Available Online at http://www.recentscientific.com

ISSN: 0976-3031

International Journal of Recent Scientific Research
Vol. 5, Issue, 12, pp.2332-2336, December, 2014

International Journal of Recent Scientific Research

RESEARCH ARTICLE

ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF BACTERIAL ISOLATES FROM SOIL SAMPLES AND THEIR POSSIBLE ROLE IN THE MANAGEMENT OF MALARIA VECTOR ANOPHELES STEPHENSI (L.)

[1]Arti Prasad, [2]Devendra Kumar, [3]Esha Sharma and *[4]Priyansh Mathur

[1]Insect Microbial and Herbal Control Laboratory, Department of Zoology, University College of Science, Mohan Lal Sukhadia University, Udaipur, Rajasthan

[2,3,4] Research Scholars, Insect Microbial and Herbal Control Laboratory, Department of Zoology, University College of Science, Mohan Lal Sukhadia University, Udaipur, Rajasthan

ARTICLE INFO

Article History:

Received 6th, November, 2014
Received in revised form 17th, November, 2014
Accepted 8th, December, 2014
Published online 28th, December, 2014

Key words:

Anopheles stephensi, Bti, B. cerus, B. Polymyxa, B. subtilis, Bacteria, Vector control

ABSTRACT

The use of entomopathogen is a feasible substitute for insect control because of their virtual specificity and lower environmental impact. The search for microbial strains against Dipterans could have an impact on mosquito control programs. Bacterial pathogens are ubiquitous in soil and water. The potential for bacteria to play a role as pathogen of vectors is an important step towards ecosafe management of vectors. In the present investigation soil bacterial fauna were isolated to assess their insecticidal activities against *Anopheles stephensi* (L) larvae. The isolated bacterial colonies from soil were characterized according to morphological, physiological and biochemical parameters. High larvicidal activity was observed by three strains of bacillus i.e. *Bacillus polymyxa*, *Bacillus cerus* and *Bacillus subtilis* amongst all the identified strains. Further *Bacillus subtilis* strain IF5 (NCBI: KJ022639) displaying the highest activity stood first against both the second and fourth larval instar larvae with LC_{50} of 1.865 and 3.361x6.5x10^7 cfu/ml respectively followed by *B. cerus* and *B. Polymyxa* (LC_{50} 2.931 and 4.305 & LC_{50} of 4.776 and 5.403 x6.5x10^7 cfu/ml). Second instar larvae were more susceptible than fourth instar for all the bacterial isolates. These findings are a valuable tool for the control of mosquitoes of medical importance, *B. subtilis* strain IF5 can be recommended for industrial production of bacterial preparations. Further work is required to assess the epidemiological impact of this finding.

INTRODUCTION

Mosquitoes are a serious threat to public health transmitting several dangerous diseases in over 2 billi in recent year's measurement of ultrasonic investigations found extensive applications in determining the physicochemical behaviour of liquid mixture. Theoretical evaluations of ultrasonic velocity give a better understanding of molecular arrangements in liquids. Several researchers[1-5] carried out ultrasonic investigations and correlated the experimental results of ultrasonic velocity with the theoretical relations of Nomoto[6], Junjie7, Van Deal and Vangeel[8], Jacobson9, Schaaf10 and Impedance dependence relation[11]. In the present communication the aforementioned relations have been used to predict ultrasonic velocity in ternary liquid mixture of tetrahydrofuran with octane and decane at temperatures of 303 K, 308 K and 313 K.

On people in the tropics (Odalo *et al*., 2005). Among the disease transmitting insects, the mosquitoes are the primary hosts for transmission of diseases like malaria, dengue, chikungunya, lymphatic filariasis, yellow fever etc., which together are responsible for several million deaths and hundreds of millions of cases every year (Chandel, *et al*., 2013). Mosquito control and personal protection from mosquito bites are currently the most important measures employed to control these diseases. Many approaches have been developed that aim to diminish mosquito menace. The use of larvicides and repellents can be an economical and practical way to prevent the transmission of these diseases to humans. The common approach for the control of mosquito vectors and reducing the transmission of human pathogens is based on the chemical insecticides (Paul *et al*., 2006). Chemical insecticides remained control which the insects quickly develop resistance which requires either increasing of dosages, or insecticide rotation. Lacking the selectivity of impact, chemical insecticides cause the death of non-target and often useful organisms. The accumulation of insecticides in natural constituents (water, soil, etc.) makes them environmentally hazardous. These shortcomings make it necessary to find new environmentally friendly methods of bloodsucking insect control. The main advantage of biological agents when compared to chemical ones is selectivity. As the extensive use of chemicals to control insect pests has been found to have detrimental effects on people and the the main

