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Toxicological Activity of Indigenous Chitinolitic *Streptomyces* Species Against *Culex pipiens* (Diptera – Culicidae)

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Abstract: Two indigenous isolates of Actinomycetes proved to induce toxicity against *Culex pipiens* larvae. The isolate *Streptomyces* 110 was more toxic than the isolate 108 with LC_{50} values 3.18±1.44 and 5.02±1.59 ml/100 ml H_2O after 72 hrs. post treatment, respectively. The isolate 110 was more active in chitin hydrolysis and the adverse effect on pupal and adult emergence or life span. Both isolates affect the body proteins and changed its configurations. The most active and toxic isolate 110 was identified as *Streptomyces griseoflavus*.

Key words: *Culex pipiens* · *Streptomyces* spp. · Insecticidal activity

INTRODUCTION

Insect transmitted diseases remain major source of illness and death [1]. Mosquitoes are the most important single group of insects in terms of public health importance, which transmit a number of diseases, such as malaria, fillaria, dengue and Japanese encephalitis causing millions of deaths every year [2]. Malaria alone kills 3 million people every year [3].

Repeated use of synthetic insecticides for mosquito control has disrupted natural biological control systems and led to resurgences in mosquito populations. It has also resulted in the development of resistance [4], undesirable effects on non-target organisms and fostered environmental and human health concern [5]. Alternative approaches to chemical insecticides for controlling vector population have become search priorities after the environmental contamination by such toxins. These have included biological and ecological control methods for limiting the destructive impact of pest population [6- 8]. Using pesticides of biological origins including bacteria, fungi, viruses and nematodes have been reported as strategies to biologically control insect pests [9]. Microorganisms are considered as a rich source of bioactive chemicals and they may be an alternative source of mosquito control agents.

Actinomycetes is an important group of microorganisms, not only as degraders of organic matter in the natural environment, but also as producers of antibiotics and other useful compounds of commercial interest [10- 11 and 12]. In addition, actinomycetes are important for the production of enzymes, such as chitinase (eg. *Streptomyces viridificans*), cellulases (eg. *Thermonospora* spp.) peptidases, proteases (*Nocardia* spp.), Xylanases (*Microbispora* spp.), ligninases (*Nocardia autotrophica*), amylases *(Thermomonospora curvata*), sugar isomerases (*Actinoplane smissouriensis*), pectinase, hemicellulase and keratinase [13].

The present study aimed to evaluate the insecticidal toxicity of indigenous type of Actinomycete isolates against *Culex pipiens* larvae and investigate the pathological and biochemical effect of such metabolites on insect tissues and proteins.

MATERIALS AND METHODS

Sampling and Collection of Actinomycete Isolates: Soil samples were collected from Beni-Suef Governorate - Egypt. The samples were collected from the rhizosphere of cultivated soil (wheat) and brought to the laboratory in sterile polythene bags for further investigations.

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Starch casein agar [14] medium was prepared and sterilized. The collected soil samples diluted and inoculated to plates including starch casein agar medium and incubated at 28 ± 2°C for seven days. Three replicates were maintained for each dilution. After incubation, *Actinomycetes* colonies purified and maintained in starch casein agar medium for further investigations.

Identification of the Actinomycetes Isolates: The Actinomycetes isolates under study were selected for further studies concerning its identification to the genus level depending on morphological criteria as mentioned in Bergey's manual of systematic bacteriology [15]. Then the most active and toxic isolate was identified to species level depending on 16-S rNA [16].

Determination of Chitinase Activity of Actinomycetes Isolates: Chitinase activity was detected using the developed method of Agrawal and Kotasthane [15]. Chitinase detection medium consisted of a basal medium comprising 0.3 g of $MgSO_4.7H_2O$, 3.0 g of $(NH_4)_2SO_4$, 2.0 g of KH_2PO_4, 1.0 g of citric acid monohydrate, 15 g of agar, 200 µl of Tween-80, 4.5 g of colloidal chitin and 0.15 g of bromocresol purple per liter; pH 4.7. Fresh culture plugs of the isolates to be tested for chitinase activity were inoculated into the medium petri plates and incubated at 28±2 °C. Purple color zone appeared due to bromocresol purple colour formation that was supplemented with media as pH indicator. As the medium containing colloidal chitin, chitinolytic isolates will break down the chitin, changing it to N- acetylglucosamine. Thus, change in pH range from acidic to basic, leading to changes of media from yellow to purple color [17]. Observations were recorded for the colour intensity and diameter of the formed zone until third day after inoculation.

