# Mosquitocidal Activity of Extracts Derived from Soil Actinomycete Isolates

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## ABSTRACT

**This study investigates the insecticidal activity of 15 extracts of actinomycete isolates (NAM-1-NAM-15) sourced from diverse soil samples because the actinomycete strains were isolated from soil only against the third instar larvae of *Culex pipiens*. The isolates were cultured on starch casein agar, purified, subjected to fermentation add then solvent extraction to obtain the crude extracts. In primary Screening of the ethyl acetate extracts at a concentration of 1.0 mg/ml, NAM1, NAM2, and NAM4 achieved 100% mortality within one hour, indicating a high acute toxicity of these three extracts, while the rest of isolates exhibited a minimal toxic effect, with mortality rates as low as 3.33%. After six hours, NAM15 and NAM13 demonstrated moderate toxicity, with mortality rates of 46.67% and 36.67%, respectively. After 24 hours, all extracts (except NAM3) reached 100% mortality, indicating their insecticidal properties. $LC_{50}$ values showed that NAM1 had the highest toxicity at 0.21 mg/ml, followed by NAM4 and NAM2 at 0.52 mg/ml and 0.60 mg/ml, respectively. Biochemical analyses revealed distinct characteristics among isolates, with NAM2 exhibiting the highest enzyme activity, particularly on lipase and protease. This research confirms the potential of local actinomycete strains as effective biological agents for sustainable mosquito control, contributing to environmentally friendly pest management strategies.**

**Keywords: Actinomycetes Isolation, *Culex pipiens*, Larvicidal activity, biological control, $LC_{50}$ values, hydrolytic enzymes.**

## INTRODUCTION

Mosquitoes are the most public health important arthropods as they are the main vectors and reasons of occurrence and spread of numerous parasitic killing diseases, such as encephalitis, yellow fever, dengue fever, chikungunya and malaria that causing death of thousands of people yearly (Rahuman *et al.*, 2009 and Borah *et al.*, 2010). Mosquito-borne diseases are a big threat in various regions worldwide, particularly the tropical and subtropical areas. *Culex pipiens* L. (Diptera: Culicidae) serves as a vector for various human infections, including the West Nile virus, Rift Valley Fever virus, and Bancroftian filariasis (Claire and Callaghan, 1999). Mosquito management in Egypt is becoming complex due to the extensive resistance of *C. pipiens* to numerous pesticides. Zayed *et al.* (2006) documented the larval and adult resistance of *C. pipiens* to several insecticides involving carbamates, organophosphates, pyrethroids and organochlorines. Moreover, synthetic insecticides were used since many decades but, they caused numerous of environmental problems for instance soil and ground water contamination as well as increasing resistance (Balkew *et al*., 2010 and Naqqash *et al.*, 2016).

In recent decades, bioinsecticides attract attention because of their biodiversity, effectiveness and relative safety to humans and environment. Biopesticides are derived from natural sources, such as plants, bacteria, fungi, and viruses and are effective for control weeds, insects, and pathogens (Harshith, 2022). Bioinsecticides derived from microbial origin are among the most active natural compounds against wide range of insects with less risk effects on non-target organisms and environment comparing with conventional insecticides (Castillo *et al.* 2000). Among microorganisms, actinobacteria are one of the most important resources of bioactive compounds that can be used as insect control agents (Oka *et al.*, 2000). Actinomycetes are a large group of aerobic, Gram-positive bacteria belonging to the order actinomycetes and the phylum actinobacteria, in addition to having a high guanine-cytosine percentage (60–70 mol%) (Kekuda *et al.*, 2010). They are known as branching filaments or hyphae, and asexual spores produced more than shape vary from cocci and rods to complex multicellular structures like the mycelium of fungi.

Actinobacteria are cosmopolitan microorganisms widely present in soil, humus, litter, water, dung, arctic ice, deep sea, rock, and as endophytes in animals and plants for parasitism or symbiotic goals (Okazaki *et al.*, 1995 and Sharma *et al.*, 2014). Basically, actinomycetes are a constant component in

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soil, especially in dry alkaline soil, at a rate more than 30% of the entire microbial population equivalent to $10^6$ to $10^9$ bacteria per gram of soil (Bhatti *et al.,* 2017 and Yadav *et al.,* 2018). Soil actinomycetes play several roles in soil through the high synthesis of antimicrobial compounds and other bioactive metabolites, which increase the competitive advantage as well as the improvement of soil fertility by supporting plant growth by plant hormone production, providing preservation against phytopathogens, and suppressing environmental stresses (Javed *et al.,* 2021 and Nagendran *et al.,* 2021).