* Corresponding author: **Priyansh Mathur**
Research Scholars, Insect Microbial and Herbal Control Laboratory, Department of Zoology, University College of Science, Mohan Lal Sukhadia University, Udaipur, Rajasthan

way for bloodsucking insect control, to environment, there is a pressing need to discover and develop new entomopathogens to these insects biologically. Therefore, bacteria discovered in this study to have entomopathogenic potency against mosquitoes can be a good alternate in vector control. Based on the effects, *Bti* have been used for over 30 years, with almost no cases of their negative effect on other organisms. There was no on occurrence of insect resistance to these agents also. Given the ability of *Bti* to samples malaria vector *Anopheles stephensi.*

Synthesize 4 types of protein toxins; it is hardly possible to predict the emergence of resistance in the future. According to WHO (2012), in future the share of biological agents for bloodsucking insect control should grow by isolation of new effective strains of bacteria, improvement of formulations, cost reduction and development of sustainable tactics of their use. For adult insect control there is still no alternative to chemical agents, however for destruction of larvae the biologics are increasingly used. These considerations confirm the relevance of the present investigations. Hence, in the present investigation an attempt was made to evaluate the toxicity of different bacterial strains isolate from collected soil

MATERIAL AND METHODS

Mass Rearing of Mosquito Larvae

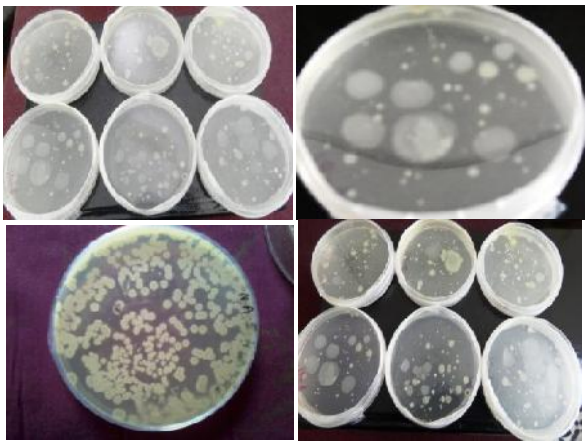

Figure 1 Isolated Bacterial Colonies

Mosquito larvae were collected from different water bodies. Species identification was carried out in Insect Microbial and herbal Control Research Laboratory, MLS University, Rajsthan, India. The mosquito larvae were identified as Anopheles stephensi (L). The collected larvae were acclimatized under proper temperature and humidity (Anjali *et al*., 2011). The strain was maintained in our laboratory as per WHO protocol.

Soil Sample Collection

The soil samples were collected randomly from different locations and were brought to the laboratory in sterile polythene bags and stored at 4˚C.We are considering here, isolation, characterization and identification of three bacterial species

Isolation and identification of Bacillus Species

Nutrient agar medium was prepared and sterilized at 121˚C in

15lbs pressure for 15 minutes. One gram of soil sample was serially diluted. 100µl of the aliquots from the 10^7 was spread over the NA plates and incubated at 28± 2˚C for seven days. After the incubation period, the bacterial colonies were purified by streaking on nutrient agar plates. The bacterial isolates were identified on the basis of morphological (colony morphology, spore morphology, and pigmentation) and biochemical properties. Morphological method consisted of macroscopic and microscopic methods. The microscopic characterization was done by cover slip culture method (Kawato and Sinobu, 1959). The observed structures were compared with Bergey's manual of determinative Bacteriology (Davies *et al*., 1999).