Extraction of Extracellular Metabolites from Actinomycetes Isolates: Actinomycete isolates that have chitinolytic activity were tested against 3rd larval instars of *Culex pipiens*. The selected isolates were inoculated into a 250 ml conical flask containing 100 ml of starch casein liquid medium and shaken at 30±2°C and 200 rpm for seven days. The cells free culture filtrates were separated by centrifugation and screened for larvicidal activity.

Insect Rearing: Larvae of *Culex pipiens* were provided from the Medical Entomology Institute and transferred to the laboratory of Entomology Department, Faculty of Science, Ain Shams University where self-perpetuating colonies were established and maintained during the present study.

Larvicidal Activity of Actinomycete Isolates: Different concentrations, 2.5, 5, 10 and 15 ml of bacterial filtrate were added to 100 ml distilled water and 20 healthy *Culex pipiens* larvae were supplied to the tested cups and incubated at 28±2 °C. Three replicates were maintained for each dilution. Mortality readings were recorded after 48 and 72 hrs. The latent effect on pupae and adults was also studied. Treated larvae with LC_{50} values for 48 hrs. transferred to distilled water for normal rearing conditions. Percentages of pupal and adult reductions were calculated. Larval and pupal and adult periods were also recorded. Data obtained were statistically analyzed using SPSS program at virgin 10.

Estimation of Total Insect Proteins: Total proteins of treated and untreated larvae were determined quantitatively using the method of Bradford [18] and measured spectrophotometrically.

Protein Analysis Using SDS Poly Acryl amide Gel Electrophoresis: Insects were homogenized with PSB then centrifuged at 4000 rpm for 20 min. at 4°C. Ten µl of each sample supernatant was mixed with three volumes of treatment buffer; (0.125 M Tris, 2% SDS, 10% glycerol, 5% 2-mercaptothanol, 0.001% bromophenol blue) and then denatured by heating at 95 ºC for 3-5 min in water bath and chilled on ice. Electrophoresis conditions and procedures were carried out as described by Laemmli [19].

RESULTS

Description of Actinomycetes Samples: Eleven isolates were determined from soil samples of Beni-Suef Governorates. The soil collected from the cultivated rhizosphere of wheat was Brown Clay soil. The isolates were encoded from 101 to 111. The isolates were grown on starch casein solid medium and the culture characteristicswere described in (Table 1).

On the basis of the morphological characters (colony morphology and pigmentation) and staining characteristics the eleven isolates were identified up to generic level to be species of *Streptomyces*.

Chitinase Activity of Isolated *Streptomyces* spp: *Streptomyces* isolates screened for their ability to hydrolyze chitin as shown in (Fig. 1, Table 2) showed that isolate 108 and 110 were active and could hydrolyze chitin effectively. Chitinase activity was observed after 24 hrs. reaching the highest activity after 72 hrs. as incubation period recording 2.3, 3.4 cm as inhibition zone for isolate 108 and 110 respectively.

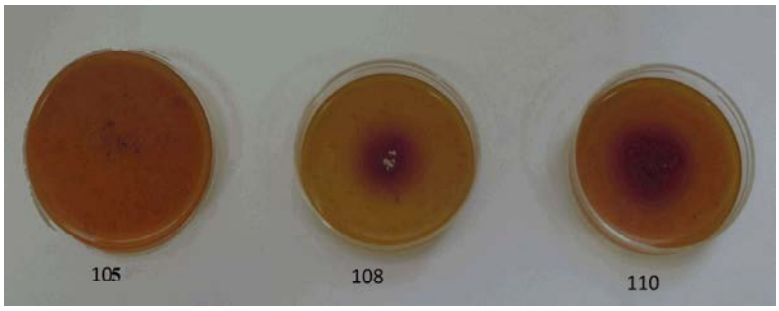


Fig. 1: Screening of chitinase activity of indigenous *Streptomyces* isolates

Table 1: Culture characteristics of Actinomycetes isolates.

