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An attempt and a brief research study to Produce Mosquitocidal toxin using Bacillus spp. (VITRARS), isolated from different soil samples (Vellore and Chittoor), by degradation of Chicken Feather Waste.

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ABSTRACT

The present modern industrial world results the problems related to the huge amount of poultry waste generated throughout the world due to industrial revolution, over population and urbanisation. The environmental burden due to such wastes is increasing at an alarming rate and thus there is a need for bioremediation. Previous treatment methods include land filling. But land filling pose problems of secondary pollutants like landfill leachate, greenhouse gases and odour. The modern treatment methods lead to the production of Mosquitocidal Toxins (Bio pesticides and Bio insecticides). For the production of Mosquitocidal toxins we used chicken feather powder (0.5%) for the preparation of bacterial culture media, on which we cultured entomopathogenic bacteria (Bacillus spp.) which completely degrades feather waste. The Bacillus spp. was screened from dumped poultry waste soils, sewage bed, river bed and pond bed of different locations at Vellore and Chittoor. The Mosquitocidal toxin is extracted and then assayed by testing on Culex larvae. The endotoxins produced are a potent larvicidal agent for mosquito. Thus reduces the overall consumption and harmful effect of commercially available mosquito repellents. Hence the treatment of feather waste can be envisaged. The screening methodology, tentative identification and Mosquitocidal nature of end product will be discussed.

Keywords: Chicken feather, Biodegradation, Bacterial toxins, Mosquito larvicide.

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INTRODUCTION

Chicken feathers are produced in a large quantity all over the world. According to [1], 18.5million thousand tons of feather waste is generated all over the world. They are very cheap bioorganic waste, which can be used as a substrate for the production of Mosquitocidal toxin. Keratins are the main chemical component of feathers. They are long chain of amino acids; two types of keratins are alpha-keratin and beta-keratin. Feathers are mainly composed of beta-keratin. Keratins are resistance to biodegradation. But we can use keratinase producing bacteria to degrade it. One such bacterium is Bacillus spp. (VITRARS). It is an entomopathogenic bacterium which produces endotoxins. These endotoxins have a larvicidal action and can be used as a mosquitocidal toxin. [4], mentioned feather waste is composed of 81% of protein, 1.3% of ash, 1.2% of fatand dry matter. Also according to [5, 6], feather consists of protein having a lot of sulphur containing amino acids (cysteine).

Malaria, filariasis, yellow fever and dengue are the major mosquito borne diseases. These diseases are mainly responsible for indisposition and death and disease-endemic countries faces major economic burden due to these diseases. According to [8], every year, around the world approx. 300 million people are struck by malaria, a lethal disease. Malaria is a risk to 2,400 million people which is 40% of world population. Uncontrolled urbanization and formation of conditions which are suited for mosquito development are the major cause of increment in mosquito-borne diseases. There are many cases of resistance development of mosquitoes towards chemical insecticides.

In India the main commercially available mosquito repellents are mosquito coil, mats, liquid vaporizer and sprays. All these products are composed of harmful chemical compounds like, Pyrethrins (causes headache, nausea and dizziness), N, N-diethyl-meta-toluamide in short DEET (causes skin irritation and has neurotoxic effect), Permethrin (causes eye irritation and has carcinogenic effect), P-mentane-3, 8-diol (causes skin redness and swelling) and Formaldehyde (causes irritation of upper respiratory tract.

According to [9], burning one mosquito coil would produce particulate matter mass equivalent to that of 75–137 cigarettes. Burning of mosquito coil also releases formaldehyde which is equivalent to release from burning 51 cigarettes.

To reduce above mentioned three problems, poultry waste degradation, mosquito-borne diseases and harmful mosquito repellent, we are producing Mosquitocidal toxin from the biodegradation of poultry waste which will ultimately reduce the overall consumption of harmful mosquito repellent.

MATERIALS AND METHODS

Screening of Microorganism- The microorganism used must be a keratinase producing and also entomopathogenic in nature; such bacteria are Bacillus spp. (VITRARS)which is arod-

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shaped and aerobic in nature. Their habitat is soil and forms endospore during adverse conditions. Entomopathogenic nature of such strain was first reported by [10]. This strain may be effective against Culex spp. and Anopheles spp.