Larvicidal bioassay

Larvicidal activity of *Anopheles stephensi* was assessed by following the standard WHO larval susceptibility test method (WHO, 2005). In brief, twenty early 2nd and 4th instar larvae were taken and treated with bacillus colonies. The serial dilutions ere done and doses were determined accordingly (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 of 6.5x107cfu/ml). Similarly the un inoculated culture medium was used as control. For each dose five replicates were maintained at a time. Additionally all the assay units were supplemented with larvae fed with a diet of finely ground brewer's yeast and dog biscuits (3:1) ratio. The LC50 values were calculated after 24 h by probit analysis (Finney, 1971). The larvae of *An. Stephensi* were collected 2nd and 4th instar larvae were used for bioassay test. A total of 100 larvae were exposed in five replicates of 20 larvae each. Experiments were conducted for 24 h at room temperature (28 ±2 ˚C). The numbers dead larvae were counted after 24 h of exposure, and the percentage mortality was reported from the average of five replicates. The dead larvae were identified when they failed to move after probing with a needle in the siphon or cervical region**:**

RESULTS & DISCUSSION

In the present study different bacillus species were isolated from soil. These isolated bacterial colonies were identified on the basis of their morphological and biochemical properti

Table 1 Preliminary Identification of Isolated Bacteria

S.No	Organism	Grams Staining	Spore Staining	Catalase Test	Oxidase Test	HLA Test
1	B.polymyxa	+ve	+ve	+ve	+ve	+ve
2	B. subtilis	+ve	+ve	+ve	+ve	
3	B. cerus	+ve	+ve	+ve	+ve	+ve

Table 2 Biochemical Test

S.No	Organism	Indole	Methylene Red	Vogas Proskauer	Citrate	Urease	H_2S
1	B.polymyxa	+ve	+ve	-ve	+ve	-ve	+ve
2	B. subtilis	+ve	+ve	+ve	+ve	+ve	-ve
3	B. cerus	+ve	-ve	+ve	+ve	+ve	-ve

Table 3 Carbohydrate FermentationTest (A-Acid, G-Gas**)**

S. No	Organism	Glucose	Manitole	Lactose	Sucrose
1	B.polymyxa	+ve	+ve	+ve	+ve
2	B. subtilis	+ve	+ve	+ve	-ve
3	B. cerus	+ve	-ve	+ve	+ve

The bioassay results revealed the toxicity level of different strains. The results of larvicidal activities of the selected strains,ie B. subtilis, B. cerus, B. polymyxa have been given in table 5 & 6.

Table 4 Confirmative Biochemical Test

S. No	Organism	StarchAgr Test	NitrateBroth Reaction	Gelatin	Casein
1	B.polymyxa	+ve	-ve	-ve	+ve
2	B. subtilis	+ve	+ve	+ve	-ve
3	B. cerus	+ve	-ve	+ve	-ve

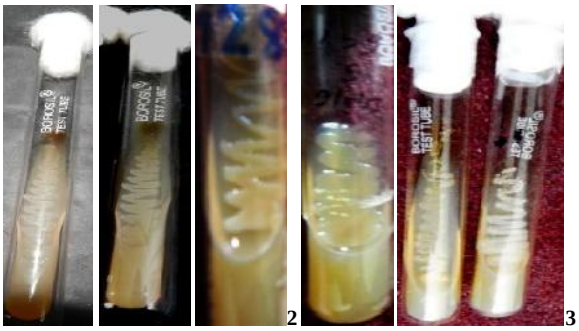

Fig 2 pure culture of *B.subtilis*(1), *B.cerus*(2), *B.polymyxa*(3)

Table 5 Relative potency of B. polymyxa, B. subtilis and B. cerus at second instar larvae of An. Stephensi

S.No	Organism	LC_{50}	Lower limit	Upper limit	Index	RR	Slope
1	B. subtilis	1.865	0.433	1.219	100	1	1.249
2	B. cerus	2.931	1.23	2.501	44.64	2.24	0.296
3	B.polymyxa	4.776	4.42	7.243	14.924	6.701	1.116

Table 6 Relative potency of B. polymyxa, B. subtilis and B. cerus at fourth instar larvae of An. Stephensi

