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Larvicidal activity of *Streptomyces cacaoi* subsp. *cacaoi*-M20 against *Culex quinquefasciatus* (III Instar)

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Abstract
Mosquito borne diseases are diseases caused by viruses, bacteria and parasitic organisms transmitted by mosquito vectors. The usage of chemical pesticides in the environment for controlling mosquitoes causes serious problem like bio-magnification of chemicals through food web. So, it is an alternative approach to find bio-pesticide from mangrove actinomycetes. Totally 25 actinomycetes were isolated by dry heat (70ºC) treatment method on SCA media, from the soil sample of the mangrove *Avicennia marina* from the back water area, Ariyankuppam, Puducherry (UT). Mosquito larvae used in this study was *Culex quinquefasciatus* (III instar). Among the 25 isolates, the isolate M20's crude extract was found to better in controlling the larvae of *Culex quinquefasciatus* mosquitoes. 100% larval mortality was noted at the concentration 500 µl. The active isolate M20 was identified as *Streptomyces cacaoi* subsp *cacaoi* with 98.6% similarity with already reported sp (NRBC 12748(T)-AB184115. The isolate M20 was examined further for their physiological and biochemical characterization, the isolate M20 was chitinase positive. The partially purified compound fraction was used to confirm the bio insecticide activity of *Culex quinquefasciatus* further by VCRC, Puducherry. There was 98% of mortality recorded in 500µl/100ml partially purified compound fraction of M20 within 24 hours. UV-Vis spectral analysis of crude extract of isolate M20 revealed the compound belonged to nucleoside antibiotics. The GC-MS of partially purified compound fraction showed 5 compounds and these may responsible for inhibiting the growth and controlling the mosquito larvae effectively. It is evident that the mangrove actinomycetes are potential for controlling mosquito borne diseases through controlling of mosquito larva effectively for human welfare.

Keywords: Mangroves, Larvicidal activity, *Culex quinquefasciatus*, *Streptomyces cacaoi* subsp *cacaoi*.M20, chitinase.

Introduction
Mosquito borne diseases are diseases caused by viruses, bacteria and parasitic organisms transmitted by mosquito vectors. They can transmit disease without being affected themselves. Nearly 700 million people get a mosquito borne illness each year resulting in greater than one million deaths. The usage of chemical pesticides in the environment for controlling mosquitoes causes serious problem like bio-magnification of chemicals through food web. To avoid this, many researchers have formulated bio-pesticides from natural sources. This is a novel approach to find the bio-insecticide from mangrove actinomycetes.
Culex quinquefasciatus mosquitoes are vectors for transmitting several tropical fevers. They are found in tropical and subtropical regions. Only the female mosquitoes bite for blood, which it needs to lay its eggs (mature). These mosquitoes are very much attracted by chemical compounds secreted by human beings especially right handed (dextrorotatary) octenol molecules. Several research works is going on to find the novel source for controlling the *Culex quinquefasciatus* larvae. El-Khawagh *et al*., (2011) [3], Sundarapandian *et al*., (2002) [11] did research to control larva of *Culex sp*, Dhanasekaran *et al*., (2010) [2] did research work on Anopheles mosquito, Janaki, (2016) [8] worked on controlling of larvae of *Aedes agypti* with mangrove actinomycetes. Actinomycetes are active source of antibiotics, besides vitamins and enzymes and such antagonistic actinomycetes of marine origin are being regularly reported (Williams *et al*., 1999) [13]. Few reports are available regarding that soil is a major source of actinomycetes (Vijayakumar *et al*., 2007; Dhanasekaran *et al*., 2008) [12, 1]. Only few reports are available pertaining to actinomycetes from mangroves (Sivakumar, 2005; Janaki *et al*., 2014) [10, 7]. Chitinase producing actinomycetes are very potential in inhibiting chitin synthesis in insects, based on this, chitinase producing actinomycetes from soil of *Avicennia marina* -mangrove

environment of Ariyankuppam, Puducherry was selected to control the growth of larvae of mosquitoes. It is an approach to find the bio-insecticidal compounds from mangrove actinomycetes.