For the screening, soil samples were collected from different locations like poultry farm(PF), sewage bed(S), river bed (R), pond bed (P) in Vellore and Chittoor (10 soil samples from each site). These samples were serially diluted.

Screening of keratinase producing bacteria can be done by baiting technique. In baiting technique, we inoculate the LB agar medium with serially diluted soil sample and place the small pieces of pre-treated feather (3Chloroform: 1Methanol).

Selective media can be prepared by using ions like Fe^{2+}, Ca^{2+}, Mg^{2+} and Mn^{2+}. Antibiotics such as streptomycin sulphate and chloramphenicol are used to screen antibiotics resistant bacteria. Other nutrients like L-arginine, thiamine and biotin are also added. Further keratinolytic nature of screened bacteria was confirmed by using production media for feather degradation.

Tentative identification of screened bacteria- Identification of Bacillus spp. (VITRARS) was done by spore staining and various biochemical tests which showed similar characteristics to Bacillus spp. (Fig. 2).

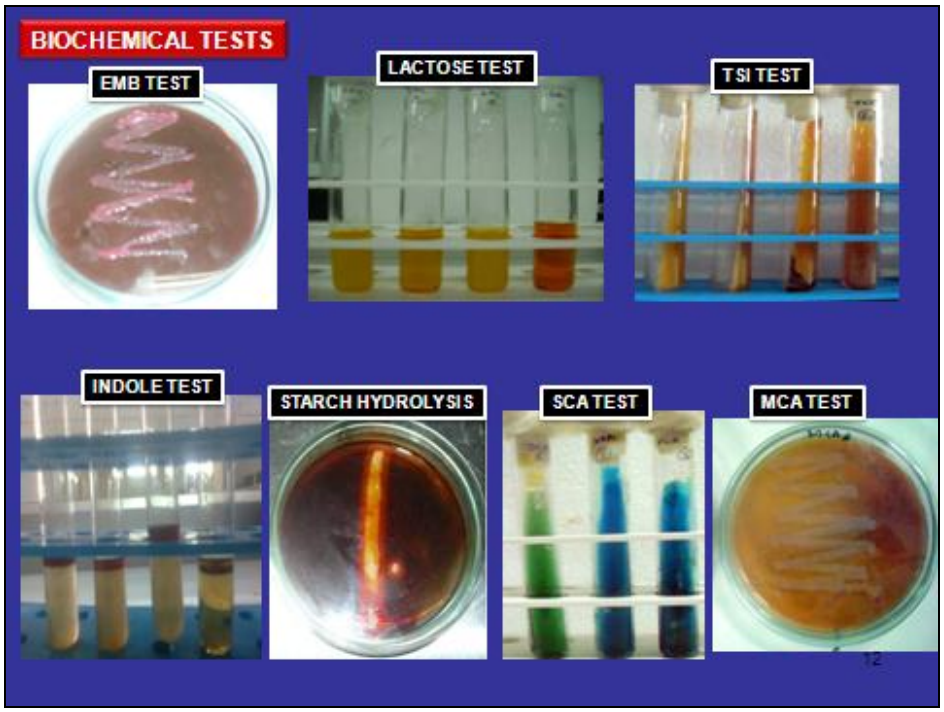

Fig. 2: Tentative identification of bacteria by biochemical tests. The tests prove that the screened bacteria were a Bacillus spp.

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Design of Bacterial culture media- Chicken feather wastes (CFW) were collected from sites near poultry farms. All the collected feathers were washed, air-dried, pre-+treated with chloroform, methanol mixture (3:1) and powdered to fine powder. This feather powder will act as a production media and can be kept at normal room temperature in air tight container.

For media preparation 10 grams feather powder was added to 2 litre of water(pH 7.5). This feather medium was poured in 6 conical flasks (inoculum from 8 best cultures + 1 control flask) of 500 ml capacity. The feather culture media was autoclaved.

Inoculum standardisation- The screened bacterium was inoculated in production media except 1 control flask (used as blank). Conical flasks were kept in orbital shaker (at 120 rev/min) to grow under room temperature (37°C). At every 12 hours optical density was measured at 670 nm using spectrophotometer. Agitation and optical density measurement is done till the sporulation stage which reaches its peak concentration at 72 hours.

