

## Biological control of *Culex pipiens* mosquito by local bacterial isolates

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### ABSTRACT

In this work, 62 bacterial and actinobacterial isolates were isolated from different soil samples and screened for their larvicidal activities against the 3<sup>rd</sup> instar *Culex pipiens* larvae. Two potent isolates that showed high mortality rate were identified by 16S rRNA gene sequencing as *Bacillus subtilis* (MH370499) and *Streptomyces griseoruber* (MH370498). Optimizing the environmental and nutritional conditions increased the mortality percentage of *B. subtilis* from 82.9% to 100% and *S. griseoruber* from 69% to be 100%

LC<sub>50</sub> and LC<sub>90</sub> of *B. subtilis* were 13.68% and 28.29%, while for *S. griseoruber* were 5.79% and 18.53% respectively. The best solvent for active substance from *B. subtilis* extraction was ethyl acetate, while for *S. griseoruber* was hexane. The GC-MS analysis of *S. griseoruber* detected the presence of antilarval compounds ambrosin and triacontane. In addition, the larvicidal surfactin produced by *B. subtilis* was detected by haemolysis.

**Key words:** Control, *Culex*, *Streptomyces*, *Bacillus*, mosquito.

### INTRODUCTION

Mosquitoes are medically important arthropod vectors that responsible for the spread of many parasitic diseases like malaria, chikungunya, encephalitis, dengue fever, yellow fever, etc.; causing thousands of deaths annually (Rahuman *et al.*, 2009; Borah *et al.*, 2010).

Even if mosquito-borne diseases represent a real health problem in the tropical and subtropical zones, no part throughout the world is safe from this danger. Besides, the cost of battling these diseases and output loss has ruined the economic growth of endemic countries (Ahmed *et al.*, 2014, 2017).

With all trials to reduce mosquito-borne diseases in different countries, these vectors are still wide spread, and even increasing in some parts of the world. Consequently, synthetic insecticides were being used for several decades however, they caused soil and ground

water contamination as well as increasing vector resistance (Balkew *et al.*, 2010; Naqqash *et al.*, 2016).

The mosquito, *Culex pipiens* is a worldwide insect introducing numerous diseases. In Egypt, it is the main vector of bancroftian filariasis worm, *Wuchereria bancrofti* (Darwish and Hoogstraal, 1981) as well as Rift valley fever virus (Conley *et al.*, 2014) and West Nile virus (Amraoui *et al.*, 2012).

Mosquito control is progressively difficult in Egypt because of the development of *Cx. pipiens* resistance to many insecticides. Zayed *et al.* (2006) reported that there were clear indications of larval resistance to organophosphate insecticides, besides the widespread adult resistance to many insecticides including: organochlorine, pyrethroid, organophosphate, and carbamate.

Although, World Health Organization (WHO) regularly demand vector information to update the

insecticides susceptibility and resistance and to evaluate current procedures for resistance tracking (WHO 1992, 2000), there is an urgent need to explore and utilize a different environmentally safe alternate to manage mosquito vectors.

Biological control has been always the most attractive alternative to chemical pesticides; by using biological organisms or their metabolites to target mosquito populations. Natural predators have been used such as the mosquito fish (Angelon and Petranka, 2002), nematodes (Sanad *et al.*, 2013) and Copepods (Pernia *et al.*, 2007).

Likewise, large screening has been started for using the oils and extracts of medicinal plants against *Cx. Larvae* (Amer and Mehlhorn, 2006; Khater and Shalaby 2008; Benelli, 2015). Furthermore, nanomosquitocides are successfully synthesized from more than 80 plant species particularly with their larvicidal properties (Benelli, 2016). These fast-growing research studies are still limited and there is much remains to be revealed about them.

On the other hand, microbial pesticides have been used for decades and some of them are considered to be effective on large-scale application. Entomopathogenic fungi have shown lethal effect on *Cx. quinquefasciatus* (Ravindran *et al.*, 2015; Popko *et al.*, 2018). Though, fungi are mostly used to target the adult mosquitoes besides, the development of fungus resistance is predicted to be much slower, however, more research is needed in order to conduct a large-scale application of fungal spores into wild mosquito adults (Darbro and Thomas, 2009).

The larvicidal toxins produced by *Bacillus thuringiensis* var. *israelensis* (*Bti*) and *Lysinibacillus sphaericus* (*Ls*) have been commercialized and used broadly in mosquito eradication programs since their discovery (Jones *et al.*, 2007; Lacey, 2007). However, the persistent usage of *Bti* has shed the light on its downside, such as

the instability of the antilarval crystal proteins in sunlight and the inability of one *Bti* to show the same lethal efficiency in all regions due to the varied mosquito strains (Poopathi and Abidha, 2010). Additionally, *Cx. pipiens* larvae became resistant to the bacterial agent *Bti* in the laboratory (Saleh *et al.*, 2003), besides their resistance to *Ls* (Nielsen-Leroux *et al.*, 1997).

Actinobacteria were also reported to produce numerous valuable bioactive compounds that can be used as mosquito larvicides such as tetranectin, avermectins and macrotetrolides (Pampiglione *et al.*, 1985; Rao *et al.*, 1990; Zizka *et al.*, 1989). Meanwhile, most of the vital metabolites were extracted from soil *Streptomyces* (Basilio *et al.*, 2003).

Therefore, this study was conducted to isolate local bacterial isolates with potential larvicidal activity against *Culex pipiens* mosquito.

## MATERIALS AND METHODS

### 1- Collection of soil samples

Seven soil samples were collected from different regions from Cairo and El-Qalyubia governorates. The polluted samples of drainage regions of a metal product factory (Teraat Al Ismailiah, Al Amireyah Ash Shamaleyah, Cairo), a chemical factory (Qalyub, El-Qalyubia) and an electric industrial factory (Teraat Al Ismailiah, Qesm Than Shubra Al Kheimah, Cairo), in addition to the rhizospheres of bean, wheat, garlic and apricot crops which were cultivated in Qalyub and Shibin El-Qanater (El-Qalyubia). All samples were collected in sterile self-sealing plastic bags.

