positive control for the motility test (Maheswaran *et al*., 2010).

2.5. Susceptibility to Antibiotics

Antibiotic susceptibility of *Bt* isolates was performed in response to commercial antibiotic ampicillin, erythromycin, cotrimoxazole and bacitracin using the disk diffusion method described by Ichikawa *et al*. (2008). The test results of antibiotic sensitivity were determined according to the inhibition zone diameter. But the absence of such a clear zone or the presence of some colonies within the clear zone indicated that the collected isolates were resistant to those antibiotics(Sarker *et al*., 2010).

2.6. Collection of FAW

About 150 FAWs of the 5th instar larvae were collected from farm in Gondar and feed on cabbage and Castrol leaf alternatively until the bioassay experiment was conducted. The larval stage of worms was determined by professionals.

2.7. The Pesticide Activity of *Bt* Isolates Against Fall Armyworm

2.7.1 Preparation of *Bt* Spore-Crystal Complex

One ml of each bacterial culture was inoculated into sterilized Luria Bertani (LB) broth enriched with salts ($g.L^{-1}$ in distilled water) 0.002g $FeSO_4$, 0.02g $ZnSO_4$, 0.02g $MnSO_4$, 0.3g $MgSO_4$ and 2g glucose to aid sporulation in 250 ml flasks and pH of media was adjusted to 7.5. Liquid cultures were incubated in an orbital shaker at 30^0C for five days until sporulation was observed, the broth culture containing spore-crystal complex was centrifuged(Lobo *et al*., 2018).Due to its more effectiveness than the crystal alone, the spore-crystal complex was prepared for each isolate to test insecticidal efficacy(Johnson and McGaughey, 1996;Rosas-Garcia, 2009;Keswani *et al.*, 2016).At each sampling the density of sporulation and crystal formation was monitored using the Smirnoff stain, the staining slides were viewed under a light microscope with an oil immersion objective (Smirnoff, 1962).

2.7.2 Viable Spores Quantification

One gram of *Bt* culture samples was inoculated into 9 ml of sterile water and shaken in an orbital shaker for 30 min. The bacterial suspensions were subjected to thermal shock (80ºC for 12 min) to kill vegetative cells. Then Samples were serially diluted and 100 µl of *Bt* isolates were plated on nutrient agar plates with triplicate plates having a medium pH of 7.2 and incubated at 30ºC for 24 hrs. The colonies formed were counted and expressed as colony-forming units per milliliter (CFU / ml). CFU/ml of isolates were calculated by using the formula, CFU/ml = (Average count) / (Dilution plated) (ml plated) (Shafer *et al*., 2015).

2.7.3 Insecticidal Test of *Bt* Isolates Against FAW

All the bacterial contents(1 gram wet cell (spore-crystal inclusion complex)) of each 21 *Bt* isolates were transferred to falcon tubes containing 10 ml of autoclaved distilled water(Shishir *et al*., 2012; Lobo *et al*., 2018). Serial dilution (10^{-1}) of each spore-crystal

inclusion was prepared through transfer 1 ml of spore-crystal inclusion from a concentrated suspension of each isolate mixed with 9 ml of sterilized distilled water with 0.01%of Triton X-100 for each bioassay (Lobo *et al*., 2018). The concentration of the spore-crystal inclusion (10^{-1}) is similar to McFarland standard 4. A treatment with no bacteria was prepared as the negative control. Cabbage and Castrol leaves were used as the food of larvae alternatively. According to the leaf dip method, an insecticidal test was conducted through the leaves were soaked in jars that contain 10 ml of diluted each spore-crystal inclusion (10^{-1}) with 0.01% Triton X-100 after the leafs washed with sterilized distilled water containing 0.1% Triton X-100 and air dry for about 10 minutes(Entomology, 2004). Then they were raised, allowed to dry on sterilized plastics and transferred to other sterilized jars. Finally, FAW worms were added to jars, which contain treated leaves. After 24 hrs, 48 hrs, 72 hrs of treatment worms mortality was verified by counting living and dead worms (Astuti *et al*., 2018). The worm that did not move when touched with a sterile stick was considered as dead (Dulmage *et al*., 1990). Triplicate was maintained for the potential isolates.

2.8. Data Collection

Insect mortality was assessed 24, 48 and 72 hrs after treatment application. A larva was considered dead if it could not move itself after being placed on its dorsal surface.

2.9. Data Analysis

The insecticidal efficiency of isolates was reported as a mean ± standard deviation of triplicate data. Data were analyzed by one-way analysis of variance (ANOVA) (Gomez and Gomez, 1984). The List significant difference (LSD) test, at $p < 0.05$, was used to determine significant differences between the means of the isolates in the SPSS statistical software Version 22.

3. RESULT

3.1. Collection, Isolation and Identification of Bacterial Isolates

A total of 15 soil samples and 3 water samples were collected from different sites of the North Gondar Zoneand Afambo District (Afar) under sterile conditions. Sampling results are shown in **Table 2**. After heat shock, a total of 102*Bt*like colonies appeared on the nutrient agar medium, out of which 21 isolates were identified as *Bt*based on purple color after Gram staining and the presence of parasporal bodies (**Figures 6 and 7**). Since the *Bt* index is defined as the number of identified *Bt* colonies divided by the total number of *Bacillus* like colonies examined, a *Bt* index of 0.2was obtained (**Table2**). Geographically, the Metema and Beyeda District showed the highest percentage of *Bt* isolates (28.5%), followed by the Mirab Armacho, Gondar (Maraki sub-town) and Afambo District (Afar) (23.8%, 9.5% and 9.5%, respectively) (**Table2).** The two reference *Bt* strains used were *B. Thuringiensis* subs. *Kurstaki* (*Btk*) and *B. Thuringiensis* var. *israelensis* (*Bti*), kindly provided by Dr. Meera Indracanti from India.

Table 1: Isolation of *Bt* isolates from soil and water samples of North Gondar (Amhara) and Afambo (Afar) regions.

District	District towns	Sample type	Noof samples analyzed	Noof *Bacillus*-like colonies examined	No of *Bt* isolate obtained	*Bt* index
Mirab Armacho	Abderafi	Soil	3	14	5	0.357
Metema	Yohannes Ketema	Soil	3	32	6	0.188
Gondar zuria	Maraki sub town	Soil	3	22	2	0.09
Beyeda	Ras dashen	Soil+water	3+3	28	6	0.2
Afambo	Dubeti	Soil	3	6	2	0.33
	Total	2	18	102	21	0.2

Bt index: No of identified *Bt* colonies divided by the total number of *Bacillus*-like colonies examined.