Isolate no.	Aerial mycelium	Substrate mycelium	Pigment production	Gram's staining
101	Pink around white	white	+ve	+ve
102	Gray with pink	Pink with white	-ve	+ve
103	Pink with white	white	-ve	+ve
104	Dark green	White to green	+ve	+ve
105	Pink	Pink to white	-ve	+ve
106	White with grey	White	-ve	+ve
107	Grey to violet	Creamy pinkish	+ve	+ve
108	Pink with white	white	-ve	+ve
109	Grey with white	Light pink	+ve	+ve
110	Black	White	+ve	+ve
111	Dark grey	White	-ve	+ve

Table 2: Chitinase activity of isolates of *Streptomyces* spp.

Isolate no.	Activity after 24 hrs. (cm)	Activity after 48 hrs. (cm)	Activity after 72 hrs. (cm)
101	□	□	□
102	□	□	□
103	□	□	□
104	0.	0.35	0.4
105	□	□	□
106	□	□	□
107	□	□	□
108	0.3	1.05	2.3
109	□	□	□
110	0.8	2.35	3.4
111	□	□	□

Table 3: Larvicidal activity of *Streptomyces* spp. 108 and 110 against *Culex pipiens* larvae

		Isolate 108				Isolate 110			
		48 hrs.		72 hrs.		48 hrs.		72 hrs.	
Conc. %	Total larvae	Dead	M%	Dead	M%	Dead	M%	Dead	M%
2.5	60*	6 (2,2,2)	10	22 (7,8,7)	36.7	15 (4,5,6)	25	29 (9,10,10)	48
5	60*	9 (3,2,4)	15	29 (9,9,11)	48.3	24 (8,9,7)	40	32 (10,11,11)	53
10	60*	15 (5,6,4)	25	35 (11,12,12)	58.3	30 (9,12,9)	50	48 (16,17,15)	80
15	60*	32 (10,11,11)	53	48 (16,15,17)	80	40 (15,13,12)	60	52 (17,17,18)	87
LC_{50}	17.48 ± 4.60	5.02 ± 1.59	8.19 ±2.05	3.18 ±1.44					
Chi	4.51	2.8	0.78	2.7					
r	0.93	0.94	0.98	0.96					
slope	1.7284 +/- 0.3403	1.3505 +/- 0.2855	1.3252 +/- 0.2873	1.5515 +/- 0.2984					

*Three replicates each for 20 larvae

Larvcidal Activity of *Streptomyces* Isolates: According to results of estimating chitinase activity, the highly active isolates number 108 and 110 were chosen to test its activity against 3rd instar larvae of *Culex pipiens*. Results as shown in (Table 3) proved the toxicity of both isolate metabolites against *Culex pipiens* larvae.

Mortality readings were positively correlated to increase in metabolite concentrations for both isolates after 48 and 72 hrs. post treatment. LC_{50} values calculated to be 17.48 ± 4.60 and 5.02 ± 1.59 ml/100ml for isolate 108 after 48 and 72 hrs. respectively, while for 110 isolate LC_{50} values were 8.19 ±2.05 and 3.18 ±1.44 ml/100ml after 48 and 72 hrs. respectively.

In detecting the latent effect of both isolates on different insect stages, as shown in (Table 4), pupal mortality increased reaching 66.67% and 71.43% after treatment with isolates 108 and 110 respectively, comparing with 13.33% for control experiment. Great reduction was recorded in percentage of adult emergence to be 33.33% and 28.57% for 108 and 110 isolates, comparing with 86.67% in case of control experiment. Total mortality percentage increased to reach 81.25% and 87.50% for isolate 108 and 110 compering with total mortality in control reached 18.75%. Treatment increase larval and pupal duration to reach 9 days and 9.6 days after treatment with 108 and 110 isolates, while untreated larvae had duration of 4.5 days. The delay in pupal duration reached 3.3 days and 2.6 days instead of 2 days in control experiment.

Estimation of Total Larval Protein after *Streptomyces* Treatment: The total protein content of larvae was estimated to be 0.2362 mg/ml for control (untreated) while treated with isolates108 and 110 were recorded 0.5387, 0.403 mg/ml respectively as shown in (Table 5).

Protein Fractionation Using SDS-Poly Acrylamide Gel Electrophoresis: Results in (Fig. 2) and (Table 6) showed fractionation of normal and treated *Culex pipiens* larvae as well as emerged pupae and adults after.

