Isolation and Identification of Microorganisms with Mosquito-Larvicidal Potential from Soils of Kolhapur and Ratnagiri Districts of Maharashtra

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Abstract

Kolhapur and Ratnagiri districts are situated in south-west Maharashtra. Both districts have different types of soil profile. These are agriculturally well explored but microbiologically unexplored areas. In current study, from soils of these areas two bacterial strains; possessing mosquito larvicidal potential were isolated and identified. The bacterial isolates were isolated from soil samples collected from areas like farms, polluted region, open grounds and forest area. The isolates were enriched and its cell free supernatants were assessed for its mosquito-larvicidal activity against Culex quinquefasciatus spp. The secondary metabolites from the effective isolates was studied for its LC_{50} and isolates were identified by 16s rRNA technique.
253 Isolates obtained from 107 soil samples out of that 8 isolates have mosquito-larvicidal activity, and two isolates have significant LC_{50} value. The isolates were identified as Lysinibacillus sphaericus strainPOB05 and Staphylococcus lentus strainPOB35.

KEYWORDS: Bacteria, Secondary metabolite, Mosquito larvae, LC_{50} assay, 16S rRNA,

INTRODUCTION

Mosquitoes are one of the life-threatening insects in the world. Their ability to carry parasite and spread disease to humans causes millions of deaths every year. In 2016, 23990 deaths have occurred in India out of 1.09 million reported cases. [World malaria report, 2017] Vector borne diseases such as malaria, dengue, chikungunya, yellow fever, west Nile virus [Caraballo,2014] filariasis, tularemia, Western equine encephalitis, Eastern equine encephalitis, Venezuelan equine encephalitis and Zika fever are transmitted by mosquitoes.

To prevent mosquito-vector transmission of diseases and improve public health, it is necessary to control mosquito larvae's. In recent years, however, mosquito control programmes have failed because of the ever increasing insecticide resistance. Most of the mosquito control programmes target the larval stage in their breeding sites with larvicides because the adulticides may only reduce the adult population temporarily. Therefore, a more efficient way to reduce mosquito population is to target the larvae. The microbial secondary metabolite can be used as an insecticidal agent. [Dhanasekaran 2014]

Microbial secondary metabolite are compound produced mainly by some microbial species and plants usually late in the growth cycle. These include antibiotics, pigments, toxins, pesticides, insecticides. [Demain, 1998] Microbial metabolites and antimicrobial substances exhibit specific insecticidal activity, bacteria pathogenic to

insects such as Wolbachia are found to reduce the susceptibility of Aedes mosquito towards the Dengue virus. Similarly Bacillus thuringiensis, Serratia through their metabolites inhibit Aedes, Anopheles and Culex mosquito larvae.

The secondary metabolites produced of microbial origin have various mode of action including changes in essential enzymes, AchE, carboxylesterase, alkaline and acid phosphatase of larvae. Some microbes produce bioactive peptides which are amphiphilic membrane active biosurfactant and peptide antibiotics with potent larvicidal activity.[Nabar 2015]

One of the most attractive potential uses of 16S rRNA gene sequence informatics is to provide genus and species identification for isolates that do not fit any recognized biochemical profiles, for strains generating only a “low likelihood” or “acceptable” identification according to commercial systems, or for taxa that are rarely associated with human infectious diseases. The cumulative results from a limited number of studies to date suggest that 16S rRNA gene sequencing provides genus identification in most cases (>90%) but less so with regard to species (65 to 83%). [Janda, 2007]

The district of Kolhapur lays in the south west of Maharashtra. It spreads across the Deccan Plateau. The farm, forest and open ground area is about 53%, 18.43 % and 1.82% of total geographical area of Kolhapur district respectively [District Socio-Economic Survey 1997-98]. There are 6 MIDC and 7 Industrial estates working in district. [DIPS Kolhapur district]