3.2. Colony Morphology, Parasporal Body and Gram Reaction Characterization

Individual colonies were obtained from 10^{-3} to 10^{-5}serial dilutions from soil and water samples. In these serial dilutions, 102 bacilli like colonies were obtained and labeled with subsequent subscript numbers. Out of these 102 colonies, 21 *Bt* isolates were having a white or milky, round, circular or irregular shape, raised, slightly raised or flat center. All 21 *Bt* isolates were Gram-positive and a parasporal body positive (**Table 3, Figures 5, 6, 7**).

Table 2: Colony morphological, parasporal body and gram staining characterization of *Bt* isolates

	Morphological and cultural features							
Isolates	**Colony Color**	**Margin**	**Size**	**Elevation**	**Oxygen requirement**	**Bacterium shape**	**G-staining**	**Parasporal body**
Ab02WRF	White	Round	Small	Raised	Aerobic	Long rod	+ve	+ve
M8B	White	Round	Small	Slightly raised	Aerobic	Long rod	+ve	+ve
A	White	Round	Small	Slightly raised	Aerobic	Long rod	+ve	+ve
MK3C’	White	Round	Large	Flat	Aerobic	Long rod	+ve	+ve
Abo3sw	White	Round	Large	Flat	Aerobic	Long rod	+ve	+ve
Ab01WRF	White	Irregular	Large	Raised	Aerobic	Long rod	+ve	+ve
W1A	White	Round	Large	Slightly raised	Aerobic	Long rod	+ve	+ve
W1D1	White	Round	Small	Raised	Aerobic	Long rod	+ve	+ve
M1W	White	Round	Large	Flat	Aerobic	Long rod	+ve	+ve
W3C	White	Round	Small	Raised	Aerobic	Long rod	+ve	+ve
Ab01y	White	Round	Large	Slightly raised	Aerobic	Long rod	+ve	+ve
MK1G	Milky	Round	Small	Flat	Aerobic	Long rod	+ve	+ve
W3A	White	Irregular	Large	Slightly raised	Aerobic	Long rod	+ve	+ve
C	White	Round	Large	Flat	Aerobic	Long rod	+ve	+ve
M111W	White	Round	Large	Slightly raised	Aerobic	Long rod	+ve	+ve
W3A’	White	Irregular	Small	Raised	Aerobic	Long rod	+ve	+ve
M8E	White	Round	Large	Flat	Aerobic	Long rod	+ve	+ve
W3B	Milky	Circular	Large	Flat	Aerobic	Long rod	+ve	+ve
Ab01B	White	Circular	Small	Flat	Aerobic	Long rod	+ve	+ve
M5B3	White	Round	Large	Flat	Aerobic	Long rod	+ve	+ve
M8C	White	Round	Small	Raised	Aerobic	Long rod	+ve	+ve

Key:

Ab01WRF,Ab02WRF,Ab03sw,Ab01y,Ab01B = Ab represent isolate from Abdrafi, 01,02,03 = numeric number represent isolated site, WRF, sw, y, B = the letter represent colony type, MK3C’, Mk1G = Mk represent isolate from Maraki, 1,3 = numeric number represent isolated site, W1A, W1D1, W3A, W3A’, W3B, W3C = isolate from Rasdashin water sample, 1,3 = numeric number represent isolated site, A, A’, B, D, C = letters represent colony type, M1W, $M_1$11WGeo, M5B3, M8B, M8C, M8E = isolate from Metema, A and C = isolates from Dubeti (Afar) soil samples, G- staining,= gram staining, +ve = positive.

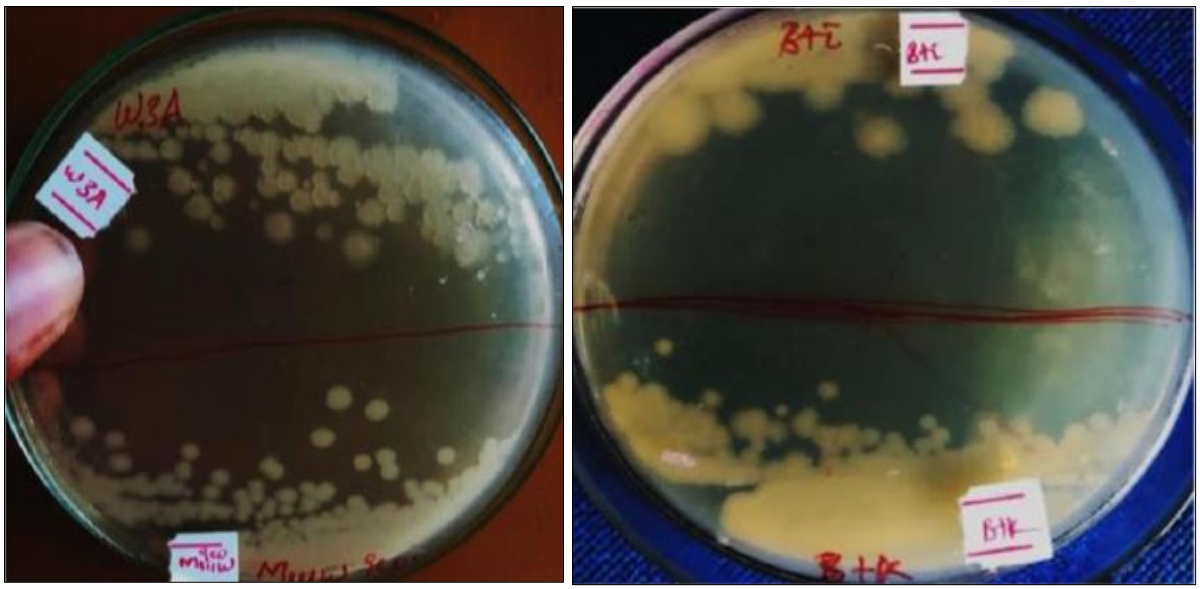


Figure 1: A purified single colony of Bacillus thuringiensis

3.3. Microscopic Observations

3.3.1 Gram Staining

Bacillus species are generally Gram-positive and rod-shaped (**Figure 6**). Gram staining was done to differentiate Gram-positive from the Gram-negative. A light microscope was used for observations. Isolates that were rod-shaped and blue indicate Gram-positive isolates, whereas isolates that did not exhibit these characteristics were discarded (**Table 3**).

