ISSN: 0975 -8542

Journal of Global Pharma Technology

Available Online at: www.jgpt.co.in

RESEARCH ARTICLE

Biological Control of Mosquitoes Cluex pipiens Using Locally Isolate of Bacillus Thuringiensis in Diyala Province

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Abstract

The study included isolating, diagnosing and studying the ability of *Bacillus thuringiensis* to kill *Culex pipiens* larvae, the samples were isolated from different places of agricultural soils in Diyala province. The results of the fermentation medium to grow the *B thuringiensis* inoculums showed that the best liquid fermentation medium was the barley extract, with 20 gm. A ratio of sucrose per liter mixed with 20 mg of the Amoxicillin antibiotic. The bacterial density in it was $221x10^6$ colony forming unit/ml. Then carry the bacterial suspension on calcium carbonate, preparation three concentrations (1, 2, 3g) of the dry preparation/liter of sterilized water. The results show that the effect of different concentrations of *B.thuringiensis* on the death rate larvae of Culex *pipiens* was significant, it was the highest value of dead larvae it was (2.52) at 3 g/L concentrations of *B. thuringiensis s*and the lowest value of dead larvae it was (0.92) at 1 g/L concentration of B.thuringiensis, at a significant level (0.05). There were also significant differences between the days, the highest value of dead larvae it was in the fourth day of treatment (2.55) and the lowest value of dead larvae it was on the first day of treatment (0.15) at a significant level (0.05). Twenty-four hours after the initiation of the treatment of larvae *Culex pipiens* with the bio product of *B.thuringiensis*, she appeared symptoms of change in behavior, slowed the moving of larvae gradually until completely stopped movement and shrunk, due to the effect of crystalline protein , which decomposes in the middle gut of the larvae and is associated with receptors in the membrane of the gastrointestinal tract, then poison enters the membrane causing the weakness of the epithelial cells and swelling and dissolution causing holes in the membrane or that the toxins of the bacteria may lead to a disturbance in the nervous system leading to paralysis in the organs responsible for feeding and then the death of larvae from hunger.

Keywords: *Bacillus thuringiensis, Bioproduct, Mosquitoes, Culex pipiens, Larvae, death.*

Introduction

Mosquito of Culexpipiens is one of the species that cause transmission of diseases which caused harm to humans and animals [1, 2]. Scientists have been interested in combating by using various chemical insecticides, but these pesticides have become a danger to humans and their environment, in addition to the target insects acquired adaptation to those toxic substances and was able to develop immunity against them [3,4]. Then the research turned to the use of biological control, using natural enemies against these lesions, Which was the most prominent and proved effective bacteria *B.thuringiensis*, Its effectiveness comes from through the production of crystalline proteins, which are in two forms the first consists of spores and crystalline protein and the second consists of crystalline protein only [5]. Later named, it is abbreviated to B.t. Products; B.t is characterized by meticulous specialization, Low cost of production and non-pollution of the environment and non-toxic to other living organisms [6, 7]. It was also characterized by widespread, widely in soil and its diseases to some harmful insects, including mosquitoes *Culexpipiens* [8, 9]. *B.thuringiensis* is bacilli, gram-positive stain, anaerobic elective belongs to a family bacillaceae, formation of spores, grow in the form of long chains.

The most important characteristic of it is its ability to produce a crystal protein or so-called delta-endotoxin during the formation of spore's stage it is responsible for the toxicity of insects, research has therefore focused on this type of bacteria for use in the field of biological control as a good insecticide for plant pests [10, 12].

Materials and Methods

Collection of Samples

Collected (25) samples from various agricultural areas in diyala province. The soil layer was removed 3-5 cm deep and 20 gm of soil was taken and then transferred to the laboratory for the isolation process.

Sampling Culture

Samples were cultured based on the method [13] depending on the germination of spores and then kill the vegetative cells by thermal treatment, which were carried out as follows: transfer 0.5 g per sample to a conical flask containing 10 ml of nutritious broth, which contains sodium acetate at a concentration of 0.25 molar [pH= 6.8], the flasks were placed in a vibrator incubator 250 cycles/min at 30 °C for four hours. All spores will germinate except spores of *B.thuringiensis*, which inhibited their growth by sodium acetate.