Lane 1, represents the configuration of normal larval proteins, which had eight protein patterns of molecular weights (M.Wt.) 73.87, 52.43, 41.80, 29.93, 24.60, 18.58, 13.16 and 5.37 (KD). Lane 2, represents the treatment after 48 hrs. with isolate (108) giving eight bands with M.Wt. 67.742, 48.706, 38.731, 30.424, 24.848, 18.585, 13.338 and 6.31(KD). Treatment with isolate 110, lane 3, reduced protein bands to seven fractions with M.Wt. "49.07, 37.57, 31.03, 24.36, 18.41, 12.80 and 6.311(KD).

More fractionation of larval proteins appeared after treatment after 72 hrs. as where protein patterns after treatment with isolate 108 showed 15 bands (lane 5) with M.Wt. 227.5, 196.67, 61, 48.70, 42.37, 38.73, 34.88, 30.90, 27.75, 23.66, 17.73, 13.16, 10.04, 7.49 and 3.06 (KD). Treatment with isolate 110 for 72 hrs. also led to more protein fractionation recording 14 bands lane 6 with M.Wt. 215, 195.83, 158.33, 118.33, 63.452, 47.961, 41.615, 34.885, 30.182, 24.485, 18.246, 13.338, 7.4918 and 2.5738 KD. Lane 4, represents proteins of untreated larvae after 72 hrs. and showed 10 bands with M.Wt. 46.843, 39.885, 30.061, 26.061, 22.477, 18.754, 13.338, 10.246, 7.6885 and 5.1311 (KD).

Lanes 7, 8 and 9 in (Table 6) represent protein patterns of untreated pupae and those formed after larval treatment with isolates 108 and 110 respectively.

Lane 7, (untreated pupae) showed protein patterns of M.Wt. 270.83, 210.83, 192.5, 152.5, 76.935, 60.255, 46.098, 40.846, 34.885, 30.667, 27.152, 23.154, 19.262, 15.538, 13.508, 7.8852 and 2.7705 (KD). While Lane 8, showed protein patterns of M.Wt. 277.5, 210, 190.83, 157.5, 134.17, 105.74, 73.871, 63.452, 45.725, 41.038, 33.731, 30.182, 28.242, 23.831, 18.923, 15.2, 8.1803, 7.6885 and 2.9672 KD representing proteins of pupae formed after larval treatment with isolate 108. But at Lane 9, the protein patterns of M.Wt. 284.17, 211.67, 190.83, 155.83, 78.161, 62.226, 45.725,40.846, 34.115, 30.424, 27.758, 24, 20.277, 19.092, 14.692, 11.82, 8.8689 and 3.1639 (KD) represents pupal proteins after larval treatment with isolate 110.

Table 4: Latent effects of *Streptomyces* spp. on stages of *Culex pipiens*.

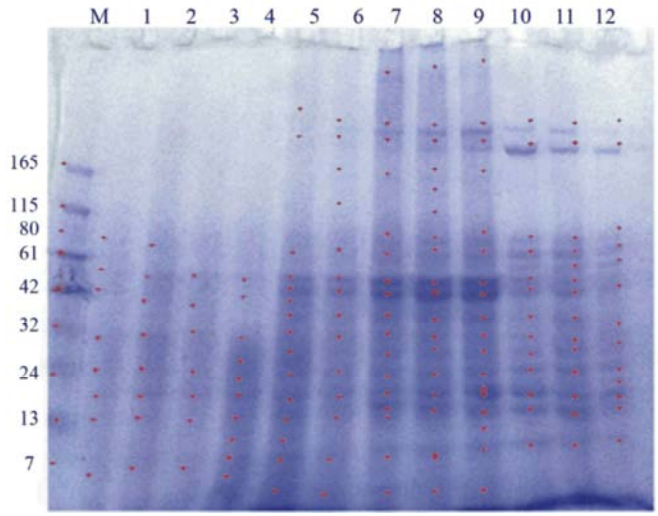
Isolate no.	Larval mortality%	Larval duration	% Pupation	Pupal mortality%	Pupal duration	Adult emergence	Total mortality%
Control	6.25%	4.5	93.75%	13.33%	2	86.67%	18.75%
108	43.75%	9	56.25%	66.67%	2.6	33.33%	81.25%
110	56.25%	9.6	43.75%	71.43%	3.3	28.57%	87.50%