### 2- Isolation and preservation of bacteria and actinobacteria

Isolates were recovered from soil samples by serial dilution technique in sterile saline solution and then 100 µl from each dilution was subcultured on proper isolation medium. For bacteria, nutrient agar plates were used and incubated at

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37°C for 24 h (Park *et al.*, 2016), while for actinobacteria, starch nitrate agar plates were used and incubated at 30°C for 7 days (El-Khawagh *et al.*, 2011).

After incubation period, the colonies were purified and maintained on agar slants for further investigations. For long term storage, the isolates were suspended in 15% glycerol solution and stored at -20°C.

### 3- Rearing of *Culex pipiens* mosquito

Egg rafts of *Cx. pipiens* were obtained from colonies maintained at the Research and Training Center of Vector Disease, Ain Shams University, Cairo. The eggs were transferred to metal white enamel plates containing dechlorinated tap water and were reared in the insectary for 3<sup>rd</sup> instar larvae collection for further larvicidal bioassay (Kamaraj *et al.*, 2009).

According to Ahmed *et al.* (1999), mosquitoes were reared in the insectary for 10 generations before any experiments. They were maintained at 24-28°C and 70–80% RH under a 12:12 L/D photoperiod. Larvae were fed a diet of grounded fish food. Pupae were transferred from the trays to a cup containing dechlorinated tap water and placed in rearing cages (30 × 25 × 15 cm) where adults emerged (Robich and Denlinger, 2005).

Adults were continuously provided with freshly prepared 10% sucrose solution by cotton pads. Seven days postemergence, the adults were deprived of sugar for 2 h, and provided with a pigeon placed on the mesh of the cage for 2 h for blood feeding by females. Adult mosquitoes were maintained under the same environmental conditions as the larvae.

#### 4- Screening of microbial isolates for their larvicidal activity

Flasks containing 100 ml of proper broth media were inoculated with 100 µl (10<sup>6</sup> CFU/ml) of 24 h bacterial cultures. After incubation periods (24 h at 37°C for bacteria and 7 days at 30°C for

actinobacteria). The cultures were centrifuged at 10000 rpm for 20 min. then the cell free supernatants were collected and used for larvicidal bioassay.

Larvicidal activity of isolates against *Cx. pipiens* was evaluated according to (Saurav *et al.*, 2013; Rajesh *et al.*, 2015). Late third instar larvae (20) were transferred to sterile cup and treated with the crude culture medium, uninoculated culture medium was used as control. The experiments were replicated three times (total treated larvae=60). Furthermore, the larvae were placed in 120 ml cups containing 100 ml dechlorinated tap water and supplemented with grounded fish food.

Based on the preliminary screening trials, the concentration of each bacterial filtrate was fixed to be 20% , while the actinobacteria filtrate concentration was 10% to give the significant effects. Dead larvae were counted after 24 and 48 h post exposure then the mortality percentage was calculated from the average of three replicates. Mortality percentage was corrected when necessary for mortality in the controls using the following Schneider-Orelli's formula (Püntener, 1981):

$$\text{Corrected Mortality Percentage} = \frac{M \% \text{ in treated plot} - M \% \text{ in control plot}}{100 - M \% \text{ in control plot}} \times 100$$

M %: mortality percentage.

The most potent bacterial and actinobacterial isolates were selected for further experiments.

### 5- Optimization of different physical and nutritional conditions for the production of antilarval compounds

#### 5.1- Effect of different physical conditions on production of antilarval compounds

The optimum temperature for maximum production of larvicidal compounds was determined by incubating the cultures at different temperatures from 20-45°C with an interval of 5°C on

nutrient broth and starch nitrate broth media and incubated for 24 h and 7 days for bacteria and actinobacteria, respectively. To detect the optimum pH, media was adjusted at different pH values (5, 6, 7, 8, and 9) using 1N NaOH/1N HCl as shown by Kunal *et al.* (2016).

To determine the optimum incubation period, the bacterial culture was incubated at 120 rpm for 12, 24, 48, 72 and 80 h, while the actinobacterial culture for 5, 7, 10, 12 and 14 days intervals at 120 rpm. The effect of agitation speed was tested by incubating the cultures in shaking incubator at different rpm (0, 50, 100, 150 and 200 rpm).

## **5.2- Effect of different nutritional conditions on production of antilarval compounds**

### **- Effect of different carbon and nitrogen sources on bacterial isolate**

As demonstrated by Devidas *et al.* (2014), basal medium was prepared with different carbon sources: sucrose, sodium succinate, mannitol, lactose and glucose. For the effect of different nitrogen sources, basal medium was used with yeast extract, beef extract, ammonium sulphate, ammonium chloride, soybean and potassium nitrate as nitrogen sources. Nutrient broth was used as control for the bioassay.

### **- Effect of different types of media on actinobacterial isolate**

The selected isolate was grown on different broth media: starch nitrate, starch casein, yeast malt extract, soybean meal broth, nutrient broth, oat meal broth and tryptic soy broth. The cultures were incubated at the best conditions according to previous assays results

All factors were tested in sequence and the result from each experiment was applied for optimizing the next one. For each parameter, the cultures supernatants were assayed as described before in screening of microbial isolates for their larvicidal activity.

## **6-Determination of the lethal concentrations of the isolates filtrates**

The isolates were grown at their optimum conditions then, the LC<sub>50</sub> and LC<sub>95</sub> were recorded by calculating the regression line employing, using probit analysis of Finney (1971). The bioassays were carried out to estimate the sublethal mosquito larvicidal concentrations of the filtrates and the morphological deformations were detected.

Five ascending concentrations were prepared as recommended by WHO (2009). Three replicates per dose were prepared and the bioassays were repeated thrice. The bacterial isolate was tested for 5%, 10%, 15%, 20% and 25% concentrations, while the actinobacteria was tested for 2%, 5%, 7%, 10% and 12% concentrations.

## **7- Extraction of active compounds**

The supernatants were dissolved in equal volumes of different solvents; ethyl acetate, ether, hexane, chloroform and acetone (Vargas, 2016). The mixtures were agitated vigorously for 1 h in a separating funnel. The solvent extract was removed, dried properly, dissolved in 20% DMSO and was used for larvicidal bioassay (Rajesh *et al.*, 2015).