The samples were then treated with heat at 80°C for three minutes to kill all the vegetative cells, and then transfer 0.1 ml of heat-treated suspensions to the dishes containing the nutrient agar and incubate at 30°C for 48 hours, and then examine the colonies by light microscope.

Diagnosis of Samples

The samples were initially diagnosed based on phenotypes, such as colony shape, color, properties, odor, and size, samples were then subjected to microscopic examination using a gram stain to know the type of bacteria and their interaction with the stain and the samples were then subjected to the green malachite to sure that the spores were present, also samples subjected to Coomassie brilliant blue and carbol fuchsin to ascertain the presence of crystalline proteins, a number of biochemical tests were then carried out for accurate diagnosis [14].

Stages Production of Bio Product to *B. thuringiensis*

Reproduction of B. thuringiensis

For the purpose of increasing the isolation which shown crystalline bodies grown in nutrient broth, then incubated at 35°C for 48 hours [15].

Preparation of Fermentation Medium Appropriate for Growth *B. thuringiensis*

By grinding 200 g of barley seeds and placed in a 1 liter flask and then complete the volume to 1000 ml of distilled water and leave for 24 hours and then filtered by a clean and sterile piece of gauze, by grinding 200 g of barley seeds and placed in a 1 liter flask and then completed to a volume of 1000 ml of distilled water and left for 24 hours and then filtered by a clean and sterile piece of gauze, and added 20 g of sucrose and autoclave sterility and then added Amoxicillin at a concentration of 20 mg /L ,then inoculated the flask with 50 ml of bacterial suspension.

Calcium Carbonate Tested as a Carrier of the *B. thuringiensis.*

Take 100 g of calcium carbonate ($CaCo_3$)and sterilize in the oven at 160°C for one hour ,after cooling it is placed in shallow and sterile utensils , and add 100 mL of pre-prepared fermentation medium which contains bacteria at the age of 48 hours, Then transfer the utensils to the oven at 35°C for five days until dry well , and grind the powder which loaded with bacteria in a sterile room, After that I prepare series of dilution (10^{-1} - 10^{-6}) for bacterial suspension which loaded on calcium carbonate in sterile test tubes.

Then transfer 1 ml of dilution (10^{-6}) to the nutrient agar dishes contains amoxicillin at a concentration of 20 mg / L., the dishes were incubated at 35°C for 48 hours [16]. The density of bacteria was estimated in one gram the bearing material to bacteria, according to equation [17].is as follows: Number of colonies growing in a 1g Bacterial = Average of colonies developing in dish x Inverted dilution.

Determine the Percentage of Fermentation Medium to the Carrier Substance to Bio-Product of *B.thuringiensis*

After proving the efficiency of sodium carbonate as a carrier to the bio-product of *B. thuringiensis*. The best ratio of fermentation medium to the carrier substance is 1: 1, by adding 100 g of calcium carbonate to 100 ml of bacterial suspension, and then placed in the

oven at a temperature of 35 °C for five days and after the end of drying grind the powder in the conditions of sterilization [16] .Preparation three concentrations (1, 2, 3 g) of the dry preparation / liter of sterilized water.

Testing the Effectiveness of Bioproduct of *B.thuringiensis* on the Larvae of the Fourth Stage to Mosquito *Culex pipiens*

The fourth phase larvae of *Culex pipiens* were obtained from the Center for transitional diseases control in the ministry of health and environment , taking 10 larvae by 3 replicates for each of dilution (1,2,3) g of the dry bioproduct (B.t.) / liter of distilled water, and 500 ml of bacterial suspension in sterile plastic containers and placed in breeding cages, then transferred to an isolated room and temperature controlled to 27 ± 2˚ m , light 10 hours and relative humidity 70-80% , and followed daily then compared with the control coefficient that was treated with calcium carbonate solution only to calculate the rate of larval death, the larvae were fed on mice chow by addition 0.05 g after grinding [18].