Table 5: Total protein concentration of *Culex pipiens* larvae after *Streptomyces* treatment

Isolate no.	Protein conc. mg/ml	protein conc. mg protein /g tissue
Control	0.2362	147.625
108	0.5387	359.133
110	0.403	251.875

Table 6: Molecular weight (M.Wt.) of protein fraction of larval, pupal and adult stages of *Culex pipiens* after larval treatment with *Streptomyces* species

Lanes:	Marker	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10	Lane 11	Lane 12
Rows	(M.Wt.)	(M.Wt.)	(M.Wt.)	(M.Wt.)	(M.Wt.)	(M.Wt.)	(M.Wt.)	(M.Wt.)	(M.Wt.)	(M.Wt.)	(M.Wt.)	(M.Wt.)	(M.Wt.)
r1								270.83	277.5	284.17			
r2						227.5							
r3							215	210.83	210	211.67	214.17	211.67	214.17
r4						196.67	195.83	192.5	190.83	190.83	187.5	189.17	187.5
r5	165						158.33	152.5	157.5	155.83			
r6									134.17				
r7	115						118.33		105.74				
r8	80	73.871						76.935	73.871	78.161	75.097	73.258	83.088
r9	61		67.742			61	63.452	60.255	63.452	62.226	62.226	62.839	67.129
r10		52.431										54.667	57.647
r11			48.706	49.078	46.843	48.706	47.961	46.098	45.725	45.725	47.961	46.471	43.863
r12	42	41.808			39.885	42.373	41.615	40.846	41.038	40.846	42	41.231	
r13			38.731	37.577		38.731							
r14	32					34.885	34.885	34.885	33.731	34.115	34.5	34.115	32.577
r15		29.939	30.424	31.03	30.061	30.909	30.182	30.667	30.182	30.424	30.182	29.697	29.212
r16					26.061	27.758		27.152	28.242	27.758	27.394	28.121	
r17	24	24.606	24.848	24.364	22.477	23.662	24.485	23.154	23.831	24	24.727	24.364	24.848
r18								19.262		20.277			21.969
r19		18.585	18.585	18.415	18.754	17.738	18.246		18.923	19.092	18.754	18.415	18.585
r20								15.538	15.2	14.692	15.369	14.523	15.877
r21	13	13.169	13.338	12.803	13.338	13.169	13.338	13.508		11.82			
r22					10.246	10.049					9.459	9.459	10.148
r23									8.1803	8.8689			
r24					7.6885	7.4918	7.4918	7.8852	7.6885				
r25	7	5.3279	6.3115	6.3115	5.1311								
r26						3.0656	2.5738	2.7705	2.9672	3.1639			



Lane 1: larval protein of untreated Cx. pipiens (48 hrs.);
Lane 2: larval protein treated with isolate 108 (48 hrs.)
Lane 3: larval protein treated with isolate 110 (48 hrs.);
Lane 4: untreated larvae (72 hrs.)
Lane 5: treated larvae with 108 isolate (72 hrs.);
Lane 6: treated larvae with 110 isolate (72 hrs.)
Lane 7: untreated pupae;
Lane 8: treated pupae with 108 isolate
Lane 9: treated pupae with 110 isolate;
Lane 10: untreated adult
Lane 11: treated adult with 108 isolate;
Lane 12: treated adult with 110 isolate

Fig. 2: Photograph of electrophoretic protein patterns of larvae, pupae and adult stages of *Culex pipiens* after larval treatment with 110 and 108 *Streptomyces* isolates

Protein fraction in (Table 6) lanes 11 and 12, represent protein of adult stage which emerged after larval treatment with isolates 108 and 110 respectively while lane 10, represents adult from untreated larvae.

The isolate number 110 that exhibited high total mortality and high chitinase activity was identified to species level depending on 16-SRNA and it was *Streptomyces griseoflavus*.

DISCUSSION

Actinomycetes have provided many important bioactive compounds of high commercial value and screened for new bioactive substances.

The secondary metabolites of actinomycetes namely tetranectin, avermectins, faerifungin, macrotetrolids and flavonoids were found to be toxic to mosquito species [20, 21].

Our native actinomycetes isolates from our Egyptian habitat proved its toxicity to mosquito larvae not only in inducing mortality rate but also in delayed the life span of different developmental stages.