Materials and methods

Isolation of mangrove actinomycetes

Soil sample was collected from the root region of the mangrove plant, *Avicennia marina (Forsk). Vierh – (Avicenniaceae)* in Ariyankuppam back water area, Puducherry. Physico-chemical nature of soil sample was analysed in soil testing laboratory, Department of Agriculture, Puducherry, India. The soil sample was subjected to dryheat (70ºC for 15 min) (Hayakawa *et al*., 1991) [4] (Janaki *et al*., 2014) pretreatment. After pretreatment, one gram soil was mixed and serially diluted in sterile water blanks. 0.1 ml of last two dilutions ($10^{-5}$and 10^{-6}) was inoculated by pour plate method (Zheng *et al*., 2000) [14] using Starch casein agar (Kuster and Williams, 1964) [9] supplemented with Fluconazole 80µg/ml and Nalidixlic acid 75µg/ml. Plates were incubated at 30 ±ºC for up to 30 days. Plates were periodically examined for actinomycetes colonies. Selected colonies were transferred to Yeast Malt Extract agar (ISP_2) slants and maintained in the same medium.

Physiological, biochemical and molecular characterization of isolate M20

Growth and activity in different pH (6, 7, 7.5, 8, 9, 10, 11, 12), temperatures (25ºC, 30ºC, 37ºC, and 45ºC), concentrations of sodium chloride (0%, 2%, 4%, 6%, 8%, 10%, 12% and 14%), Production of extra cellular enzymes like chitinase (Hsu and Lockwood, 1975) [5] was also tested. For the 16sRNA sequencing analysis of M20, the purified PCR products of approximately 1,400bp were sequenced by using 2 universal primers: 518F 5'CCAGCAGCCGCGGTAATACG 3', 800R 5' TACCAGGGTATCTAATCC 3'. Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied Bio Systems, USA). The isolate M20 was identified and phylogenetic tree was constructed.

Partial purification of methanolic crude extract by column chromatography

Using Chloroform: methanol: acetic acid (8.5:1.5: 0.2 ml) as the mobile phase two colour spots were resolved in both in ascending paper chromatography and TLC. Since methanol extraction produced highest activity, the culture filtrate (1-litre) was extracted in methanol, after drying, produced six gram of dark brown oily residue. The residue was mixed with silica gel and applied to the silica gel column (230–400 mesh) and eluted with chloroform: methanol: acetic acid (85:15:2 ml) to give 2 active fractions. The yellow compound fraction was used for further investigation.

Ultra Violet-Visible Spectrum analysis

UV-Visible spectral analysis of partially purified methanol fraction was carried out by using Hitachi U-2010 Spectrophotometer, Wavelength Range: 200 nm to 800 nm.

GC-MS analysis of partially purified methanol compound fraction

The partially purified compound fraction (sample) was dissolved in methanol and 1 µl volume of sample was injected in the GC-MS equipment: THERMO GC - TRACE ULTRA VER: 5.0, THERMO MS DSQ II with column : ZB 5 - MS Capillary standard non -polar column and its dimension : 30 Mts, ID : 0.25 mm, Film : 0.25 µm using the helium as the carrier gas. The carrier gas flew 1.0 ml/min. The oven temperature programme was initially 70ºC and this was raised to 260 ºC at rate of 6ºC/min. The instrument was set to analysis the compounds in the sample from Low Mass (m/z): 50 to High Mass (m/z): 650, the total run time for the analysis of sample from isolate M20 was 37.50 min.

Bioassay of methanolic partially purified active fraction of M20 against the larvae of *Culex quinquefasciatus* (III instar)

Initially the larvicidal activity was tested with larvae of *Aedes aegypti* mosquito in the laboratory by using culture filtrate of M20. Its percentage of larval mortality, Pupation, Pupal mortality, Adult emergence, Pupal malformation was noted (Janaki 2016) [8]. Based on the larvicidal activity of culture filtrate of M20 against *Aedes aegypti*, it was decided to confirm the larvicidal activity of partially purified compound fraction with larvae of other mosquito and larvicidal activity was confirmed by Vector Control Research Centre, Puducherry.

Culex mosquito larvicidal activity was examined in Vector Control Research Centre, Unit of Microbiology and Molecular biology, Puducherry with methanolic partially purified active fraction of isolate M20. The 0.450 mg were reconstituted in 2 ml sterile distilled water and used for assay. Each bioassay cup (wax coated paper cup 125 ml capacity) contained 100 ml of chlorine free tap water and 25 numbers of III instar larvae. Eight dosages were used 10µl, 20µl, 30µl, 40µl, 50µl, 60µl, 70µl and 500 µl with control in duplicate. Experimental set up was left for 24 hours. The number of live larvae was counted and % mortality was calculated after 24 hours.