Results

Isolation and Diagnosis *B. thuringiensis*

The total number of isolated samples (25) samples, eight samples (32%), negative growth of bacterial culture and 17 samples (68%) showed positive growth of bacterial culture, of which 9 isolates (52.9%) diagnosed under sex *B.thuringiensis* , Rod-like shaped, Chains , Gram- positive as shown in Figure (1), formation elliptic spores sub-terminal and crystalline proteins when pigmented with Coomassie brilliant blue and carbol fuchsin pigment ,a characteristic of *B.thuringiensis* bacteria that distinguish them from other species , as shown in Figure (2, 3) the isolates were subjected to a set of biochemical tests shown result in Table (1).

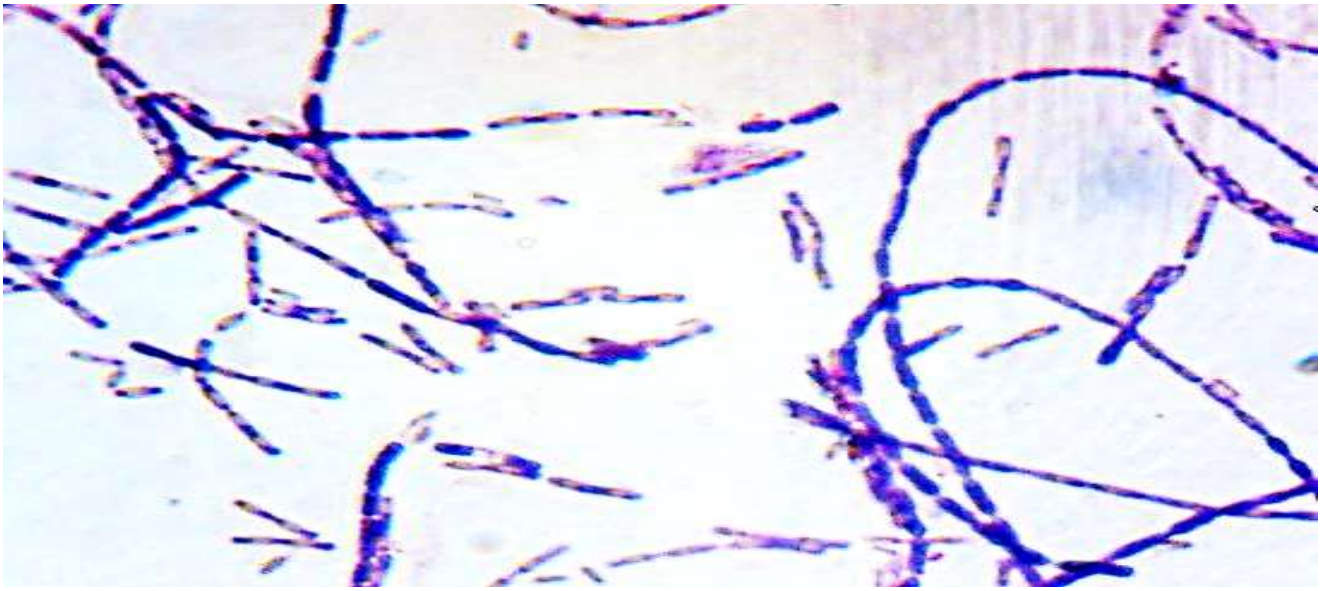

Figure 1: The shape of the bacterial cell after Pigmentation by gram stains Fewer than 100 X lens (Oil Immersion)

