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Biocontrol potential of Bacillus species isolated from soil against mosquito larva

Tushar Kadam, Prajakta Pavtekar and Meena Chittapure
Department of Biotechnology, MGM Institute of Biosciences and Technology,
MGM University, Chhatrapati Sambhajinagar, India

***Corresponding Author's E-mail:** tusharkadam631@gmail.com **Mob.:** +91- 9766558889

A B S T R A C T

A major challenge for achieving successful mosquito control is overcoming insecticide resistance. The potential of Bacillus species isolated from the black soil sample as control strategy of mosquitoes and monitoring of larva susceptibility was investigated in this research. Larvicidal activity of Bacillus spp. The isolated organisms were confirmed by on the basis of different biochemical tests and microscopic examination. From the soil we can isolate four different forms of the Bacillus species that labeled as (Bacillus1, Bacillus2, Bacillus3, and Bacillus4). The larvicidal activities was analyzed by the mortality rate and morphology changes in larva, were the dilution of bacterial culture from 101 to 105 at different time interval at 2, 4, 6 hrs. After the time of incubation the mosquito was killed in tubes that mean the bacillus spp. was showing the larvicidal activity against mosquito larva. From this study we concluded that the Bacillus spp. was showing vary potent biolarvicide that bring about mortality of mosquito larvae at short duration of time.

Keywords : *Biolarvicide, Mortality, Bacillus spp., Insecticidal.*

1. Introduction :

Mosquitoes are vectors of various disease causing agents, responsible for transmission of pathogens causing more life threatening and debilitating human diseases than any other organism. Over one million people worldwide die from mosquito borne diseases which include malaria, filariasis, yellow fever, chikungunya and dengue fever yearly within disease-endemic countries [1-4].

Chemical insecticides provide benefit in food production, human health and have proven very effective at increasing agriculture and forestry productivities. However, uncontrolled use of chemical insecticides has resulted in irreparable damage to environment [5]. Continuous use of chemical-based insecticides has resulted in the development of resistance, detrimental effects on non-target organisms and human health problems [4]

Consequently, they suggested the need for alternative control measures which leaves biological control as a viable alternative to chemical control. Microbial insecticides are especially valuable because their toxicity to non-target animals and humans is extremely low and a crucial part of integrated pest management [1].

Bacillus thuringiensis is an important insect pathogen which is highly toxic to mosquito larvae and related

dipterans [6-8]. Bacillus thuringiensis is selectively active on pests and less likely to cause resistance hence it is considered beneficial to humans, animals and plants and alsoas a suitable replacement to chemical pesticides in many countries [9-12].

Bacillus Species are a Gram-positive facultative anaerobe and spore forming saprophytic soil bacterium. The toxicity is attributed to an endotoxin, which is made of proteins that are produced and assembled when the bacteria sporulate [6]. Bacillus thuringiensis during the sporulation produces one or more proteinaceous parasporal crystals, recognized as delta-endotoxin. This crystal protein under alkaline condition of midgut of insects, gets solubilized, and then activated by intrinsic protease into an active toxin that selectively binds specific receptor in the cell membrane, leading to poreformation and consequent insect larvae death [9].

Most Bacillus thuringiensis preparations available in the market contain spores with parasporal inclusion bodies composed of δ (delta) - endotoxins. In commercial production, the crystals and spores obtained from fermentation are concentrated and formulated for spray on application according to conventional agriculture practices. There are many strains of Bacillus thuringiensis having insecticidal activity against insect orders (eg.Lepidoptera, Diptera, Homoptera, Mollaphage, and Coloptera). Only a few of them have been commercially developed [5].

The earliest record of dengue fever was found in the year of 1992 in Chinese Encyclopedia. Its incidence has increased over time and major factor that contribute to the expansion of global shipping industry in 18th and 19th century causing is spread to new geographic areas, rapid unplanned urbanization south Asia after world war2 caused the increased transmission on dengue virus serotypes resulting in the hyper endemicity and lack of vector control measures [5].

