International Journal of Science and Research (IJSR)
ISSN: 2319-7064
ResearchGate Impact Factor (2018): 0.28 | SJIF (2018): 7.426

Isolation and Identification of *B. thuringiensis* from Different Agro-Ecology of Ethiopia

Denberu Kebede[1], Solomon Yilma[2]

Ethiopian Institute of Agricultural Research, Ambo Agricultural Research Center, P.O.Box 37, Ambo, Ethiopia

Abstract: ***Agricultural pests had and still have a significant role in the recurrence of famine in developing countries. Biological means of controlling pests proved to be a reasonable and safe alternative and/or supportive to chemicals. Bacillus thuringiensis (Bt) is found to produce pro-toxins which have shown insecticidal capacity to larvae of different orders including Lepidoptera. Searching in different environments for new Bt strains with insecticidal potentiality increases the current existing strains. Hence, the study was aimed on isolation and identification of Bt isolates from soil samples collected in different agro-ecologies. Sodium acetate heat treatment technique were used for selective isolation of Bt. Cultural, microscopic and selected biochemical tests were used for further characterization of the obtained isolates. Sixty-nine pure isolates were obtained from seventy-four analyzed soil samples from which 29 isolates based on morphological similarities were selected for characterization. Most of the isolates had morphological (colony color, shape, elevation and margin) similarities. Elliptical or spherical endospore shape were observed among the different isolates and most of them (75%) possessed elliptical endospore and the rest 25% have spherical endospore shape. There was a difference among isolates in hydrolyzing esculin indicated that there are strain variations. From the characterized isolates almost all except 09Bt-2 were suspected to be different strains of B. thuringensis and B. sphaericus. The obtained isolates should be screened for larvicidal activity of different insect pest and exploited for their cry gene content using polymerase chain reaction (PCR) since their toxicity is directly related with the content of cry genes.***

Keywords: *Bacillus thuringiensis*, endospore, Ethiopia

1. Introduction

Agricultural pests attack many different crops world-wide (Berlinger, 1986). They had and still have a significant role in the recurrence of famine in developing countries. Over the last fifty years, the use of chemical pesticides has been the method of choice to control insect pests. However, many problems associated with their use came to existence (Harstack and Witz 1983) Biological means of controlling pests proved to be a reasonable and safe alternative and/or supportive to chemicals. Studies have shown that many entomopathogens can infect and attack insects. These pathogens include bacteria, fungi, protozoa, viruses and nematodes (Gleave, *et al*., 1993). Of the bacteria pathogenic to insects, the spore-forming bacilli are viewed as having the highest potential for the use in the management of pest population (Dulmage 1981).

Among the spore-forming bacilli, the species *Bacillus thuringiensis* (*Bt*)is found to produce pro-toxins which have shown insecticidal capacity to larvae of different orders including Lepidoptera (Angus, 1968), Diptera (Carlberg, 1986). Some strains showed toxicity to other orders of insects like Homopteran, Hymenopter and Orthoptera (Feitelson et al., 1992). The *Bt* products have shown the best success in most of the *Heliothis* management programs and many other pests world-wide as well as a number of insects of public health (Barjac, 1978; Armstrong etal.,1985). Nevertheless, many difficulties encountered its use (Cannon, 1993). There is a need for new strains from different environments that have high insecticidal activity with broad spectra for different targets, or extension of host range of the existing strains through introduction of new crystal protein genes or through conjugal mating to transfer the large plasmid containing the endotoxin genes to other strains to produce a conjugate which has the host range of both the parents (Gonzalez et al., 1982). It has been indicated that screening of *B. thuringiensis* is important in utilizing this bacterium for microbial control of insect pests. Several of the newly discovered *Bt* strains are considered twenty times as potent as present

The use of *Bt* has advantages because it is suited to industrial production, less sensitivity to ultraviolet radiation and adverse weather conditions, increased persistence in the field and non-toxic to humans. Currently, these bacteria-based products account for about 90% of the worldwide market for biological control agents. It has been indicated that screening of *Bt* is important in utilizing this bacterium for microbial control of pests (Padua et al., 1984). It is obvious that searching in different environments for new *Bt* strains with insecticidal potentiality will add to the current existing strains. In Ethiopia no attempts were made to isolate strains of this important bacterium form various environmental samples. However, the existing and newly emerging insect pests have great challenges for Ethiopian agriculture, the use of *Bt* to reduce the damaging effects of insect pests is low. Analyzing Specimens/samples collected from different agro-ecological areas will increase the chance to get potent strains of *Bt*.

