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ISOLATION AND CHARACTERIZATION OF A NOVEL BACTERIUM, *Bacillus thuringiensis* var. *israelensis* VCRC-B651 EXHIBITING HIGH EFFICACY TO CONTROLMOSQUITO VECTORS

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ABSTRACT

The aim of this study was to isolate novel mosquitocidal bacteria from clayey soil. The soil samples were collected during the period 2021 to 2022 from an agricultural field of Tamil Nadu (India). The samples were processed for isolating the bacteria on nutrient yeast extract salt medium. The bacterial cell mass was harvested after 72 h of lyophilisation. Bioassay was carried out with the bacterium against larval species of *Culex quinquefasciatus, Aedes aegypti* and *Anopheles stephensi.* Whole genome sequence (WGS) analysis (Illumina) was done to identify the strain. Growth dynamics, biochemical characteristics, and toxicity assays with non-target aquatic organisms were also done. Based onWGS analysis the new isolate was identified as *Bacillus thuringiensis* serovar *israelensis*. The strain was coded as VCRC-B651. The isolate exhibited effective toxicity against *A. aegypti, A.stephensi,* and *C. quinquefasciatus* with LC_{50} values of 0.005, 0.009 and 0.009 mg L^{-1}, respectively, and LC_{90} values of 0.009, 0.02 and 0.018 mg L^{-1}, respectively. The isolate exhibited higher mosquitocidal activity as compared to the WHO reference strain (*Bti* H14). No adverse effect on aquatic non-target organisms was observed. The new isolate was highly efficacious in controlling the mosquito larvae.

Keywords: *Bacillus thuringiensis* serovar *israelensis*, bioassays, clay soil, growth dynamics, mosquito larvae, whole genome sequence

INTRODUCTION

Mosquito vectors harbour bacteria, viruses, protozoans, and nematodes, and spread diseases [mosquito-borne diseases (MBD)] to humans and animals (Niang *et al.,* 2018). Some of the MBDs like heartworm, Zika, West Nile fever, chikungunya, Japanese encephalitis, Rift Valley fever, malaria, dengue, etc. are more life-threatening. Each MBDs is transmitted by different mosquito species vectoring different pathogens from different host(s) (human/animal) having a unique transmission cycle. These diseases may cause short- or long-term sickness, and probably cause deaths in human and animals, thereby inflict economic losses and prove global burden. In wildlife, these diseases may even lead tobiodiversity loss (Asigau and Parker, 2018; Folly *et al.,* 2020).

Augmenting globalization, pollution, urbanization, and climate change have made mosquito vectors to evolve and adapt to the changing environment. This causes serious impediments in controlling mosquito vectors. In mosquito control interventions, the emphasis is laid on to reduce the

mosquito breeding source as it kills/eradicates the immatures before reaching the adult stage (Joshi *et al.,* 2005; Asigau and Parker, 2018). Presently, chemical and biological pesticide approaches are mostly practiced to control mosquito vectors. However, due to the resistance development and environment hazards affecting non-target organisms in the surroundings, the chemical pesticides have emerged as a global issue (Wilson *et al.,* 2020). Worldwide, huge efforts are on to explore and isolate potential mosquitocidal bacteria (Wilson *et al.,* 2020). *B. thuringiensis israelensis* (*Bti*) and *Lysinibacillus sphaericus* (*Ls*) are broadly in use as bio-pesticides for mosquito control. *Bti* produces crystal toxin proteins that are effective in killing many pests like chironomid midge, black flies, mosquitoes, etc. (Federici *et al.,* 2010). *Ls* produce binarytoxins (Bin) that have a larvicidal effect on mosquito species (Filha *et al*., 2014). However, *Ls* has developed resistance in many countries and led to the omission of its use as mosquito control agent (Wirth *et al.,* 2010). Consequently, attention is focussed on the isolation of new bacteria from natural sources for mosquito control (Poopathi *et al.,* 2013). Soil being a natural source of diverse microorganisms, hence the current research was focused on to find and utilize effective alternative strategies like microbial biocontrol agents which may be eco-friendly and cost-effective. This study attempted to isolate novel bacteria from agricultural soils to control mosquito vectors.