The present investigation on Biocontrol potential of Bacillus species isolated from soil against mosquito larva will be conducted with following objectives.

a. To do production of mosquito larva from water.

b. To Isolate Bacillus species from soil.

c. To perform biochemical analysis of the Bacillus species.

d. To evaluate the insecticidal activity of Bacillus species against the mosquito larva.

Comparative analysis is to be conducted for different soil samples with Bacillus species against the mosquito larva.

2. Literature of review:

The attempts has been made in this chapter to review the work done in past on this aspect of present investigation by eminent scientist in India and abroad. Authors have worked on the evidence on alternative control of mosquito which is not only ecofriendly but also has negligible risk of mosquito developing resistance towards it also worked on producing the biopestiside that reduced environmental pollution [1].

Authors concluded that Bacillus thuringiens is a very potent biolarvicide that brings about mortality of mosquito larva at short duration of time that mechanism beside Bacillus thuringiensis was secreted the crystalline protein inside larva the block mid gut metabolism and result larva was dead [6]. show that Bacillus thuringiens is isolated from organic rich soil sample in zario has promising larvicidal potential in the control of green quifascitus larvae hence reducing number of adult mosquito that same as vector diseases [3]. Authors observed that the use of moderate amount of chemical fertilizer that showing the positive impact on the environment and the human health but in case its uncontrolled use that resulted in irreparable damage to environment that leading to soil, water pollution that positive factors for the insect related diseases [5]. Author conducted that ento-mopathogenic bacteria are present in natural environment of Lahore and screening of more number of samples may yield different and even more toxic strains of bacteria study reported that the discovery of a strain

of Bacillus subtilis and signals towards further exploration of other bacterial strains that can be used to control dengue vector in different parts of the world [9].

3. Methodology:

3.1 Collection of Soil Sample:

The soil samples were taken at a depth of 10 cm below the soil surface, after scrapping of the surface material with sterile spatula; the sample was placed in a sterile plastic bag and transferred to the laboratory for isolation of Bacillus species from the sample. Black soil samples were collected from four different locations from the garden.

3.2 Isolation of Bacillus species from soil:

Acetate selection method described by Travers et al. (1987) which inhibit non- spore forming bacteria was used. The medium contained: 10g Tryptone, 0.5g yeast extract and 5g Nacl. Prepared in an Erlenmeyer flask. Half a gram (0.5 g) of each soil sample was added to 10 ml of the broth medium. The broth was buffered with 0.25 M sodium acetate. The mixture was vortexes vigorously and incubated for 24 hours on a rotary shaker at a speed of 160 rpm at $37^0C \pm 2^0C$. At the end of this time, 0.5 ml of each soil suspension was drawn into test-tube and o pasteurized in a water bath at 80^0C for 10 minutes to kill vegetative cells and non-spore forming bacteria. After cooling at room temperature, the mixture was then serially diluted with sterile distilled water of 4.5 ml in five folds. A volume of 0.1 ml of each dilution (10^{-1} to 10^{-5}) was streaked on nutrient agar medium. Plates were incubated at 37^0C for 24 hours.

3.3 Screening of bacteria:

The morphological characteristics of the colonies formed on nutrient agar media were studied taking their colour, texture, elevation and margin into account. Each suspended colony resembling to that of Bacillus species were purified repeatedly subculture on selective media i.e. NA and preserved further study.

3.4 Identification and Characterization of bacterial strains:

The bacterial isolates were identified on the basis of classification schemes published in Bergeys manual of determinative bacteriology reported by William and co-scientists in 1994.