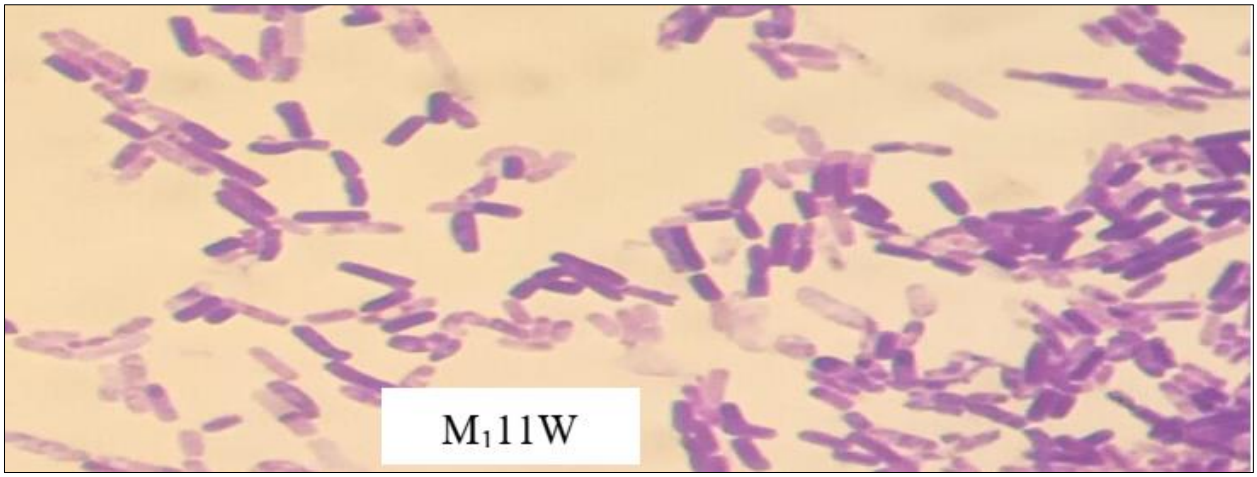


Figure 2: Zoomed microscopic image of gram staining,the normal microscopic image found inthe Appendix Figure 1 (image A)

3.3.2 Parasporal Body Staining

The insecticidal crystals formed by parasporal bodies(Cry and Cyt proteins) are the principal characteristic that differentiates *Bt* from *B. cereus* as well as other species of the *B. cereus* group(Zeleke W. Tenssay *et al.*, 2009; Federici, 2013). Gram-positive isolates were then subjected to further screening of the parasporal body through Coomassie blue staining (**Figure 7**). This method has a higher resolution compared to phase-contrast microscopy. Thus, samples which have a parasporal body can be easily identified. 21 isolates were taken up the Coomassie brilliant blue stain (CBB) and had parasporal bodies during the sporulated phase and autolysis phase (**Table 3, Figure 7**).

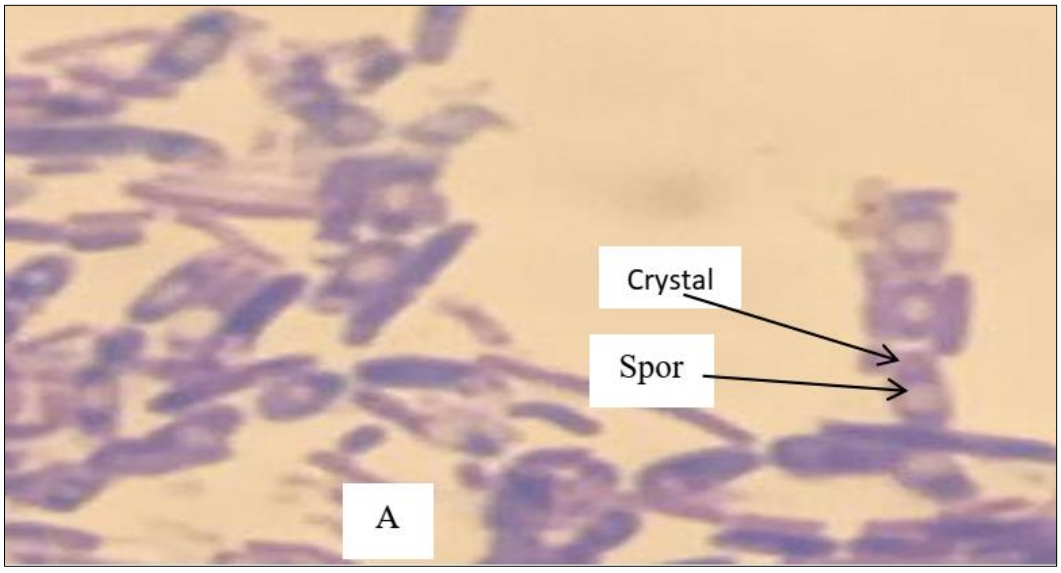


Figure 1: Zoomed microscopic image of parasporal bodies, the normal microscopic image found in the Appendix Figure 1 (image C)

3.4. Biochemical Typing

As shown in **Table 4**below and **Figure 8,** the various biochemical tests for 21 *Bt* isolates were conducted and the results obtained are presented. All isolated *Bt* were positive for catalase activity and indole test,47.6% of isolates ferment only glucose while others ferment lactose or sucrose, 80.95% of *Bt* could utilize citrate, 90.47% positive to MSA, 76% were starch hydrolysis test positive, 85.7% oxidase-positive, 38% methyl red test positive, 80.95% of isolates were urease negative, 81% were motile and 95% isolate were hemolytic. However, all 7 insecticidal *Bt* isolates (100%)

exhibited active motility, hemolytic activity, starch hydrolysis activity, catalase, indole and MSA positive.

Table 3: Biochemical typing

Code of strains	Indole test	Methyl red	oxidase	TSI		MSA	Citrate	Urase	Starch	Motility	Hemolytic
				G-ferm	L/S-ferm						
Ab02WRF	+	-	+	-	+	+	-	+	+	+	+
M8B	+	-	+	-	+	+	+	-	-	+	+
A	+	-	+	+	-	+	-	-	+	+	+
MK3C’	+	-	+	+	-	-	+	-	+	+	+
Ab03sw	+	-	+	+	-	+	+	-	+	+	+
Ab01WRF	+	+	+	-	+	+	+	+	+	+	+
W1A	+	+	+	-	+	+	+	-	-	-	+
W1D1	+	-	+	+	-	+	+	-	+	+	+
M1W	+	+	-	+	-	+	+	-	+	+	+
W3C	+	+	+	-	+	+	+	-	+	+	+
Ab01y	+	-	+	-	+	+	+	-	+	+	+
Mk1G	+	-	+	+	-	+	+	+	-	+	+
W3A	+	-	+	+	-	+	+	-	+	+	+
C	+	+	+	-	+	+	-	-	+	+	+
$M_1$11WGeo	+	-	+	-	+	+	+	-	+	+	+
W3A’	+	-	+	-	+	-	+	-	+	-	+
M8E	+	-	-	+	-	+	+	-	+	+	+
W3B	+	+	-	+	-	-	+	-	-	+	+
Ab01B	+	+	+	-	+	+	+	-	+	+	-
M5B3	+	-	+	-	+	+	-	-	-	-	+
M8C	+	+	+	+	-	+	+	+	+	-	+

Key: TSI = triple sugar iron, G-ferm = glucose fermentation, L/S-ferm = lactose or sucrose fermentation, MSA =mannitol salt agar test, + = positive, - = negative