## **8- Preliminary identification of the active compounds**

### **-Gas Chromatography (GC - MS)**

GC-MS analysis the crude extracts was performed using Agilent 7890B gas chromatograph equipped with Agilent 5977A mass spectrometer, at the central laboratory of faculty of Science, Ain Shams University, Egypt. In which, one microliter sample was injected and mass acquisition commenced after 3 minutes.

Helium gas was used as the carrier gas at a constant flow rate of 3 ml/min. The injector and MS transfer line temperature were set at 300°C and the oven temperature was programmed at an initial temperature 40°C for 3 min. to

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reach eventually 300°C for 5 min with an increasing 20°C/min. gradient.

### -Detection of surfactin production

The production of surfactin was detected by erythrocytes haemolysis for the selected bacterial isolate. The bacterial isolate was streaked on sterile blood agar plates containing defibrinated sheep blood (5% v / v). The plates were incubated at 37°C for 24 and 48 h (Geetha and Manonmani, 2010).

## 9- Identification of the isolates

### 9.1 Cultural characteristics

The selected bacterium was grown at 30°C on nutrient agar for 24 h then colony morphology and growth patterns were observed. Morphological and cultural characteristics of the selected actinobacterial isolate was studied by inoculation onto different International *Streptomyces* Project (ISP) media such as; starch nitrate agar, starch casein agar, yeast -malt extract agar (ISP2), oat meal agar (ISP3), inorganic salts-starch agar (ISP4), and nutrient agar. The cultures were incubated at 30°C for 7 days to determine the color of substrate, aerial mycelia and any soluble pigments produced by the isolate as well (Shirling and Gottlieb, 1966).

### 9.2 Microscopic examination

#### -Light microscopy

Gram stain was carried out for the bacterial isolate according to Cheesbrough (1989). While, actinobacteria was Gram stained and examined for shape and the mycelium structure and arrangement of spore on the mycelium (William and Cross, 1971).

#### -Electron microscopy

This was carried out to examine the spore surface and mycelium for actinobacteria by scanning electron microscope (JSM -5500 LV) at the Fungal and Biotechnological Center, Al- Azhar University, Egypt.

### 9.3 Identification of potent isolates by 16S rRNA gene

Colony PCR was carried out to amplify the 16S rRNA gene for the selected isolates. PCR was performed using Fermentus Dream Taq Mastermix according to instruction manual. The universal primers PA (5'-AGAGTTTGATCCTGGCTCAG-3') and 517R (5'-ATTACCGCGGCTGCTGG-3') (Heuer *et al.*, 1997) to amplify 500 bp. PCR was performed using thermal cycler (Applied biosystem 2720). The PCR conditions were adjusted to 5 min for initial denaturation at 94°C and then 35 cycles of 1 min at 94°C, 1 min at 54°C, and 1 min at 72°C, and finally 10 min at 72°C. PCR products were purified by Qiagen extraction kit according to the manufacturer instructions before applying to DNA sequencer. Sequencing was performed by automated florescent dye terminator sequencing method originally developed by using (DYEynamic ET Terminator Cycle Sequencing Kit, Amersham Pharmacia Biotech. ABI 3130) at the Animal Health Research Institute, El Dokki, Giza, Egypt.

## 10 Statistical analysis

The study data were done by Excel for windows program Microsoft office 2007. All data were evaluated by calculation of the mean (M) and standard deviation (SD). Mortality percentages were calculated after 24 h and 48 h after treatment as the mean of three replicates. The data were evaluated using probit analysis (LdP Line, copyright 2000by Ehab Mostofa Bakr, Cairo, Egypt); determining the LC<sub>50</sub>, LC<sub>90</sub>, and other statistical values.

## RESULTS

### 1- Isolation of bacteria and actinobacteria

From 7 different soil samples collected from El-Qalyubia and Cairo governorates, 62 isolates were isolated, 46 bacterial isolates and 16 actinobacterial

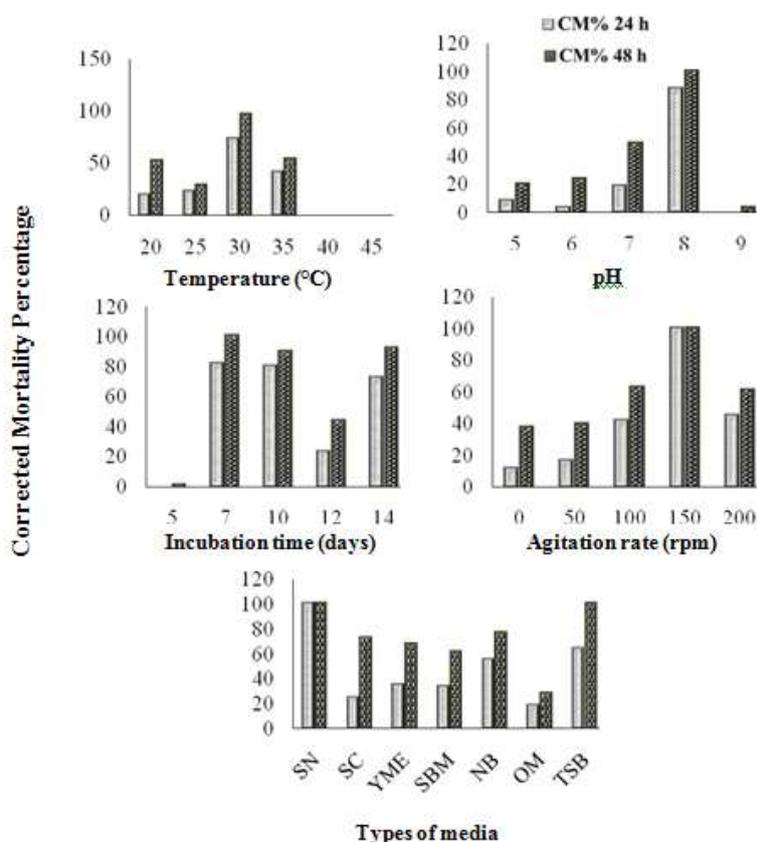
isolates, according to the difference in morphological and cultural characters.