3.5 Gram staining:

A small portion of colony from each streak plate was selected with the help of sterile inoculating loop and transferred to a clean slide having a drop of sterile water on it. The samples were air-dried and heat fixed by passing through flame several times. Each smear was first covered with crystal violet dye for one minute and washed with water. Then the smear was covered with gram iodine for thirty seconds and again rinsed with alcohol. At the end, smear was covered with counter-stain gram saffranin for one minute and washed with water. After drying, slides were examined by 100X oil immersion microscopy. During this examination, grouping, gram stain results and morphology of the cells were recorded. Smears with violet colour were labeled as gram-positive and pink or red smears were labeled as gram-negative.

3.6 Spore staining:

Bacillus species can form endospore in order to survive in hostile conditions. For spore staining, small portion of bacterial colony from pure culture was picked and transferred to a clean slide and heat fixed by

passing through flame several times. The slide was placed over boiling water and malachite green was applied over the smear and heated for about fifteen minutes. After that slide was rinsed with water and counter-stain saffranin was applied for about one minute and again rinsed with water. The slide was air dried and examined under microscope. Endospores were green, whereas vegetative cells were pink.

3.7 Catalase test:

For catalase test, colonies from streak plate were selected and transferred through a sterile inoculating loop to a clean slide. Hydrogen peroxide was applied to the bacterial colony on the slide. Catalase positive bacterial colony resulted in the appearance of bubbles within 5 to 10 s of hydrogen peroxide application. Bacterial colony with no bubbling was catalase negative.

3.8 Motility test:

Motility test agar medium was prepared by mixing enzymatic digest of gelatin, beef extract, sodium chloride and agar. Final pH of the medium was maintained at 7.3 and then medium was autoclaved at 121 °C for 15 min and transferred into the sterile test tubes. Tubes were inoculated by stabbing through center of the medium with inoculating needle to approximately one-half theDepth of the medium. Inoculated tubes were incubated at 37°C for 24 h. After 24 h diffused growth spreading from the line of inoculation was observed in motile bacterial cultures. Non-motile organisms grew only along the line of inoculation.

3.9 Voges proskeur test:

Voges proskeur stock solution was prepared and transferred to test tubes and autoclaved. The test tubes were inoculated with bacterial cultures and incubated at 30 °C. Inoculated tubes were examined for acetoin production after 2, 4 and 6 days. For VP test, 1 mL solution from test tube was transferred in another sterile test tube and then 18 drops of 40% KOH and a small amount of creatine was added. Tubes were allowed to stand for 15 min before interpreting the results. VP positive bacterial colonies gave pink or red colour at the surface of the medium showing the presence of acetoin whereas VP negative colonies gave yellow or no colour at the surface of the medium.

3.10 Indole test:

Tryptone broth was prepared, transferred in test tubes and autoclaved. The test tubes were inoculated with a small amount of a pure culture and incubated at 37°C for 24 to 48 h. After incubation 5 drops of Kovács reagent was added directly to the tube to test for indole production. Tryptone broth having indole positive bacterial colonies were indicated by the formation of a pink to red color in the reagent layer on top of the medium within seconds of adding the reagent. In indole negative culture the reagent layer was yellow or slightly yellowish green.

3.11 Starch hydrolysis:

Preparation of starch agar medium that autoclaved at 121^0C after autoclaving it pour in petriplates allowed to solidify after solidification that striking of the bacterial culture and incubation at 37^0C for 24hrs. In next day observed the microbial growth on plate after add iodine solution on plates and observed the result.

3.12 Mannitol broth fermentation:

Phenol red Mannitol broth medium was prepared and autoclave after transferring into tube at pH of the

medium was adjusted to 7 before autoclaving. Add loopful suspension in tube and incubated at 37^0C for 24 hrs. After a day incubated culture was received and observed colour changes that yellow colour was positive test while red colour was negative test.

3.13 Breeding of Mosquito Larvae:

Water containers were left to stand in an open o space at ambient temperature of about 30^0C for seven days to facilitate lying of eggs by the mosquito. The water container was monitored daily to observe the emergence of the larvae. The larvae of the female anopheles mosquitoes were harvested using sieve and placed in a moistened cotton-wool to preserve them before use [10].

