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IDENTIFICATION AND CHARACTERIZATION OF MOSQUITOCIDAL TOXINS FROM *BACILLUS CEREUS* VCRC-641

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ABSTRACT

***Bacillus cereus* VCRC 641 was found to be an alternate larvicidal bacterial agent which was newly isolated from fresh water fish, *Clarias batrachus* (walking cat fish). The protein responsible for mosquito toxic effect was separated and purified by Sephacryl S-200 column chromatography and NATIVE PAGE. It was observed that a unique protein with molecular weight of 70kDa was the responsible factor for mosquito killing effect. Further analysis of the protein by liquid chromatography-mass spectrometry (LC-MS) it was identified as "Uncharacterized Protein". The length of the uncharacterized protein was around 158bp. The uncharacterized protein was expected to have a molecular mass of 70 kDa, 51 amino acids, confidence score of 78.45 and mass of 206772.57. Therefore, in the present study we report for the first time that uncharacterized protein (70kDa) was the responsible factor for the mosquito killing effect.**

Key words: *Bacillus cereus*, native page, LC-MS, SDS-PAGE, uncharacterized protein, amino acids, Trypsin, mosquitocidal, toxins, vector control, bio assay, *Clarias batrachus*

Mosquitoes are a medically important blood-sucking insect vector belonging to the phylum Arthropoda that transmits a variety of life-threatening parasitic and viral diseases such as dengue fever, filariasis, West Nile fever, yellow fever, malaria, and Japanese encephalitis. Each year, over 4 lakh deaths related to malaria were reported. The majority of malaria affects are children under the age of five (WHO, 2018). According to a recent WHO estimate, more than 15 million people worldwide have filarial lymphedema, and 25 million males have hydrocele. There are 36 million persons who are affected by these illness symptoms (WHO, 2021). In India alone, it contributes 44.3 % (7.44 million by filarial lymphedema, 12.88 million from hydrocele out of general 31.26 million filaraemics) of the global filariasis burden (WHO, 2014). Infected *Aedes* mosquitoes, principally the *Aedes aegypti* mosquito but sometimes other species, transmit the dengue virus to people (WHO, 2021). The flavivirus category includes the dengue virus along with other arboviruses that cause yellow fever and Japanese encephalitis. There are four different viral strains that cause dengue (DENVI, DENV2, DENV3, and DENV 4). Each of the 4 dengue virus strains has a different host immunological reaction to the disease infection. Currently, a novel dengue virus serotype that shares some genetic similarities with DENV2 but differs genetically from the three previous dengue serotypes (DENV1, 3, and 4) was found in Malaysia in 2013 (Mustafa et al., 2015; Normile, 2013). Dengue is one of the persistent illnesses carried by mosquitoes in Africa and Asia, along with malaria and filariasis. According to the most recent WHO estimate, 96 million new dengue cases are recorded annually, and 390 million people have been exposed to the dengue virus. Researchers have developed and documented various synthetic insecticides for control and management of mosquito vectors in recent years; however, these pesticides cause some ecological problems and are hazardous to non-target organisms. But microbial agents are environmentally safe alternatives to chemical pesticides for combating mosquito resistance and focusing on specific mosquito species. *Bacillus sphaericus* and *B. thuringiensis israelensis*, both sporulative, gram-positive bacteria, are well-known biological agents for controlling mosquito larvae. *B. sphaericus* is found to produce binary toxins (51kDa, 42kDa) responsible for mosquitocidal activity against *Culex* species. 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). Under these circumstances, *Bacillus cereus* VCRC-641 was isolated newly from the fresh water fish, namely *Clarias batrachus* and in the present study

we report the characterization of new toxin responsible for mosquito killing effect.

MATERIALS AND METHODS

In order to obtain the whole protein profile of the newly isolated mosquitocidal bacteria *Bacillus cereus* VCRC 641 was subjected to Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) analysis. The wells were loaded with samples, and the experiment was run beginning at 100V up to the sample dye edge touched the separating gel. Following that, gel performed at 200V for 2 hours. Coomassie Brilliant blue used for staining for overnight and then de-stained. Then de-stained gel was documented using Syngene gel documentation system. It is indispensable to identify and characterize the toxin (s) present in the new isolate (*B. cereus* VCRC-641) to understand the mosquitocidal effect. The cell pellet was quantified and one gram of wet cell pellet was mixed with 25 ml of 0.5% PBS. Further the suspension was homogenized using mechanical homogenizer (REMI, Made in India) then it was subjected to sonication (process time 10 min with pulse on time 10 sec and pulse off time 10 sec) under cold condition in sonicator (Biomatrix, Made in India). The sonicated mixture was centrifuged at 9000 rpm for 20 mins. Then the aqueous phase (cell lysate) was carefully separated and subjected to column chromatography for separating toxic fractions. The cell lysate was loaded onto the Sephacryl-S200 column with 0.5X PBS as a mobile phase. Totally 160 fractions of 1 ml each were collected in 1.5 ml eppendorf centrifuge tubes. Later, preliminary bioassay was carried out to check the mosquitocidal activity of these fractions.

