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Unraveling the presence of multi-class toxins from *Trichodesmium* bloom in the Gulf of Mannar region of the Bay of Bengal

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ABSTRACT

Trichodesmium is an enigmatic bloom forming, non-heterocystous cyanobacterium reported most frequently in the coastal waters of India. However, the toxigenic potential of this globally significant N_2 fixing cyanobacterium has not been characterized. In this study, we report for the first time the presence of potent multi-class neurotoxins such as Anatoxin-a, Saxitoxins, Gonyautoxin and hepatotoxins like MC-LR, MC-YA from a bloom material of *Trichodesmium* sp. MBDU 524 collected at the Gulf of Mannar region. Toxins were determined using liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS) analysis of HPLC purified aqueous and solvent fractions. Molecular phylogenetic analysis through 16S rRNA gene sequencing showed the close relationship with *Trichodesmium erythraeum* clade. The toxigenic potential was validated through brine shrimp toxicity assay and showed 100% mortality after 48 h of incubation. The results suggest the potential toxigenic and environmental impacts of *Trichodesmium* bloom sample from the Gulf of Mannar region.

1. Introduction

Trichodesmium is a filamentous, non-heterocystous and marine diazotrophic cyanobacterial genus of the family Oscillatoriaceae. *Trichodesmium* group forms extensive surface bloom, generally described as "Sea-Sawdust" covering kilometers of ocean in tropical and subtropical regions (Capone et al., 1997, 2005; Carpenter and Romans, 1991). The genus *Trichodesmium* is recognized as a unique primary producer, aiding in balancing nitrogen (N_2) flow through fixation of atmospheric N_2in global ocean and sustaining marine life through cycling of key nutrients such as carbon and N_2 (Carpenter and Capone, 2008). It has been reported that the genus *Trichodesmium* is composed of, so far, eight species, *Trichodesmium erythraeum*, *T. thiebautii*, *T. hildebrantii*, *T. aureum*, *T. contortum*, *T. havanum*, *T. pelagicum* and *T. tenue* (Hynes et al., 2012). Of all the species, *T. erythraeum* is the most commonly found bloom-forming species in world Ocean including Indian coastal waters (Capone et al., 1998; D'Silva et al., 2012).

The *Trichodesmium* bloom creates discoloration of waters and reported to cause mortality of fishes (Endean et al., 1993) and pearl oysters (Negri et al., 2004). The coastal *Trichodesmium* blooms have been demonstrated to possess toxic effects on copepod *Acartia tonsa* (Guo and Tester, 1994) and humans (Tamandare fever) (Satô et al., 1963). Due to the advancement of chromatographic methods integrated with mass analyses (Poon et al., 1993), in recent years, *Trichodesmium* sp. have been shown to produce different type of toxins, including the ciguatoxin-like toxin, palytoxin and 42-hydroxy-palytoxin from New Caledonia lagoon (Kerbrat et al., 2010, 2011), trichotoxin, a novel chlorinated compound, from western Gulf of Mexico (Schock et al., 2011) and Saxitoxin from southwestern South Atlantic Ocean (Detoni et al., 2016). The difficulty in laboratory culturing of *Trichodesmium* hinders the identification and characterization of toxins and natural products from this important genus of the phytoplankton community.

Among all the species, *T. erythraeum* is the common bloom-forming species in coastal waters of India. With the enhancement in algal monitoring programs, the distribution of *Trichodesmium* blooms was reported until recently from various coastal areas of India, including the Arabian Sea (Parab and Matondkar, 2012; Jabir et al., 2013), Cochin back waters (Martin et al., 2013) and Bay of Bengal (Shetye et al., 2013). These blooms had both direct and indirect effects on the coastal waters and affected fisheries, other marine life and humans as well. More than 40 mortality cases caused by *Trichodesmium* bloom have been reported along the east

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and west coast of India (D'Silva et al., 2012). There are accumulating evidence on the distribution, biogeochemical and ecological significance of *Trichodesmium* blooms in Indian coastal waters available since 1965, when Wood (1965) reported *Trichodesmium* blooms for the first time in the Indian Ocean (Thajuddin and Subramanian, 2002; D'Silva et al., 2012). However, reports dedicated to the toxic nature, toxins produced and the molecular mechanism behind toxicity remains very sparse.

In the present study, we reported for the first time the evidence of toxins producing *Trichodesmium* blooms, identified as *Trichodesmium* sp. MBDU 524, near coastal areas of the Gulf of Mannar region of the Bay of Bengal. The purpose of the research work was to characterize the toxins produced by *Trichodesmium* sp. MBDU 524 through liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS) analysis. The toxicity level of HPLC purified extracts of *Trichodesmium* sp. MBDU 524 by brine shrimp toxicity assay was also performed.

2. Materials and methods

2.1. Collection of Trichodesmium bloom samples

The bloom sample of *Trichodesmium* sp. MBDU 524 was collected from coastal areas of the Pudumadam region (9.27702° N; 78.993845° E) at the Gulf of Mannar, Ramnad district, Tamilnadu in August 2015. The bloom water samples were collected in screw top centrifuge tubes, polythene bags, 2 L bottles and 20 L plastic containers and transported to the Bharathidasan University, Tiruchirappalli, Tamilnadu on the same day. On arrival at the laboratory, the samples were observed initially under a light microscope for morphological characterization of *Trichodesmium* and for the presence of other plankton species. The trichomes were hand-picked manually using spatula to get rid of attached sea grass and other debris. The hand-picked trichomes were washed couple of times in trays containing filtered sea water (0.2-μm-pore size Whatmann membrane filter) until pure trichomes seen under microscope. Finally, the cleaned trichomes that were transferred to filter sterilized sea water were used for further studies.