4. Results and discussions:

The morphological characteristic and the biochemical result of the isolated four different Bacillus species from soil as given in Tables 1-7.

Table 1. Biochemical and Morphological characteristics of isolated Bacillus species 1

Biochemical tests	Result
Gram staining	Positive
Spore staining	Positive
Shape	Rod shape
Motility test	Motile
Indole test	Negative
Vp test	Positive
Catalase test	Positive
Starch hydrolysis	Positive
Mannitol fermentation test	Positive

Table 2. Biochemical and Morphological characteristic of isolated Bacillus species 2

Biochemical tests	Result
Gram staining	Positive
Spore staining	Positive
Shape	Rod
Motility test	Motile
Indole test	Negative
Vp test	Negative
Catalase test	Positive
Starch hydrolysis	Positive
Mannitol fermentation test	Negative

Table 3. Biochemical and Morphological characteristic of isolated Bacillus species 3.

Biochemical tests	Result
Gram staining	Positive
Spore staining	Positive
Shape	Rod
Motility test	Motile
Indole test	Positive
Vp test	Positive
Catalase test	Positive
Starch hydrolysis	Negative
Mannitol broth fermentation test	Positive

Table 4. Biochemical and Morphological characteristic of isolated Bacillus species 4

Biochemical tests	Result
Gram staining	Positive
Spore staining	Negative
Shape	Rod
Motility test	Non motile
Indole test	Negative
Vp test	Negative
Catalase test	Positive
Starch hydrolysis	Positive
Mannitol broth fermentation test	Negative

Table 5. Bioactivity of the Bacillus species isolated from soil against mosquito larva at 2 h

Cultural Dilutions	No. of larva added	Bacillus.1		Bacillus.2		Bacillus.3		Bacillus.4	
		Live	Dead	Live	Dead	Live	Dead	Live	Dead
Control	2	2	0	2	0	2	0	2	0
10-1	2	0	2	0	2	0	2	1	1
10-2	2	1	1	0	2	1	1	2	0
10-3	2	2	0	1	1	0	2	1	1
10-4	2	0	2	0	2	1	1	0	2
10-5	2	1	1	1	1	2	0	1	1

Table 6. Bioactivity of the Bacillus species isolated from soil against mosquito larva at 4 h

Cultural Dilution	No. of Larva added	Bacillus.1		Bacillus.2		Bacillus.3		Bacillus.4	
		Live	Dead	Live	Dead	Live	Dead	Live	Dead
Control	2	2	0	2	0	2	0	2	0
10-1	2	0	2	0	2	0	2	1	1
10-2	2	1	1	0	2	0	2	2	0
10-3	2	2	0	1	1	0	2	1	1
10-4	2	0	2	0	2	0	2	1	1
10-5	2	0	2	0	2	1	1	0	2

Table 7. Bioactivity of the Bacillus species isolated from soil against mosquito larva at 6 h

Cultural Dilution	No. of Larva added	Bacillus.1		Bacillus.2		Bacillus.3		Bacillus.4	
		Live	Dead	Live	Dead	Live	Dead	Live	Dead
Control	2	2	0	2	0	2	0	2	0
10-1	2	0	2	0	2	0	2	0	2
10-2	2	0	2	0	2	0	2	0	2
10-3	2	0	2	0	2	0	2	0	2
10-4	2	0	2	0	2	0	2	0	2
10-5	2	0	2	0	2	0	2	0	2