Endotoxin extraction- Cell biomass is separated by centrifugation at 12,000 rpm for 20 min at 4°C. Cell biomass is washed twice each with 0.1M saline solution and ddH_2O at 12,000 rpm for 10 min at 4°C. At last they were washed with phenyl methyl sulphonyl fluoride, PMSF (protease inhibitor). Endotoxins from the spores are extracted by disruption of cells using 1g Alumina and performing sonication.

Bioassay- For the bioassay of endotoxins against Culex spp. firstly the larvae were collected from stagnant water bodies. After the collection larvae were used for susceptibility test against endotoxin.

In an enamel cup of 50 ml capacity 10 larvae were added. In each type of endotoxin different volumes of endotoxin were added (0ml, 1ml, 2ml, 3ml, 4ml, and 5ml), enamel cup without endotoxin was used as control. All cups were incubated at room temperature for 48 hours. Mortality of larvae was observed for each cup and % of larval mortality was calculated by:

$$\% \text{ of larval mortality} = \frac{\text{number of dead larvae}}{\text{total number of larvae}} \times 100$$

RESULTS

Screening of Microorganism- Bacterial spp. was screened using the selective media (Fig. 1). The colonies were **smooth**, with **circular form, convex elevation** and **Entire margin.** After Gram staining and endospore staining the cells were analyzed microscopically. The Colonies were rod shaped, Gram negative and endospore forming (Fig. 3). The screened Bacillus spp. is resistant to antibiotics like chloramphenicol and streptomycin. Slants were prepared to store the pure culture of isolated colonies.

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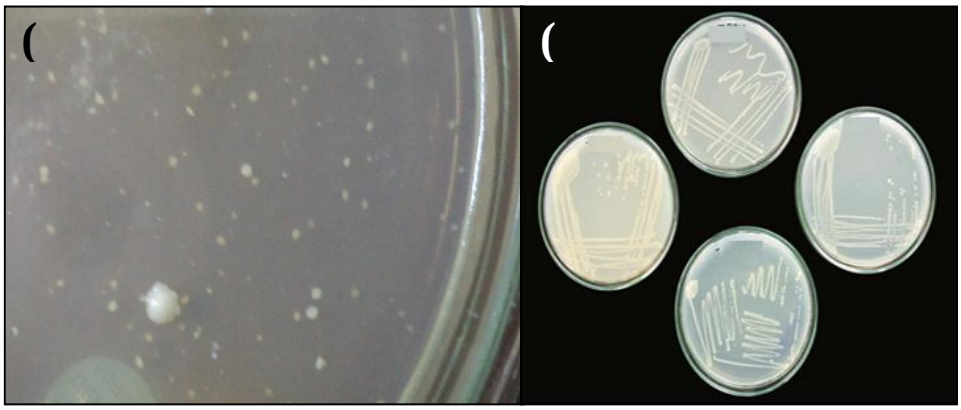

Fig. 1:*Bacillus spp.* was screened using selective media. (a). Colonies selected from soil sample. (b). Isolated pure colonies were obtained by streaking colonies with