## 2- Screening of larvicidal effect of the isolates

All isolates were screened for their activity against *Cx. Pipiens* 3<sup>rd</sup> instar larvae. The mortality percentages ranged between 50 to 82.9% for bacteria and 50 and 69% for actinobacteria. The maximum activity was demonstrated by 2 isolates BAC (bacteria) and SC10 (actinobacteria) which were selected for further experiments.

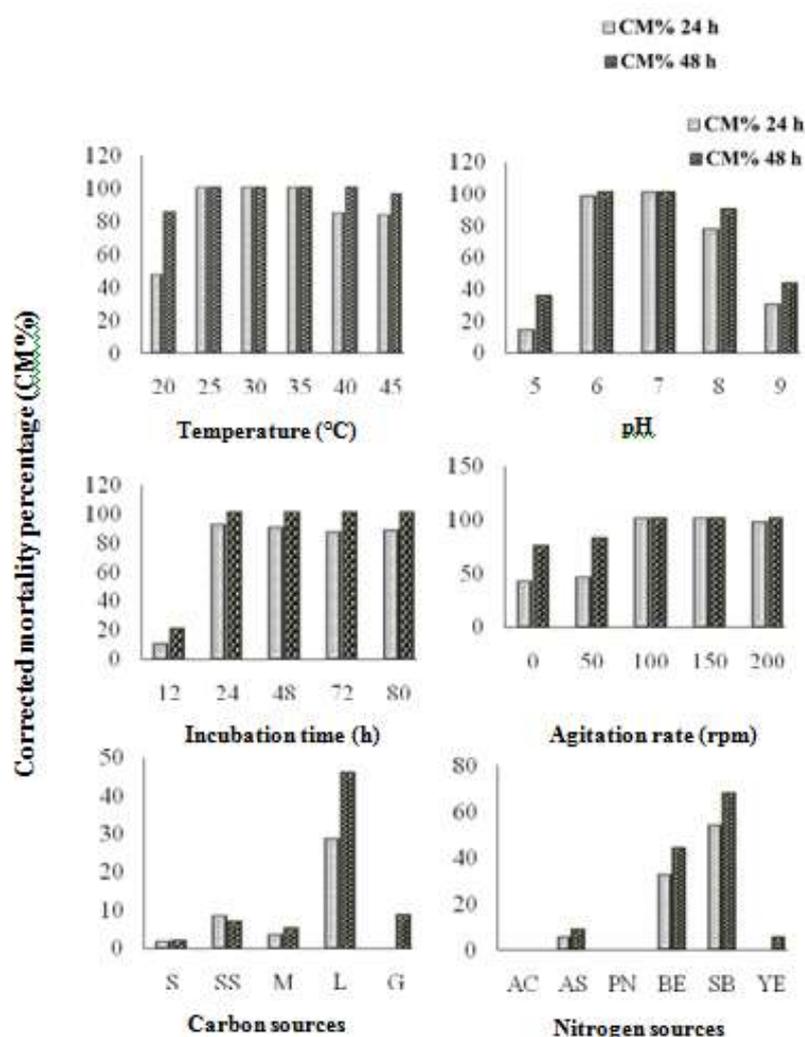
## 3-Optimization for production of larvicidal compounds by the selected isolates

The optimum conditions for larvicidal activity by the isolates were carried out using the mortality percentage as an indicator. The best conditions were 30°C; pH 8, 150 rpm agitation speed for 7 days on starch nitrate medium for actinobacteria (SC10). While for bacteria, the best conditions were 25°C , pH 7, 100 rpm agitation speed for 24 h incubation. Using basal medium with lactose carbon source and soybean as a nitrogen source demonstrated 45.7% and 67.1% mortality percentages, respectively (Figs 1 & 2).



**Fig. (1):**Optimization of growth conditions and larvicidal active compounds production of SC10. SN: Starch nitrate medium, SC: Starch casein, YME: Yeast malt extract, SBM: Soybean meal, NB: Nutrient broth, OM: Oat meal, TSB: Tryptic soy broth.

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**Fig. (2): Optimization of growth conditions and larvicidal active compounds production of BAC. Carbon source: S: Sucrose, SS: Sodium succinate, M: Mannitol, L: Lactose, G: Glucose. Nitrogen sources: AM: Ammonium chloride, AS: Ammonium sulphate, PN: Potassium nitrate, BE: Beef extract, SB: Soybean, YE: Yeast extract.**

#### 4- Determination of the lethal concentration $LC_{50}$ and $LC_{90}$ of BAC

Statistical data of  $LC_{50}$  and  $LC_{90}$  were represented in Tables (1 & 2). The results indicated that the effect on larval mortality was dose-dependent and plotted

by linear relationship in Figure (3). The low concentration 5% caused the least mortality percentage, while on increasing the supernatant concentration, the mortality increases gradually to 98% at 25% BAC concentration.

**Table (1): Toxicities caused by different concentrations of BAC against *Cx. pipiens* larvae.**

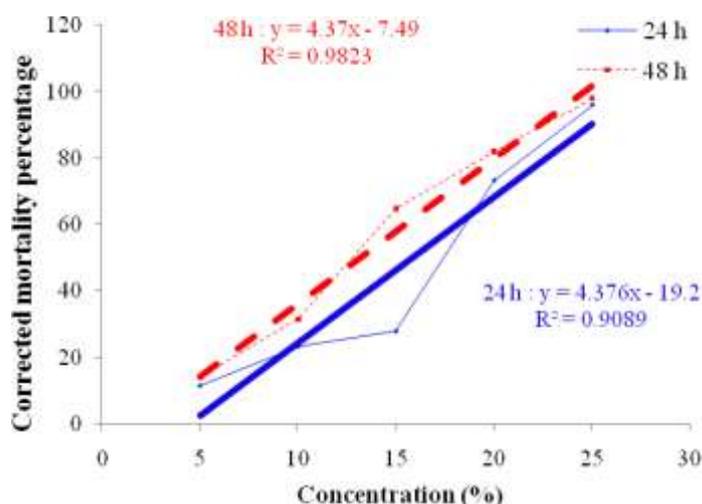
	LC <sub>50</sub>	LCL	UCL	Toxicity Index	RR	Slope	Chi	r	LC <sub>90</sub>
<b>After 24 h</b>	13.68	-	-	83	1.2	4.06	67.7	0.8	28.3
<b>After 48 h</b>	11.37	8.05	14.5	100	1	3.81	11.8	0.9	24.6

LCL: Lower confidence limit; UCL: upper confidence limit; LC<sub>50</sub>: Lethal conc. (the conc. killing 50% of test larvae); LC<sub>90</sub>: Lethal conc. (the conc. killing 90% of test larvae); RR: Resistance Ratio.