2. Materials and Methods

Soil Sample Collection

Surface soil were removed and around 200gr of soil samples were scraped off using aluminum scoop at the depth of 2 to 5 cm. The samples were placed in a clean plastic bags aseptically, and brought to bacteriology laboratory of Ambo Agricultural Research Center (AmARC) for analysis of *Bt*. The soil samples were collected from different areas of three regions; SNNP, Oromia and Afar at different altitudes representing the three major agroecological zones: lowland, midland and highland.

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Isolation of *B. thuringiensis* from soil
Sodium acetate heat treatment method as described by Travers *et al*., (1987) was used for selective isolation of *Bt* from the soil. From each soil sample 1g was added to10 ml of sterile nutrient broth medium in a 125ml flask buffered with 0.25 M sodium acetate for selective inhibition the *Bt* spores. The flasks were shake for 4hrs on a rotary shaker at 150 rpm at 30°c. The mixture was heat treated in a water bath at 80ºc for 15 minutes. A volume of 0.1 ml of the suspension was spread plated on pre dried nutrient agar medium and the plates were incubated at 30°c for 48 to 72hrs. The plats were sub cultured using nutrient broth and the pure isolates were preserved using 20% glycerol for further characterization.

Identification
Cultural, microscopic and biochemical tests were performed to characterize the obtained isolates. For Cultural characteristics colony color, shape, margin, and elevation were considered.

Light microscopy for cell morphology and endospore staining
The pure *Bt* isolates were inoculated on NA media and examined for cell morphology and presence of endospore. The *Bt* isolates were aseptically smeared on glass slides and heat fixed. The slide was then suspended over a water bath with some sort of porus paper over it, so that the slide is steamed. Malachite green was applied to the slide, which can penetrate the tough walls of the endospores, staining them green. After five minutes, the slide was removed from the steam, and the paper towel was removed. After cooling, the slide was rinsed with water for thirty seconds. The slide was then stained with diluted safranin for two minutes, which stains most other microorganic bodies red or pink. The slide was then rinsed again, and blotted dry. After drying, the slide was viewed under a light microscope with oil immersion. Cell shape, endospore shape and position were examined and recorded.

Biochemical characterization
Biochemical testes including KOH solubility, catalase, oxidase, starch hydrolysis, motility, and esculin hydrolysis were done following standard procedures as described by Goszczynska *et al*, (2010). All the time of biochemical tests a 24 to 48hrs old culture was used.

Catalase activity: Cultures of the isolates were growing on nutrient agar medium for 24 – 48 hours and colonies were picked with sterile loop and mixed with 3% H_2O_2. Production of gas bubbles indicate presence of catalase enzyme**.**

Potassium hydroxide (KOH) solubility test: KOH solubility test was carried out by mixing a 24-48hr old bacterial culture into a drops of potassium hydroxide solution (KOH) (3%w/v) on a glass slide until an even suspension was obtained. The formation of mucoid thread when the loop is lifted from the slide indicated the bacterium is gram negative.

Starch hydrolysis test: 5g soluble starch was added in nutrient agar (NA) and a 24hr old bacterial culture were streaked at the center using inoculating loop. The plates were incubated at 30°c for 2-5 days. The plats were flooded with iodine solution and a clear zone was recorded for amylase activity.

Motility test: A test tube containing semi solid motility agar media were stab inoculated using sterile inoculating needle. The spreading of bacteria from the stab line were visually observed after 48-72hr incubation period at 30°c.

Oxidase test: Cytochrome oxidase enzyme activity were detected by smearing a 24-48hr old bacteria colonies using a wood stick on whatman No. 1 filter paper moistened with a drops of fresh oxidase reagent (1% NNN'N-tetramethyl-p-phenylene-diamine).

Esculin hydrolysis: Esculin agar media (composition: esculin 1.0g/li, ferric citrate 0.5g/l, peptone 10g/li, and agar powder 15g/li) were inoculated by streaking the cultures. The plates were incubated at 30°c for 2 to 5 days and examined daily for the blackening of the plates around the growing colonies indicating hydrolysis of esculin.