Results and Discussion

Isolation and maintenance of actinomycetes

The wet pH of mangrove soil sample collected from *Avicennia marina* was 7.7. The soil analysis results showed that there were very low available Nitrogen, P_2O_5 and Cu. Micro-nutrients like Zn and Fe were high in their available form, Mn was medium. Totally 25 actinomycetes were isolated from soil sample of *Avicennia marina* by dry heat (70ºC for 15 min) pretreatment method. Dry heat method yielded bioactive actinomycetes for antimicrobial activity. The isolated actinomycetes were subcultured in yeast malt extract agar-ISP2.

Physiological, biochemical and molecular characterization of M20

Observed the growth and activity of M20 in pH 7, 7.5, 8, 9 and 10 with maximum activity in pH 7.5. Maximum growth and activity was observed in 30ºC. Growth and activity was noticed from 0%-10%, maximum activity observed in 6 & 8% of sodium chloride. The isolate was chitinase positive. The sequence was submitted to Gene Bank with the accession No. KP872910. Phylogenetic analysis of 16S rRNA gene (1400bp) of M20, species of *Streptomyces* was carried out with 18 different reference species of *Streptomyces* available in the Gene Bank database. The isolate M20 branched along with *Streptomyces cacaoi subsp cacaoi* (NRBC 12748(T)-AB184115 in the analysis.

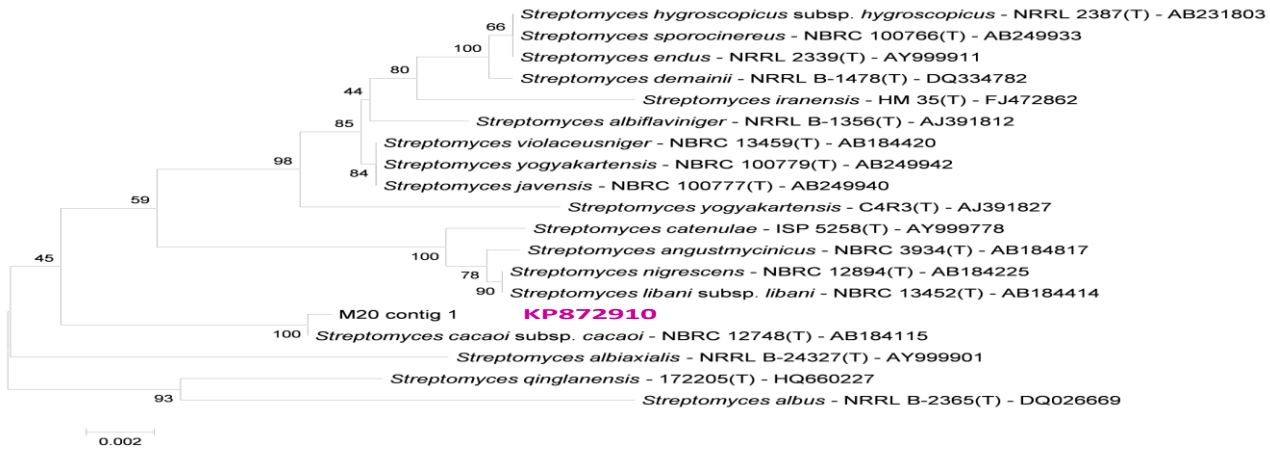


Fig 1: Phylogenetic tree based on the 16S rRNA sequence homology of M20

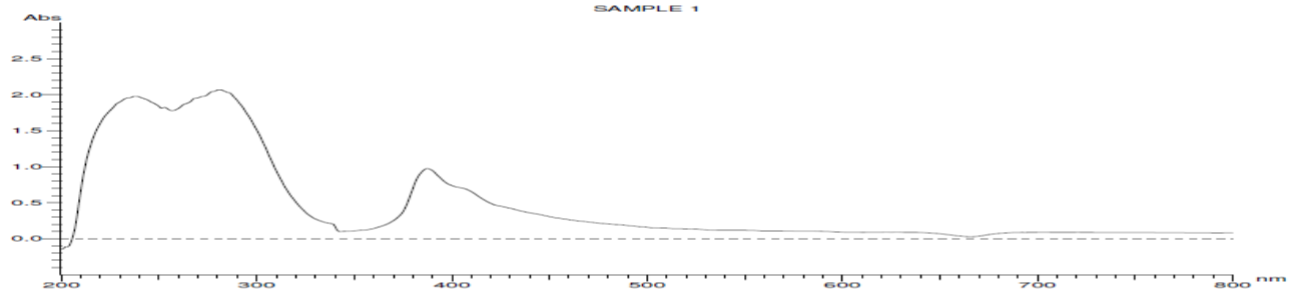
Neighbour-joining tree based on 16S rRNA sequence showing the phylogenetic relationship between the isolate M20 and other closely related species of the genus *Streptomyces*