**Table (2): BAC lethal doses (LD<sub>50</sub> and LD<sub>90</sub>) values on 3<sup>rd</sup> instars of *Cx. pipiens* after 24 h and 48 h.**

Conc. of BAC	No. of Treated Larvae	After 24 h		After 48 h	
		M%	CM%	M%	CM%
<b>5%</b>	60	11.6%±0.3%	11.6%	18.3%±0.6%	14%
<b>10%</b>	60	23.3%±1.2%	23.3%	35%±1%	31.5%
<b>15%</b>	60	28%±1.7%	28%	66.6%±1.2%	64.8%
<b>20%</b>	60	73.3%±0.3%	73.3%	83.3%	82%
<b>25%</b>	60	96%±0.6%	96%	98%±0.3%	98%

M%: Mortality Percentage; CM%: Corrected Mortality Percentage

**Fig. (3): Regression plot showing the linear relationship between the lethal concentrations of BAC filtrate, and mortality percentages of the 3<sup>rd</sup> instar *Cx. pipiens* larvae at 24 h and 48 h post-treatment.**

#### 5- Determination of the lethal concentration LC<sub>50</sub> and LC<sub>90</sub> of SC10

Statistical data of LC<sub>50</sub> and LC<sub>90</sub> are represented in Tables (3 & 4).

Tabulated results of the isolate showed that on high concentrations, mortality percentages were greater, except for the high conc. of SC10 that caused lesser

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activity (81.6%) in all the bioassays performed. Figure (4) showed the regression lines at 24 h and 48 h post-treatment.

**Table (3): Toxicities caused by lethal concentrations of SC10 on *Cx. pipiens* larvae.**

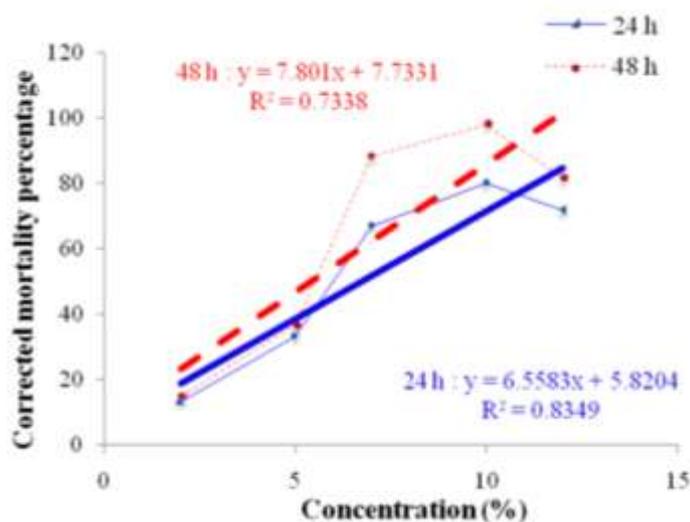
LCL: Lower confidence limit; UCL: upper confidence limit; LC<sub>50</sub>: Lethal conc. (the conc. killing 50% of test larvae); LC<sub>90</sub>: Lethal conc. (the conc. killing 90% of test larvae); RR: Resistance Ratio.

	LC <sub>50</sub>	LCL	UCL	Index	RR	Slope	Chi	r	LC <sub>90</sub>
After 24h	5.8	-	-	79.8	1.2	2.53	13.4	0.9	18.5
After 48h	4.6	-	-	100	1	3.23	42.6	0.8	11.5

**Table (4): Lethal doses of SC10 filtrate (LD<sub>50</sub> and LD<sub>90</sub>) against *Cx. pipiens* after 24 h and 48 h**

Conc. of SC10	No. of Treated Larvae	After 24 h		After 48 h	
		M%	CM%	M%	CM%
2%	60	13.3%±0.8%	13.3%	15%±1.1%	15%
5%	60	33.3%±0.3%	33.3%	36.6%±0.3%	36.6%
7%	60	67%±2%	67%	88.3%±1.4%	88.3%
10%	60	80%±0.8%	80%	98%±0.3%	98%
12%	60	71.6%±0.5%	71.6%	81.6%±1.8%	81.6%

M%: Mortality Percentage; CM%: Corrected Mortality Percentage.



**Fig. (4): Regression plot showing the linear relationship between the lethal concentrations of SC10 filtrate and mortality percentages of the 3<sup>rd</sup> instar *Cx. pipiens* larvae at 24 h and 48 h post-treatment.**

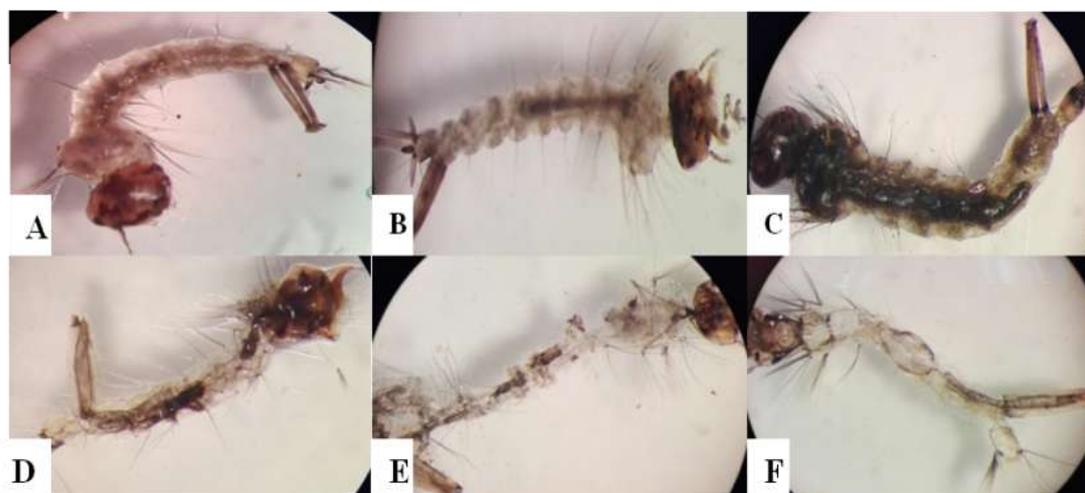
### 6- Effect of the larvicidal active compounds of SC10 and BAC on the morphology of the larvae