Previous research indicated that actinomycetes metabolites exhibit a broad range of biological activity against bacteria, fungi, viruses, nematodes, insects and herbicidal properties. Of the 22,500 bioactive chemicals derived from microorganisms, about 50% of the recognized natural antibiotics are sourced from actinomycetes. Streptomyces species create around 7,600 chemicals among actinomycetes (Miyadoh, 1993). Furthermore, actinomycetes are recognized as prolific makers of antibiotics, fungicides, bactericides, herbicides, insecticides, and acaricides. They can be utilized on target crops as culture filtrate, spore suspension, wettable powder, emulsifiable concentration, and wettable granules (Aggarwal *et al.,* 2017).

Many investigations have documented the toxic effects of actinomycete extracts against several pests, including *S. littoralis* (Osman *et al.,* 2007 and El-khawaga & Megahed, 2012); aphid sp. (Benbelkhir *et al.,* 2023); and *C. pipiens* (Kassem *et al.,* 2018 and Kim *et al.,* 2020). In addition, several metabolites from actinomycetes were employed widely as important agricultural products, such as spinosad, avermectin, emamectin, milbemycin, and polynactins, towards a variety of insect pests (Copping and Menn, 2000).

In this investigation, 15 actinomycete isolates have been isolated from soil samples. The isolates were fermented, and the extracts of isolates examined for the larvicidal activity against the 3rd instar larvae of *Culex pipiens*.

## MATERIALS AND METHODS:

### Chemicals

Spinosad (TC 98%) was provided by Kafr El-Zayat Pesticides and Chemicals Company, Egypt.

All other chemicals and solvents were of reagent grades and were obtained from reputed companies.

### Insects

A laboratory strain of *Culex pipiens* (Diptera: Culicidae) larvae were obtained from Medical Insect Institute, Agricultural Research Center, Dokki, Giza, Egypt. Insects were reared for several generations under controlled conditions at temperature of 27 ± 2°C, relative humidity 70 ± 10% and 12-12 light-dark regime. Adults were kept in wooden cages and daily provided with sponge pieces soaked in 10% sucrose solution for feeding. After this period the females were allowed to feed a blood meal from a pigeon host. Plastic cup containing dechlorinated tap water was placed in the cage as a place for egg laying. The obtained egg rafts were picked up from the plastic cups and transferred into plastic pans containing of dechlorinated tap water. The hatching larvae were provided daily with fish food as a diet (Kasap and Demirhan, 1992). The third instar larvae of the *C. pipiens* were used for the bioassay test.

### Soil Samples Collection

Soil samples were collected at 5 cm depth from various sites in Behera and Alexandria governments (Table 1). Soil samples (500 g) were placed in clean, tidy, dry and sterile polythene bags and transported in Ziploc bags to the laboratory (Saadoun *et al.,* 1999 and Arasu *et al.,* 2009). Samples were preserved at 4°C until starting work and some samples were stored at -40°C (Upright Freezer, Climas) for long period storage. The isolation of actinomycetes was carried out by the standard serial dilution method (Valli *et al.,* 2012 and Chaudhary *et al.,* 2013). To remove any solid material and unwanted items, such as plant or crop parts, roots, and stones that impede the solubility process, the soil samples were sieved. 10 g of each soil sample were added to separate conical flasks to create stock solutions, which were then suspended in 100 ml of either sterilised physiological saline (NaCl, 0.9%) or sterile distilled water. For every soil sample, the serial dilution procedure was used to create additional dilutions from the stock solution as follows: One millilitre of the original solution was aseptically removed and mixed with nine millilitres of sterile distilled water or regular saline solution (NaCl 0.9%), resulting in a final dilution of $10^{-1}$ and then $10^{-2}$. The final dilutions of $10^{-3}$ and $10^{-4}$ were produced using a similar method.

### Isolation of actinomycetes

To prevent bacterial and fungal contamination, respectively, the sterile medium was supplemented with chloramphenicol (34 mg/l), ampicillin (65 mg/l), clotrimazole (275 mg/l), and thioconazole (95 mg/l) after cooling but before solidifying. After cooling to roughly 45°C, the medium was then transferred into petri dishes. Using a sterile micropipette, 1 ml of the soil sample suspensions in the following dilutions ($10^{-1}$, $10^{-2}$, $10^{-3}$, and $10^{-4}$) were applied to each petri dish. Lastly, the plates were incubated aerobically for 7–14 days at 30°C, with periodic monitoring during the incubation period (Arasu *et al.,* 2008 and Kumar

*et al.*, 2014a, b). Actinobacterial colonies were separated and stored on starch casein agar media for further examination after incubation. The cross-streak method was used to cross-streak isolated actinomycetes (Oskay, 2009). Purified bacterial colonies displaying actinomycete-like characteristics were obtained through repeated streaking on starch casein agar plates. After being moved from the plates to starch casein agar slants, a few pure actinomycetes colonies were cultured for growth at 30°C. Following the incubation period, the slants containing pure isolated actinomycetes were kept in Eppendorf tubes at -40°C for a long time by adding 20% (v/v) glycerol.