MATERIALS AND METHODS

Soils from paddy fields in Pallikuppam village, Vellore district,Tamil Nadu, India (12.9935° N, 79.1534°E) were examined. The soil samples were collected from three different soil depths, *viz.*, 0, 5 and 10 cm soil depth in sterile tubes. The samples taken from different levels of all experimental sites were pooled into 135 samples. The sample tubes were sealed and kept at 4˚C until further processing in laboratory (Kumar *et al.,* 2012). A known quantity of soil (1 g) was thoroughly mixed in sterile distilled water (10 mL) and autoclaved at 121^0C for 15 min in test tubes and serially diluted to 10^{-4} fold under aseptic conditions. Then 0.1 mL of seriallydiluted sample was spread in pre-solidified nutrient yeast extract salt mineral medium (NYSM) agar plates. NYSM broth was prepared by mixing yeast extract (0.5 g), NaCl (50 g), beef extract (3 g), peptone (5 g), glucose (5 g), and mineral solution containing $CaCl_2$, $MnCl_2$ and $MgCl_2$ (10 mL) in 1 L distilled water (pH 7.5). The diluted soil samples were not heated before plating to increase the range of expected mosquitocidal bacteria (from both Gram-positive and Gram-negative groups). The plates were incubated at 30°C for 24 h. Then the growths of different bacterial colonies were observed and single separate colonies picked up using a sterile metal wire loop and inoculated aseptically in 10 mL NYSM broth in test tube. These were then incubated at 30˚C in an incubator shaker (Scigenics Biotech LT4676, India) at 250 rpm for 72 h.

Preliminary bioassays with mosquito larvae

The preliminary screening was carried out through bioassays. The bioassay was conducted in a wax-coated paper cup (150 mL capacity) filled with 100 mL tap water (chlorine-free),with each cup containing 25 laboratory-reared larvae of mosquitoes *viz*., *Culex quinquefasciatus, Aedes aegypti* and *Anopheles stephensi*. The late 3rd instar larvae were obtained from the Mosquito Rearing and Colonization Unit of ICMR-VCRC, Puducherry (India). Toxicity tests were carried out under a 12:12 h light/darkness photoperiod while maintaining room temperature in the laboratory. Doses of 1 and in control biscuit and yeast in 1:2 ratio) was added to the bioassay cups. Two positive control NYSM + larval food, and larval food alone were used. The larval mortality from respective cups was recorded after 24 h. The percentage mortality of larvae was calculated as follows:

$$\text{Mortality (\%)} = \frac{\text{Dead larvae counted}}{\text{Total larvae exposed}} \times 100$$

Abbot's formula was cross checked, if the controls showed < 5% mortality (Abbot, 1925):

$$\text{Mortality (\%)} = \frac{\text{Mortality (\%)in treatment} - \text{Mortality (\%) in control}}{100 - \text{Mortality (\%)in control}} \times 100$$

From the aforesaid preliminary screening, only the isolates showing 100% larval mortality were considered as highly potential isolates from soil for further analysis.

Extensive/detailed bioassays

The screened most potential bacterium was streaked in an agar plate through quadrant streaking method and incubated for 24 h to check its purity. Slant culture was made after 24 h from the colonies with similar morphology. From slant culture, a loopful of bacterial colony was picked up and inoculated in NYSM broth (10 mL) and kept in an incubator shaker for 12 h (mother culture). The mother culture (10 mL) was inoculated in a 2 L Erlenmeyer flask containing 500 mL NYSM and kept in an orbital shaker for 72 h at room temperature (37˚C) with constant agitation (250 rpm). The culture was centrifuged at 10,000 rpm for 20 min in a high-speed refrigerated centrifuge (Hitachi CR22III, Japan), and cell pellet (cell mass) harvested and freeze-dried using lyophilizer (Christ Alpha 1-2 LD plus, Germany).

The stock solution was prepared from the lyophilized powder (5 mg) by homogenizing it with 10 mL water (Remi RQT 124A, India). Bioassay was carried out using seven different doses with each one replicated four times (WHO, 2005). The experiment was repeated three times on different days. Positive controls were maintained.Moribund larvae (if any) were counted as dead. After 24 h of exposure, bioassay readings were noted. The live larvae were counted to record the percent mortality. Regression analysis-SPSS (16.0 version software) was statistically applied to estimate the lethal concentrations (LC_{50} and LC_{90}).

Bioassay with non-target aquatic organisms

Non-target aquatic organisms *viz*., tilapia fish (*Oreochromis mossambica***)**, damselfly larvae (*Zygoptera* larvae), water bugs (*Lethocerus*), water striders (*Gerridae*), back swimmer (*Notonectidae*), snails (*Physa*), and tadpoles (frog) were collected from irrigation canals, paddyfields and ponds. They were acclimatized in laboratory, and bioassay carried out using suitable doses (0, 1, 10, 20, and 30 times of LC_{90} dose). Mortality was noted after 24 and 48 h. With adequate replicates, bioassay was repeated 3 times at different time interval (Wipfli *et al.*, 1994).

