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ISOLATION OF A NOVEL MOSQUITOCIDAL BACTERIUM *BACILLUS CEREUS* VCRC-641 FROM FRESH WATER FISH *CLARIAS BATRACHUS*

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ABSTRACT

In this study, search for new mosquitocidal bacterium was attempted from the gut content of the fresh water fish *Clarias batrachus* (walking cat fish) which resulted in the isolation of a highly potential mosquitocidal bacterium. The isolate was identified as *Bacillus cereus* VCRC 641 by *Bacillus* species specific molecular markers *ilvD, pur* and *pycA* gene sequence analysis. Laboratory mosquito larval toxicity assay (bioassay) against mosquito larvae revealed that this new isolate was highly potent against *Culex quinquefasciatus followed by Aedes aegypti* and *Anopheles stephensi*. The lethal concentration values at LC_{50} for these three mosquito species were 0.001, 0.004 and 0.007mg/ L respectively. Therefore, in the present study, this is the first report that a highly potential mosquitocidal bacteria of *B. cereus* VCRC 641 was isolated from the gut content of *Clarias batrachus* and suggested that it can be used for mosquito vector control program.

Key words: *Bacillus cereus*, midgut, *Clarias batrachus*, bioassays, *Culex quinquefasciatus*, *Aedes aegypti*, *Anopheles stephensi*, *ilvD*, *pur*, *pycA*, mosquitocidal, vector control, biopesticides.

Nearly 7,00,000 fatal cases have been reported per annum due to vector borne diseases (WHO, 2020). As a result of globalization, urbanization and climate change, the vector breeding has been enhanced and posing a challenge to vector control strategies (Bhatt, 2013; Lessler, 2016; WHO, 2019). Application of chemical and biological pesticides has been one of the promising approaches used to control mosquito vectors for many decades. Resistance to chemical pesticides has emerged as a global issue and it has a severe impact on public health around the world. Hence there is an immediate need to search for new biocontrol agent. Generally, bio pesticides have advantages such as cost-effectiveness, eco-friendly, biodegradable and safe to non-target organisms (Senthilnathan, 2020). Controlling of mosquito vectors by biological control methods by utilising bacteria is an ideal alternative to chemical insecticides. Enormous efforts have been made by the researchers to isolate and identify the mosquitocidal bacteria (Park et al., 2007; Federici et al., 2010; Wilson et al., 2020). *Bacillus thuringiensis israelensis* (*Bti*) is a unique bacterial agent, produces protoxins (δ-endotoxins) during sporulation, which are typically used as commercial bio-pesticides and are effective against mosquito, black fly, and chironomid midge larvae (Federici et al., 2010; Margalith, 2000). Besides, *Bacillus sphaericus* is a gram-positive endospore forming bacteria found to produce binary toxins (51kDa, 42kDa) responsible for mosquitocidal activity against *Culex* species (George et al., 2019). Despite these facts, due to the resistance developed by *B. sphaericus*, many nations have abandoned its application for vector control measures (Wirth et al*.,* 2010). Therefore, an essential need to search for new mosquitocidal bacteria from different natural sources is mandatory (Poopathi et al., 2013). In the current research, an attempt was made to identify new mosquitocidal bacteria from the gut content of fresh water fishes in the Union Territory of Pondicherry, India. This is a new approach and was not made earlier by researchers and therefore will provide a stage for figuring out the genomic diversity of mosquitocidal bacteria from the fresh water environment.

MATERIALS AND METHODS

Five species of freshwater fishes were investigated in this study namely, *Oreochromis mosambicus*, *Labeo rohita, Catla catla, Clarias batrachus and Channa punctata* were collected from fish market in Mettupalayam, Puducherry, India. The gut content was removed cautiously and preserved in sterile vials containing 30% glycerol for further use (Mani et al., 2014). Samples were processed using the normal serial dilution method (Radhika et al., 2011). Then the sample

was streaked on agar LB media plates and single pure bacterial colony inoculated into Luria Bertani broth (LB) and incubated overnight at 250 rpm. A 500 ml Erlenmeyer flask was used to inoculate 10µl of this culture, which was then cultured for 72 hours at 250 rpm in an orbital shaker. The cell mass was collected and kept in deep freezer (-80^{0}C) overnight for freeze-dried in a lyophilizer. A biochemical study was done using Hi*Bacillus* identification kit to identify the enzymes which were produced by the bacterium.