2.2. Genomic DNA isolation

The genomic DNA was isolated from cleaned trichomes of *Trichodesmium* sp. MBDU 524 using DNAeasy Blood and Tissue kit (Qiagen, Germany) according to manufacturer's instruction. The extracted genomic DNA was quantified using Spectro UV-VIS Double PC8 Auto Cell Scanning spectrophotometer (Labomed, Inc., USA.). The quality of the DNA was verified by agarose gel electrophoresis according to standard procedures (Sambrook et al., 1989).

2.3. Morphology and molecular identification of Trichodesmium bloom samples

The taxonomic identity of *Trichodesmium* trichomes was determined by morphological criteria (Desikachary, 1959; Rippka et al., 1979), PCR amplification and cycle sequencing of 16S rRNA gene. The 16S rRNA gene was amplified using the cyanobacterium-specific 16S rRNA gene primers as described previously (Thajuddin et al., 2010). The fragments of the 16S rRNA gene was amplified using a forward primer A2F (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer S17R (5′-GGCTACCTTGTTACGAC-3′). PCR reaction was carried out in a total volume of 50 μL containing 100 pmol of each primer, 0.2 mM of dNTPs, 100 ng of cyanobacterial genomic DNA and 1.25 U of Dream Taq DNA polymerase (Thermo scientific Dream Taq DNA polymerase # EP0702) using Veriti 96-well thermal cycler (Applied Biosystems, USA.). The PCR cycle includes - initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 92 °C for 45 s, annealing at 58 °C for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 7 min. The amplified and purified PCR product (GeneJET PCR Purification Kit, Thermo Scientific, USA.) was sequenced using an automated DNA sequencer (Applied Biosystem, USA.). Cycle sequencing was performed in 1× sequencing buffer (Big Dye Terminator v3.1 cycle sequencing kit; Applied Biosystems, USA.) containing 10 pmol of forward and reverse primer, 1 μL Big Dye Ready Reaction mix (Applied Biosystems, USA.), and 50 ng of template DNA in a total volume of 10 μL. The cycle sequencing products were denatured for 2 min at 94 °C and separated on an ABI PRISM 310 genetic analyzer (Applied Biosystems, USA.). The 16S rRNA gene sequence determined in this study was analyzed for cyanobacterial identity using BLASTn, submitted to the GenBank under the accession number, KX002033. For phylogenetic analysis, sequences obtained from GenBank were aligned with our sequence using MUSCLE (v3.8.31) and then a maximum likelihood (ML) tree was constructed using PhyMl (v3.1/3.0 aLRT) HKY85 substitution model. The phylogenetic tree was graphically represented by MEGA (v7.0).

2.4. Preparation and purification of cyanobacterial extracts

The cleaned trichomes of *Trichodesmium* sp. MBDU 524 were poured into the big tub, the floating trichomes were carefully taken using spoons and poured into several centrifuge tubes. The samples were centrifuged at 2000 rpm for 15 min at room temperature (RT). The supernatant was discarded and the pellets were pooled and further used for extraction. The pellets were dissolved in 100% methanol and kept in shaking for 24 h. The mixture was centrifuged at 3000 rpm, 15 min at RT. The supernatant was carefully transferred to another tube and the pellet was re-extracted with 100% methanol and kept again in shaker for 24 h. The mixture was centrifuged at 3000 rpm, 15 min at RT and the supernatant was collected. The procedure was repeated until the pellet became colorless. The supernatants collected at each extraction were mixed together and filtered through Whatmann No. 1 filter paper to remove any cell debris. An equal volume of dichloromethane and water was added to the pooled supernatant. The mixture was shaken manually for 1 min to form an emulsion. After centrifugation, (14,000 rpm, 10 min, RT) the upper phase (water/methanol) and lower phase (dichloromethane) was collected separately and evaporated. The final residues of both upper aqueous and lower solvent phase were dissolved to HPLC eluent, 45% acetonitrile (ACN) in water for purification of crude extract.

The purification of crude extract was carried out with Shimadzu CLASS-VP V6.13 SP2 HPLC. The column used was C18, which was eluted isocratically 0.8 mL min^{-1} with 45% ACN in water at ambient temperature. A fraction of the single peak from each phase eluting from 2 to 5 min, was collected and evaporated to dryness. The final residue was dissolved in 100% methanol and further used for mass detection.

2.5. LC-ESI-MS analysis

LC-ESI-MS analysis was carried out using Surveyor Plus LC system (Thermo Fisher Scientific, CA, USA.) coupled to LCQ Fleet ion trap mass spectrometer equipped with a diode-array detector. Chromatographic separation was performed with a Thermo scientific Hypersil™ C18 column (50 × 2.1 mm, 3 μm particle size) maintained at 150 °C. The column was eluted with a binary solvent system of water (solvent A) and ACN (solvent B) at a constant flow-rate of 0.2 mL min^{-1}. The injection volume was 10 μL for each sample. Mass spectrometry detection was carried out in a positive