Microscopic and biochemical studies

The colony characters of VCRC-B651 such as shape, margin, opacity, and colour were observed. For microscopic studies of vegetative and sporulation stage of bacteria, simple safranin staining and Schaeffer-Fulton staining was done, respectively (Schaeffer and Fulton, 1933). Gram's staining was done in vegetative cells. A biochemical test was done to detect the use of different carbohydrate sources (arabinose, trehalose, mannitol, and sucrose) and other components like (malonate, citrate, arginine, nitrate reduction, and catalase) using Hi *Bacillus* identification kit. It is a combination of 12 tests, based on the pH changes and substrate utilization.

Bacterial growth, protein, biomass and SDS-PAGE

The isolate from glycerol stock was inoculated in NYSM (10 mL) and grown for 12 h as pre-culture. Then, 200 µL pre-culture was inoculated into 15 test tubes containing 10 mL NYSM broth and incubated at 37^0C in an orbital shaker for 90 h with constant agitation (250 rpm). After every 6 h, one tube was picked up and stored at 4˚C. After 90^{th} h, the turbidity of cultures was measured using spectrophotometer SP-75 UV-VIS (Sanyo, UK) at 650 nm using NYSM broth as control.

Protein quantification was done with SP-75 UV-VIS spectrophotometer (Sanyo,UK) at 595 nm using standard bovine serum albumin (Lowry *et al.*, 1951; Bradford, 1976). For biomass analysis, the cultures with different growth periods were centrifuged (10,000 rpm, 15 min) and wet and dry biomass quantified (Poopathi *et al.*, 2013; Mardhiah *et al.*, 2015). Electrophoresis (sodium dodecyl sulphate polyacrylamide gel electrophoresis) was carried out from bacterial cultures to visualize the expression of protein profiles. The 10 mg cell pellet (derived from different culture times 6 to 90 h) was mixed

with sample loading buffer (SLB) and electrophoresis carried out (Genei, India) (Laemmli, 1970). The protein profiles were visualized after staining.

Bacterial identification

DNA from the newly isolated bacterium was done (bacterial genomic DNA Kit, GenElute™, Sigma-Aldrich, USA). Whole Genome Sequence (Illumina - next generation sequencing) for bacterial identification was carried out (Rabha *et al.*, 2023).

RESULTS AND DISCUSSION

Screening and selection of potential bacterial strain

During field survey 675 clay soil samples were collected from different soil depths in agricultural land of Pallikuppam village, Vellore, Tamil Nadu (India). The serial dilution of soils, plating and incubation of soil samples on growth medium (24 h) depicted the presence of several colonies of bacteria. Homogenous colonies were identified and segregated. Bacterial colonies were inoculated in NYSM broth and 72 h culture used for preliminary bioassay screening against the larvae (late 3rd instar) of *Culex quinquefasciatus, Anopheles stephensi*. and *Aedes aegypti*. Three bacterial isolates showed toxicity and the most potential isolate was selected for further studies. The lethal concentrations (50 and 90%) against *A. aegypti* were 0.76 and 0.96 mg L^{-1}, respectively. In present study the isolated strain *Bacillus thuringiensis* serovar *israelensis* VCRC-B651 showed more efficacy against the *A. aegypti* (Table 1). Recently, Hemaladkshmi *et al.* (2023) identified *B. thuringiensis israelensis* VEVP-60 from red soil and was moderately effective against the mosquito larvae, such as, *A. aegypti*. *An. stephensi* and *Cx. quinquefasciatus*.

Table 1: Mosquitocidal toxicity of *Bacillus thuringiensis israelensis*, VCRC B651

Mosquito species	Slope	Intercept	LC_{50}		LC_{90}		χ^2 (df)
			mg L^{-1}	*LCL-UCL	mg L^{-1}	*LCL-UCL	
Aedes aegypti	0.028	- 1.431	0.005	0.0045-0.0054	0.009	0.009-0.013	204.14
Anopheles stephensi	0.012	-0.913	0.009	0.0083-0.010	0.020	0.018-0.0219	169.37
Culex quinquefasciatus	0.015	-1.416	0.009	0.009-0.0101	0.0183	0.017-0.0192	114.23

*LCL-UCL = Lower confidential limit to Upper confidential limit

The DNA extracted from the isolate was subjected to Whole Genome Sequence (Illumina) and the strain was identified as *Bacillus thuringiensis* subspecies *israelensis*. The history of the origin of the bacterial strain was submitted to MCCF (Microbial Culture Collection Facility, VCRC) and the reference code was documented (VCRC B651). The WGS for identification of bacterial strain (*Bti*) was meticulously followed the principles of work reported earlier (Doggett *et al.*, 2013).