3. Results and Discussion

Isolation of B. thuringiensis
A total of 74 soil samples were collected from different areas of the three regions. The samples were taken from river shores, swamps and different vegetations including cereals and vegetables. The altitude of sampling sites ranges from 342masl at Afambo, Afar and 2883masl at Kebun, SNNP regions. All the collected soil samples were analyzed for isolation of *Bt* and 69 pure isolates were obtained and preserved for further research works. The obtained isolates were expected to lay on two species of genus *Bacillus* i.e *Bt* and *Bacillus sphaericus* (*Bs*). This is because the method used in this study was sodium acetate heat treatment technique which is a noble method for selective isolation of *Bt* and *Bs*. Once the soil sample is inoculated to nutrient broth enriched with sodium acetate all soil bacteria both spore former and none spore formers were germinated except spores of *Bt* and *Bs* which is selectively inhibited by sodium acetate. Then after all the vegetative cells were killed by heat treatment except spores of *Bt* and *Bs*. *Bacillus thuringiensis* naturally occurs in the soil, dead and plant surfaces and it produces a large crystal toxin during sporulation which is toxic to many insect pests (Ammouneh *et al*., 2011). However, its natural occurrence is not uniform over different sample types. Assaeedi *et al*. (2011) obtained 65% *Bt* isolates from dead insect whereas only in 5% from the soil samples.

Characterization and identification
Among the 69 pure *Bacillus* spp. cultures 29 representative isolates were further characterized for some selected biochemical tests. Most of the isolates had morphological similarities in colony color, shape, elevation and margin (Table 1 and figure 1). The colony color of 88.8 % isolates were white and almost all except 09Bt-2 had flat colony elevation. Similarly, 89.7% of the isolates had irregular colony morphology. Hence this indicates that there are strain variation among isolates. The result is in agreement with the study conducted by Rabha et al.(2017)

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Figure 1: White, flat and irregular morphological characteristics of pure isolate on NA

Table 1: Cultural characterization of selected isolates of *B. thuringiensis*

Isolate code	Cultural characteristics on NA			
	Colony color	Colony shape	Colony margin	Elevation
44Bt-2	White	irregular	Erose	flat
02Bt-2	White	irregular	Erose	flat
22Bt	White	irregular	Erose	flat
42-Bt	White	irregular	Erose	flat
08Bt-2	White	irregular	Erose	flat
40Bt	White	irregular	Erose	flat
24NBt	White	irregular	Erose	flat
10Bt-1	White	irregular	Erose	flat
25Bt	White	irregular	Erose	flat
24Bt	cream	irregular	erose	flat
18Bt	White	irregular	Erose	flat
06Bt-1	White	irregular	Erose	flat
21Bt-2	white	regular/small	Erose	flat
26Bt	White	irregular	Erose	flat
44NBt-1	White	irregular	Erose	flat
17Bt-2	White	irregular	Erose	flat
34Bt	White	irregular	Erose	flat
9NBt	White	irregular	Erose	flat
22NBt	White	irregular	Erose	flat
12Bt	White	irregular	Erose	flat
24Bt-2	White	irregular	Erose	flat
43Bt-2	White	irregular	Erose	flat
36Bt-3	White	irregular	Erose	flat
07Bt	White	irregular	Erose	flat
19Bt	cream	regular	entire	flat
25NBt	cream	irregular	Erose	flat
11Bt	cream	irregular	erose	flat
21Bt-1	White	irregular	Erose	flat
09Bt-2	cream	regular	entire	raised

As the results indicated in table 2 all the tested isolates were gram positive except 09Bt-2 and the cells of all the isolates had rod shape. Light microcopy was used to determine the shape and position of endospores. Two endospore shapes, elliptical and spherical, were examined using light microscopy after the spores stained with malachite green (figure 2E). Similar study conducted by Rabha et al.(2017) also confirmed the presence of endospore among the collected Bt isolates.

Table 2: Light microscopy and biochemical characterization of selected *Bacillus* spp. isolates