Treatments of larvae with the different filtrates concentrations, showed morphological deformations such as general thoracic deformation, extended

body, neck elongation and abdomen pigmentation. Higher concentrations demonstrated advanced levels of deformation such as with degradation of larval cuticle layer, full decomposition of the body and head deformation (Figs. 5 & 6).



**Fig. (5):** Showing the morphology of 3<sup>rd</sup> instars *Cx. pipiens* larvae after the treatment with the SC10 supernatant at different concentration. (A) Control, (B) 2%, (C) 5%, (D) 7%, (E) 10%, (F) 12%. Magnification: 40X



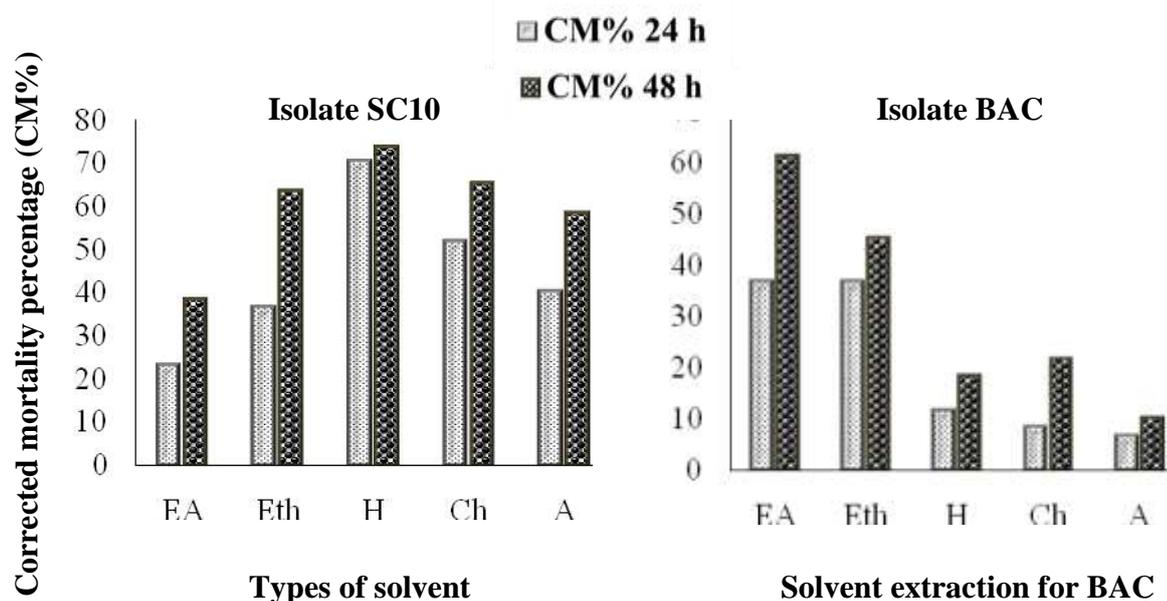
**Fig. (6):** Showing the morphology of 3<sup>rd</sup> instars *Cx. pipiens* larvae after treatment with the supernatant of BAC at different concentration. (A) Control, (B) 5%, (C) 10%, (D) 15%, (E) 20%, (F) 25%. Magnification: 40X

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### - Extraction of larvicidal active substances by solvents

Ethyl acetate solvent was the best solvent for extraction of the active

compounds from BAC achieving 61% mortality percentage, while hexane was the best for SC10, showing the highest mortality (73.3%) (Fig. 7).



**Fig. (7): Solvent extraction for SC10 and BAC isolates. EA: Ethyl acetate, Eth: Ether, H: Hexane, Ch: Chloroform, A: Acetone.**

### 8- Preliminary chemical analysis of the active compounds (GC-MS) analysis

GC-MS analysis of hexane extracts for SC10 supernatant showed different compounds. The major compounds were identified based on the seven highest peaks are Benzene, (1-butylheptyl) (10.03%); Benzene, (1-pentylloctyl) (7.11%); Benzene, (1-pentylheptyl) (6.53%); Benzene, (1-ethyldecyl) (6.48%); Benzene, (1-butylloctyl) (6.39%); Benzene, (1-ethylnonyl) (6.21%) and Benzene, (1-pentylnonyl) (6.12%).

Other minor compounds were identified as: Ambrosin (0.232%) and Triacotane (0.06%).

### - Detection of surfactin production

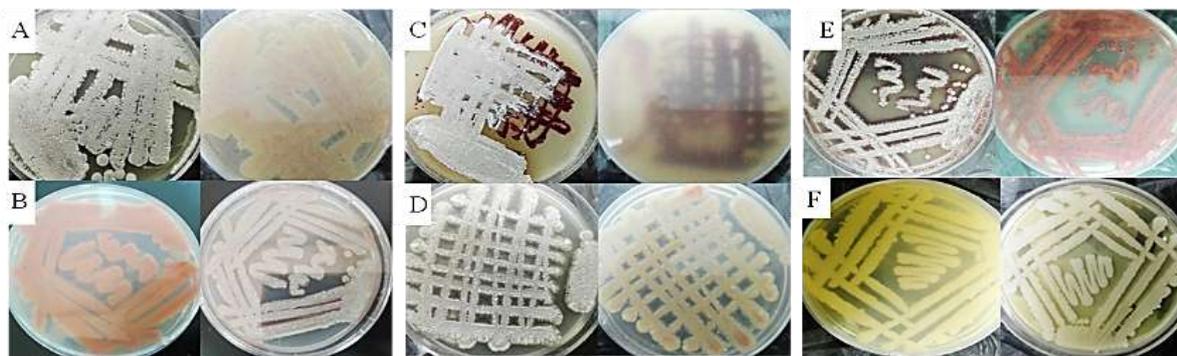
The bacterial isolate (BAC) was

inoculated on blood agar medium and incubated for 24 h at 37°C. Zones of beta-hemolysis surrounding the colonies indicating production of surfactin.