In present study, the isolated bacterial strain of *Bacillus thuringiensis* subspecies *israelensis* VCRC B651 showed a significant effect on controlling the mosquito larvae (Table 1). Comparative analysis of the results showed that *A. aegypti* was highly susceptible, followed by *An. stephensi* and *Cx. quinquefasciatus.* The relative trend in the toxicity was graded as: *A. aegypti* > *An. Stephensi* > *Cx. quinquefasciatus*. Further, the comparative analysis with WHO reference strain *Bti* H14 showed that the new isolate was more potent. The lethal concentration values (LC_{50}) of new (*Bti* VCRC B651) and reference (*Bti* H14) strains against *Cx. quinquefasciatus* were 0.009 and 0.018 mg L^{-1}, respectively (Table 2). The observations depicted drastic variation in toxicity between the isolates (*Bti* VCRC B651 and *Bti* H14). Poopathi *et al.* (2014) have earlier reported that some *Bacillus* species of *Bti* and *B. sphaericus* showed differential toxicity between the bacterial strains and also between the mosquito species. *Cx. quinquefasciatus* was highly susceptible larvae than other species of *A. aegypti* and *An. stephensi*. Therefore, the results in present study are in accordance with the earlier work (Poopathi *et al.*, 2014). To confirm the target

Table 2: Comparative analysis of toxicity of new isolate (*Bti*) with reference strain (*Bti* H-14)

Bacterial strains	Mosquito species	LC50 (µg mL^{-1}) (LCL -UCL)
Bti VCRC B651	*Culex quinquefasciatus*	0.00961 (0.0095-0.0101)
(Isolate from clay soil, India)	*Anopheles stephensi*	0.00919 (0.0083 - 0.0100)
	Aedes aegypti	0.00501 (0.00459- 0.0054)
Bti H-14	*Culex quinquefasciatus*	0.018 (0.017 - 0.0186)
(WHO reference strain)	*Anopheles stephensi*	0.008 (0.0079-0.0089)
	Aedes aegypti	0.009 (0.0082-0.0097)

specific *i.e.*mosquito specific characteristic of *Bti* VCRC B651, and to prove that the bacterial strain does not have any adverse effect on test aquatic organisms, bioassays were carried out on non-target organisms, such as damselfly larvae (*Zygoptera*), waterbug (*Lethocerus*), common snail (*Physa*), tilapia (*Oreochromis mossambica*) and black swimmer (*Notonectidae*) as damselfly larvae (Zygoptera), water bug (*Lethocerus*), and tadpoles (frog). After 24 and 48 h exposure to the bacterial toxins (*Bti* VCRC B651), no mortality was observed in non-target organisms. These observations are in agreement with the earlier reports on the non-efficacy of bacterial bio-pesticides against aquatic non-target organisms (Manikandan *et al.*, 2023).

The colony morphology of bacterial isolate *Bti* VCRC B651 revealed that the colony was circular, dry flat, rough, irregular margins with dull white colour. The shape during vegetative and sporulation stages was rod (filamentous) and elliptical, respectively. The strain was endospore-forming, aerobic but at the same time facultative anaerobic. Other morpho-physiological characteristics revealed the bacterium to be sporangial and crystal-forming in nature (Fig. 1). Biochemical characterisation of this mosquitocidal bacterium (*Bti*) when tested with *Bacillus*-specific Hi *Bacillus* Identification Kit showed that the strain was positive in reaction to trehalose and catalase tests, but negative to Voges-Proskauer, citrate, glucose, mannitol, arginine, sucrose, malonate, o-nitrophenyl-β-D galactopyranoside (ONPG) and arabinose tests. A delayed reaction was observed for nitrate reduction test. These biochemical characteristics showed that the strain was closely similar to *B. cereus* and *B. thuringiensis* as per the guidelines of the kit for data interpretation.