Gram staining and endospore staining also done. Preliminary bioassay was conducted using late 3rd instar laboratory reared larvae of *Aedes aegypti, Culex quinquefasciatus,* and *Anopheles stephensi* collected from Mosquito Rearing and Colonization (R&C) unit, VCRC, Puducherry, India. The bacteria showing potential larvicidal activity was further subjected to extensive toxicity bioassays to determine the lethal concentrations (LC_{50} and LC_{90}). The 25 late third instar larvae from three mosquito species were introduced (WHO, 1985). Seven doses from 0.0125mg/L to 0.000096 mg/L were made and three replicates for each dose were used for bioassay experiment with appropriate controls. After 24 hours, the alive larvae were counted. The results were finally analysed by probit analysis ($P \geq 0.05$) and the LC_{50} and LC_{90} values were determined. Non-target aquatic organisms such as *Physa, Dapnia cephalad*, *Chironomonas* larvae, *Rana hexadactyla* (Tadpoles), *Poecillia reticulate* and *Oreochromis mossambica* collected from ponds, rice fields, and canals were subjected to toxicity assays. The non-target organisms were treated with sub lethal doses (LC_{50}) of *Bacillus cereus* VCRC 641 and then mortality was observed after 24 hrs of exposure and recorded (Wipfli and Merritt, 1994). Each of the experiment (including the control) was conducted three times for statistical significance and average LC_{50} values were calculated and tabulated (Abott, 1925).

Genomic DNA from *B. cereus* was extracted using Sigma Gen Elute kit. Polymerase chain reaction was carried out with *Bacillus* species specific gene markers (Priest et al., 2004), such as *ilv*D, *pur* and *pyc*A primers and cycling conditions were optimized. PCR amplicon was subjected to 2% agarose gel and observed under Syngene gel documentation system (Poopathi and Abidha, 2007, 2014). *ilvD*, *pur* and *pycA* amplicon of the isolate was purified with PCR purification kit (QIAGEN, USA) and cycle sequencing was done using BigDye terminator V3.1 kit (Applied biosystem) then purified by using Macherey Nagel Nucleoseq purification column. Sequencing was carried out in 3130XL Genetic Analyzer facility (Applied Biosystem), VCRC, Pondicherry. Chromatogram was analyzed using Chromas software (Version 2.01). Sequence was subjected to nucleotide blast (BLAST program, NCBI). Maximum likelihood Phylogenetic tree was constructed with 1000 bootstrapping by MEGA program using K2P method (MEGA 10.2.6) and species identification was done (Poopathi and Abidha, 2004, 2014). The genbank accession numbers were received for this new isolate. To determine the growth, protein and biomass production of *B. cereus* VCRC 641, samples were analysed at every 6 hour intervals up to 72 hours (Poopathi et al., 2014; Abubakar, 2015) (Bradford, 1976).

RESULTS AND DISCUSSION

In the present study, screening of potential mosquitocidal bacterial agent from the gut content of five fresh water fishes were screened. Two hundred gut samples from these fishes were examined to isolate mosquitocidal bacteria. Twelve bacterial samples were shown mosquitocidal activity from preliminary mosquito toxicity screening. Out of 12 bacterial samples studied, only one bacterium from the gut content of fish *Clarias batrachus* was shown potential mosquitocidal activity against *An. stephensi, Cx. quinquefasciatus,* and *Ae. aegypti.* From the last few years, an attempt was made to isolate new mosquitocidal bacteria from natural sources for the control of mosquito vectors. Since, 1950 onwards, the widespread use of chemical insecticides has caused the mosquito population to become resistant (Hemingway and Ramson, 2000). In order to minimise vector mosquitoes, chemical pesticides can be replaced with spray formulations based on the toxins produced by bacteria that kill mosquitoes in a safe and environmentally friendly manner. The isolated bacterial colonies appear as circular, flat, wet, pale whitish in colour with smooth edges on the LB agar plates. Microscopic examination of the slides showed that mosquitocidal isolate was gram positive, rod shaped bacteria and the spore appears green, oval shape while the vegetative cells appear pink.