**Identification of the selected actinomycetes**

The morphological and biochemical identification of the selected isolates were examined by methods described by Shirling and Gottlieb (1966).

**Morphological characteristics**

The cultural and growth properties of the isolates were assessed using the method of Shirling and Gottlieb (1966) on starch casein agar (SCA) medium, following incubation at 30°C for 7 days. Colony morphology was assessed based on growth intensity, growth pattern, colony color, aerial and substrate mycelium color, and pigmentation on SCA.

**Biochemical identification**

Qualitative biochemical assays were conducted in accordance with conventional protocols as stated by Akond *et al.* (2016) and Topatan and Kati (2022).

**Fermentation:** For seven days, a loop of slant culture of actinomycete isolates was cultivated on starch casein agar until the plate was almost completely covered in hyphal growth. After that, 250 ml Erlenmeyer flasks filled with 125 ml of starch casein broth were inoculated with fresh mycelium fragments. Well-grown culture agar cylinders, about 6 mm in diameter, were cut and put into the sterile conical flasks. Following sterilization, small pieces of new growth were added to the medium. To create secondary metabolites in the liquid medium, inoculated flasks were incubated for 14 days at 30°C on a shaker incubator set to 180 rpm. For every isolate separately, fermentation was carried out in eight conical flasks with a volume of one liter (Gao *et al.*, 2015).

**Extraction Process**

**Primary Extraction**: Primary extraction was carried out using a straightforward technique as described by Gao *et al.* (2015) and Vijayakumar *et al.* (2010). Using a sterile funnel, mycelial growth was separated from the filtrate or supernatant in this procedure. The method, known as filtration, required discarding the mycelium after all aliquots involved with the mycelium were removed. The filtrate was centrifuged for 15 minutes at 4°C and 10,000 rpm. The filtrate was subsequently used for bioassay experiments and stored at 4°C for further evaluations.

**Extraction of secondary metabolites:** In accordance with Liu *et al.* (2008), cultures of actinomycetes grown in a shaker incubator for 14 days in starch casein broth fermentation media were harvested by grinding the hyphae and adding ethyl acetate in a 1:1 ratio with the culture broth. Ethyl acetate solvent was used in the extraction process according to its polarity (Demain and Fang, 2000). The actinomycetes' culture broths were filtered through a cloth sheet to produce cell-free supernatant, and the hyphae were pulverized in a blender or homogenizer. After two hours of extraction at room temperature at 180 rpm on a shaker or 30 minutes at 30°C using a sonication device, an equal volume of filtrate broth and ethyl acetate solvent were added. To make sure that the solvent was distributed evenly throughout the broth and to increase separation efficiency, the crude extract and solvent mixture was shaken vigorously for five minutes before being separated into distinct layers using a separatory funnel. At a temperature of no more than 44°C, the organic layer was gathered, dried, and concentrated under vacuum using a rotary evaporator. After the first processes, a second extraction using ethyl acetate was performed on the aqueous layer. Following the solvents' drying and evaporation, the crude extracts were moved from the rotary evaporator flask to clean, closed vials by progressively adding a different solvent, such as ethyl acetate, acetone, or chloroform, in tiny amounts (roughly 1 ml), that had a high rate of evaporation at room temperature. Using a micropipette, the crude extract was transferred to the prepared vials after the solvents had been added gradually to dissolve it. We call this technique quantity transfer. To allow the solvents to completely evaporate and leave behind the precipitate of the absolute crude extract, the vials containing the solvent and crude extract mixes were allowed to sit at room temperature. To ensure their complete drying, these vials were subsequently put in a desiccator filled with $CaCl_2$. All extracts were kept at 4°C for additional assessment and analysis after being tested for insecticidal activity.

**Bioassay**

**Preliminary screening for the insecticidal activity against larvae of *culex pipiens* using dipping technique**

The larvicidal activity was determined by following the method of World Health Organization protocol with slightly modification (WHO, 1996 and Rahuman *et al.*, 2000). The third-instar larvae of *C. pipiens* were used to test the crude extracts of 15

actinomycetes isolates. 30 mg of crude extract was weighed and diluted in 30 ml of distilled water to produce one concentration, which was 1.0 mg/ml (1000 mg/l). The crude extracts were sonicated for 15 minutes at 32 °C to completely dissolve them in water. Three duplicates of the experiment each held ten larvae in plastic cups with a final capacity of ten ml. Extract was not used in the preparation of the control cups. After 1, 6, 12, and 24 hours of treatment, the death rate was noted.