Ratnagiri district situated to the west of Kolhapur district and it is separated by Sahyadri hills. The district is located on the western coast of India. Over 85% of the land surface in the district is hilly. 46.02% area is under cultivation. The district consists of 8 industrial areas. [DIPS Ratnagiri district]

Soil inhabit diverse group of living organisms. Microorganisms like bacteria, fungi, actinomyces and algae are widely distributed in soil. [Khalid, 2016] As microbiologically Kolhapur and ratnagiri districts soil is unexplored. In current study, with quest of bacteria possessing mosquito-larvicidal activity, a number of soil samples were collected from the Kolhapur and Ratnagiri districts to isolate bacteria. The bacterial isolate were allowed to produce secondary metabolite, whose mosquito-larvicidal activity were checked by bioassay. Finally potent bacterial isolates were identified by 16S r RNA method.

MATERIAL AND METHODS

1] Enrichment and Isolation of microorganisms from soil samples collected from Kolhapur and Ratnagiri districts of Maharashtra state, India.

107 soil samples were collected from different ecological niche like polluted area, Farm area, Forest area and Open ground area of various regions of Kolhapur and Ratnagiri districts of Maharashtra state, India. About 5gm of 5-10cm depth soil samples were collected and enriched using sterile Nutrient Broth and incubated at different temperature ranges to obtain thermophiles and mesophiles from the soil samples. The enriched broth was streaked on solid nutrient agar plate and then Plates were incubated at their enrichment temperature and the different colonies were selected and streaked to obtain pure cultures. The pure cultures were stored at 10°C till further use.

Each isolate was subjected to secondary metabolite production in their respective broth media and incubated for 15 days at the respective temperature. After incubation the cell free biomass was separated by using refrigerated centrifuge. The supernatant

obtained which was the cell free supernatant was diluted and used for primary screening.

2] Primary Screening of isolates for its mosquito-larvicidal assay:

The Culex quinquefasciatus spp. mosquito larvae was taken in tap water in disposable cups and cell free supernatant was added and kept for 48 hrs. The number of mosquito larvae killed in 48 hours was recorded.[WHO pesticide evaluation scheme,2005] The cell free supernatant which shows 100% killing effect in the primary screening were selected for further study. The selected isolates were subjected to production of cell free supernatants, while the secondary metabolite was then extracted by using Ethyl Acetate. The secondary metabolite was further studied for its $LC_{50.}$

3] Determination of LC_{50} value of effective isolates.

The Lethal concentration 50% [LC_{50}] of secondary metabolite was determined according to WHO guidelines for laboratory testing of mosquito larvicides. The dried secondary metabolite residue was dissolved in 1 ml of Dimethyl Sulfoxide [DMSO] and various 10 fold serial dilutions were prepared.

The Culex quinquefasciatus spp. mosquito larvae were taken in tap water in disposable cups and the dilutions of secondary metabolite was added and kept for 48 hrs. The numbers of mosquito larvae killed in 48 hours were recorded. The dilution of the secondary metabolite under study which killed 50% of Culex quinquefasciatus mosquito larvae was considered as LC_{50} of those secondary metabolites. 1% Temephos was used as a positive control whereas distilled water was used as negative control in experimental set-up.[WHO 2005] The LC_{50} value of the secondary metabolite were calculated by probit analysis. [Finney DJ. 1971].The secondary metabolite effective against the Culex quinquefasciatus mosquito larvae in lower concentration were selected for identification.

4] Identification of the selected isolate by 16S rRNA method.

The selected effective isolate was identified by 16S rRNA technique. Initially the solid medium purified colonies were employed for DNA extraction. The extracted DNA sample then polymerized by Polymerase Chain Reaction [PCR] technique. A 20 base pair forward primer and 22 base pair reverse primer used. The forward primer namely 27F had sequence “AGAGTTTGATCMTGGCTCAG” whereas reverse primer 1492R had sequence “TACGGYTACCTTGTTACGACTT.” The PCR were carried out in three stages as Denaturation, Annealing and Extension. The denaturation step were carried out at 94°C for 3 minutes whereas annealing and extension at 50℃ for 60 sec and 72° C for 10 mins.