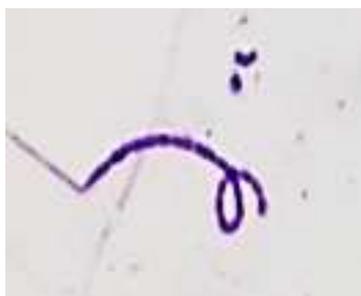
### 9- Identification of the isolate SC10

Isolate SC10 showed light grey colour colonies with pale-yellow to reddish orange at the reverse side of colony and yellow to red pigment (Fig. 8).

Light microscopy showed that the SC10 had spiral spore chains. SEM revealed that the spores had smooth surface (Figs. 9 & 10). Partial sequence of 16S rRNA gene revealed that the isolate SC10 was identified as *Streptomyces griseoruber* and the nucleotide sequence was submitted in *Gen Bank* under accession number MH370499.



**Fig. (8):** Colony morphology of actinobacterial isolate SC10. (A) Starch nitrate; (B) Starch casein agar; (C) Oat medium; (D) Insoluble salt starch agar; (E)Yeast malt agar; (F) Nutrient agar.



**Fig. (9):** Light microscope of spore chains of the actinobacterial isolate SC10 (X 1000).



**Fig. (10):** Scanning electron (SEM) of isolate SC10 (X 10,000).

#### 10- Identification of the bacterial isolate BAC

BAC isolate was Gram-positive spore forming rods (Fig. 11). Partial

sequence of 16S rRNA gene revealed that the isolate BAC was identified as *Bacillus subtilis* and submitted to Gen Bank, NCBI under accession number MH370498.



**Fig.(11):** Light microscope of BAC isolate (X400).

#### DISCUSSION

Soil is always screened for newer, safer and even more effective microbial strains to be used in insect control programs. It is highly advisable to apply

bioinsecticides that cause no harm for the environment and other non-target organisms.

Sixty two bacteria and actinobacteria have been isolated from

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random soil locations in Egypt including locally cultivated lands and contaminated sites.

Among the forty-six bacterial isolates (74.1%) and sixteen actinobacterial isolates (25.8%), 12.9% have shown antilarval activities against *Culex pipiens* larvae. González *et al.* (2013) had isolated 881 bacteria from different soils and found only nine isolates (1%) that had displayed the highest lethal activities against *Cx. quinquefasciatus*.

The two isolates BAC and SC10 in the present study have been selected according to their significant larvicidal effects against *Cx. pipiens* larvae. The actinomycetal isolate SC10 was identified as *Streptomyces griseoruber*, while the bacterial one BAC was identified as *Bacillus subtilis*. They were chosen for further investigations to optimize their bioactive metabolites production.

The results showed that *B. subtilis* (BAC) exhibited the best mortalities in the temperature range from 25°C to 35°C with no significant change in activity on decreasing and increasing temperature; indicating that *B. subtilis* tolerate the variation in temperature. The optimum pH values were 6 and 7, while the larvicidal activity was at the maximum after 24 h incubation and the produced metabolites were stable for up till 80 h on shaking incubator at 100 rpm speed. Lactose and soybean were the best carbon and nitrogen sources for the production of active agents.

Geetha and Manonmani (2010) had indicated that the crude surfactin produced by *B. subtilis* was effective against mosquito; it had a pupicidal property and was not affected by increasing the temperature in addition to the variation of pH values.

Vijayalakshmi *et al.* (2013) stated that lactose had increased the growth and enzyme production of *B. subtilis*; in addition Devidas *et al.* (2014) demonstrated that soybean flour was more supporting for the biomass and production of toxins of *B. thuringiensis* than

ammonium sulphate. The results also support other's findings, in which agitation rates for *Bacillus* sp. in the range of 180 and 220 rpm was preferable (Genckal and Tari, 2006 ; Potumarthi *et al.*, 2007).

On the other hand, *S. griseoruber* (SC10) had the highest larvicidal effect at 30°C with pH 8, at 150 rpm shaking incubator for 7 days of culture incubation. Starch nitrate was the best medium for *S. griseoruber* to produce the antilarval metabolites. The results are in accordance with those obtained by Ababutain *et al.* (2012), who noted that *Streptomyces* sp. achieved the highest antimicrobial activity after incubation for seven days. The current result agrees also with Zhicheng *et al.* (1993) who reported that the chitinase, produced by *Streptomyces* sp., had the optimum temperature at 30°C.

Whereas, Anwar *et al.* (2014) had reported that the range of 7.5- 8.5 was the optimum pH for *Streptomyces* spp. growth to exhibit a complete mortality against *Cx. quinquefasciatus* larvae. However, Carrillo and Gómez-Molina (1998) found that the optimum pH for chitinase activity of *Streptomyces griseoruber* 202 was at pH 4.6 and 40 °C.

Zhou *et al.* (2018) indicated that 200 rpm was the optimal speed for *Streptomyces kanasensis* ZX01 in glycoprotein GP-1 production. However, the present results are consistent with El-Khawagh *et al.* (2011) who studied the production of insecticidal agents from actinobacterial isolates against the 3<sup>rd</sup> instar *Culex pipiens* larvae. The study demonstrated the highest mortalities by culturing in starch nitrate media.