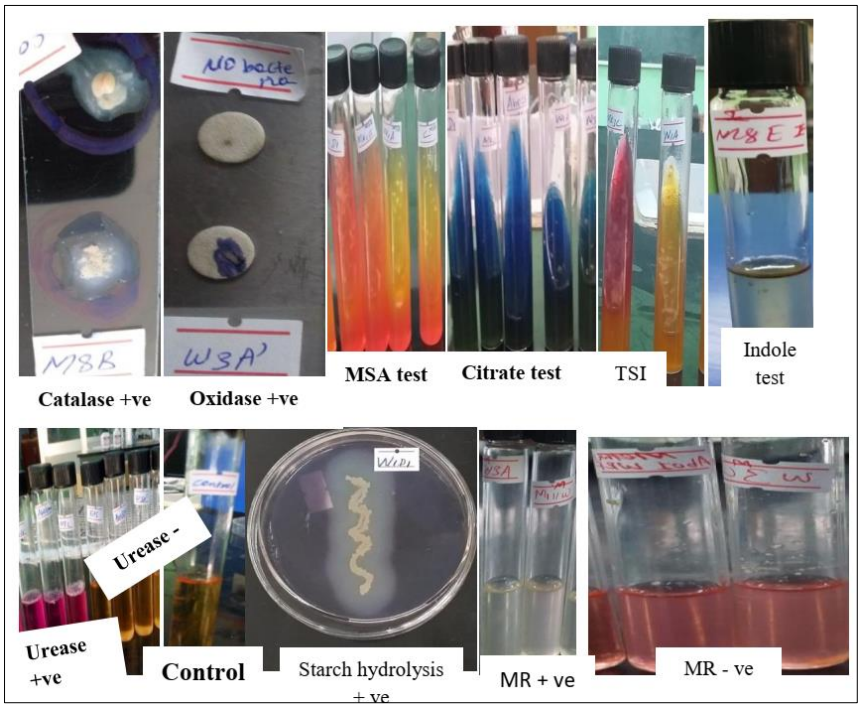


Figure 4: Some images of biochemical test result, MR =methyl red, +ve = Positive, -ve = Negative

3.5. Motility, Hemolytic and Antibiotic Susceptibility

Motility test indicated that 81% of *the Bt* isolates were motile. Likewise, hemolytic assays indicated 95% of the 21 native *Bt* isolates were hemolytic. Antibiotic susceptibility testing was conducted to the best 7insecticidal potential *Bt* isolates, including reference strains (*Bti* and *Btk*). The test indicated that all *Bt* isolates, including *Bti* and *Btk* (reference strains), were resistant to ampicillin and cotrimoxazole. Whereas 77.8% of *Bt* isolates, including *Bti* and *Btk* (reference strains), were sensitive to erythromycin and bacitracin except for W3C and Ab02WRF isolates (**Table 5 and Figure 9**).

Table 4: Antibiotic susceptibility of potential *Bt* isolates, including reference strain *Bti* and *Btk*

Isolates	A	Ab01WRF	Ab02WRF	M8E	$M_1$11W	W3A	W3C	*Bti*	*Btk*
Ampicillin	R	R	R	R	R	R	R	R	R
Erythromycin	S	S	R	S	S	S	R	S	S
Cotrimoxazole	R	R	R	R	R	R	R	R	R
Bacitracin	S	S	R	S	S	S	R	S	S

Key: R-resistant, S- Sensitive

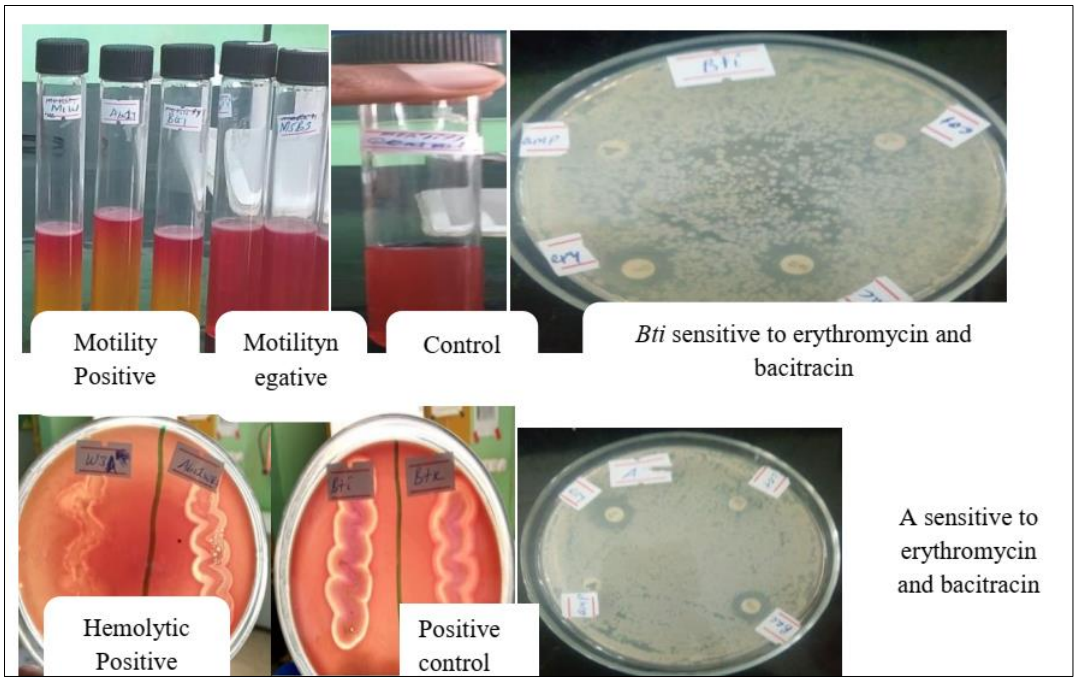


Figure 5: Motility, hemolytic and antibiotic susceptibility

3.6. Viable Spores Quantification

The concentration of viable spore formulation of the 7 *Bt* isolates was estimated by counting the number of colonies and expressed in CFU/ml(**Table 6**). CFU/ml of isolates were calculated by using the formula, CFU/ml = (Average count) / (Dilution plated) (ml plated)(Shafer *et al*., 2015). Best isolates such as W3C and M8E had $12x10^8$ and $5x10^8$ spore/ml respectively. However, *Btk* and *Bti* (reference strains) had the highest spore number as compared to isolates.

Table 5: Spore count of isolates

Strains	Ab02WRF	M8E	A	Ab01WRF	$M_1$11W	W3A	W3C	*Bti*	*Btk*
Cfu/ml at 10^{-5}	$15x10^6$	$17x10^6$	$61x10^6$	$19x10^6$	$31x10^6$	$90x10^6$	$47x10^6$	$100x10^7$	$120x10^7$
Cfu/ml at 10^{-6}	$8x10^8$	$13x10^7$	$36x10^7$	$10x10^7$	$14x10^7$	$55x10^7$	$26x10^7$	$70x10^8$	$75x10^8$
Cfu/ml at 10^{-7}	$4x10^8$	$5x10^8$	$18x10^8$	$6x10^8$	$5x10^8$	$25x10^8$	$12x10^8$	$55x10^9$	$63x10^9$

3.7. Collection FAW Worms

About 162 FAWs of the 5th instar larvae were collected from maize sellers in Gondar town and feed on cabbage and Castrol leaves alternatively until the bioassay experiment was conducted (**Figure 10**).