The product formed during PCR amplification was purified as follows. Removed unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore).The PCR product was sequenced using the 27F/1492R primers. Sequencing reactions were performed using an ABI Prism Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme). Later Sequencing of the 16S rRNA gene was carried out by sequencing PCR product. The PCR products were precipitated and denatured with formamide and sequenced by model ABI 3730 automatic sequencer. The alignment of all the 16S rRNA gene sequences was done using CLUSTAL W. The primer used for sequencing PCR were 16F 536, 16F 704, 16R 343, 518F, 800R. The sequence of the PCR product was compared with known 16S rRNA gene sequences obtained from Gen Bank to determine similarity value and distance matrix.

The Phylogenetic tree was constructed using 500 base pair aligned sequences by the neighbor joining method using MEGA7. Obtained partial 16S rRNA sequences were submitted to the gene bank.[Castresana,2000, Dereeper2008, Edgar 2004 and Talavera, 2007]

RESULTS AND DISCUSSION

Amongst the different microorganisms inhabiting the soil ecosystem, bacteria are the most abundant and predominant organisms. These bacteria during their life cycle produced secondary metabolite when it enters in stationary phase. As there are many problems associated with the extensive use of chemical insecticides leading to insects acquire resistance to the chemicals. Chemical residues pose environmental hazards and health concern. Thus microbial insecticides are seen as an alternative means of pest control to reduce our dependence on chemical insecticides.

In current study 107 soil samples were collected from the Kolhapur and Ratnagiri districts of Maharashtra state, India. The four regions were selected for soil sample collection viz. farm area, forest area, open ground area and polluted area. After enriching these 107 soil samples 253 bacteria comprising of 120 mesophiles and 133 thermophiles were obtained. The results are presented in fig.1

The highest numbers of bacterial isolates were obtained from open ground region, 96 isolates were obtained out of which 49 were mesophiles and 47 were thermophiles. The lowest numbers of bacterial isolates were obtained from polluted area which comprises of 24 mesophiles and 26 thermophiles. Farm area and forest area soil samples processing gave 55 and 52 bacteria respectively. The lowest numbers of mesophiles were found in farm area.

The cell free supernatants were screened for its mosquito-larvicidal potential against Culex quinquefasciatus larvae. About 8 potent isolates were obtained having mosquito larvicidal potensial. The secondary metabolite was extracted from the 08 effective isolates; the data is presented in table no 1.

Out of the 8 effective isolates, 4 isolates obtained from Farm area and 2 isolates each from polluted and open ground area. None of the isolates belongs to forest area. Except POB35, remaining all isolates obtained from soils of Kolhapur district. The number of thermophiles is 3 times greater than mesophiles.

The LC_{50} assay was conducted to estimate the mosquito-larvicidal concentration of secondary metabolite. Bioassay was carried out using secondary metabolite in the range of 4000 ppm to 62.5ppm. The LC_{50} and LC_{90} values of the secondary metabolite obtained against 3rd instar Culex quinquefasciatus mosquito larvae. These values were calculated by Probit analysis, for that IBM SPSS statistics 23 spread sheets were used. For a correct LC_{50} and LC_{90} values of isolate, respective Chi-Square goodness of fit value must be above 20. Both the two isolate namely POB05 and POB35 showed LC_{50} below 2000ppm and LC_{90} below 4000ppm, whereas other isolates have higher LC_{50} and LC_{90} values. The FMB09 shows highest LC_{50} and LC_{90} values.