S.No	Oganism	LC_{50}	Lower limit	Upper limit	Index	RR	Slope
1	B. subtilis	3.361	0.433	2.145	70.33	1	1.021
2	B. cerus	4.305	3.805	5.586	33.250	1.78	0.623
3	B.polymyxa	5.403	4.442	9.415	16.780	3.34	1.862

In all the treatments, the percentage mortality increased with increase in concentrations with B. subtilis treatment showing the highest percentage mortality and lowest LC_{50} value.. Although, there was significant difference in percentage mortality of B.cerus and B. polymyxa at 24 h of exposure (Figure 3), at 48 h of exposure to all the treatments, gradual increase in percentage mortality was recorded and results from each treatment were significantly different from each other (Figure 4).In Figure 5, decrease in percentage mortality of the inoculated mosquito larvae was observed. This could be as a result of decrease in the ingestion rate due to the age of the larvae. During this present study, the mosquito larvae showed greatest susceptibility to B. subtilis when compared to the other tested bacterial isolates. Toxin concentration of 4 to 5 folds of B. polymyxa was necessary to induce the same effect of 50% mortality (LC_{50}) on the larvae when compared to B. subtilis while 2 folds of B.cerus concentration will cause the same effect and resistance ratio (RR) showed that the three tested organisms in second instar varied. B.cerus showed that the resistance ratio (RR) values was 2.24 folds above that of B. substilis olds of B. polymyxa was necessary to induce the same effect of 50% mortality (LC_{50}) on the larvae when compared with B. subtilis while 2 folds of B. cerus. Concentration will cause the same effect and RR values of B.cerus were 1.78 folds above that of B. substilis while B. polymyxa was 3.34 folds above Bacillus substilis in fourth instar. The observed difference in the susceptibility might be due to their ingestion rate (Sun *et al.*, 1980). Hence more mortality of the mosquito larva was recorded in the second instar treatments.

Isolate Bacillus subtilis having potential to kill larve of An.stephensi was sent for genetic level identification to Xcleris Lab Ahmedabad India for strain identification. The isolate was identified as a Bacillus subtilis strain IF 5 (GenBank Accession Number: KJ022639) based on nucleotide homology and phylogenetic analysis (Fig 5). And we registered these bacteria in NCBI ACCESSION NUMBER is KJ022639 while B. Polymyxa was 6.70 folds above B. substilis. Toxin concentration of above 3 f

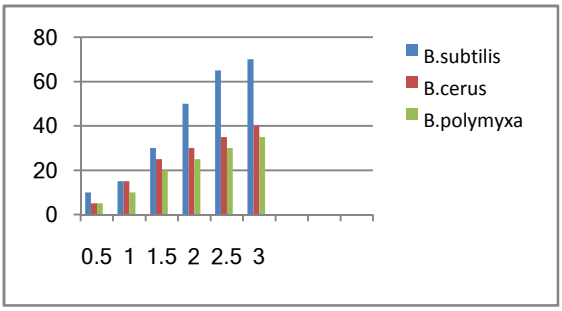

Figure 3 Effect of different concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 of 6.5×10^7 cfu/ml) of bacterial isolates on the mortality of 2nd instar Anopheles stephensi larvae at 24 h.

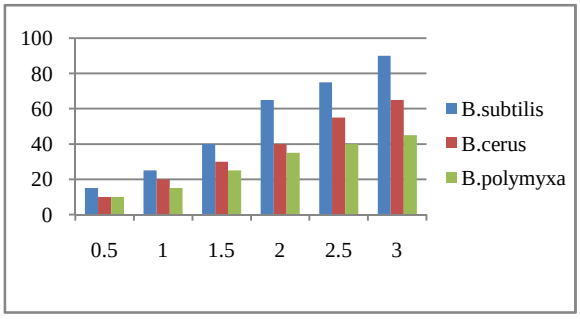

Figure 4 Effect of different concentrations (0.5. 1.0, 1.5, 2.0, 2.5, 3.0 of 6.5×10^7 cfu/ml) of bacterial isolates on the mortality of 2nd instar Anopheles stephensi larvae at 48 h.