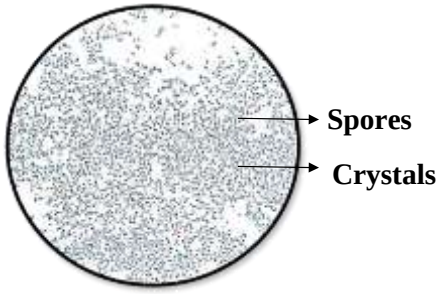

Fig. 1: Safranin-Malachite green spore staining of *Bti* VCRCB651

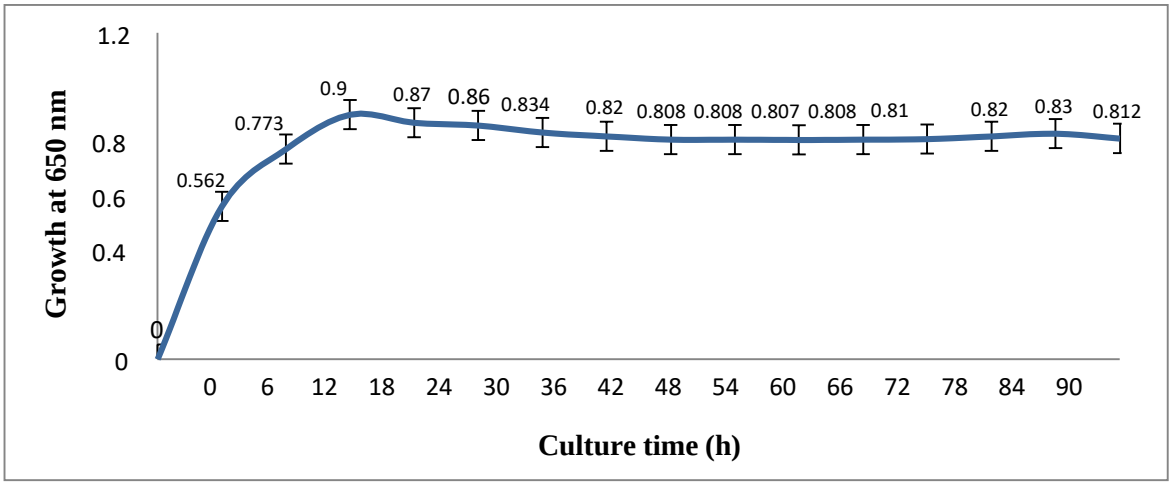

Fig. 2: Growth pattern of VCRC-B651

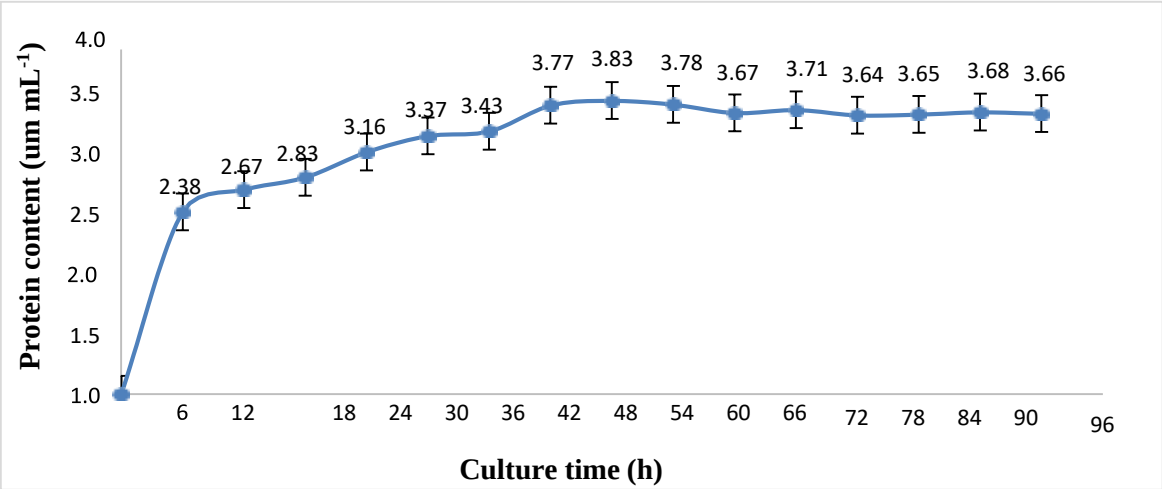

Fig. 3: Protein content of *Bti* VCRC-B651

Burgeoning literatures showed that the mosquitocidal bacterial strains have similar morphological characters especially with respect to the size and structures (Poopathi *et al.*, 2014; Hemaladkshmi *et al.*, 2023; Manikandan *et al.*, 2023). However, some biochemical characteristics differed between the strains. As the isolated bacterium was endospore-forming, Gram-positive and aerobic, an extensive study on its growth pattern, protein content, biomass production, and expression of protein profiles was investigated. As shown in Fig. 2, the culture turbidity/density of themedium isan indication of bacterial growth. The bacterium started growing steadily from lag phase to stationary phase. Up to 42 h there was rapid multiplication and steady growth were observed, thereafter, growth showed insignificant variation. Complete sporulation was attained at 90 h. A similar trend was also observed in protein synthesis up to 42 h and thereafter it was steady (Fig. 3). Biomass was the indication of the synthesis of crystal mosquitocidal toxins and in the present study uniform biomass production was noticed (Fig. 4 and 5). The results in this study revealed straight relationship between bacterial growth,