The isolate 110 was more potent than the isolate 108, as comparing LC_{50} values to be 8.19±2.05 and 17.48±4.60 ml /100 ml H_2O for the isolates 110 and 108 respectively after 48 hrs.

At 72 hrs. exposure time, the isolate 110 was still more potent to record LC_{50} values to be 3.18±1.44 ml /100 ml H_2O compering with LC_{50} values for isolate 108 to be 5.02±1.59 ml /100 ml H_2O.

On comparing chitinase activity of highly larvicidal isolates, results in (Table 2) proved that the isolate 110 was more active in hydrolyzing chitin (2.35 cm, an inhibition zone and 1.05 cm for 110 and 108 isolates respectively). *Streptomyces* chitinase was suggested to have a complex functional role in hydrolyzing chitin and cell walls [22]. Chitinolytic enzymes have been considered important in the biological control of pests and parasites [23].

These results were confirmed by delaying the growth rate of different stages of *Culex pipiens* (Table 4). *Streptomyces* chitinase could hydrolase insect chitin. The sugar moiety of peptidoglycan of and chitin has a basic structure which composed of β (1-4) glucosamine acetate residues as a repeating unit. *Streptomyces* chitinase can hydrolyze chitin with high affinity toward the glycosidic linkage between adjacent aminosugar residues [24]. Chitin formation inhibition, in insects, is usually achieved either by affecting the catalytic site of the synthase or by interfering at the sulfhydril-sensitive sites of the synthase during polymerization of acetyl-glucosamine residues [25, 26]. Insect cuticle consists largely of chitin, so chitinase producing *Streptomyces* could be involved in insect control strategy. Production of chitinases was used as the criteria for the selection of potential biocontrol agents in insect control [26]. The process of cuticular chitin deposition is coordinated with ecidysteroid regulated molting (ecdysis) during insect metamorphosis, this explain the delay in duration of larval and pupal stages. Major protein subunits of chitin synthase were produced to be integrated with epidermal cell proteins [27]. Treatment with *Streptomyces* metabolites may enforce insects to accelerate forming such units to help entering pupal phase. This may explain increase in total protein content after treatment (Table 5) where larval body proteins increased than normal to be 359.13 and 251.87 mg\g tissue after treatment of larvae with isolates 108 and 110, comparing with control experiment with 147.62 mg\g tissue.

Protein fractionation after 72 hrs. exposure time to isolate 108 proved the synthesis of new proteins, specially of high molecular weights as fractions of 227.5, 196.67 (KD) (Table 6 and lane 5) and 215, 195.83, 158.33, 118.33(KD), after treatment with isolate 110 (Table 6 and lane 6) and protein bands ranged from (61- 63.45 KD). These bands were completely absent in control larvae. It seems that treatment with *Streptomyces* isolates stimulate the immune response to activate other genes coded for protein synthesis of new types which may be consumed for recovery from toxic treatment, or may help to escape to the pupal phase. But similar bands appeared in pupal stage (Table 6 lane 8) which confirms the formation of such proteins in treated larval stage to escape toxicity by trying to enter the pupal stage.

These molecules may be the proteins of *Streptomyces* metabolites, chitinase enzyme, for example has about 60 KD as molecular weight extracted from *Streptomyces* sp. [22].

Emerged pupae, after larval treatment with isolate 108 showed special proteins of molecular weights 134.17 and 105.44 (KD). These units did not appear in control or even treatment with isolate 110. These proteins specified the metabolites of isolate 108. While protein of M.Wt. 284.17 and 20.27 (KD) specified toxicity by metabolite of isolate 110. Protein fraction of 54.66 (KD) specify the treatment with both *Streptomyces* metabolites (Table 6 lane 8, 9). In case of emerged adult no great changes could be realized. Bands of M.Wt. ranged from (54.66- 57.64 KD) appeared only after treatment with 108 and 110 isolates (Table 6 lane 11, 12).

CONCLUSION

A marked insecticidal activity by two native chitinolytic *Streptomyces* isolates was detected during this study. Metabolite of such species proved its toxicity to larvae of *Culex pipiens* the cytotoxic effect of such metabolite extended to the pupal and adult stage. *Streptomyces* metabolites affect the insect portions in a dramatic way.

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