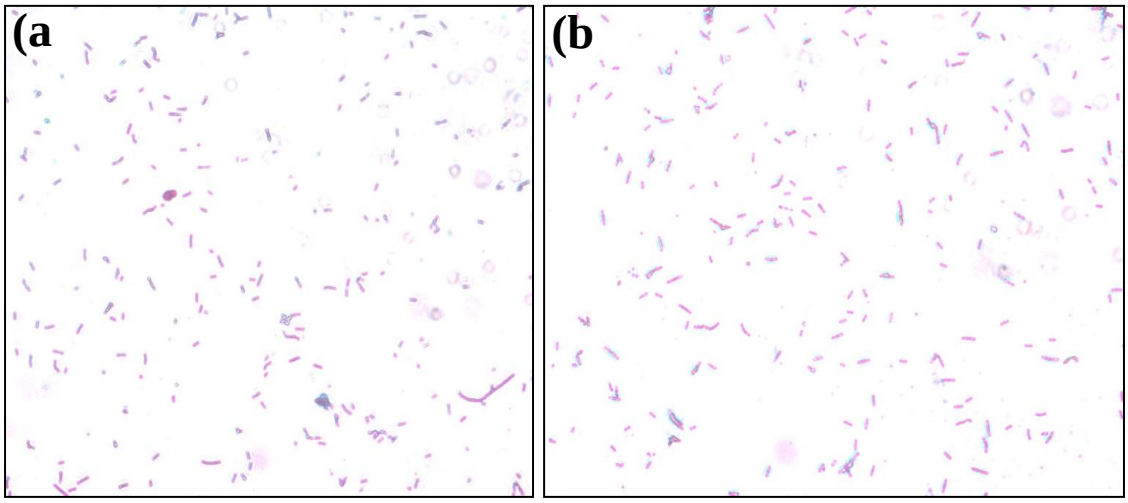

Fig. 3:Isolated Gram Negative Bacillus spp. (a) *VITRARS1.* (b) *VITRARS2.*

Keratinolytic activity- All the screened bacteria were added to production media composed of feather (Fig. 4). After analyzing the keratinolytic activity two samples from each site were selected with maximum activity (Table 1).

Growth pattern- Initially, culture growth rate was highest and the peak cell density reached after 48 hours. At the peak density the OD ranges from 2 to 2.5. After 48 hours the endotoxin is released by the disruption of cell biomass. Till 72 hours all the cell will be disrupted and at that point the suspension will have maximum concentration of endotoxin (Fig. 5).

Mortality Rate- For Culex spp. larvae % mortality in the case of PF (bacteria from Poultry farm soil) was highest at all the volumes of endotoxin, that means endotoxin produced by bacteria at poultry farm is more effective endotoxin followed by endotoxin produced by bacteria at sewage bed, river bed and pond bed (Fig. 6). The two strains with maximum activity were named VITRARS1 (from PF1) and VITRARS2 (from S2) (Fig. 7).

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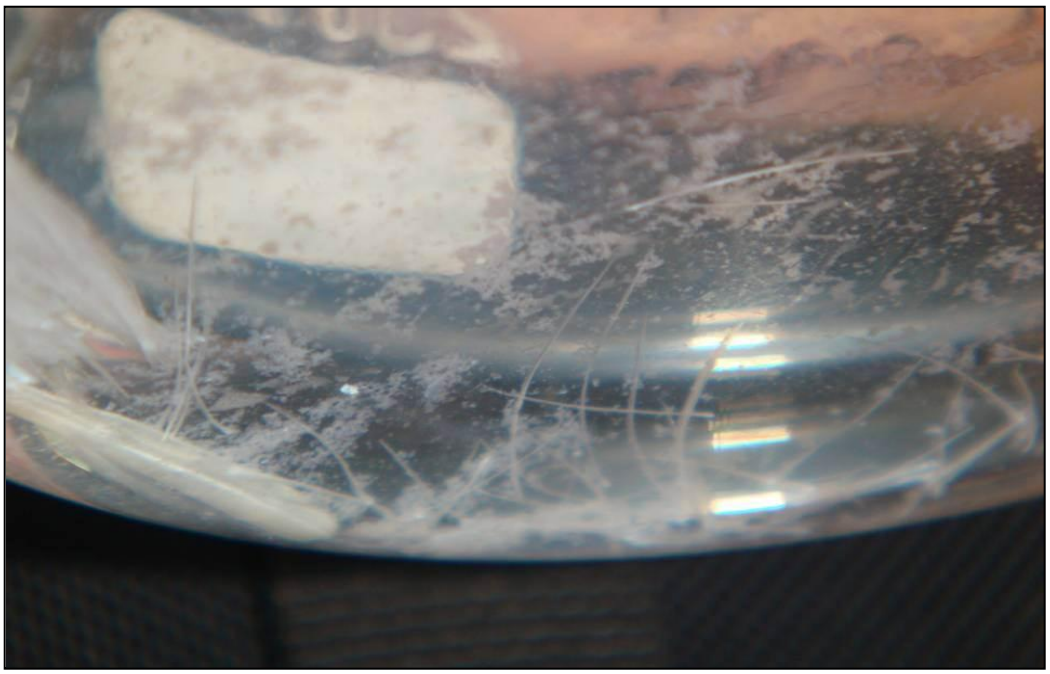

Fig. 4:Confirmation of keratinolytic nature of screened bacteria (from Poultry farm soil) by production media for feather degradation. This strain of bacteria was named as *Bacillus spp. (VITRARS)* and was used as a potential strain for mosquitocidal toxin production.