Bacillus Species 1: The Bacillus Species -1 tests are shown in Figs.1, 2

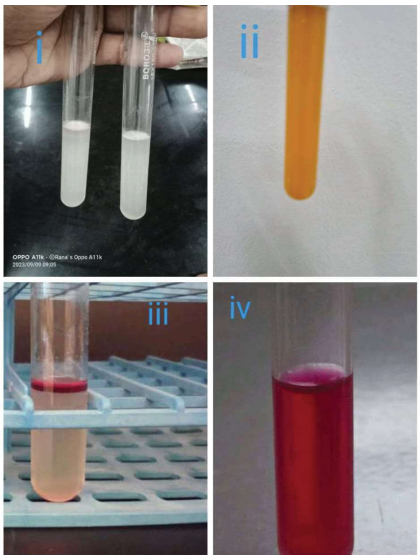

Fig.1 i) Motility test, ii) Mannitol fermentation, iii) Indole test, iv) VP test

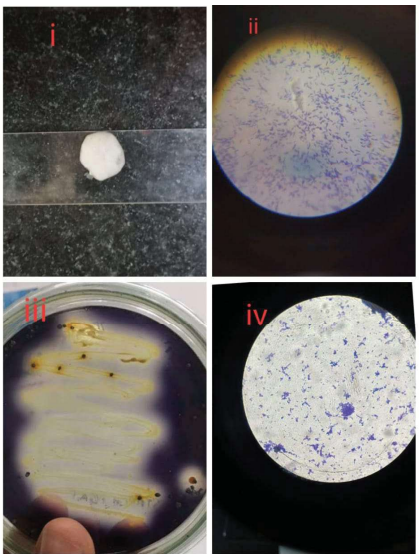

Fig.2 i) Catalase test, ii) Spore staining, iii) Starch hydrolysis, iv) Grams staining

Bacillus Species 2 : The Bacillus Species -1 tests are shown in Figs.3, 4

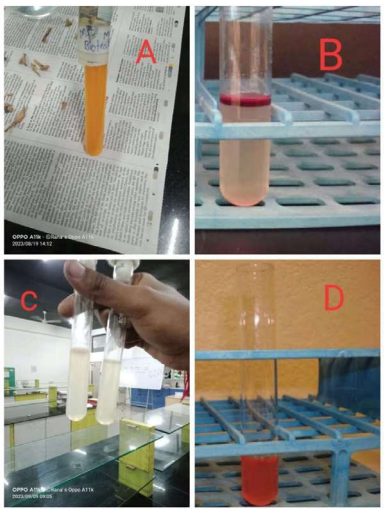

Fig.3 i) Mannitol fermentation, ii) Indole test iii) Motility test, iv) VP test

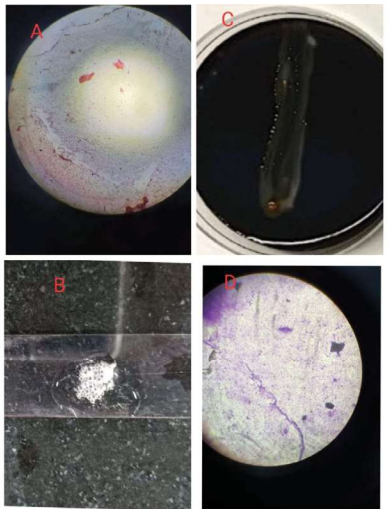

Fig.4 i) Spore staining, ii) Catalase test iii) Starch hydrolysis, iv) Grams staining