**Secondary screening of NAM-1, NAM-2, and NAM-4 for insecticidal activity against larvae of *culex pipiens***

In the preliminary screening test, the extracts of the three cultures, NAM-1, NAM-2, and NAM-4, showed the greatest effectiveness against the third instar of *C. pipiens* at a range of concentrations. To figure it the $LC_{50}$, serial dilutions of the chosen extract were made at concentrations ranging from 0.03 to 1.00 mg/ml. Ten larvae of the third instar of *C. pipiens* were placed in plastic cups with ten millilitres of a mixture of extract and dechlorinated tap water for each replication of the concentration test, which was carried out in triplicate. After receiving treatment for 1, 6, 12, and 24 hours, mortality rates were recorded.

**Statistical data analysis**

The data were statistically analyzed for each experiment by subjecting to analysis of variance of one way (ANOVA) using COSTAT program 6.311software. Student-Newman-Keuls at $p = 0.05$ was used to test the differences among the treatments. The $LC_{50}$ values were determined according to Finney (1971).

## RESULTS AND DISCUSSION

**Isolation of actinomycetes**

On starch casein agar as a growth medium, fifteen actinomycetes isolates were grown and purified from various soils sources (soils, plants, and trees). The isolates were given codes (NAM-1- NAM-15) based on source of sample (Table 1). In some cases, the same sample with different dilutions gave two or three actinomycete isolates.

According to the current study, actinomycetes were isolated from unanalyzed soil samples that collected from various, climates, and locations. This produced unique isolates with a range of characteristics. Similar investigation has shown that soil and climate have an impact on actinomycete diversity, using a variety of isolation media such as starch casein agar (P*rashith et al.,* 2012), starch nitrate agar (El-Khawagh *et al.,* 2011), and yeast-malt extract agar (Amelia-Yap *et al.,* 2023). Starch casein agar has been identified as one of the most effective media for isolating actinomycetes (Al-Hulu, 2013 and Khattab *et al.,* 2016). Each isolate has its own unique characteristics, such as color, texture, growth, and shape. These results are supported by Harir *et al.* (2018), who found that actinobacteria are widely distributed in nature and are significant as a rich source of several bioactive secondary metabolites.

**Table 1. The code and soil sample source of actinomycetes isolates.**

| Code of isolate | Soil sample source |
|---|---|
| NAM1 | Rambler plant field |
| NAM 2 | Rambler plant field |
| NAM 3 | Clovers field |
| NAM 4 | Decomposed dungs |
| NAM 5 | Agricultural drainer |
| NAM 6 | Water channel |
| NAM 7 | Rambler plant field |
| NAM 8 | Water channel |
| NAM 9 | Banana tree 2 |
| NAM 10 | Agricultural drainer |
| NAM 11 | Banana tree 2 |
| NAM 12 | Banana tree 2 |
| NAM 13 | Water channel |
| NAM 14 | Water channel |
| NAM 15 | Banana tree1 |

A = The number of soil sample which refers to soil location and name.

Additionally, the variety of actinomycetes associated with climate, region, and dilution was demonstrated by isolation from soil samples (Kitouni *et al.,* 2005; Thawai *et al.,* 2008; Solecka *et al.,* 2012; Anandan *et al.,* 2016; Khattab *et al.,* 2016; Bhatti *et al.,* 2017 and Gomes *et al.,* 2018).

**Insecticidal activity of actinomycetes**

The insecticidal activity of 15 actinomycete isolates (NAM-1–NAM-15) obtained from different soils, plants, and trees (Table 2) against *Culex pipiens* larvae was investigated in this work. NAM-1, NAM-2, and NAM-4 caused 100% mortality after one hour in primary screening at 1.0 mg/ml, whereas most other isolates had little effects (3.33% mortality) (Table 2). Six hours later, isolates NAM-10 and NAM-14 showed 36.67% and 33.33% mortality, respectively, whereas isolates NAM-15 and NAM-13 indicated moderate toxicity (46.67% mortality). Conversely, NAM-3 continued to be ineffective. Mortality rates sharply rose after twelve hours, with isolates NAM8, NAM-9, and NAM-10 reaching 63.33%, 76.67%, and 76.67% mortality, respectively. While NAM-3 was not toxic, others, such as NAM-13, NAM-15, NAM-14, NAM-12, and NAM-11, achieved 90–93.33 percent mortality. Nearly every extract showed 100% mortality within 24 hours, indicating potent insecticidal effects. Overall, all actinomycete extracts, except NAM-3, displayed potent activity against mosquito larvae, with NAM-1, NAM-2, and NAM-4 being the most potent as inducing 100% mortality after one hour of exposure. The toxicity of all extracts of all isolates increased with increasing the exposure time. Prior studies have recognized the role of actinomycetes in biological pest control. This study evaluated the mosquitocidal activity of 15 actinomycete isolates, revealing that all isolates (except NAM3) had rapid and acute toxic effects on mosquito larvae, particularly after 24 hours of exposure. Given that mosquitoes transmit serious diseases like malaria and dengue fever, finding new bioactive compounds for mosquito control is crucial.