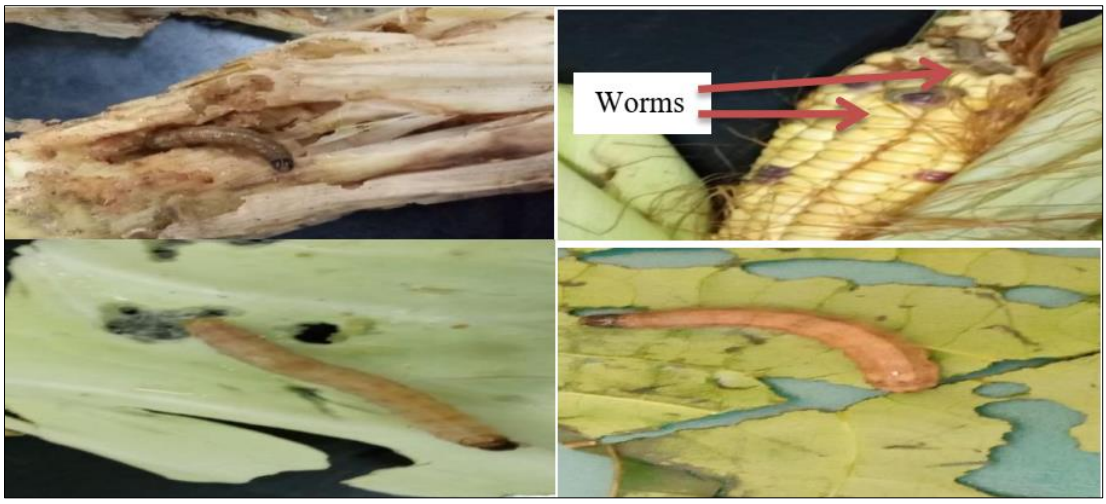


Figure 6: Collection of worms and feed on cabbage and Castrol leaves

3.8. Toxicity of *Bt* isolates to Larvae of FAW

For the evaluation of the insecticidal activity of the different isolates of *Bt* on FAW, all the isolates were subjected to the leaf smear method of bioassay against the 5thinstar larvae of FAW. Out of 21 isolates, 7 isolates were selected as potential isolates at the first trial of treatment and Triplicate was maintained for these 7 potential isolates. The mortality percentage was recorded after 24 hrs, 48 hrs and 72 hrs of feeding with *Bt* culture as described in the materials and methods. The mortality of FAW was observed in all of the 7 potential *Bt* isolates immediately after 24 hrs of the *Bt* treatment. At 48 hrs, the highest % mean mortality of FAW was recorded in the treatments of W3C and M8Eisolates (100.00 ± 0.00) these are followed by Ab01WRF, W3A, $M_1$11w, A, Ab02WRF which revealed 77.80± 19.22, 66.70 ± 0.00, 77.80± 19.22, 55.56± 19.28 and 55.56±19.28 mortality respectively. The other five isolates were killing 100 ± 0.00 of FAW at 72 hrs (**Table 7, Figures 11 and 12**). A high significant difference in efficacy among the isolates was found at 48 hrs (P = 0.004) but at 24 and 72hrs (P = 0.469 and 0.463 respectively), there was no significant difference among isolates. The ANOVA tables were shown below in the **AppendixTable1**.

Table 6: Mean percent mortality of FAW larvae 24, 48 and 72 hrs after the application of *Bt* suspension

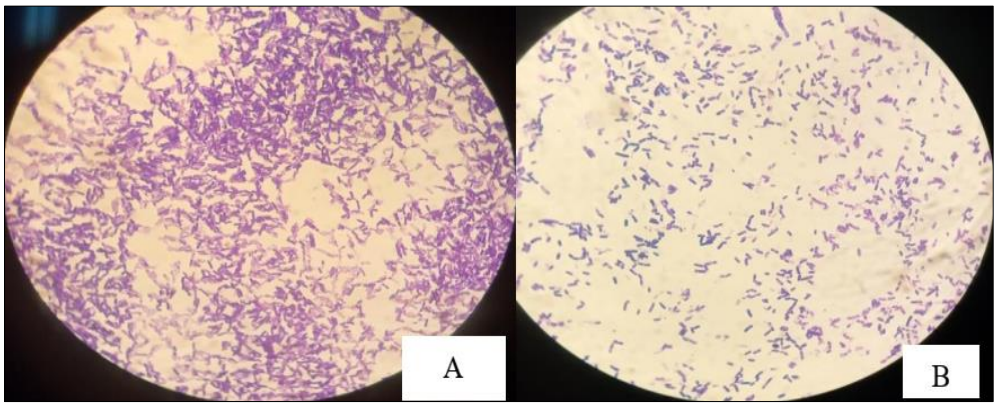
Isolates	Mortality (%) ± SD		
	% of larval death after 24hrs	% of larval death after 48 hrs	% of larval death after 72 hrs
A	33.30± 0.00^{a}	55.56 ± 19.28 ^{a}	100.00 ± 0.00 ^{a}
Ab01WRF	33.30± 0.00 ^{a}	77.80± 19.22 ^{ab}	100.00 ± 0.00 ^{a}
Ab02WRF	33.30 ± 0.00 ^{a}	55.56 ± 19.28 ^{a}	100.00 ± 0.00 ^{a}
M8E	44.43 ± 19.28^{b}	100.00 ± 0.00^{b}	--
M111W	33.30 ± 0.00 ^{a}	77.80 ± 19.22 ^{ab}	100.00 ± 0.00 ^{a}
W3A	33.30 ± 0.00 ^{a}	66.70 ±0.00 ^{a}	100.00 ± 0.00 ^{a}
W3C	33.30 ± 0.00 ^{a}	100.00 ± 0.00^{b}	--
Bti	33.30 ± 0.00 ^{a}	55.56 ± 19.28 ^{a}	88.90 ± 19.22 ^{a}
Btk	33.3± 0.00 ^{a}	66.7± 19.28 ^{a}	100.00 ± 0.00 ^{a}
	P = 0.469	P = 0.004	P = 0.463
	CV = 18.6%	CV = 19.7%	CV = 7.38%