The effect of entomo-pathogenic bacteria could be significantly at variance against different species of mosquito larvae (Balakrishnan *et al.*, 2015). In the current study, the mortality percentage increased with increase in concentrations of the metabolites of *B. subtilis* treatment. It was found that the LC<sub>50</sub> was 11.37% and LC<sub>90</sub> was 24.64%. The high LC values against *Cx. pipiens* were also parallel to

the findings reported by Kovendan *et al.* (2011) who defined the antilarval effects of *B. thuringiensis* against the third instar larvae of *Cx. quinquefasciatus* with 10.212% and 15.887% as LC<sub>50</sub> and LC<sub>90</sub> values, respectively.

Singh and Prakash (2012) have studied the effect of *S. citreofluorescens* against the third instar of *Cx. quinquefasciatus* larvae and found that LC<sub>50</sub> and LC<sub>90</sub> were 100 µl/ml and 309.2 µl/ml, respectively. In the present study, the lethal concentrations for *S. griseoruber* were calculated as LC<sub>50</sub>=57.9 µl/ml and LC<sub>90</sub>=185.3 µl/ml.

The larvicidal activity of *B. subtilis* (BAC) metabolites on the mosquito larvae exhibit remarkable features of intoxication with the appearance of blackish color pigmentation and neck elongation as well as the disintegration of the larval body.

Ahmed *et al.* (2014) suggested that the extensive damage on the midgut epithelium might be the main reason in the death of *Cx. pipiens*, in addition to midgut paralysis, disrupting the function of midgut and feeding cessation, caused by *B. thuringiensis* according to Ahmed *et al.* (2017).

Similarly, the larvae treated with *S. griseoruber* (SC10) exhibit the same manner of deformation in addition to a detrimental effect on the cuticle layer and head deformation.

This observation agreed with the findings of some authors who had recorded alike symptoms such as deformed abdomen body extension and the elongation of neck. (Tripathi *et al.*, 2002; Khater and Shalaby, 2008; Benzina *et al.* 2018).

In the present investigation, the best solvent to extract the active compounds from *B. subtilis* was ethyl acetate, while hexane was the best for *S. griseoruber*.

The GC analysis was performed for the ethyl acetate extract of *S. griseoruber*, identifying major and

minor compounds. Two vital compounds were detected; ambrosin and triacontane.

It was demonstrated by Satyan *et al.* (2012) that triacontane was the bioactive compound responsible for the toxicity of *Excoecaria agallocha* against *Cx. quinquefasciatus* larvae within 18–24h.

Moreover, it was indicated that ambrosin is considered as the most active sesquiterpenes isolated from the herbal plant *Ambrosia maritima* and according to Abdelgaleil (2010) it may serve as new natural compound for *Cx. pipiens* control. In addition to some other studies confirmed the lethal effects of *Ambrosia maritima* against different mosquito species (El Sawy *et al.*, 1986; Eisa, 2010).

Although Carrillo and Gómez Molina (1998) had studied chitinase production by *S. griseoruber* 202 in media containing insect exoskeleton powder; to the best of our knowledge, no reports have been done on the insecticidal activity of the *S. griseoruber* against the mosquito.

The larvicidal activity of some *Streptomyces* spp. against *Cx. pipiens* have been demonstrated (Zizka *et al.*, 1989; Huamei *et al.*, 2008; El-Bendary *et al.*, 2010; El-Khawagh *et al.*, 2011).

The current results specify that the presence of ambrosin and triacontane in the cell free extract could have a synergetic effect on the larvae with significant lethal activity.

Concerning the antilarval activity of *B. subtilis*, its incubation on blood agar, showed yellow zones around the colonies indicating the presence of the lipopeptide surfactin (Balakrishnan *et al.*, 2015).

The exact mode of action is still needed to be clarified. However, Revathi *et al.* (2013) had revealed the impacts of secondary metabolites of *B. subtilis* on the levels of essential enzymes, Acetylcholin esterase (AChE), carboxylesterase, alkaline and acid phosphatase of larvae of *A. aegypti*.

In addition Das and Mukherjee (2006) and Geetha *et al.* (2007) had

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demonstrated that the cyclic lipopeptides of iturin, surfactin and fengycin produced by *B. subtilis* with the potent larvicidal and pupicidal properties.

Youssef *et al.* (2004) have tested the surfactin production by *B. subtilis* strains through haemolysis in addition Geetha and Manonmani (2010) have mentioned that *B. subtilis* producing surfactin which was lethal to mosquitoes and cause haemolytic zones on blood agar medium.

### Conclusion

In conclusion, supernatants of *S. griseoruber* and *B. subtilis* resulted in complete mortality of 3<sup>rd</sup> instar of *Culex pipiens* at high concentrations. Active metabolites could be extracted and used in stagnant water bodies which are known to be the breeding grounds for the mosquitoes. Further studies are needed before field application for investigating the safety of the applied metabolites to human and the environment as well as determination of the mode of action of each of the compounds.

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### المكافحة البيولوجية لبعوضة الكيولكس ببيئز بواسطة عزلات بكتيرية محلية

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### المستخلص

في هذه الدراسة تم عزل 62 بكتيريا و أكتينوبكتيريا من عينات تربة مختلفة وقد تم مسحهم لنشاطهم المضاد ليرقات الطور الثالث ل *Cx. pipiens*. تم تعريف اقوى عزلتين والتي أظهرت أعلى معدلات إماتة من خلال التتابع الجيني بواسطة 16S rRNA الى *Streptomyces griseoruber* (MH370498) و *Bacillus subtilis* (MH370499). وقد أدى تحسين الظروف البيئية والغذائية إلى زيادة نسبة الإماتة من 82.9% إلى 100% بالنسبة إلى *B. subtilis* ومن 69% إلى 100% بالنسبة إلى *S. griseoruber*.  
 وقد كانت LC<sub>50</sub> و LC<sub>90</sub> ل *B. subtilis* 13.68% و 28.29% على التوالي, بينما كانت 5.79% و 18.53% ل *S. griseoruber* على التوالي. كان ethyl acetate أفضل مذيب لإستخلاص المواد الفعالة من *B. subtilis* بينما hexane ل *S. griseoruber*. كشف تحليل GC-MS عن وجود ambrosin و triacontane كمواد مضادة اليرقات. ايضاً عن طريق انحلال الدم تم الكشف عن إنتاج surfactin من *B. subtilis* كمضاد لليرقات.