The results presented in Table (3) highlight the efficacy of selected actinomycete extracts against the 3rd instar larvae of *Culex pipiens*. Among the isolates, NAM-1 exhibited the highest toxicity with an $LC_{50}$ value of 0.21 mg/ml and an $LC_{95}$ of 0.73 mg/ml after 24 hours. NAM-2 and NAM-4 showed potent toxicity with $LC_{50}$ values of 0.60 and 0.52 mg/ml, respectively, and $LC_{95}$ values of 1.03 and 1.04 mg/ml. However, these extracts were less toxic compared to the reference insecticide spinosad with an $LC_{50}$ value of 0.01 mg/ml and an $LC_{95}$ of 0.03 mg/ml.

Studies by El-Bendary *et al.* (2010) and Kassem *et al.* (2018) align with these findings, reporting significant mortality rates in *C. pipiens* larvae.

**Table 2. The insecticidal activity of actinomycetes extracts at 1.0 mg/ml against the 3rd instar larvae of mosquito *Culex pipense* after different exposure periods using dipping technique***

| Isolate No | (Mean % mortality ± SE) at different intervals | | | |
|---|---|---|---|---|
| | 1h | 6h | 12h | 24h |
| Control | 0.00±0.00$^{b}$ | 0.00±0.00$^{d}$ | 0.00±0.00$^{c}$ | 0.00±0.00$^{c}$ |
| NAM1 | 100.00$^{*}$±0.00$^{a}$ | 100.00±0.00$^{a}$ | 100.00±0.00$^{a}$ | 100.00±0.00$^{a}$ |
| NAM 2 | 100.00±0.00$^{a}$ | 100.00±0.00$^{a}$ | 100.00±0.00$^{a}$ | 100.00±0.00$^{a}$ |
| NAM 3 | 0.00±0.00$^{b}$ | 0.00±0.00$^{d}$ | 0.00±0.00$^{c}$ | 0.00±0.00$^{c}$ |
| NAM 4 | 100.00±0.00$^{a}$ | 100.00±0.00$^{a}$ | 100.00±0.00$^{a}$ | 100.00±0.00$^{a}$ |
| NAM 5 | 3.33±3.34$^{b}$ | 13.33±6.67$^{cd}$ | 80.00±5.78$^{ab}$ | 80.00±5.78$^{b}$ |
| NAM 6 | 0.00±0.00$^{b}$ | 23.33±3.34$^{bcd}$ | 86.67±6.67$^{ab}$ | 93.33±3.34$^{a}$ |
| NAM 7 | 0.00±0.00$^{b}$ | 23.33±12.03$^{bcd}$ | 63.33±6.67$^{b}$ | 100.00±0.00$^{a}$ |
| NAM 8 | 3.33±3.34$^{b}$ | 20.00±0.00$^{cd}$ | 76.67±14.55$^{ab}$ | 100.00±0.00$^{a}$ |
| NAM 9 | 0.00±0.00$^{b}$ | 16.67±3.34$^{cd}$ | 76.67±3.34$^{ab}$ | 100.00±0.00$^{a}$ |
| NAM 10 | 3.33±3.34$^{b}$ | 36.67±6.67$^{bc}$ | 80.00±5.78$^{ab}$ | 100.00±0.00$^{a}$ |
| NAM 11 | 3.33±3.34$^{b}$ | 30.00±5.78$^{bc}$ | 83.33±8.83$^{ab}$ | 100.00±0.00$^{a}$ |
| NAM 12 | 3.33±3.34$^{b}$ | 23.33±8.83$^{bcd}$ | 90.00±5.78$^{a}$ | 100.00±0.00$^{a}$ |
| NAM 13 | 3.33±3.34$^{b}$ | 46.67±3.34$^{b}$ | 93.33±3.34$^{a}$ | 100.00±0.00$^{a}$ |
| NAM 14 | 3.33±3.34$^{b}$ | 33.33±8.83$^{bc}$ | 90.0±5.78$^{a}$ | 96.67±3.34$^{a}$ |
| NAM 15 | 3.33±3.34$^{b}$ | 46.67±8.83$^{b}$ | 90.0±5.78$^{a}$ | 100.00±0.00$^{a}$ |
| $LSD_{0.05}$ | 6.83 | 15.48 | 15.78 | 15.78 |

*Mean values within a column sharing the same letter are not significantly different at the 0.05 probability level.