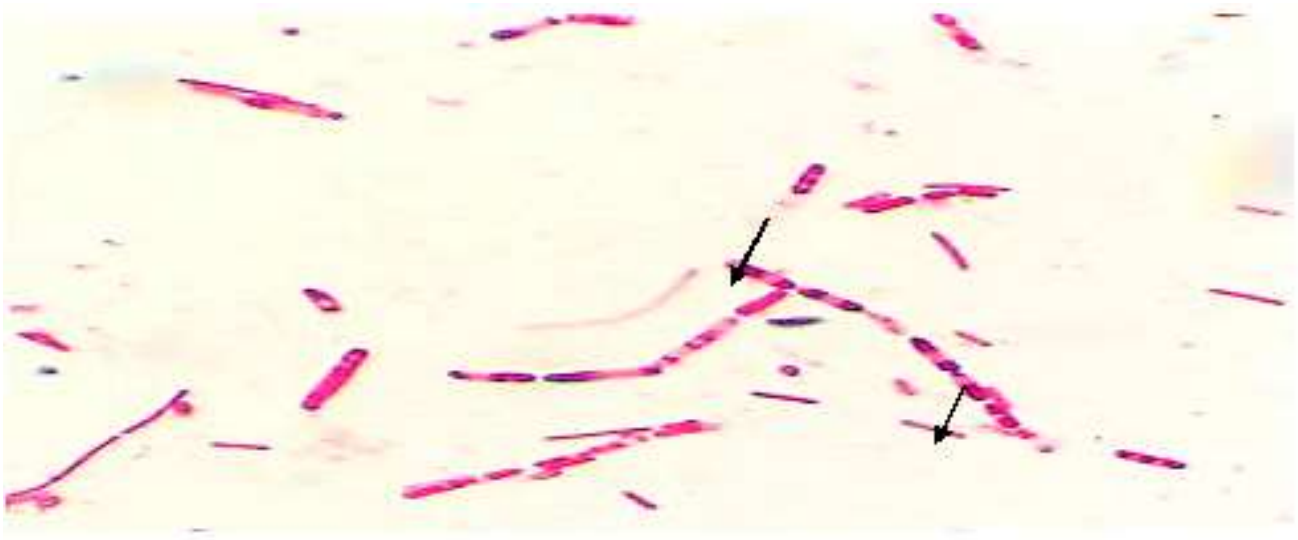

Figure 2: The shape of sub- terminal spores (pigment Carbol fuchsin)

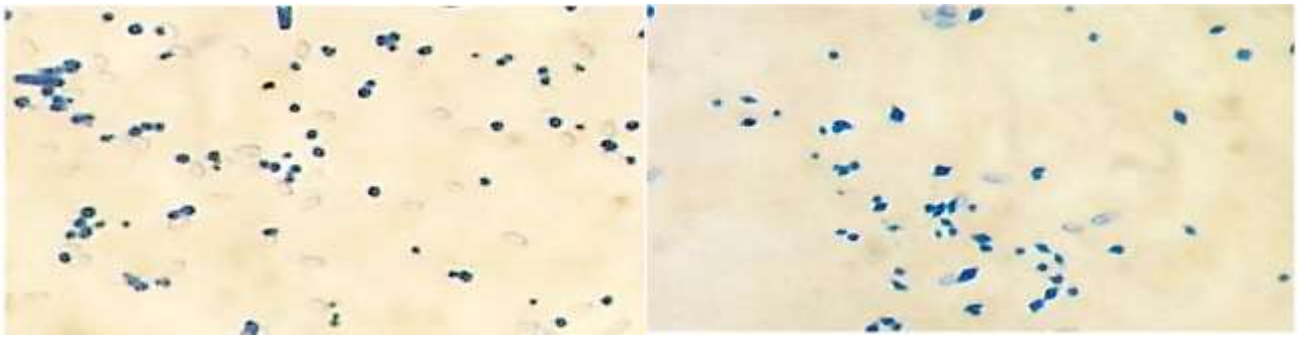

Figure 3: Forms of different crystal protein (pigment CCB)

Table 1: Biochemical tests for *B. thuringiensis*

Test	Number of positive isolates (+)	Number Negative isolates (-)
Gram stain	**9**	**0**
Malachite green	**9**	**0**
carbolfuchsin pigment	**9**	**0**
Coomassie brilliant blue(CCB)	**9**	**0**
Motile test	**9**	**0**
Catalase test	**8**	**1**
Oxidase test	**8**	**1**
Citrate test	**5**	**4**
Urease test	**9**	**0**
Indole test	**0**	**9**
Voges–Proskauer(VP)	**9**	**0**
Methyl Red(MR)	**7**	**2**
Nitrate test	**8**	**1**
Oxidation fermentation test	**9**	**0**
Growth in salt concentration 10%	**8**	**1**

Production of Bioproduct from *B.thuringiensis*

The results of the determination *B.thuringiensis* density in the prepared medium of barley seeds were 221 × 10^6 colonies / ml formation unit, this is in similar nearly with the findings of the research (16), where the density of *B.thuringiensis* was 232 × 10^6 colonies / ml formation unit, The results showed that calcium carbonate ($CaCo_3$) high efficiency as a carrier to cells of *B.thuringiensis.*