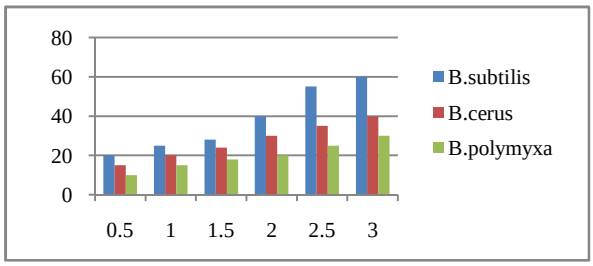

Figure 5 Effect of different concentrations (0.5. 1.0, 1.5, 2.0, 2.5, 3.0 of 6.5×10^7 cfu/ml) of bacterial isolates on the mortality of 4th instar Anopheles stephensi larvae at 48 h.

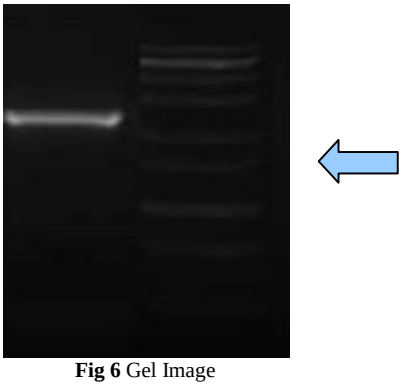

Fig 6 Gel Image
Lane 1: 16S rDNA amplicon band
Lane 2: DNA marker

1. SAMPLE_8F_S6073_023_E03.ab1: Data obtained with Forward primer
2. SAMPLE_1492R_S6073_021_F03.ab1: Data obtained with Reverse primer

SAMPLE_8F_S6073_023_E03 (522bp)

GCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACAC
GTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGG
GAAACCGGGGCTAATACCGGATGGTTGTTTGAACCG
CATGGTTCAAACATAAAAGGTGGCTTCGGCTACCAC
TTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTG
AGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGA
CCTGAGAGGGTGATCGGCCACACTGGGACTGAGACA
CGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAAT
CTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCC
GCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCT
GTTGTTAGGGAAGAACAAGTACGGTTCGAATAGGGC
GGTACCTTGACGGTACCTAACCAGAAAGCCACGGCT
AACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGG
CAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTC
GCAGGCGGTTTCTTAAGT

SAMPLE_1492R_S6073_021_F03 (930bp)

ACTTCGGGCGTTACAAACTCTCGTGGTGTGACGGGC
GGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGC
ATGCTGATCCACGATTACTAGCGATTCCAGCTTCACG
CAGTCGAGTTGCAGACTGCGATCCGAACTGAGAACA
GATTTGTGGGATTGGCTTAACCTCGCGGTTTCGCTGC
CCTTTGTTCTGTCCATTGTAGCACGTGTGTAGCCCAG
GTCATAAGGGGCATGATGATTTGACGTCATCCCCAC
CTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTG
CCCAACTGAATGCTGGCAACTAAGATCAAGGGTTGC
GCTCGTTGCGGGACTTAACCCAACATCTCACGACAC
GAGCTGACGACAACCATGCACCACCTGTCCCTCTGC
CCCCGAAGGGGACGTCCTATCTCTAGGATTGCCAGA
GGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTC
GAATTAAACCACATGCTCCACCGCTTGTGCGGGCCC
CCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTAC
TCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCA
CTAAGGGGCGGAAACCCCCTAACACTTAGCACTCAT
CGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGT
TCGCTCCCCACGCTTTCGCTCCTCAGCGTCAGTTACA
GACCAGAGAGTCGCCTTCGCCACTGGTGTTCCTCCA
CATCTCTACGCATTTCACCGCTACACGTGGAATTCCA
CTCTCCTCTTCTGCACTCAAGTTCCCCAGTTTCCAAT
GACCCTCCCCGGTTGAGCCGGGGGCTTTCACATCAG
ACTTAAGAAACCGCCTGCGAGCCCTTTACGCCCAAT
AATTCCGGACAACGCTTGCCACCTACGTATTACCGC
GGCTGCTGGCACGTAGTTAG

Fig 6: 16S rRNA sequence of Bacillus subtilis species.