GenElute™ (SIGMA ALDRICH) bacterial genomic DNA kit method was used for isolating the genomic DNA from the bacterial isolate. The extracted Genomic DNA was used for amplification of *ilvD, pur* and *pycA* genes. The size of the *ilvD, pur* and *pycA* encoding gene was 556bp, 536bp, and 550bp respectively. The sequence was analysed using Chromas, and the consensus sequence was generated with Bio-Edit (Version 7.0.9.0). The phylogenetic tree was created

using a maximum likelihood model with 1000 bootstrap replications, and MEGA software was used to apply the Kimura 2-parameter model to the isolate. The strain was finally identified as *B. cereus* by species specific markers *ilvD* (NCBI: OK030837), *pur* (NCBI: OK030839) and *pycA* (NCBI: OK030838). The *ilvD* phylogenetic tree of the consensus sequence VCRC 641 displayed 100% similarity with *B. cereus* strain (Fig. 1.). Similar phylogenetic tree based on *pur* and *pycA* has been closely associated or clustered with *B. cereus* strain. Overall, phylogenetic tree based on three different molecular markers revealed that the *B. cereus* VCRC 641 strain has shown close similarity with *B. cereus*. Earlier publication by Priest et al*.,* 2004 reported that

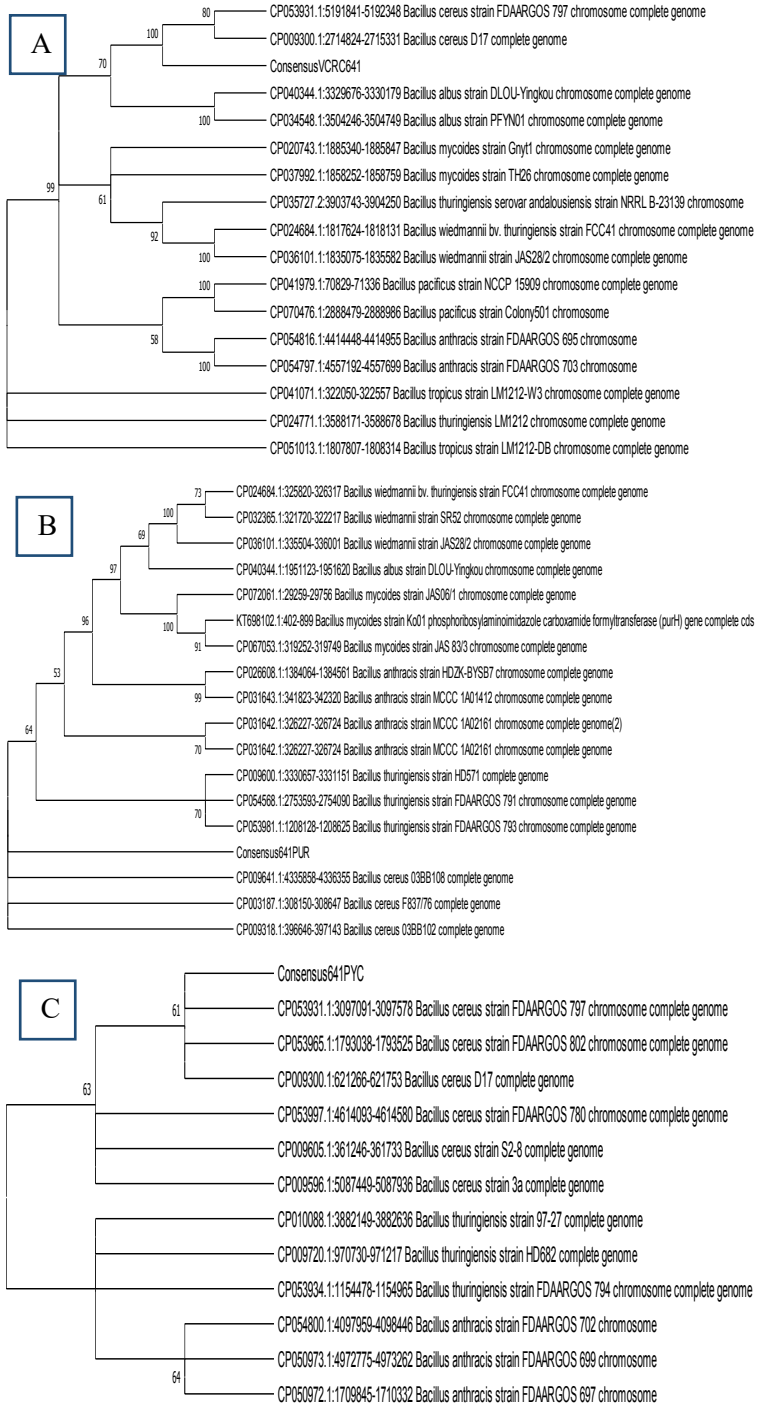

Fig. 1. Maximum likelihood Phylogenetic tree of VCRC 641 – A-*ilvD,* B-*pur* and C-*pycA* gene using Kimura 2 parameter model

the molecular markers such as *ilvD*, *pur*, and *pycA* used to determine the populace arrangement and progression of the *B. cereus* group. From the similar study, it was reported that utilising multilocus sequence typing (MLST) with *ilvD*, *pur*, and *pycA* and characterized 47 *B. cereus* food-borne isolates (Cardazzo et al., 2008).