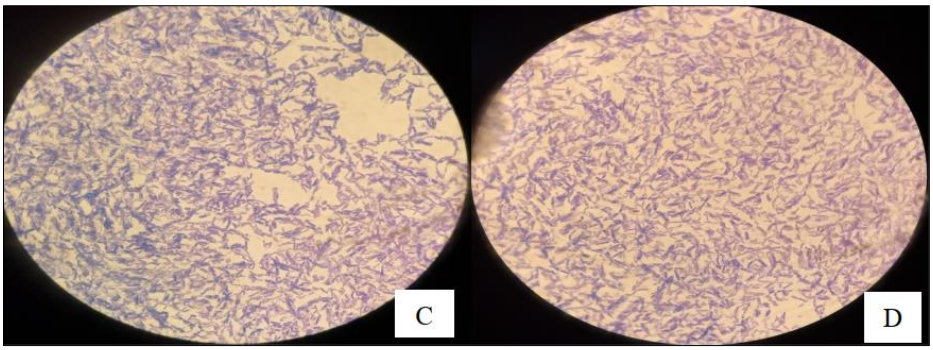
Key:

Mortality is expressed as mean ± standard deviation; P values were 0.469, 0.004, and 0.463 at 24 hrs, 48 hrs and 72 hrs respectively. Mean followed by the same letters in each column are not significant. 3 larvae of FAW were exposed to each *Bt* isolates; all tests were carried out in triplicate.

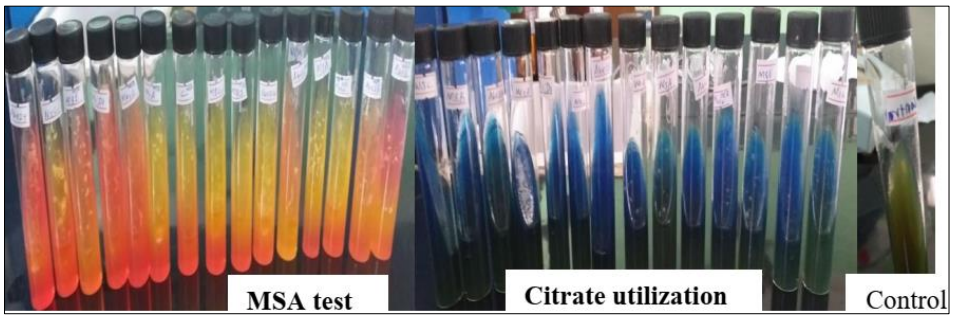
Figure 7: The insecticidal activity of *Bt* isolates result images, images A and B were taken after 48 hrs, C was taken after 72 hrs.

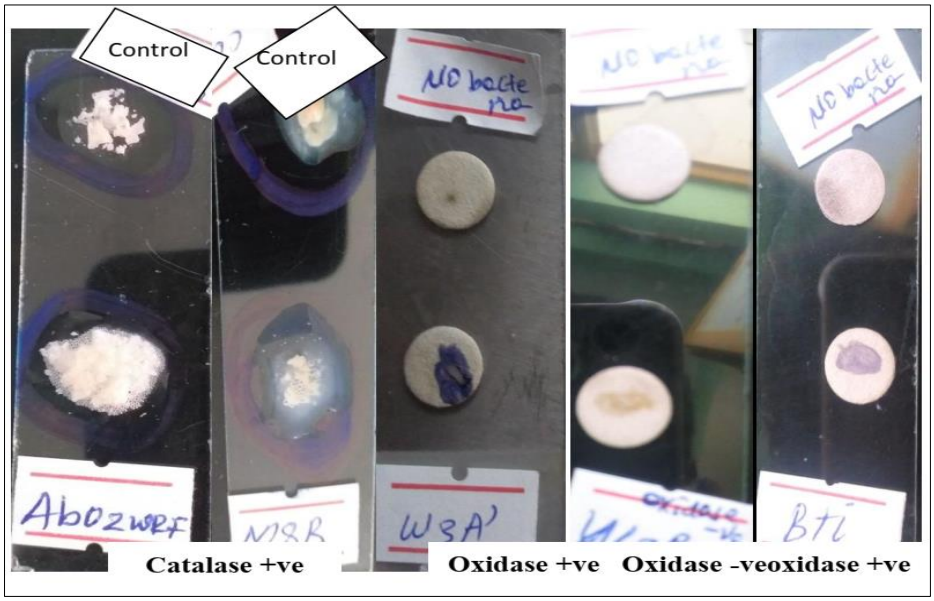
APPENDIX

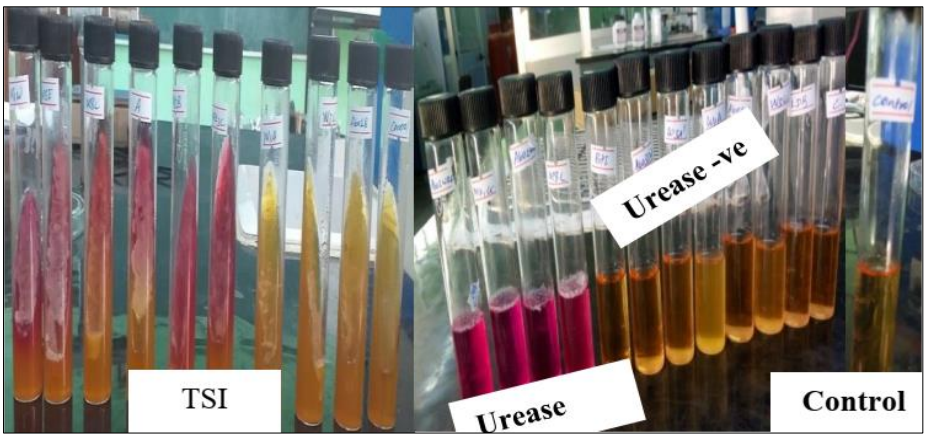


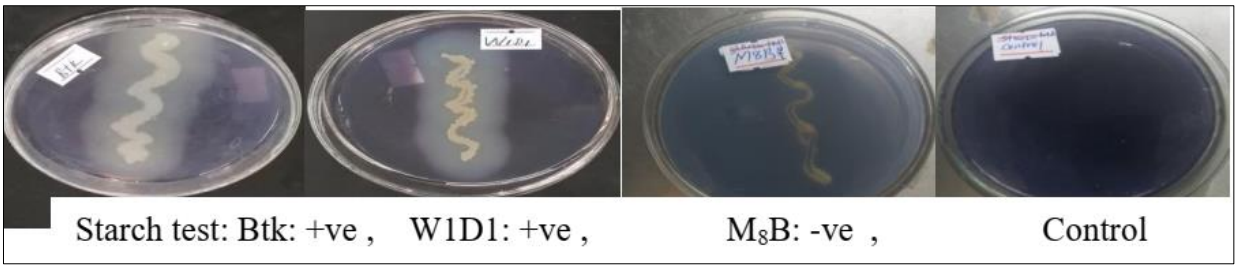


Appendix Figure 1: Microscopic images of gram staining (A and B) and CBB staining (C and D)