By considering minimum LC_{50} for mosquito-larvicidal activity two isolates were selected for further study. These two isolates are POB 05 and POB 35. They having LC_{50} value against Culex quinquefasciatus mosquito larvae is 1131ppm, 927ppm respectively. These two isolates were identified by 16S rRNA technique. The phylogenetic trees were constructed by Maximum Likelihood Method. The phylogenetic tree for POB05 and POB35 is given in figure no 2 and 3 respectively. It

is found that POB 05 belongs to Lysinibacillus sphaericus strain and POB35 belongs to Staphylococcus lentus strain.

After processing all soil samples, numbers of different bacterial isolates were obtained having mosquito-larvicidal activity. Out of that, two effective isolates having highest mosquito-larvicidal potential were identified by 16S rRNA technique.

Conclusion

The 253 bacterial isolates were obtained by enrichment of 107 soil samples. These bacteria were allowed to produce secondary metabolite, which was harvested and checked for mosquito-larvicidal activity. The primary screened 08 isolates were further studied by LC_{50} assay. POB05 and POB35 were found to be most effective in LC50 study. These effective secondary metabolite producers were identified by 16S rRNA technique. These two isolates identified as Lysinibacillus sphaericus strain POB05 and Staphylococcus lentus strain POB35. The Lysinibacillus sphaericus strain POB05 were obtained from soil sample collected from Kolhapur district whereas Staphylococcus lentus strain POB35 from Ratnagiri district. So in the present study, bacterial isolates having mosquito-larvicidal potential were isolated and identified successfully from soils of Kolhapur and Ratnagiri districts of Maharashtra state, India.

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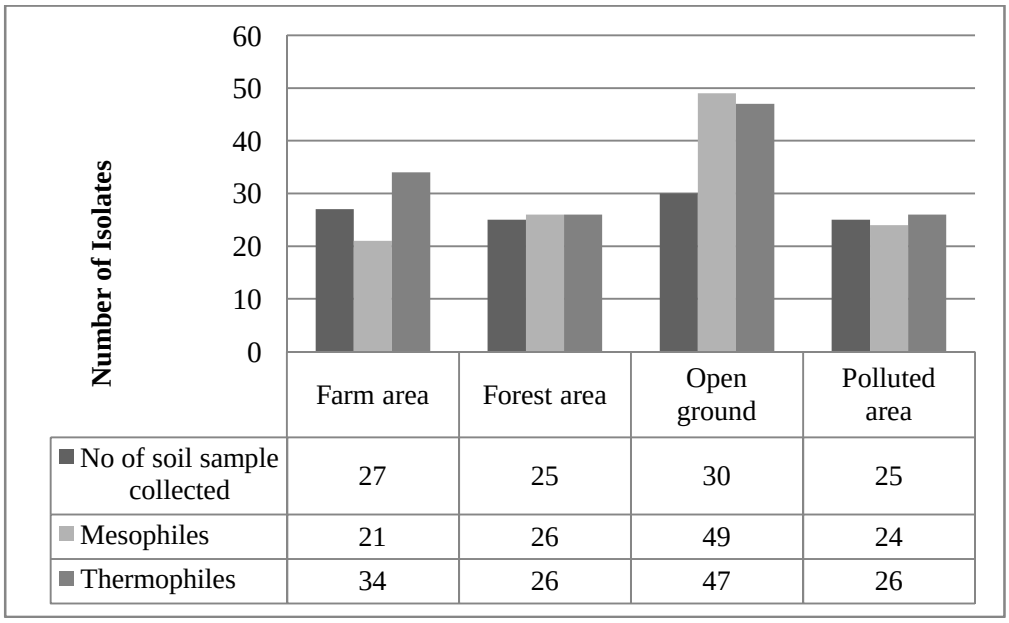
Table no 1 :Data of selected bacterial isolates for LC50 analysis