Further, 12% Native PAGE gel electrophoresis was run with the mosquitocidal fractions and toxic protein bands were observed in the gel. Finally, the protein bands were seen under Gel Doc system (SYNGENE, Germany) and photographs of the gel was taken. The derived protein band was sent for further characterization of this toxic protein by LC-MS analysis from Rajiv Gandhi Centre for Biotechnology (RGCB), Trivandrum, India. The excise gel band was subjected to trypsin digestion using de-staining solution: 100 mM acetonitrile + ammonium bicarbonate (1:1, v/v). Extraction buffer: 5% acetonitrile + formic acid in 2:1 ratio. Liquid chromatography system (Instrument): nano ACQUITY UPLC® chromatographic system (Waters, Manchester, UK). Acquisition software: MassLynx4.1 SCN781 software, Waters. Binary solvent system: (0.1% CH_2O_2 in H_2O) Solvent A and (0.1% CH_2O_2 in C_2H_3N) Solvent B (Table 1).

RESULTS AND DISCUSSION

The polypeptide profile present in the newly isolated bacteria (*Bacillus cereus* VCRC 641) was analysed by SDS-PAGE and the result showed that the protein profile ranged from 15–200 kDa (Fig. 1).

Table 1. Liquid chromatography- Mass Spectrometry

Peptide count	Unique peptides	Confidence score	Mass	Description	TR1	TR2
3	3	15.0	19454.5	Azoreductase OS=*Bacillus cereus* OX=1396 GN=azr PE=4 SV=1	670.5	675.5
2	2	10.1	16615.1	Nucleoside diphosphate kinase OS=*Bacillus cereus* OX=1396 GN=ndk PE=3 SV=1	403.5	323.0
1	1	4.5	38346.1	Glycosyl hydrolase OS=*Bacillus cereus* OX=1396 GN=acm PE=4 SV=1	597.3	524.2
3	3	10.0	124145.4	LPXTG-motif cell wall anchor domain protein OS=*Bacillus cereus* AH1134 OX=405533 GN=BCAH1134_0567 PE=4 SV=1	635.7	734.4
19	19	78.4	20672.5	Uncharacterized protein OS=*Bacillus cereus* VD107 OX=1053229 GN=IIM_02704 PE=4 SV=1	23261.8	24749.9
4	4	13.4	115621.2	LPXTG-domain-containing protein cell wall anchor domain OS=*Bacillus cereus* HuB1-1 OX=1053207 GN=IGE_05002 PE=4 SV=1	1180.8	1156.3

Totally 160 fractions each 1ml were collected in 1.5 ml Eppendorf centrifuge tubes. Preliminary bioassay of these fractions was carried out to find the mosquitocidal activity. From the results it was observed that 11th to 75th cell fractions exhibited toxicity against mosquito larvae (*Aedes aegypti, Culex quinquefasciatus,* and *Anopheles stephensi*). 12% Native-PAGE gel was run with the mosquitocidal fractions and a prominent band of 70kDa protein was observed (Fig. 2). In the Rajiv Gandhi Centre for Biotechnology, (RGCB), Trivandrum, India, the LC-MS analysis was used to further characterise this mosquitocidal toxic protein, and it was identified as "Uncharacterized protein" of *B cereus* VCRC641. Results from the Liquid chromatography–Mass Spectrometry showing highest confidence score of 78.4, TR1 and TR2 value of 23261.8 and 24749.9 hits to "Uncharacterized protein" of *Bacillus cereus* strain (Table 1). One similar research demonstrated that the *Bacillus thuringiensis israelensis* (*Bti)* produced the mosquitocidal toxins Cry4A, 4B, 11A, and Cytolitic1A in a parasporal body (Federici et al., 2006). Crystal (Cry) and mosquitocidal (Mtx) toxins are the main types of toxins that *Bti* and *B. sphaericus* generates, and they have different compositions and synthesis times (Poopathi and Archana, 2012). Two polypeptides with molecular masses of roughly 51 and 42 kDa make up the *B. sphaericus* crystal toxin (Poopathi and Abidha, 2009; Charles and Nielsen-LeRoux, 2000). All these bacterial strains generate crystalline-endotoxin, which is only found in Coleoptera and Diptera (Hofte and Whitley, 1989, Poopathi and Tyagi, 2002). While the 42 kDa component confers toxicity, the crystal toxin