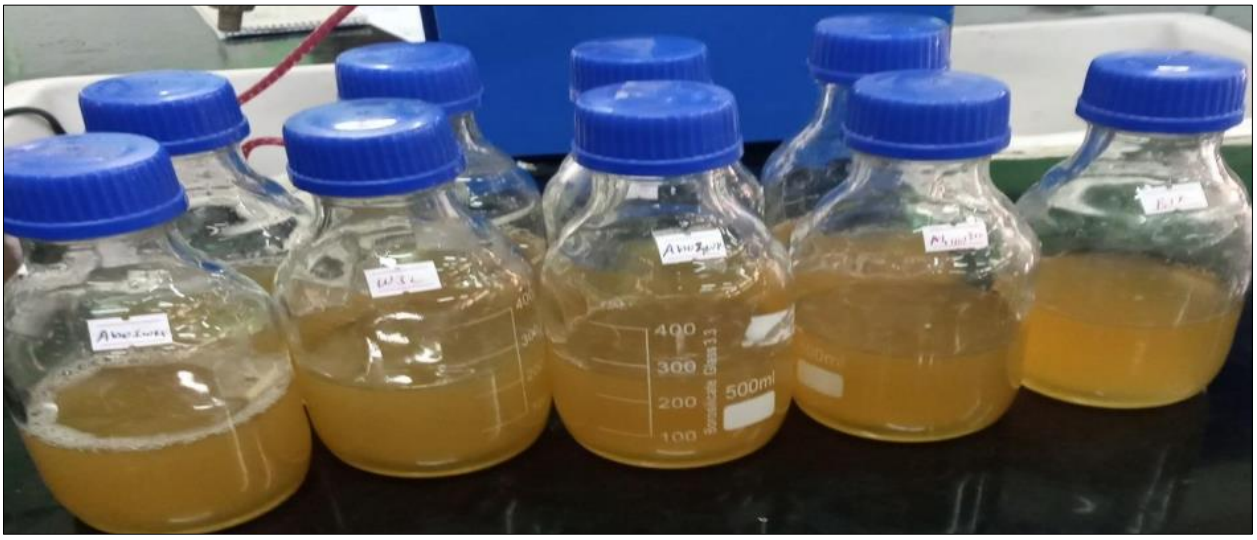








Appendix Figure 2: Some additional biochemical test images



Appendix Figure 3: Preparation of *Bt* isolates culture

Appendix Figure 4: Images of working in a laboratory, cabbage soaked in *Bt* suspension, dried on sterilized plastic and transferred to baby food jars for insecticidal activity test

Appendix Table 1: Analysis of variance showing Mean percent mortality of FAW larvae 24, 48 and 72 hrs after application of *Bt* suspension in laboratory test 24 hrs

ANOVA					
Source of variation	**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Between Groups	330.536	8	41.317	1.000	.469
Within Groups	743.707	18	41.317		
Total	1074.243	26			

Appendix Table 2: Analysis of variance showing Mean percent mortality of FAW larvae 24, 48 and 72 hrs after application of *Bt* suspension in laboratory test 48 hrs

ANOVA					
Source of variation	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7485.107	8	935.638	4.540	.004
Within Groups	3709.640	18	206.091		
Total	11194.747	26			

Appendix Table 2: Analysis of variance showing Mean percent mortality of FAW larvae 24, 48 and 72 hrs after application of *Bt* suspension in laboratory test 72 hrs

ANOVA					
Source of variation	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	316.826	6	52.804	1.000	.463
Within Groups	739.260	14	52.804		
Total	1056.086	20			

4. DISCUSSION

Bacillus thuringiensis bacteria are present in the soil, dead larvae, sand, leaves, water, or dust from stored grains(Konecka *et al.*, 2012). It accounts for 90% of all commercial bio-insecticides used to control pests (Naster *et al*., 2002). Studies declared that wild strains isolated from environmental samples can synthesize insecticidal crystals that show higher activity against insect pestsin a manner comparable to commercial *Bt* strains (Konecka *et al.*, 2012). This implies that it is possible to isolate potential *Bt* strains from different micro-environment and use to control insect pests.

In this study, a total of 21 *Bt* isolates were successfully isolated from 18 samples, which often contained more than one *Bt* isolates from the same sample except for Rasdashin soil samples (no isolated *Bt*) that may due to washed away during staining. But from Rasdashin water sample about 6 isolates were obtained. This is in line with Anderson (2011) that *Bt* can be isolated from soil and water environments. The present study also shows that it is possible to isolate *Bt* isolates from different agro-climatic zones in Ethiopia in line with Zeleke W. Tenssay *et al*., (2009). But the amount of *Bt*index of the study area across the world may be different. This may be related to climate and geographic conditions(Apaydin *et al*., 2005). However, the total *Bt* index (**0.2**) of the present study was in agreement with previous finding Mahalakshmi *et al*., (2012) and El-kersh *et al*., (2012), but it is much greater than the report by Zothansanga *et al*., (2016) (**0.012**), from shifting cultivation habitat in India and nearly fourfold less than Martin and Travers (1989) report, found the highest *Bt* index as 0.85 in the soil samples collected from Asia.

Based on heat-resistance and colony morphology alone, it was possible to discriminate 102*B. Cereus-Bt-like bacilli*. Out of these, 21 isolates contained crystal protein inclusions. This is in line with Kampfer (1991), who reported that it is difficult to discriminate *Bt* from *Bacillus cereus* based on the colony morphology and almost all biochemical reactions (Kampfer, 1991). Many studies rely on crystal inclusions as the only characteristic feature that distinguishes *Bt* from *B. cereus* (Zeleke W. Tenssay *et al*., 2009; Federici, 2013). *Bt* formsparasporal crystals and spores during the stationary phase, which is a nutritionally deficient phase; and the crystals would be toxic and could kill the larvae of mosquito (Renganathan *et al*., 2011).

The colonial morphology of almost all the native *Bt* isolates of the present study was a white or milky, round, circular or irregular shape, raised, slightly raised or flat center. This is highly in agreement with the colonial characterizations described by El-kersh *et al*., (2012).The microscopic observations show that all the 21isolates used were long-rod shaped, Gram-positive, produced endospore and crystal protein. This indicated that the isolates were *Bt*. This study is in consistent with Jyothi and Priya (2018) andAdeyemo *et al*., (2018) reports.