Isolates	Isolate code	Light microscopy				Physiological characterization				
		Gram type	Cell shape	Endo shape	Endo location	Catalase	Oxidase	Esc-hydro	Motility	Star- hydro
B. thuringiensis	44Bt-2	+ve	rod	elliptical	terminal	+ve	+ve	-ve	None-motile	+ve
B. thuringiensis	02Bt-2	+ve	rod	elliptical	terminal	+ve	+ve	-ve	Motile	+ve
B. thuringiensis	22Bt	+ve	rod	elliptical	terminal	+ve	+ve	+ve	Motile	+ve
B. thuringiensis	42-Bt	+ve	rod	elliptical	terminal	+ve	+ve	-ve	Motile	+ve
B. thuringiensis	08Bt-2	+ve	rod	elliptical	terminal	+ve	+ve	-ve	Motile	+ve
B. thuringiensis	40Bt	+ve	rod	elliptical	terminal	+ve	+ve	-ve	Motile	+ve
B. thuringiensis	24NBt	+ve	rod	elliptical	terminal	+ve	+ve	+ve	Motile	+ve
B. thuringiensis	10Bt-1	+ve	rod	elliptical	terminal	+ve	+ve	-ve	Motile	+ve
B. thuringiensis	25Bt	+ve	rod	elliptical	terminal	+ve	+ve	-ve	Motile	+ve
Bacillus sphericus	24Bt	+ve	rod	spherical	terminal	+ve	+ve	-ve	Motile	+ve
B. thuringiensis	18Bt	+ve	rod	elliptical	terminal	+ve	+ve	-ve	Motile	+ve
B. thuringiensis	06Bt-1	+ve	rod	elliptical	terminal	+ve	+ve	-ve	Motile	+ve
B. thuringiensis	21Bt-2	+ve	rod	elliptical	terminal	+ve	+ve	-ve	Motile	+ve
B. thuringiensis	26Bt	+ve	rod	elliptical	terminal	+ve	+ve	-ve	Motile	+ve
Bacillus sphericus	44NBt-1	+ve	rod	spherical	terminal	+ve	+ve	-ve	Motile	+ve
Bacillus spheicus	17Bt-2	+ve	rod	spherical	terminal	+ve	+ve	-ve	Motile	+ve
B. thuringiensis	34Bt	+ve	rod	elliptical	terminal	+ve	+ve	-ve	Motile	+ve
B. thuringiensis	9NBt	+ve	rod	elliptical	terminal	+ve	+ve	+ve	Motile	+ve
B. thuringiensis	22NBt	+ve	rod	elliptical	terminal	+ve	+ve	-ve	Motile	+ve
Bacillus sphericus	12Bt	+ve	rod	spherical	terminal	+ve	+ve	-ve	Motile	+ve
B. thuringiensis	24Bt-2	+ve	rod	elliptical	terminal	+ve	+ve	-ve	Motile	+ve
B. thuringiensis	43Bt-2	+ve	rod	elliptical	terminal	+ve	+ve	+ve	Motile	+ve
Bacillus sphericus	36Bt-3	+ve	rod	spherical	terminal	+ve	+ve	-ve	Motile	+ve
B. thuringiensis	07Bt	+ve	rod	elliptical	terminal	+ve	+ve	-ve	Motile	+ve
B. thuringiensis	19Bt	+ve	rod	elliptical	terminal	+ve	+ve	-ve	Motile	-ve
Bacillus sphericus	25NBt	+ve	rod	spherical	terminal	+ve	+ve	-ve	Motile	+ve
Bacillus sphericus	11Bt	+ve	rod	spherical	terminal	+ve	+ve	-ve	Motile	-ve
B. thuringiensis	21Bt-1	+ve	rod	elliptical	terminal	+ve	+ve	-ve	None-motile	+ve
ND	09Bt-2	-ve	rod	Absent	Absent	+ve	-ve	+ve	Motile	+ve