Compound separation

The oily solid obtained from the methanolic crude extract was subjected to silica gel column chromatography (230–400 mesh), and eluted with chloroform: methanol: acetic acid (85:15:2 ml) and 2 active fractions were separated.

Ultra Violet- Visible Spectrum of partially purified yellow compound fraction

The maximum absorbance of partially purified yellow compound fraction was (λ_{max}) at 387.0, 282.0 and 239.0 nm (Figure 2).



Peaks

Peak #	Start (nm)	Apex (nm)	End (nm)	Height (Abs)	Area (Abs*nm)	Valley (nm)	Valley (Abs)
1	800.0	387.0	342.0	0.976	89.050	342.0	0.099
2	342.0	282.0	257.0	2.071	108.654	257.0	1.780
3	257.0	239.0	200.0	1.979	83.569	200.0	-0.146

Fig 2: Ultra Violet- Visible Spectrum of partially purified yellow compound fraction

UV-Vis spectral analysis of methanolic crude extract of M20 has shown 4 peaks and maximum absorbance at 406 nm followed by 291 nm. UV-Vis spectral analysis of methanolic partially purified yellow compound fraction of M20 has shown 3 peaks and maximum absorbance at 284 nm.

Analysis of partially purified compound fraction through Gas Chromatography - Mass spectrometry.

There were 4 compounds were detected from GC-MS analysis with the retention times 6.71, 26.05, 32.40 and 37.61 (Figure 3). Alkanes like nonadecane, hentriacontane and tetratetracontane were detected with high peak height and area. 7-Aminoheptamide, N-methyl-N-[4-(pyrrolidinyl)-2-butynyl]- was detected with low peak area. These compounds have antifungal, antibacterial, anticancer and insecticidal properties.

Table 1: Compounds detected through GC-MS analysis

S.no	R.Time	Compound Name	M. Formula	M. Weight
1	6.71	7-Aminoheptamide,N-methyl-N-[4-(pyrrolidinyl)-2-butynyl]-	$C_{16}H_{29}N_{30}$	279
2	26.05	Nonadecane	$C_{19}H_{40}$	268
3	32.40	Hentriacontane	$C_{31}H_{64}$	436
4	37.61	Tetratetracontane	$C_{44}H_{90}$	618

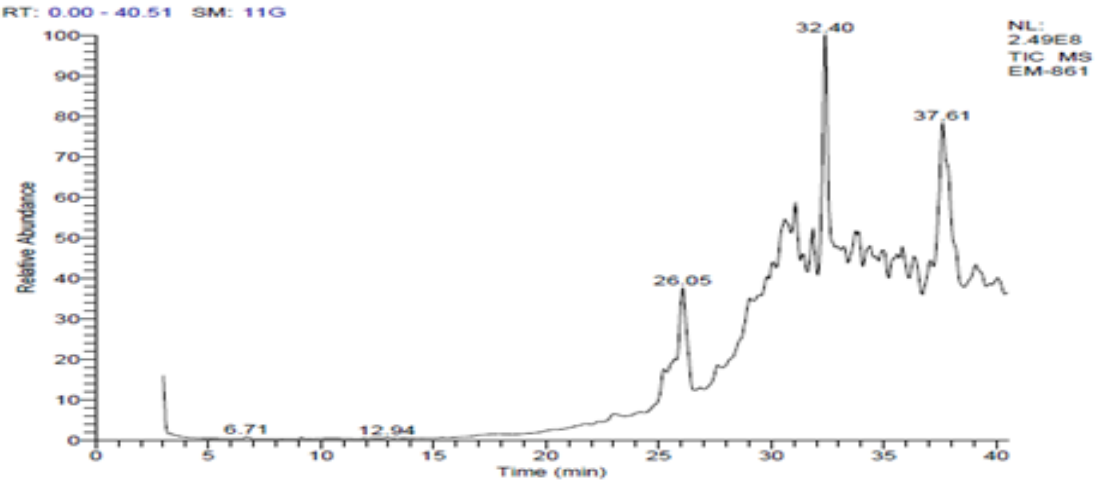


Fig 3: Chromatogram of Partially purified compound fraction of isolate M20

Bioassay of Partially purified compound fraction of isolate M20 against the larvae of *Culex quinquefasciatus* (III instar)