Biochemical characteristics are one of the important methods for the classification of organisms. This is mainly based on the various biochemical reactions taking place in their metabolic and normal pathways (Eswarapriya *et al*., 2010). *Bt* isolates showed great variation in their biochemical reaction. About 80.95% of isolates were utilize citrate,90.47% MSA test positive, 76% starch hydrolysis positive, 47.6% of isolates ferment only glucose while others ferment lactose or sucrose, 85.7% oxidase-positive, 62% methyl red test negative, 80.95% of isolates were urease negative, 81% were motile and 95% isolates were hemolytic, all 21 isolated *Bt* were positive for catalase activity and indole test. This study result differs from El-Kersh *et al*., (2016)report for citrate, MSA test and 100% differ for indole test but similar for starch hydrolysis, motility, hemolytic and urease test, which was conducted in Saudi Arabia. According to Chatterjee *et al*., (2007) report, all isolates were negative to the indole test. But in this study, all isolates were positive for the indole test as mentioned in the result portion. Therefore, the strains which are isolated across the world show the different biochemical test result.

In this study, an antibiotic susceptibility test was conducted for the 7 potential insecticidal *Bt* isolates based on the above method and result in all *Bt* isolates were resistant to ampicillin and cotrimoxazole. But they were sensitive to erythromycin and bacitracin except for W3C and Abo2WRF.Insecticidal *Bt* isolates of this study were 100% Ampicillin resistant. This finding is in agreement with those previously reported studies by Bouba-Adji *et al*., (2014), Bautista and Teves (2013) and Sarker *et al*., (2010). All potential insecticidal *Bt* isolates were sensitive to erythromycin with only the exception of 2 *Bt* isolates, this result similar to El-kersh (2011) and Bouba-Adji *et al*., (2014) reports. These isolates were also sensitive to Bacitracin except W3C and Abo2WRF, which is supported by Chatterjee *et al*., (2007) report.

The concentration of viable spore formulation of the potential 7 *Bt* isolates was estimated through the above methods and the number of colonies was counted and expressed in CFU/ml. The numbers of colonies (spores) of reference strains (*Btk* and *Bti*) were higher than isolates. However, the isolates W3C and M8E had smaller CFU/ml as compared to reference strains (*Btk* and *Bti*), their insecticidal activity was high. This result confirm the studies WHO(1999) and Keswani *et al.*, (2016), bacterial spore counts do not necessarily reflect the insecticidal activity of a *Bt* strain or *Bt* product because the number and amount of ICPs produced per bacterial cell can vary.

In this study, a bioassay was carried out for the detection of the toxicity of the different isolate of *Bt* against the 5th instar larvae FAW by the leaf smear method. Out of 21 isolates, 7 isolates had the potential to kill FAW within 72 hrs and they (100%) exhibited active motility, hemolytic activity, starch hydrolysis activity, indole, catalase and MSA positive. Hemolytic and motility activity of the 7 insecticidal *Bt* isolates were 100% positive, the result similar to the study El-Kersh *et al*., (2016) that all 23insecticidal*Bt* isolates (100%) exhibited active motility and hemolytic activity. According to Bouillaut *et al*., (2005), motility positive is an indirect indicator of virulence and biological activity of *Bt* strains. This study result confirms the Bouillaut *et al*., (2005) report because the 7 selected potential insecticidal isolates were motile. Though the observations of mortality were recorded in 24, 48 and 72 hrs after treatment of *Bt* culture, the final observation (72 hrs after treatment) was recorded as the mortality rate of FAW larvae increased with an extended time. This may be due to the delayed action of *Bt* toxin as the toxin production is connected to the sporulation cycle of *Bt* (Andrup *et al*., 2010).

Though the mortality was observed immediately after one day of the *Bt* treatment, maximum mortality was observed on the second and third days. The highest mortality (100 ± 0.00) was observed on the second day for the isolates M8E and W3C, and more than 55.56% mortality was observed for the isolates, A, Ab01WRF, Ab02WRF, $M_1$11W and W3A. On the third day, more than 89% of mortality was observed for all isolates, including reference strains (*Btk* and *Bti*) except M8E and W3C since they were killed on the second day (48 hrs). A high significant difference in efficacy among the isolates was found at 48 ($P = 0.004$) but at 24 and 72 hrs ($P = 0.469$ and 0.463), there was no significant difference among isolates. The isolates (M8E and W3C) can be used commercially because 2 isolates had the best insecticidal activity and the others had similar effects as compared to reference strains (*Bti* and *Btk*).

In the present study, 2 isolates had 100% potential insecticidal activity at 48 hrs. This finding is better than Cerqueira *et al.*, (2016) report, only 4 out of 52 isolates had 80-90% mortality and other isolates were less than 60% mortality at 48 hrs of exposure. This may due to larval stage different b/n the present study (5th larval instar) and the report (3rd instar) and concentration of *Bt* suspension, the concentration of the present study was (10^{-1}) similar to McFarland standard 4. Voracious feeding habit and consuming more leaf area treated with the insecticide cause high mortality (Hakeem and Akhtar, 2016).Mostly the older larval stages causing higher damage proportioned to over 70% of the overall damage (Assefa, 2018). Therefore, High % mortality may be observed on 5th instar due to its higher feeding than 3rd instar.

The bioassay result of this study was similar to Dias *et al*., (1999) found that out of 25 *Bt* isolates, only eight had high toxicity towards FAW larvae. The positive control (*Bti* and *Btk*) result of the present study similar to Valicente and Fonseca's (2004) report, evaluated *Bt* subsp. *Tolworthi* against 2 day old larvae of FAW for 24, 48, and 72 hrs of exposure. The highest mortality was observed at 72 hrs exposure.

According to a Zeleke W. Tenssay *et al*., (2009) study, which is conducted from soil samples of different agro-climatic zones of Ethiopia, 12 killed 100% of the larvae within 24 hours, and 12 killed 100% *Anopheles arabiensis* larvae within 48 hours. But in this study, only 2 isolates killed 100% FAW at 48 hrs and no isolates which killed 100% FAW at 24 hrs. This may be due to the order of FAW (*Lepidoptera*) and *Anopheles arabiensis (Diptera)* is different and the methods used for bioassay in the present study and Zeleke W. Tenssay *et al.*, (2009) study is different, this may cause for the different result observed.

5. CONCLUSION

In this study, a total of 21 *Bt* isolates were isolated from soil and water samples collected from study sites, which represent the different agro-climatic zone. This confirmed the wide distribution of *Bt* isolates across different ecologies. The *Bt* index of the study sites was different. From 102 *B. cereus* - *Bt* - like *bacilli*, 21 *Bt* isolates were obtained which contained crystal protein inclusions. Out of 21 *Bt* isolates, 7 isolates had a potential

insecticidal activity against FAW. The highest mortality (100%) was observed for M8E and W3C isolates at 48 hrs, but others had similar insecticidal efficiencies as compared to reference strains (*Btk* and *Bti)*. These 7 isolates were resistant to ampicillin and cotrimoxazole, but they were sensitive to erythromycin and bacitracin except W3C and Abo2WRF.The results obtained in this study confirmed the efficiency of the *Bt* in controlling FAWs. Therefore, Screening of soil and water samples from different sources and habitats could be useful to obtain *Bt* isolates with potential insecticidal activity.

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