Bacillus Species 3 : The Bacillus Species -1 tests are shown in Figs.5, 6

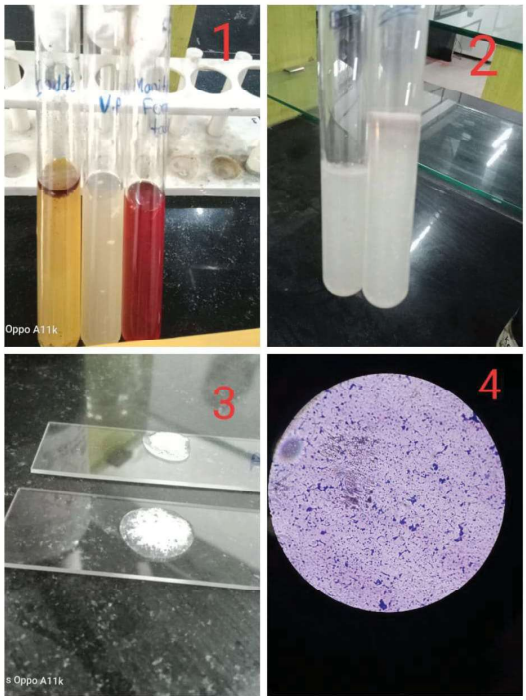

Fig.5 i) Indole, VP, Mannitol test, ii) Motility test iii) Catalase test, iv) Grams staining

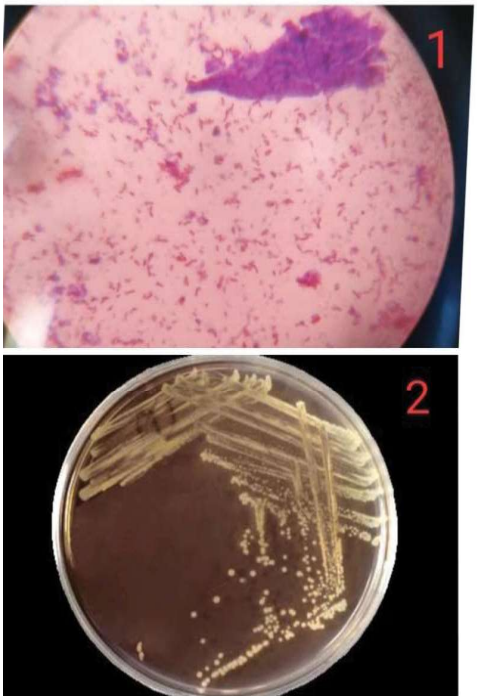

Fig.6 i) Spore staining, ii) Starch hydrolysis

Bacillus Species 4 : The Bacillus Species -1 tests are shown in Figs.7, 8

Fig.7 i) Motility test, ii) Indole, VP, Mannitol test, iii) Spore staining, iv) Grams staining

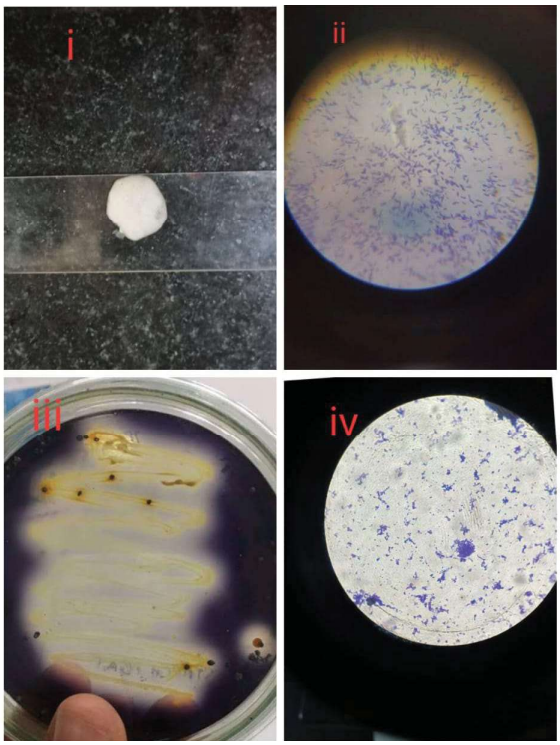

Fig.8 i) Catalase test, ii) Spore staining iii) Starch hydrolysis, iv) Grams staining