Larvicidal bioassay was carried out against laboratory reared mosquito species with *B. cereus* VCRC 641. The mosquito larval toxicity assay (laboratory bioassays) revealed that the larvicidal activity of this isolate was highly potent against *Culex quinquefasciatus, Aedes aegypti,* and *Anopheles stephensi*. The LC_{50} and LC_{90} mg/L values were 0.001, 0.004, and 0.007 and 0.003, 0.005 and 0.009 respectively. Among these three mosquito species, *Culex quinquefasciatus* showed more susceptibility followed by *Aedes aegypti* and *Anopheles stephensi.* From the study, it was clear that the new isolate *B. cereus* VCRC 641 showed promising mosquitocidal activity against the mosquito vectors and has not shown any toxic effect against non-target aquatic organisms (Table 1). One similar research demonstrated that *B. cereus* M413 and C32, two mosquito-killing bacteria, were discovered in sediment samples collected from a ditch and a wooded area, respectively. The results revealed that *B. cereus* M413 is mostly toxic to *Oc. taeniorhynchus* (LC_{50} = 0.089 mg/ L) whereas *B. cereus* C32 is to *Cx. quinquefasciatus* (LC_{50} = 0.082 mg/ L). *B. cereus* M413 had no effect on *A. aegypti* or *Cx. quinquefasciatus* and had a low toxicity on *An. quadrimaculatus* (LC_{50} = 1.155 mg/L). Interestingly, it had no effect on the closely related species of *Oc. taeniorhynchus*, *Ae. Aegypti*, Ae*. aegypti* and *An. quadrimaculatus* did not exhibit any toxicity in response to *B. cereus* C32, while *Oc. taeniorhynchus* did promising toxicity (LC_{50} = 0.739 mg/ L) (Park et al., 2009). Concurrent report revealed, *B. cereus* (VCRC-B549 and VCRC-B550) were screened from excreta of arid-birds and LC_{50} values of bacterial strain against *Cx. quinquefasciatus* and *An. stephensi* were 0.13 and 0.107 mg/ L for *B. cereus* VCRC-B549; 0.101 and 0.12 mg/ L for *B. cereus* VCRC-B550. These two isolates from excreta of arid-birds has not shown mosquitocidal activity against *A. aegypti* larvae (Poopathi et al., 2014). According to a recent investigation, a marine *B. cereus* (VCRC B540) that kills mosquitoes was found in a marine red snapper fish's intestines (*Lutjanus sanguineous*) (Mani et al., 2014) *B. cereus* VCRC B540 had LC_{50} values of 0.004, 0.006, and 0.01 mg/ L against *Cx. quinefasciatus*, *An. stephensi*, and *Ae. aegypti*, respectively.

Similarly, *B. cereus* VCRC 641 used in the present study was more potent to any of the mosquito species tested such as *A. aegypti, Cx. quinquefasciatus,* and *An. stephensi,* the LC_{50} values were 0.004, 0.001, and 0.005 mg/ litre respectively. The relative trend of mosquito toxicity of *B. cereus* is described in this study as *Cx.*

Table 1. Mosquitocidal toxicity values (LC_{50} and LC_{90}) of *Bacillus cereus* VCRC-641 lyophilized cell pellet against mosquito larvae and non-target organisms

Bacterial Strain	Mosquito species	LC_{50} (mg/ L) (90% UCL-LCL)	LC_{90} (mg/ L) (90% UCL-LCL)	χ^2 (df)
Bacillus cereus VCRC-641	*Culex quinquefasciatus*	0.0019 (0.0045-0.0011)	0.0036 (0.0515-0.00249)	88.6
	Anopheles stephensi	0.0050 (0.0062-0.00411)	0.0095 (0.0123-0.0078)	85.2
	Aedes aegypti	0.0040 (0.0050-0.0033)	0.0074 (0.0096-0.0069)	104.54
	Non target species			
	Chironomonas larvae	-	-	-
	Dapnia cephalata	-	-	-
	Physa (Common snail)	-	-	-
	Oreochromis mossambica (Tilapia)	-	-	-
	Poecillia reticulata (Guppy)	-	-	-
	Rana hexadactyla tadpole (Pond frog)	-	-	-