Various actinomycetes have demonstrated high larvicidal activity against different mosquito species, suggesting a broad potential for biocontrol strategies. The obtained data have shown that all isolates possessed acute and rapid toxic effect with variable degrees of potent with the concentration. However, previous studies revealed that other actinomycetes isolates had insecticidal activity against *C. pipiens* (El-Khawagh *et al.,* 2011). The insecticidal activity of actinomycetes isolates against other *Culex* mosquitos, such as *C. quinquefasciatus* was reported by Singh & Prakash (2012) and Amelia−Yap *et al.* (2023). Moreover, actinomycetes isolates has been reported to possess insecticidal activity against *Aedes aegypti* (Kekuda *et al.,* 2012).

In addition to Samri *et al.* (2017) investigated the insecticidal potential of actinomycetes against the larvae, pupae and adults of the Mediterranean fruit fly *Ceratitis capitata* pronounced four actinobacteria isolates were significantly active against the first-instar larvae, and nine were active against the medfly adult, while no significant mortality was obtained against the third-instar larval and pupal stages. Valinomycin, produced by *Streptomyces griseus* var. *flexipertum* var. nov., was shown to exhibit insecticidal activity with $LC_{50}$ value of 2-3 ppm for mosquito larvae and 35 ppm for Mexican bean beetle larvae (Heisey *et al.,* 1988). And an antibiotic Aculeximycin, isolated from *Streptosporangium albidum*, was shown to exhibit mosquito larvicidal activity with an $IC_{50}$ value of 0.66 ppm (Ikemoto *et al.,* 1983).

**Morphological identification of NAM-1, NAM-2 and NAM-4 isolates**

Thee isolates that showed the highest insecticidal activity against the larvae of *C. pipiens* were subjected to morphological and biochemical identification. After 7 days of incubation, NAM-2 and NAM-4 demonstrated more prolific growth than NAM-1, indicating a higher biomass production capacity. The isolates exhibited distinct mycelium colors: NAM-2 had brown mycelium, likely due to melanin-like pigments, while NAM-1 and NAM-4 showed pale yellow and yellow mycelium, respectively, suggesting different pigment types. The aerial mycelium varied as well, with NAM-2's white mycelium contrasting the colored mycelium of NAM-1 and NAM4, reflecting differences in sporulation characteristics. None of the isolates produced diffusible pigments, which may be beneficial in applications where pigment production is undesirable. Additionally, differences in colony texture were noted, NAM-1 had a velvety texture, NAM-2 was filamentous, and NAM-4 was leathery. All isolates were Gram-positive, typical of actinomycetes, with thick peptidoglycan cell walls (Table 4). These morphological differences suggest potential variations in secondary metabolite production and bioactive compound exploration.

Observations on starch casein agar plates also revealed NAM-1's pale yellow substrate and aerial mycelium with good growth, while NAM-4 had abundant growth with yellow mycelium.

**Table 3. The insecticidal activity of the selected actinomycetes extracts NAM1, NAM2, NAM4 and Spinosad against the 3rd instar larvae of mosquitos *Culex pipense* after 24 hours of treatment.**

| Isolate. No | $LC_{50}$ (mg/ml) (95% confidence limits) | $LC_{95}$ (mg/ml) (95% (confidence limits) | Regression equation | Slope±SE | $X^2$ |
|---|---|---|---|---|---|
| NAM1 | 0.209 (0.145- 0.300) | 0.730 (0.441-1.217) | Y=-7.02+3.02x | 3.02±0.59 | 2.05 |
| NAM2 | 0.602 (0.536- 0.677) | 1.028 (0.824-1.285) | Y=-19.66+7.07x | 7.07±2.27 | 1.58 |
| NAM4 | 0.516 (0.435- 0.613) | 1.038 (0.774-1.393) | Y=-14.70+5.42x | 5.42±1.91 | 1.01 |
| Spinosad | 0.009 (0.006- 0.001) | 0.032(0.019 - 0.056) | Y=6.07+2.98x | 2.98±0.57 | 2.00 |

**Table 4. Morphological characteristics of selected isolates of actinomycetes isolates (NAM1, NAM2 and NAM4) after 7 days of incubation.**

| Features | NAM1 | NAM2 | NAM4 |
|---|---|---|---|
| Growth behavior | Good | Abundant | Abundant |
| Color of substrate mycelium | Pale yellow | Brown | Yellow |
| Color of aerial mycelium | Pale yellow | White | Yellow |
| Diffusible pigment | - | - | - |
| Texture | Velvety | Filamentous | Leathery & sticky |
| Gram staining | + | + | + |

NAM-2 stood out due to its wide, dense brown vegetative mycelium and white aerial mycelium. Mycelium color, branching, spore arrangement, and structure were further examined using oil immersion (100X).