Test the Efficacy of the bio Product of *B.thuringiensis* on the Fourth Phase Larvae to Mosquitoes *Culex pipiens.*

The results in Table (2) and Figure (1,2) show that the effect of different concentrations of *B.thuringiensis* on the death rate of fourth-stage larvae of *Culex pipiens* was significant, it was the highest value of dead larvae it was (2.52) at 3 g/L concentration of *B. thuringiensis* and the lowest value of dead larvae it was (0.92) at 1 g/L concentration of *B.thuringiensis*, compared to the control coefficient which amounted to (0.2) at a significant level (0.05).There were also significant differences between the days ,the highest value of dead larvae it was in the fourth day of treatment (2.55) and the lowest value of dead larvae it was in the first day of treatment (0.15) compared to the control coefficient which amounted to (0.3) at a significant level (0.05).

Table 2: Effect of Concentrations and time in the rate of death the larvae of the fourth stage to mosquitoes *Culex pipiens*

Concentration Of bacteria gram/ Liter	Rate of death				Effect the Concentration of B.t. product
	1 - Day	2-Days	3-Days	4-Days	
1gm/ L	0	0.1	1.5	2.1	0.925
2gm/ L	0.1	0.3	2	3.4	1.45
3gm/ L	0.5	2.1	3	4.5	2.525
Control	0	0	0	0.2	0.05
Effect of days	0.15	0.625	1.625	2.55	L.S.D to Concentration of B.t =2.48
L.S.D of days	2.29				

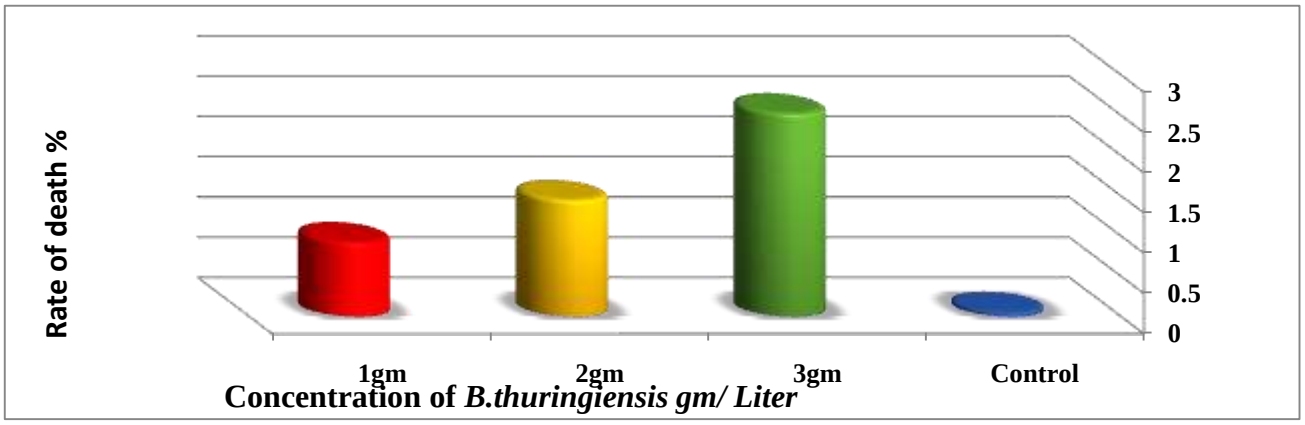

Figure 1: Effect of *B.thuringiensis* concentrations in the rate of death the larvae of the fourth stage to mosquitoes *Culex pipiens.*