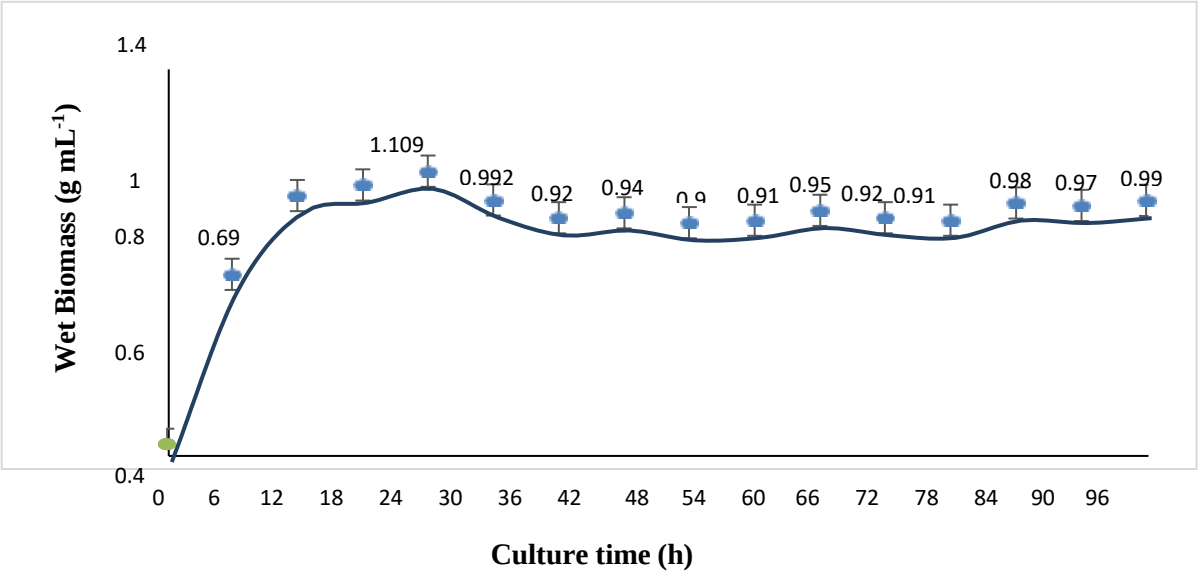

Fig. 4: Wet biomass production of *Bti* VCRC-B651

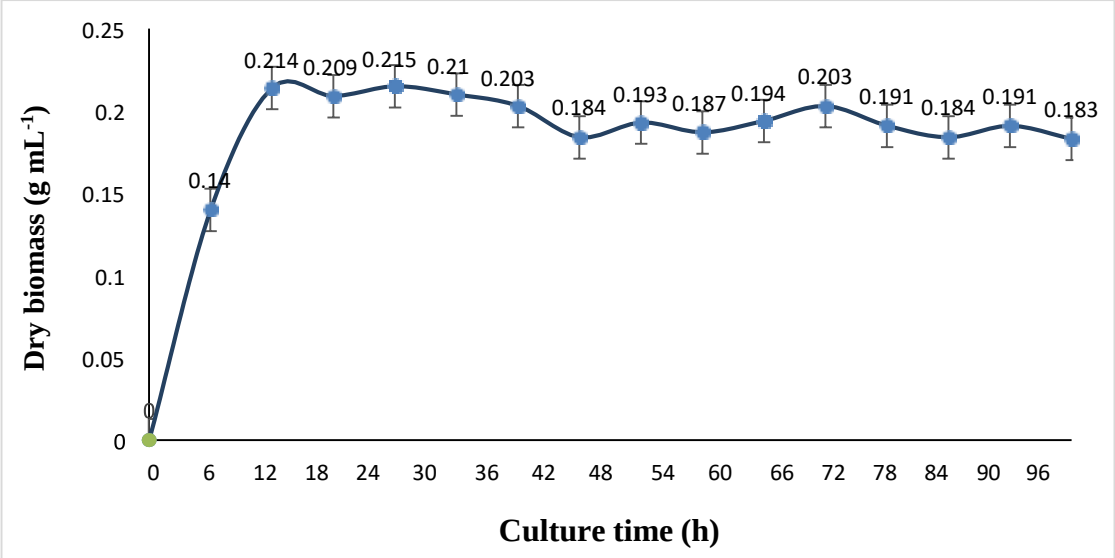

Fig. 5: Dry biomass production of *Bti* VCRC-B651

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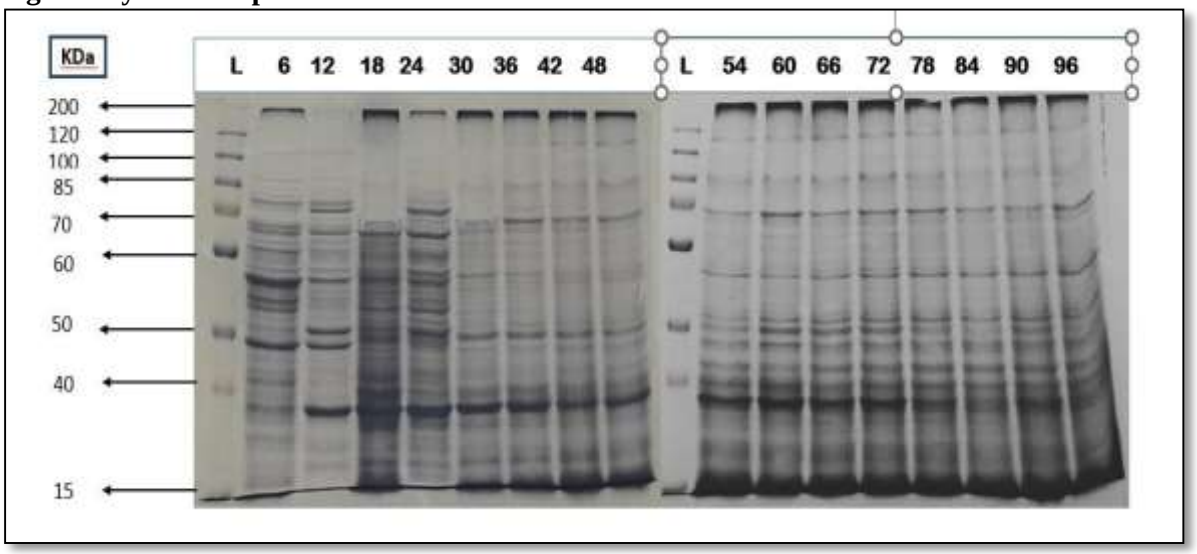

Fig. 6: SDS-PAGE: protein profile expression from *Bti* VCRC-B651

protein synthesis, and biomass yield. Similar trend in growth, protein, and biomass production were seen in other strains of *Bti*, *B. sphaericus B. cereus* etc. (Mani *et al* 2014; Manikandan *et al.*, 2023; Abhisubesh *et al.*, 2023). Expression of protein in SDS-PAGE depicted that up to 42 h the protein profile in the form of protein band expression started increasing and then remained constant (Fig. 6). These physiological reactions withinthe bacterial cells are its unique features, as there was a direct correlation among bacterial growth, protein synthesis, biomass prodction, and protein (polypeptide) profiles. The toxic polypeptide (s) responsible for mosquitocidal effect from these proteins expressed with special emphasis to different bacterial growth is yet to be identified. The unique polypeptides namely surface layer protein (51.7 kDa) and uncharacterized protein (70 kDa) were identified as mosquitocidal from *B. cereus* strains (VCRC-540 and VCRC-641) (Mani *et al.*, 2018; Manikandan *et al*., 2023).

Mosquito vectors are potentially responsible for the transmission of many deadly diseases to humans. Mosquito control is the most essential public health aim (Dahmana and Mediannikov, 2020). The frequency of MBDs is growing in countries where it is already a serious issue. Due to the increased globalization, urbanization, and the effects ofglobal warming there is a rise in the mosquito vector population (Caminade *et al.,* 2019). The application of chemical pesticides for controlling mosquito vectors in long-term results of resistance emergence in mosquito vectors and also causes environmental hazard (Senthil-Nathan, 2020). Consequently, adaptation of the mosquito to climate change and urbanization and the cross-transmission of MBDs in man and animals are responsible at various levels for augmented mosquito proliferation (Joshi and Miller, 2021). Under these situations, there is a need to follow proper vector surveillance to monitor mosquito breeding and take proper interventions at the appropriate time to curtailthe spreading of diseases by mosquitoes (Fournet *et al.,* 2018). So this also emphasizes theurgent need to use an alternative vector control method that is eco-friendly, cost-effective,and does not affect non-target populations in the environment.