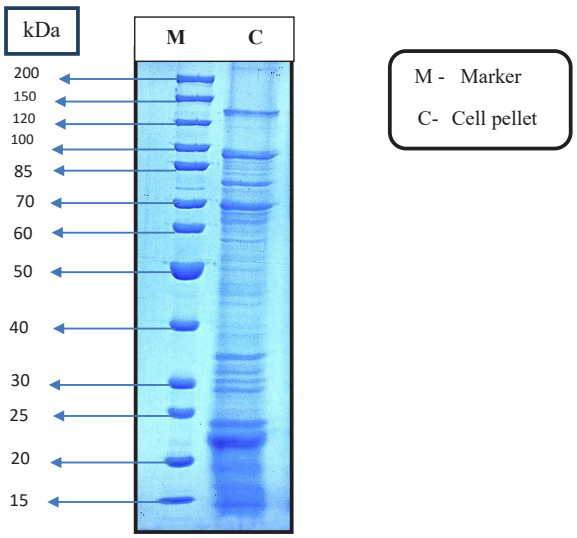

Fig. 1. Protein profile of *Bacillus cereus* VCRC 641 lyophilized cell pellet

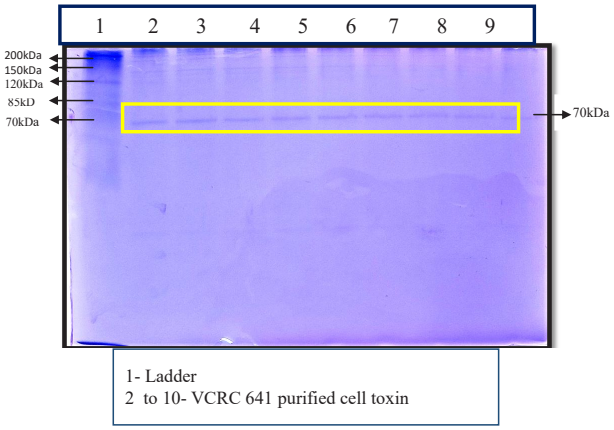

Fig. 2. Native PAGE for *Bacillus cereus* (VCRC-641) purified cell toxin

component has synergistic efficacy then its process of action necessitates precise binding to the larval gut wall receptor (Oei et al., 1992).

Due to the S-layer protein on their outer surface, *B. sphaericus* has lately been linked to high perseverance even in polluted ponds and industrialized effluent. S-layer also contributes to the pathogenicity of many bacteria, particularly those belonging to the *Bacillus* species clusters *B. cereus* and *B. anthracis*, and this indication provides a starting point for the investigation of new isolates that include S-layer protein. Allievi et al. (2014) discovered that S-layer protein has a significant part in larvicidal activity. Finally, it was determined that S-layer was a factor in the killing of *Culex* and *Aedes* larvae by testing it against spore crystal protein and S-layer protein. *Cx. quinquefasciatus*, *An. stephensi*, and *Ae. aegypti* were the three main mosquito vectors that *B. cereus* VCRC B540, which was isolated from marine fish, demonstrated entomopathogenic activity against these mosquitoes. Additional examination of the toxin using M/S MALDI-TOF discovered S-layer protein was the mosquitocidal factor. According to the preceding reports, S-layer protein from *Bacillus cereus* may have a significant role in mosquitocidal activity. In the current study, mosquitocidal effect of *Bacillus cereus* VCRC 641 was due to a specific mosquitocidal polypeptide with molecular weight of 70kDa and it was identified as "Uncharacterized protein". Recently, similar observations have been made on varies strains of *B. cereus* isolated from a variety of habitats, including silt, dead mosquito larvae, clinical samples, and ditch (Haynes et al., 2011; Poopathi et al., 2014). The spore-coat proteins of *B. cereus* VB17 (180 and 160 kDa) and *B. cereus* VB24 (130 kDa), two of these strains, have shown mosquito harmful action (Haynes et al., 2011).

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AUTHOR CONTRIBUTION STATEMENT

SM has contributed in conducting a thorough literature search, compiling and interpreting the results, data collection, data analysis and interpretation, drafting the article, critical revision of the article. KA has contributed in selection of a relevant journal with proper parameters and participation in writing appropriately. BB has contributed to the setting up of the toxicity experiment. VA has aided in the extraction of genomic DNA, gathering of non-target organisms for toxicity testing from paddy fields. PH has contributed in article collection, data tabulation, article framing, reference formatting. SM has contributed in the collection of literature, checking grammar and tabulating of data. JL has contributed to study 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 manuscript, literature check-up, facilitating the update about experts in the field. SP has 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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