* +ve= positive to the test, -ve= negative to the test, ND= not determined

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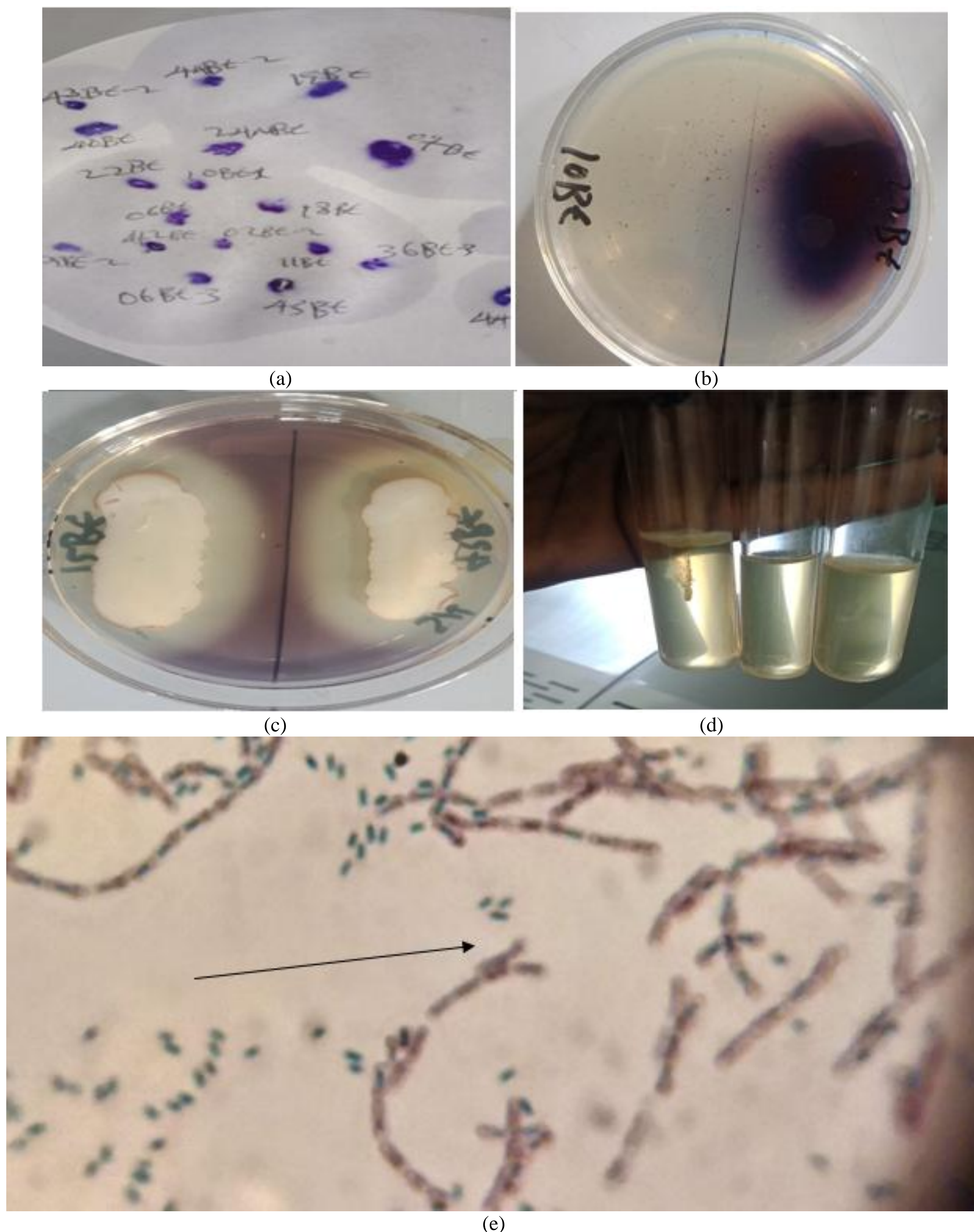

(a) (b)

(c) (d)

(e)

Figure 2: Physiological tests for selected isolates

A: oxidase test (Blue black color indicated for the +ve reaction), B: Esculin hydrolysis test (the dark black color indicated for the +ve reaction), C: starch hydrolysis test, D: motility test E: light microscopy of endospore staining, the arrow indicates green stained endospores released from the cell. A similar observation was reported in China (Zhu et al. 2009; Patel et al. 2013) and other parts of India (Letowski et al. 2005). Most of the isolates (75%) possessed elliptical endospore and the rest 25% had spherical endospore shape. However, most of the isolates were motile and could hydrolyze starch, only 17.2% of the isolates could digest of esculin (figure 2C and D).

4. Conclusion

The majority of the samples collected for isolation and identification of *B.thuringensis* are positive to the test. This indicates that soil is rich in *B.thuringensis, Bacillus sphericus* and other species of the genus bacillus used for insect pest management. The findings of this study

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confirmed the isolates possessed two kinds of spore shapes indicating the chance of getting *B. sphericus* which is also used for the management of insect pests other than *B. thuringiensis*. From the characterized isolates almost all except 09Bt-2 were suspected to be different strains of *B. thuringiensis* and *B. sphericus.* The obtained isolates should be screened for larvicidal activity on larvae of different insect pest which are known constraints of crop production. Toxicity of *B. thuringiensis* strains is directly related with the content of *cry* genes. Hence, the isolates should be exploited for their *cry* gene content using polymerase chain reaction (PCR) to determine the larvicidal activity. The toxins from effective isolates therefore is mass produced and applied for management of economically important insect pests.

5. Acknowledgements

We are grateful to Ethiopian Institute of Agricultural Research and Ambo Agricultural Research Centre for providing the necessary financial fund and facility to pursue this work.

Conflict of interest: The authors declare that they have no competing interests

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