The isolate M20 was actively degrading the chitin in chitinase activity tested for checking its efficiency in excellular enzyme production. The mosquitoes have the chitinous covering in their body as the protective layer, also they need chitin for completing its metamorphosis, without chitin the larvae cannot form pupae. Since, the isolate degraded the chitin; the culture filtrate of isolate M20 was bio-assayed initially against the larvae of *Aedes aegypti* mosquitoes and finally the bio insecticide activity was confirmed with partially purified compound fraction of M20 against *Culex quinquefasciatus* (III instar).

To confirm the larvicidal activity of isolate M20, the partially purified compound fraction was given to Vector Control Research Centre, Puducherry. It was noted that, the concentration of the partially purified compound fraction increased, the mortality of larvae of *Culex quinquefasciatus* (III instar) also increased. There was 98% of mortality recorded in 500µl/100ml partially purified compound fraction of M20 within 24 hours (Table 2).

Synthesis of chitin is very important for insects to get full development and cuticle formation for their protection. Some of the insects act as vectors for spreading harmful diseases, such type of insects can be controlled with the help of chitin lysing enzyme secreted by the actinomycete antagonists. The isolate -*Streptomyces cacaoi* subsp. *cacaoi.* M20 synthesis such enzyme extracellularly, the compound from the isolate M20 was subjected for mosquito larvicidal activity. The partially purified compound fraction was tested against larvae of *Culex quinquefasciatus* (III instar) and culture filtrate was tested against larvae of *Aedes aegypti*.

Table 2: Bioassay of partially purified compound fraction of M20 against the larvae of *Culex quinquefasciatus (III instar)*

S.no	Dose(µl)	Mortality (%)
1.	10	50
2.	20	58
3.	30	62
4.	40	64
5.	50	70
6.	60	72
7.	70	78
8.	500	98

When the concentration of the partially purified compound was increased, the mortality of larvae of *Culex quinquefasciatus* (III instar) was also increased respectively. There was 98% of mortality recorded in 500µl/100ml partially purified compound of isolate M20 within 24 hours for larvae of *Culex quinquefasciatus* (III instar) and conditions of larvicidal activity. The isolate M20 actively involved in chitin inhibiting activity with the production of polyoxins and heptaene group of antibiotics (UV-VIS analysis). This may be a reason for control of larvae of mosquito. Sundarapandian *et al*., (2002) [11] had proved *Culex quinquefasciatus* larvicidal activity of 3 actinomycetes out of 44 screened for the activity, *Streptomyces* -98-7 showed good larvicidal activity; El-Khawgh *et al*. (2011) [3] had proved the larvicidal activity of *Culex pipiens* using a *Streptomyces sp.* It is important to carry out field trial of larvicidal compounds as in the form bio-pesticides from *Streptomyces cacaoi* subsp. *cacaoi*. M20 for human welfare.

Conclusion

Vector borne diseases are serious problems in tropical and subtropical regions. The indiscriminate use of chemically synthesized pesticides in controlling mosquitoes causes major environmental problems. Finding the better bio-pesticide compounds are challenging work in the developing countries. It is a novel approach to find better bio-pesticide from the mangrove actinomycetes, because, everyday mangroves face lot of fluctuations in their life style. They survive in the high stressed salty, heavy metal stressed environment. Recycling of nutrients take place fastly in these areas. These are the some of the reasons for isolating the novel actinomycetes from the mangrove area to control the vector borne diseases with the help of their bio-insecticidal properties. In our study, the isolate-*Streptomyces cacaoi* subsp *cacaoi*.M20 has insecticidal property with chitin degrading capacity, above that the alkanes like nonadecane, hentriacontane and tetratetracontane were detected with high peak height and area. 7-Aminoheptamide, N-methyl-N-[4-(pyrrolidinyl)-2-butynyl]- was detected with low peak area. These compounds have antifungal, antibacterial, anticancer and insecticidal properties, these may responsible for inhibiting the growth and controlling the mosquito larvae effectively. It is evident that the mangrove actinomycetes are potential for controlling mosquito borne diseases through controlling of mosquito larvae effectively for human welfare.

Acknowledgement
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