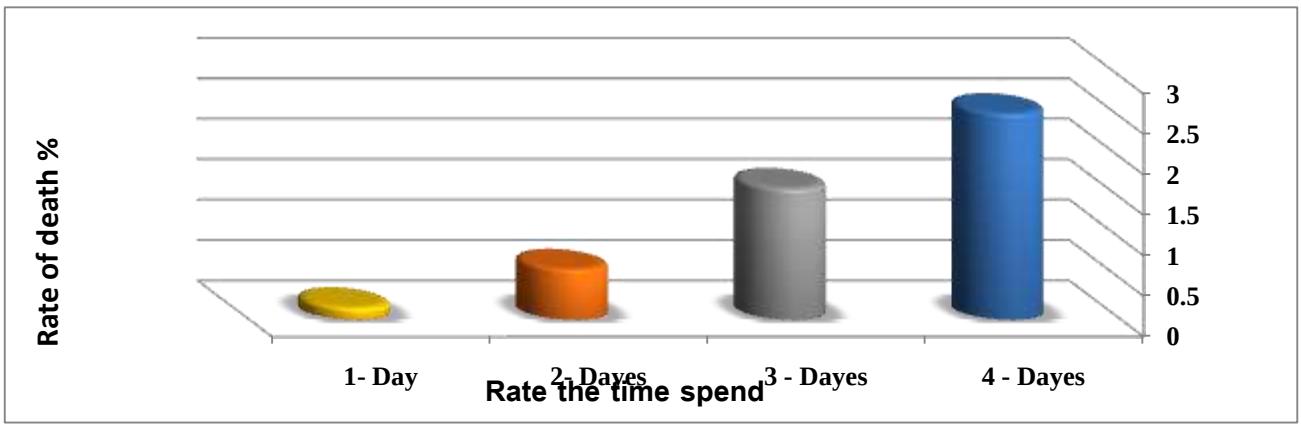

Figure 2: Effect of time at the rate of dead the larvae of the fourth stage to mosquitoes *Culex pipiens*

Discussion

The prevalence of *B.thuringiensis* in different ecosystems is attributed to the formation of spores, which tolerant to Cruel environmental conditions such as low temperatures (up to-20°C) and high temperatures (up to 80°C), they also grow in drought conditions as well as in sandy soils where the humidity is more than 10 % and grow well when humidity is 36% [19]. The growth of *B. thuringensis* in the fermentation medium of barley seeds is due to its ability to exploit the contents of these seeds efficiently to ensure get the requirements of nutrition such as carbohydrates, fats, proteins, and minerals as well as some important vitamins[19].

The results showed that calcium carbonate (CaCo3), as a good carrier of bio product of *B.thuringiensis*, as it is a natural material that provides an appropriate medium for the stability of bacterial cells and their spores on the surface of calcium molecules, as well as to make the medium tends to basal when hydrolyzed, which is a positive point for the growth of *B.thuringiensis*[19]. Twenty-four hours after the initiation of the treatment of larvae *Culex pipiens* with the Bioproduct of *B.thuringiensis*, she appeared symptoms of change in behavior, slowed the moving of larvae gradually until completely stopped movement and shrunk, we can be sure of vitality by prick and urged her to movement, then the color of the larva begins to change towards the lions slowly and she then stops eating and dies.

The abstention of larvae from food is due to the effect of crystalline protein , which decomposes in the middle gut of the larvae and is associated with receptors in the membrane of the gastrointestinal tract, then poison enters the membrane causing the weakness of the epithelial cells and swelling and dissolution causing holes in the membrane or that the toxins of the bacteria may lead to a disturbance in the nervous system leading to paralysis in the organs responsible for feeding and then the death of larvae from hunger [20].

The results of the present study showed that the percentage of larval death is increased by increasing the concentration of bio product to *B.thuringiensis* as well as increasing the percentage of death over time. This is consistent with the findings of researcher [21].

The difference in the velocity of the larvae death; may be due to the readiness of the bacteria and their formation of crystalline

protein, which leads to the paralysis of the digestive tract and the degradation of the wall, and then easy invasion of bacteria to the cavity, while bacterial spores need longer to germinate within the digestive tract of insect larvae and then protein crystallization to play their role in killing.

Conclusions

- The best liquid fermentation medium for grows *Bacillus thuringiensis* the barley extract, with 20 gm. a ratio of sucrose per liter mixed with 20 mg of the Amoxicillin antibiotic.
- That the calcium carbonate (CaCo3), as a good carrier of bio product of *B. thuringiensis* ,as it is a natural material that provides an appropriate medium for the stability of bacterial cells and their spores on the surface of calcium molecules.
- percentage of larval death is increased by increasing the concentration of bio product to *B. thuringiensis*.
- Percentage of larval death is increased by increasing for time.

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