Since there is a huge demand to develop new eco-friendly bacterial mosquitocidal agents from natural sources, exploratory researches are in progress globally. Soil is an excellent natural source (reservoir) for many microbes having potential to control mosquito vectors (Kumar *et al.,* 2012). Many noteworthy mosquitocidal bacteria like *Lysinibacillus sphaericus (Ls), B. thuringiensis israelensis (Bti), B. cereus, B. subtilis, B. circulans, Clostridium bifermentans, Pseudomonas fluorescens, B. brevis, B. alvei, Chromobacterium, Aneurinibacillus* sp. *Xenorhabdus* and *Photorhabdus* have been isolated from natural sources (Geetha *et al.,* 2007; Kumar *et al.,* 2012; Ramirez *et al.,* 2014; Da Silva *et al.,* 2020). Most of these mosquitocidal bacteria belong to the *Bacillus* group. On the whole, the most effective way to decrease the MBDs incidence is to manage the mosquito population, primarily by applying bio- pesticides on their breeding sites (Benelli *et al.,* 2016). The two most recognized biopesticides are: *B. sphaericus* and *B. thuringiensis* var. *israelensis*, which were employed due to their ability to kill mosquitoes by producing endotoxins. The principal factor in *L. sphaericus* is "Bin toxin" (51 and 42 kDa proteins) present in the endotoxin. Whereas in *Bti*, it is due to the presence of complex toxic crystal proteins that include Cyt1A, Cry4A, Cry4B, and Cry11A (Silva-Filha *et al.,* 2021). Despite the valuable role of endotoxins from *Ls* and *Bti*, to control mosquito vectors, regrettably, the recent development of resistance to *Ls* against the filarial vector of *Cx. quinquefasciatus* had impeded its application (Rao *et al.,* 1995).

In present study efforts were made to isolate novelbacteria with high larvicidal potential. Soil is a huge reservoir of many types of microorganisms that has the potential to be used for many industrial and medical applications (Prasad *et al.,* 2012). Considering the importance of soil resources, for the mosquito control program, a field survey was carried out from 2021 to 2022 in various districts of Tamil Nadu, India. In the experimental district, clay soil from paddy fields wasidentified as rich source of microbes. The clay soil majorly encompasses tiny sand particles with colloidal, sticky substances. From this clay soil, a novel bacterium was isolated and identified through WGS, as *B. thuringiensis* serovar *israelensis* VCRC-B651.This strain was highly effective against the dengue vector of *Aed. aegypti*. It also has an effect against the malarial and filarial vectors of *An. stephensi* and *Cx. quinquefasciatus*. No adverse effect against non-target organisms was proved. More significantly, this new isolate was comparatively more efficient than the internationally recognized reference strain of WHO (*Bti* H14). Therefore, it is suggested that the isolated strain may be useful for future researchers and industrialists for better improvement of mosquito control in present scenario of resistance development in mosquito population. Biochemical analysis revealed that the strain wasclosely similar to *B. cereus* and *B. thuringiensis.* The authors have earlier proved that these strains have proficient in toxic effect against mosquito larvae (Poopathi *et al.,* 2014; Mani *et al.,* 2015; Manikandan *et al.,* 2023). Therefore, the present isolate *Bti* VCRC B651 appears one of the potential strain. The principal factor (toxin) responsible for the mosquitocidal effect is under investigation. The physiological characteristics of this strain revealed the uniqueness of direct correlation of growth, protein synthesis, biomass production, and expression of polypeptides during vegetative and stationary phases of bacterial growth. These observations corroborate with our earlier works (Poopathi *et al.,* 2014, 2015). Further,

the expression of polypeptides from the isolate was consistent upto 42 h and thereafter started increasing till completion of sporulation (90 h). This may be due to the reason of more protein requirements at the time of production of spores. Further, zymogram analysis is required to confirm this observation.

Conclusion: A novel mosquitocidal strain of *B. thuringiensis israelensis* VCRC- 651 was isolated from the clay soil of an agricultural field for the first time in South India.The salient feature of the strain is to control mosquito larvae subjecting with minimal dosage compared to the reference strain. The isolate is not hazardous to the environment as well as the aquatic organisms. Therefore, the investigated isolate (*Bti* VCRC B651) in the current study, may be useful for futuristic researchers to develop appropriate formulation in the current scenario of resistance by the mosquitoes in the field condition.

Conflict of interest: All authors declare there was no conflictof interest.

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