The ideal method of controlling mosquitoes is by targeting them at the stage at which they are vulnerable i.e., the larval stage. Therefore, mosquitoes can be controlled by application of insecticides to their habitat, where, the mobility of the larvae is low (Wiseman & Chapagain, 2005). The use of synthetic-based pesticides over the past few years has increased the resistance toward the vectors (Rodriguez *et al*., 2002), thus, instilling the need for finding alternative ways to control mosquitoes.

Biological control is the use of natural enemies to deal with mosquito populations. There are several types of biological control including the direct introduction of parasites, pathogen and predators to target mosquitoes (Kenneth, 1995) or by using the dead spores of varieties of the natural soil bacteria which are used to interfere in the digestion systems of larvae. These spores were no longer effective after the larvae turn into pupae because they stop eating (Walker and lynch, 2007). Bacterial larvicides have been used for the control of nuisance and vector mosquitoes for more than two decades. The discovery of bacterium like bacillus, which is highly toxic to dipteran larvae, has opened the possibility of its use as a potential biolarvicide in mosquito eradication program worldwide (Kalfon *et al*., 1984). An important alternative measure to chemical insecticides is biological control measure which involves the regulation of pest population using natural control agents such as predators, nematodes and microbial insecticides (Merritt *et al*., 2005). It is the use of one biological organism to control another; releasing bacteria, fungi or arthropods to limit pest infestation (Weinzierl *et al*., 2005) noted that the organisms used in microbial insecticides are essentially non toxic and non pathogenic to non target organisms. The safety offered by microbial insecticides is their greatest strength. Bacteria and fungi have been shown to kill mosquitoes to varying degrees (Orduz *et al*., 1991; Su *et al*., 2001). Bacillus thuringiensis var isrealiensis (BTI) and B. sphaericus are being used in worldwide field test designed to control mosquito's population (Philip *et al*., 2001). These microorganisms have their own limitations which include low persistence of the bacteria larvicidal crystal protein in warm environment as a result of sunlight inactivation. The direct use of Bti strain also has its drawbacks as its cells do not exhibit stable in their environment. In addition, the strains of mosquito existing in a particular region appear different from that in another place. Thus one Bti strain may not be effective for use in all regions where mosquitoes are problematic. Therefore the isolation of other bacterial strain with larvicidal activity having a broad host range specifically, stable habitation and non hazardous properties is desired.

Bacillus is an important group that produces many biologically active compounds and secondary metabolites. They play an important role in biological control of many insects especially dipterans (Hussain *et al*., 2002: Gadelhak *et al*., 2005). Strong larvicidal activities against culex and anopheles larvae for bacillus have been reported by many workers (Sundarapandian *et al*., 2002). Hence isolation of such bacterial strain for the future use in mosquito control can be a welcoming step in the field of vector control.

CONCLUSION

Mosquito borne diseases are extensively spreads in the world population and influenced the global economy also. Consequently it should be eradicated from the world through the usage of the non-polluted mosquitocidal agents like microbial metabolites. Bacillus exhibited its effect against mosquito Anopheles stephensi. So it can be used as, an alternative insecticides because they are free from harmful effects on the environment. Further studies needed for identification the active compounds that can be used in broad spectrum for controlling insects and also determination the mode of action of these compounds.

Acknowledgement

We are thankful to Department of Zoology, College of

Science Mohan Lal Sukhadia University Udaipur (Rajasthan) for providing necessary laboratory facilities. We are also thankful to Deputy Director Regional Disease Diagnostic Center, Department of Animal Husbandry, Udaipur.

Conflict Of Interest Statement

We declare that we have no conflict of interest.

References

Anjali, C.H., Sudheer Khan, S., Goshen, K.M., Magdassi, S.,Mukherjee, A. & Chandrasekaran, N. (2011). Formulation of water dispersible nanopermethrin for larvicidal applications.Ecotoxicology and Environmental Safety 73, 1932–1936.