Sr. No.	Isolate	Type	Place	Region
1	OGB 26	Thermophiles	Kolhapur	Open ground
2	OGB 50	Thermophiles	Kolhapur	Open ground
3	POB 05	Mesophiles	Kolhapur	Polluted area
4	POB 35	Mesophiles	Ratnagiri	Polluted area
5	FMB 12	Thermophiles	Kolhapur	Farm area
6	FMB 09	Thermophiles	Kolhapur	Farm area
7	FMB 53	Thermophiles	Kolhapur	Farm area
8	FMB 14	Thermophiles	Kolhapur	Farm area

Table no 2: LC_{50} Value of 8 isolates against Culex quinquefasciatus mosquito larvae PROBIT model : PROBIT (p) = Intercept + BX100

Isolate	Coeffcient	Intercept	Std error of coefficient	Std error of Intercept	Chi-Square goodness of fit	Significance	LC_{50}	Expt. LC_{50}	LC_{90}
POB 05	0.001	-1.338	0.000	0.090	20.197	0.001	**1131**	**1000**	2216
POB 35	0.001	-1.142	0.000	0.086	85.450	0.000	**927**	**1000**	1969
OGB 50	0.001	-1.170	0.000	0.076	57.200	0.000	**2001**	**2000**	4194
OGB 26	0.001	-1.756	0.000	0.101	81.666	0.000	**3383**	**4000**	5852
FMB 12	0.001	-1.573	0.000	0.090	58.721	0.000	**2227**	**2000**	4042
FMB 09	0.000	-2.165	0.000	0.133	27.229	0.000	**4740**	-	7545
FMB53	0.000	-1.563	0.000	0.092	52.169	0.000	**4122**	-	7502
FMB 14	0.000	-1.013	0.000	0.073	80.688	0.000	**2636**	**2000**	5972

Figure no 1: Comparative study of number of isolates obtained from different regions.



	Farm area	Forest area	Open ground	Polluted area
No of soil sample collected	27	25	30	25
Mesophiles	21	26	49	24
Thermophiles	34	26	47	26

Figure no 2: Molecular Phylogenetic analysis by Maximum Likelihood method for **POB05**

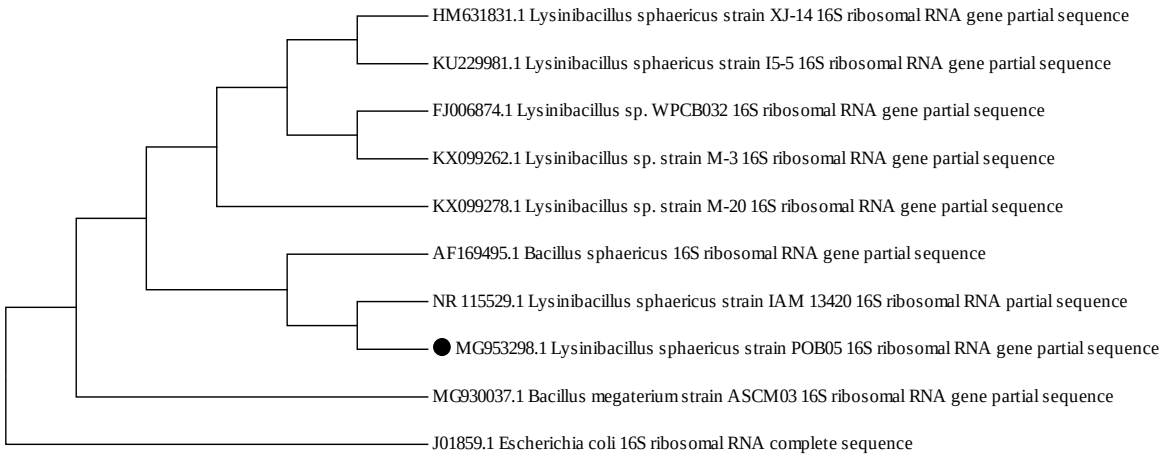


Figure no 3: Molecular Phylogenetic analysis by Maximum Likelihood method for **POB35**