SOIL SAMPLES		MAXIMUM KERATINOLYTIC ACTIVITY	MAXIMUM PERCENTAGE OF MORTALITY FOR Culex spp. LARVAE
Poultry Farm	25	PF1, PF2	VITRARS1 (PF1)
Sewage Bed	25	S1, S2	VITRARS2 (S2)
River Bed	25	R1, R2	-
Pond Bed	25	P1, P2	-

Table 1: Strain selected with maximum percentage of mortality of larvae from 100 soil samples. The strain selected were also having maximum keratinolytic activity.

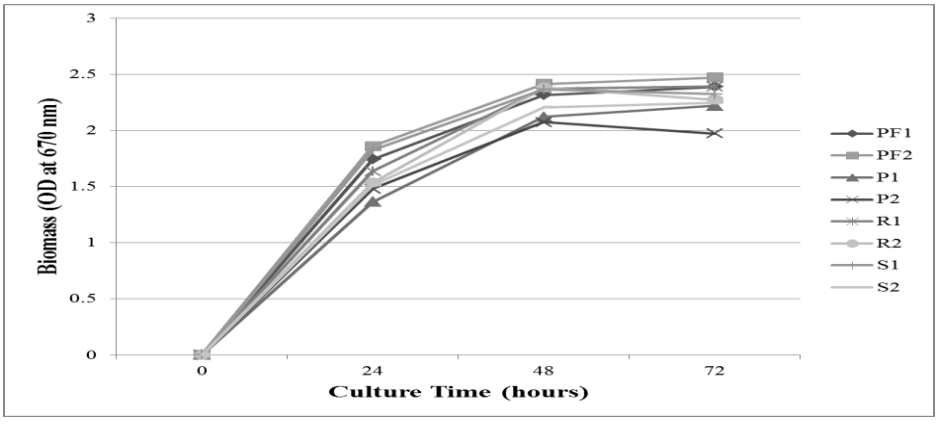

Fig. 5: Graphical representation of growth pattern of screened Bacillus spp. from different sites at intervals of 24 hours.

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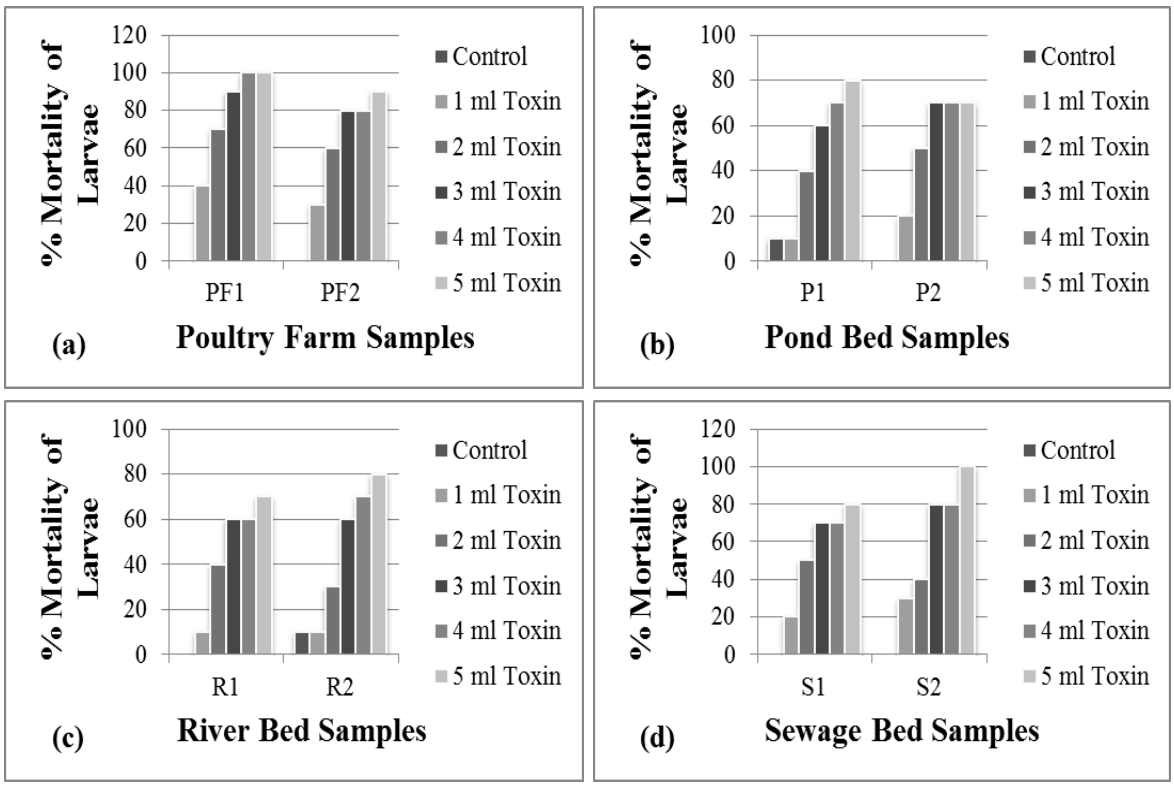