Chandel K, Mendki MJ, Parikh RY, Kulkarni G, Tikar SN. (2013). Midgut Microbial Community of Culex quinquefasciatus Mosquito Populations from India. PLoS ONE 8(11): e80453. doi:10.1371/journal.pone.0080453.

Davies, C. and B. Williams. (1999) Genus Bacillus in Bergeys manual of systematic bacteriology sneath, PH. Ed Williams and Wikins Company Baltimore, 102:77-88.

Finney, D.J, (1971). Probit Analysis. Cambridge Univ. Press, Cambridge, 333.

Gadelhak, G.G. EL-TARABIL, Y. Kh. A. and F.K. AL-Kaabi (2005). "Insect control using chitinolytic soil actinomycetes as biocontrol agents", Int. J. Agri. Biol, 7 (4): 627–63.

Hussain, A.A. Mostafa, S.A. Ghazal, S.A. and. Ibrahim, S.Y. (2002) "Studies on antifungal antibiotic and bioinsecticidal activities of some actinomycete isolates". African J. Mycol. Biotechnol, 10: 63–80.

Kalfon, A. Charles, J.F Bourgouin, C. De Barjac H, (1984)"Sporulation of Bacillus sphaericus 2297: an electron microscope study of crystal like inclusion, biogenesis and toxicity to mosquito larvae", J Gen Microbiol 130: 893–900.

Kawato M, Shinobu, R (1959). "A simple technique for the microscopical observation, memoirs of the Osaka University Liberal Arts and Education", 114, 1959.

Kenneth, T.M, (1995.)PART111:INTERACTION31, "A Comparative Study of the ability of fish to Catch Mosquito Larvae Rice-fish culture in china", International Development Research Center. ISBN, , 1-55250-313-5.

Merritt, R.W. Lessard, J.L. Wessell, K.J. Hernandez, O. Berg, M.B, Wallace, J.R. Novak, J.Ryan, A J. Merritt, B.W. (2005)Mosq Control Assoc, , 21,201–212.

Odalo, J., Omolo, M. Malebo, H. Angira, J. Njeru, P. Ndiege, I. Repellency of essential oils of some plants from the Kenyan coast against Anopheles gambiae. Acta.Tropica. 2005, 95: 210-218.

Orduz, S. Axtel, R.C (2002)."Compatibility of Bacillus thuringiensis var. israeliensis and B. sphaericus for managing Anopheles mosquito larvae. Proceedings of the 3th International Conference on Biopesticides", Apr., Kuala Lumpur, Malaysia, 1991, 102–111.

Paul, A., Harrington, L.C. and Scott, J.G. (2001). Evaluation of novel insecticides for control of dengue vector Aedes aegypti (Diptera: culicidae). J. Medi. Entomo.2006, 43: 55-60. Philip, R.S. Clin Microbiol Rev, 14, pp208–226.

Rodriguez, M.M., Bisset, J., Ruiz, M. & Soca, A. (2002)Crossresistance to pyrethroid and organophosphorus insecticides.

Su X, Zuo F, Guo Q, Huang J, Chen TX.(2001) A report on a mosquito killing fungus, Pythiumcarolimanium. Fungal Diversity,, 7: 129-113.

Sun C, Georghiou GP, Weiss K. (1980) Toxicity of Bacillus thuringiensis var.isrealensis to mosquito larvae variously resistant to conventional insecticides. Mosquito news, 40: 614-618

Walker, K. and Lynch, M (2007) "Contribution of Anianopheles larvae control to malaria suppression in tropical Africa: review of achievements and potential". Med. Vet. Entomol, 21(1), 2–21.

Weinzierl R, Henn T, Koehler PG, Tucker CL. (2005) Microbial Insecticides Entomology and Nematology Series, ENY-275 (IN081), Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida.http://edis.ifas.ufl.edu..

WHO (2005) Guidelines for laboratory and field testing of mosquito larvicides, CDS/WHOPES/GCDPP/05.13.

Wiseman, Z. & Chapagain, B.P. (2005). Larvicidal effects of aqueous extracts of Balanites aegyptiaca (desert date) against the larvae of Culex pipiens mosquitoes. African Journal of Biotechnology 4(11), 1351–1354.

****