(-No mortality found)

quinquefasciatus, followed by *A. aegypti,* and *An. stephensi*. This relative trend toxicity was in justification with *Bti* H14 (Federici et al., 2010). In a similar study reported the relative trend of toxicity was observed that *Cx. quinquefasciatus* found to be more susceptible with *B. cereus* VCRC-B549 and *An. stephensi* more susceptible with *B. cereus* VCRC-B550. *B. cereus* VCRC-B540 has shown more susceptibility towards *Cx. quinquefasciatus* followed by *An. stephensi* and *Ae. aegypti.* (Poopathi et al., 2014). Biochemical assays demonstrated that the isolate had a close similarity to *B. cereus*. The growth range of bacteria (*B. cereus*) was directly proportional to the culture time, the optical density of culture from 6 to 72 hr was ranged from 0.507 to 1.65. The turbidity, bio-mass production and protein content of *B. cereus* VCRC 641 was gradually increasing from 6 to 72 hr. The dry bio mass yield of bacteria was 0.24 mg/ ml at 6 hr and gradually increased to 0.752 mg/ ml at 72 hr. Similar pattern was observed in protein content from 6 to 72 hr. Mani et al., (2014) stated that the marine isolate *B. cereus* VCRC-B540 has shown significant growth pattern from 6 to 72hr with the optical density of 0.1 to 1.5 (Fig. 2). Whereas, dry biomass yield and protein content also gradually increased from 6hrs to 72 hr (Fig. 3). The dry bio mass yield of *B. cereus* VCRC-B540 at the end of 72 hr was 0.5 mg/ml. Similar kind of pattern was observed in

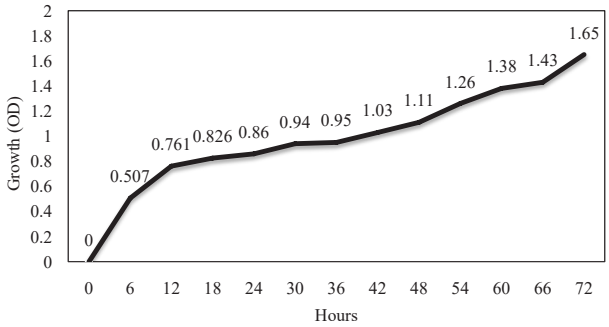

Fig. 2. Growth pattern of *Bacillus cereus* VCRC 641

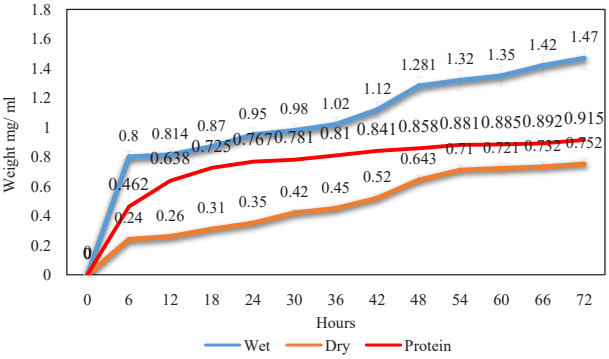

Fig. 3. Bio mass yield and protein concentration of *Bacillus cereus* VCRC 641

protein content from 6 to 72 hr. In the present study, a new mosquitocidal bacterium, namely, *B. cereus* was isolated from the fresh water (*Clarias batrachus*). This strain was highly potential against filarial vector of *Cx. quinquefasciatus,* followed by other two mosquito species (*Ae. aegypti, An. anopheles*) with promising toxicity. These toxicity effects are comparatively higher than the *Bti* H-14.

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AUTHOR CONTRIBUTION STATEMENT

SM has contributed in conducting a thorough literature search, compiling and interpreting the results, design of the work, data collection, data analysis and interpretation, drafting the article, critical revision of the article. JL has contributed in selection of a relevant journal with proper parameters and participation in writing appropriately. PH has contributed in setting up of the toxicity experiment. BB has aided in the extraction of genomic DNA, compilation of data. VA has contributed in molecular work. KG has contributed in article collection, data tabulation, article framing, reference formatting, rectifying plagiarism, and cleaning of glassware. SM has contributed in collecting of literature, structuring the document, checking grammar mistakes and tabulating of data. KA has contribution to the study of bacterial growth patterns and collecting of non-target organisms. AM has contributed in interpretation of the author's assigned job, preliminary screening, and data-results verification. KV has contributed in preliminary review of contributed in setting background information, data completion, paper review, current information, and scientific connections, Conception of idea, manuscript overview, correction and finalization. SM has written the manuscript. All authors have read and approved the manuscript.

CONFLICT OF INTEREST

No conflict of interest.

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