Fig. 6: Graphical representation of percentage mortality of culex spp. Larvae to toxins from different soil samples at different volume.

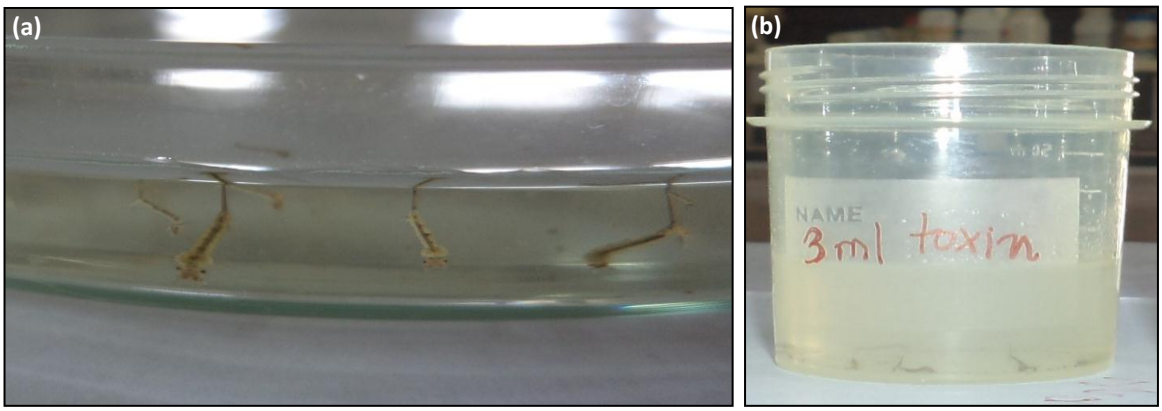

Fig. 7:Bioassay of mosquitocidal toxin on Culex spp. larvae. (a) Culex larvae (Alive) floating on the surface of water through siphon (breathing tube) before addition of mosquitocidal toxin from VITRARS. (b) After the addition of mosquitocidal toxin Culex larvae died and deposited to the bottom.

On the initial introduction of endotoxin the % mortality increased at large rate but on further addition the increase in % mortality rate was slow.

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DISCUSSION

An environmental threat is caused by poultry processing industries. Such industries are dumping their waste (chicken feather) in the environment in large quantities. Land filling, burning, production of natural gas and fertilizer are the primitive methods to remove the bulk feather waste.

The present aim of the study is to utilize the entire chicken feather waste (CFW), for the production of commercial product (mosquitocidal toxin). Since the microorganism was capable of growing on feather media, we can say that composition of feather waste is a self-sufficient medium and will allow the growth of degrading bacteria without addition of any other specific nutrients.

Bacillus spp. (VITRARS)provides effective alternatives to wide range of larvicides without harming the environment. When we spray chemical larvicides in aquatic environment it kills the aquatic animal also and reduces biodiversity in aquatic ecosystem. But the Mosquitocidal toxin from VITRARS1 and VITRARS2 has no ill effects on human and non-target organisms thus increase the biodiversity. It effectively reduces the population of mosquito, thus the overall consumption of harmful commercially available mosquito repellent is reduced. This reduces many human problems which arise from mosquito repellent.

The VITRARS1 and VITRARS2screened from soil enriched CFW has many advantages like complete degradation of feather waste; avoid environmental contamination, increased shelf life, easy preservation, transportation and convenience in handling and application. This method is highly economical as it utilizes cost free chicken feather waste.

This technology upholds triple benefits of complete removal of Chicken feather waste from nearby poultry farm sites, synthesis of Mosquitocidal toxin, and reducing the overall consumption of harmful mosquito repellent.

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