The pigmentation of actinomycetes isolates was recorded as negative, aligning with findings from Ganesan *et al.* (2017). However, other studies report various cultural characteristics, some of which closely resemble to our data, while others differ significantly. Actinomycetes share structural similarities with fungi, particularly in mycelium and spore production, as noted by Anandan *et al.* (2016). Actinobacteria, a group of unicellular microorganisms within the bacteria kingdom, are typically aerobic and form substrate and aerial mycelium. They reproduce through binary division or spore/conidia formation, with sporulation occurring via fragmentation or segmentation of the aerial mycelium. Li *et al.* (2016) observed that substrate mycelium can vary in size, shape, and thickness, with a wide range of colors such as white, yellow, orange, red, and brown. These colors are produced by hyphae, and the pigments can be water- or fat-soluble. Abbas (2006) documented that actinomycetes isolates can form colonies with extensively branched vegetative mycelium and branching aerial hyphae, while Chukwuneme *et al.* (2020) observed white, gray, and brown aerial mycelium, along with various substrate mycelium colors such as brown and yellowish-brown. Naligama *et al.* (2022) noted colony morphologies featuring greyish white or white aerial mycelium, with reverse side pigments in colors like yellowish-orange and green.

In addition, Naligama *et al.* (2022) reported that colony morphology as greyish white and white aerial mycelium sometimes with non-pigmentation and other times yellowish orange, yellowish green and yellowish grey for different reverse side pigments. Sometimes the vegetative and aerial mycelium possessed the same color of mycelium as our isolates NAM-1and NAM-4 produced a single color as well as found both vegetative and aerial mycelium resulted the same colors, except white (Pérez-Corral *et al.*, 2022). Our isolates, NAM1 and NAM4, displayed consistent colors for both vegetative and aerial mycelium, similar to findings by Pérez-Corral *et al.* (2022). Many studies have confirmed the importance of Gram-positive staining in identifying actinomycetes (Mehta and Jadeja, 2022). However, growth behavior and pigmentation of actinomycetes strains can vary across studies due to differences in media and isolate characteristics. These unique features are essential for the identification and characterization of actinomycetes.

**Table 5. Qualitative biochemical identification of selected isolates of actinomycetes (NAM1, NAM2 and NAM4) after 7 days of incubation.**

| Enzyme | Substrate | Reagent | Positive reaction sign | NAM1 | NAM2 | NAM4 |
|---|---|---|---|---|---|---|
| Amylase | Starch 1% | Iodine solution | Clear zone | +++ | +++ | +++ |
| Catalase | 3 % hydrogen peroxide $H_2O_2$ | - | Oxygen bubbles | +++ | +++ | +++ |
| Caseinase | Casein | HCl solution | Clear zone | +++ | +++ | +++ |
| Urease | Urea 1% | Phenol red | Deep pink | + | +++ | ++ |
| Methyl red | Glucose | pH | Red | - | - | ++ |
| Lipase | Tween 20 1% | $CuSO_4$ solution 1% | Blue hales | + | +++ | ++ |
| Protease | Gelatin 1.5% | Mercury chloride solution | Pale zone | + | +++ | ++ |
| Cellulase | Carboxymethyl cellulose | Congo red dye + NaCl solution | Yellow hales | + | +++ | +++ |
| Pectinase | Pectin 1% | CTAB solution | Pale zone | - | + | - |
| Gelatinase | Gelatin agar | Freezing for 1 h | Fail to solidify | +++ | +++ | - |
| Oxidase | TMPD (tetramethyl-phenylenediamine dihydrochloride) | _ | Black zone | +++ | +++ | +++ |
| $H_2S$ | Protein | Lead acetate solution | Black precipitate | - | - | - |
| $NO_3$ reduction | Nitrate | Sulfanilic acid + α-naphthylamine | Red precipitate | + | - | +++ |

**Biochemical identification of NAM-1, NAM-2 and NAM-4 isolates**

The biochemical identification of selected actinomycete isolates, NAM-1, NAM-2, and NAM-4, was performed through qualitative enzyme activity tests. Each isolate was inoculated onto growth media containing specific substrates for various enzymes and incubated at 30°C for seven days. After incubation, specific reagents were added to detect enzyme presence. Most of the tested enzymes were hydrolytic, including amylase, catalase, caseinase, and oxidase, all of which exhibited positive reactions (Table 5). The presence of amylase was indicated by a clear zone after iodine was added, while catalase activity was shown by oxygen bubble formation with hydrogen peroxide. However, all isolates tested negative for hydrogen sulfide production and methyl red tests. Among the isolates, NAM-2 demonstrated the highest enzyme activity, particularly for lipase, protease, cellulase, and gelatinase, while NAM-1 showed limited enzymatic activity beyond core enzymes, suggesting a more specialized role. NAM-4 exhibited moderate to high activity across most enzymes, particularly in methyl red and nitrate reduction tests.