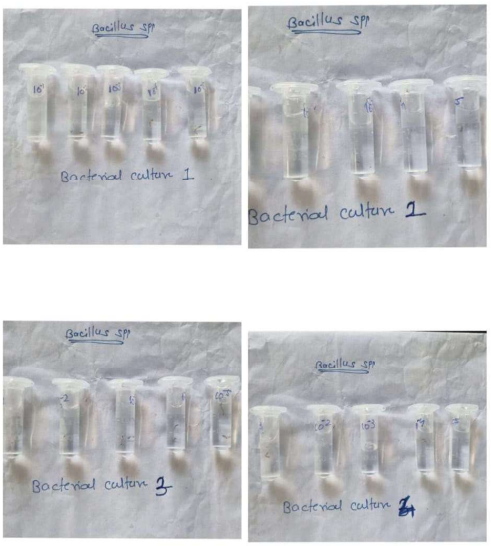

Fig.9 Larvicidal activity testing

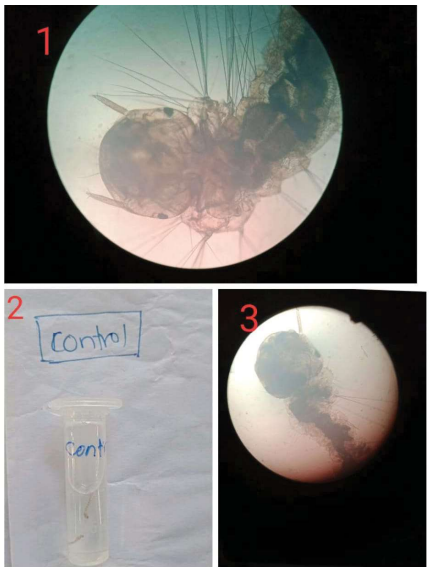

Fig.10 i) Live larva, ii) Control, iii) Dead larva

Bacillus species is a Gram positive, spore forming soil bacterium that produces insecticidal protein during sporulation. For example the Bacillus thuringiensis is the most widely used microbial control agent in the developed world. Biological pesticides based on Bacillus spp. are becoming increasingly important in pest management programs, accounting for 80-90% of all biological pest control agents used worldwide. Authors isolated the four Bacillus species from the soil because the soil is the natural resource for all microbial isolates. The microbial strain was identified and characterized on the basis of their morphology and biochemical tests that including Indole test, VP test, Starch hydrolysis test, Mannitol fermentation test etc. In case of the Bacillus species all isolates have pyramidal crystal protein while others have cuboidal forms. The unique characteristic that differentiates Bacillus from other species that formation crystal parasporal matrix. According to the Kampfer (1991) reported that the difficult of differentiates Bacillus thuringiensis and Bacillus cereus that both showing the same biochemical and morphological characters. Researchers stated that analytical methods like DNA homology, Pyrolysis, Gas chromatography etc. that failed to differentiate two species. In this study toxicity was demonstrated by death at a specified period of time and change in morphology of the larva. For determination of the larvicidal effect on mosquito, authors serial diluted the four different culture of Bacillus that labeled as (Bacillus 1, 2, 3, 4) from 10^{-1} to 10^{-5}. This culture was placed into the micro centrifuge tubes then each tube added two cultivated mosquito larva for 2hrs, 4hrs, 6hrs. After 2 hrs., Bacillus culture 1 was showing the maximum amount of larva death. After 4 hrs of incubation Bacillus culture 2 and 3 shows maximum amount of death (Table 1-7). After the 6hrs all the larva has been dead in all four different Bacillus culture tube. For each time the sample tube was compared with the control tube (with distilled water and mosquito larva). Authors observed that as time goes on increasing, the death of larva also increases.

5. Conclusions :

The results obtained in this study clearly demonstrated the efficiency of the Bacillus species in controlling mosquito larva. The use of Bacillus species as a Biocontrol agent against mosquito larva is preferred as it is environmentally friendly and does not deplete the ozone layer unlike the regular pesticides used in killing mosquitoes in most countries.

It is still necessary to search for more microbial toxins to control insect orders which have the ability to

develop resistance against selected insecticides. Screening of soil samples from different sources and habitats may be useful to obtain Bacillus strain with broader host ranges and novel crystal proteins.

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