The enzymatic test results were documented to evaluate each strain's ability to analyze specific substrates, determining positive or negative reactions based on hydrolytic halos. The secretion of extracellular and intracellular enzymes by actinomycetes plays a significant role in various applications, including polymer hydrolysis, chemical synthesis, soil decontamination, biological disease control, and organic matter decomposition, thereby promoting plant growth (Anandan *et al.,* 2016). Additionally, extracellular enzymes may serve as secondary metabolites, such as antibiotics (Aggarwal *et al.,* 2017).

Previous studies have identified a range of enzymes from various genera of actinomycetes, highlighting their potential in biotechnological applications. For instance, enzymes like pronase from *Streptomyces griseus* and kerase from *Streptomyces fradiae* are used in the commercial production of biotechnology products (Pandey *et al.,* 2000).

Our findings align with reports from Minotto *et al.* (2014), who noted the positive enzymatic activity of actinomycetes isolates across different enzymatic tests. Actinomycetes are particularly adept at breaking down complex materials and recalcitrant polymers, such as lignocellulose, cellulose, pectin, and chitin, which are not utilized by other microorganisms. Swarna and Gnanadoss (2020) assessed 25 isolates for enzymatic activity, revealing that only five were dominant in the tested reactions. Biochemical characteristics, including the degradation of casein and the hydrolysis of urea, were also examined, with positive results for casein degradation. Additionally, Sowmya and Ramalingappa (2022) conducted various biochemical tests, including catalase and oxidase tests, and observed similar patterns of enzyme activity among their isolates.

Conclusion, the actinomycete extracts demonstrated significant potential for mosquito larval control, with varying levels of toxicity depending on the isolate. This research supports the idea that actinomycetes are valuable sources for developing new biopesticides, as their secondary metabolites show promise against a range of insect species, highlighting their importance in pest management strategies.

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## الملخص العربي

## النشاط الإبادي للبعوض لمستخلصات الأكتينوميسيتات المعزول من التربة

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تستهدف هذه الدراسة اختبار النشاط الإبادي الحشري لخمس عشرة مستخلصاً من عزلات الأكتينوميسيتات-NAM (1 إلى NAM-15) المأخوذة من عينات تربة متنوعة لأن عزلات الأكتينوميستات عزلت من عينات تربة فقط ضد الطور اليرقي الثالث لبعوضة *Culex pipiens*. تم تنمية العزلات على بيئة أجار الكازين والنشا، وتم تنقيتها وتخمرها ثم يضيف مذيب الاستخلاص للحصول على المستخلصات الخام. أظهرت التجارب الأولية أن مستخلصات الإيثيل أستيت عند تركيز ١,٠ مجم/مل، أن أكثر المستخلصات فعالية هي راشح العزلات NAM1 وNAM2 وNAM4 بنسبة موت ١٠٠% بعد ساعة واحدة من المعاملة، مما يشير إلى سمية حادة عالية لهذه المستخلصات الثلاثة، في حين أظهرت بقية العزلات تأثيراً سمياً ضئيلاً، حيث وصلت معدلات الموت إلى ٣،٣٣% فقط. يليها راشح سلالة NAM15 وNAM13، حيث كانت معدلات الموت ٤٦،٦٧% و٣٦،٦٧% على التوالي بعد ٦ ساعات من المعاملة. وبعد ٢٤ ساعة من المعاملة أظهرت جميع المستخلصات باستثناء (NAM3) نسبة موت بلغت ١٠٠%، مما يدل على خصائصها الفعالة كمبيدات حشرية. وأظهرت قيم $LC_{50}$ أن NAM1 كانت الأكثر سمية (٠،٢١ مجم/مل)، يليها كل من NAM4 وNAM2 بقيم ٠،٥٢ مجم/مل و٠،٦٠ مجم/مل على التوالي. كما أظهرت التحليلات البيوكيميائية لتلك العزلات خصائص مميزة لكل منها، حيث أظهرت NAM2 أعلى نشاط إنزيمي، خاصةً في إنزيمات الليبيز والبروتييز. تؤكد هذه الدراسة على إمكانيات سلالات الأكتينوميسيتات المحلية كعوامل بيولوجية فعّالة للتحكم المستدام في الباعوض (للمكافحة المستدامة للباعوض)، مما يساهم في الإستراتيجيات الصديقة للبيئة لمكافحة الآفات.

**الكلمات المفتاحية:** عزل الأكتينوميسيتات، كيولكس بيبنس، النشاط الإبادي لليرقات، المكافحة البيولوجية، قيم $LC_{50}$، إنزيمات التحليل المائى.