# Isolation and Characterization of Microcystin-Producing *Microcystis aeruginosa* MBDU 626 from a Freshwater Bloom Sample in Tamil Nadu, South India

A. M. P. Anahas, M. Gayathri, and G. Muralitharan

**Abstract**

Toxin-producing cyanobacteria are a worldwide threat to both human and animal health. Microcystins (MCs) are the most commonly occurring toxins produced by bloom-forming cyanobacteria, especially *Microcystis* sp. This study describes the occurrence of bloom-forming toxigenic *Microcystis aeruginosa* MBDU 626 from Manjalar Dam, Theni District, Tamil Nadu, South India. Two microcystin (MC) variants, MC-LR and [D-Asp$^3$] MC-LR were identified from the isolated strain using high-performance liquid chromatography and gas chromatography coupled mass spectrometry (GC/MS). Four peptides such as aeruginosin, microginin, kasumigamide and anabaenopeptin were also co-produced along with these MC variants. Our results show that the presence of cyanobacterial toxins in essential water resources requires rapid remedial action and needs to develop a national program for regular monitoring of toxigenic blooms in freshwater bodies of South India, in general, Tamil Nadu, in particular.

**Keywords**

Cyanobacteria · *Microcystis aeruginosa* · Microcystin-LR · Manjalar dam · Theni · Tamil Nadu · South India

## Introduction

Cyanobacteria (blue-green algae) are the prominent cause of water blooms in eutrophic lakes and drinking water reservoirs worldwide (Carmichael 1994; Sivonen 1996). Toxic bloom-forming cyanobacteria have been reported causing animal death and also adversely affecting human health (Carmichael 1994, 2001; Codd et al. 1997). Microcystins (MCs) are the most commonly encountered cyanotoxins (Sivonen

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1996). *Microcystis*, *Anabaena*, *Planktothrix*, *Nostoc*, *Hapalosiphon*, *Anabaenopsis*, etc., are common MC-producing cyanobacterial genera (Carmichael 1992; Sivonen and Jones 1999). However, majority of the MCs-producing blooms are dominated by *Microcystis* (Codd 1999; Kabernick et al. 2000; Lehman et al. 2005; Li et al. 2007; Dai et al. 2008; Xu et al. 2008).

The general structure of MC is cyclo-(D-alanine-*X*-D-MeAsp-*Z*-Adda-D-glutamate-Mdha), where D-MeAsp is D-erythro-*β*-methyl-aspartic acid, and Mdha is *N*-methyldehydroalanine (Mdha). *X* and *Z* are variable L-amino acids (Sivonen and Jones 1999). To date, more than 90 structural variants of MCs have been reported (Zurawell et al. 2005; Wood et al. 2008). The most common MC congener (MC-LR) is characterized by the presence of leucin (L) and arginin (R) as L-amino acids in positions 2 and 4 ($Xaa^2$ = L: Ala, $Yaa^4$ = R: Arg) (Gulledge et al. 2002). Since the first elucidation of MC structure by Botes et al. (1984), extensive structural characterizations of other MC variants have been the subject for many studies (Sivonen et al. 1990; Namikoshi et al. 1992a, b; Luukkainen et al. 1994; Namikoshi et al. 1995; Sano and Kaya 1995, 1998; Beattie et al. 1998) and resulted in the identification of different structural variants of MCs to date. MC-LR is the most toxic and widely encountered MC variant for which World Health Organization (WHO) set a guideline value of 1 μg $L^{-1}$ for drinking water (WHO 1998). Based on the review of all the toxicity data, the International Agency for Research on Cancer (IARC) classified MC-LR as a potential carcinogen (Group 2B) (Grosse et al. 2006).

Beside MCs, various other linear and cyclic oligopeptides such as aeruginosins, anabaenopeptilides, cyanopeptolins, anabaenopeptins and microginins are found within the genus *Microcystis* (Namikoshi and Rinehart 1996). As like MCs, the structures of these peptides generally include unusual amino acids residues, such as 3-(4-hydroxyphenyl)-lactic acid (Hpla) and 2-carboxy-6-hydroxy-octahydroindole (Choi) in aeruginosins, or 3-amino-6-hydroxy-2-piperidone (Ahp) in cyanopeptolins, *β*-amino-*α*-hydroxy-decanoic acid in the linear microginins (Neumann et al. 1997; Fukuta et al. 2004; Harada 2004; Welker et al. 2004a, b).

In fact, no consistent hypothesis has been developed so far to explain the high structural variability and patchy distribution of cyanopeptides (Welker et al. 2006). This is partly due to the still very limited knowledge on the occurrence of individual peptides and peptide classes in environmental samples (Fastner et al. 2001). These peptides have been found to exhibit a wide range of biochemical and pharmacological activities (Fastner et al. 2001; Bister et al. 2004; Welker et al. 2004a).

While there have been lengthy investigations regarding the occurrence of toxic cyanobacteria in many countries, there are only a few reports on their occurrence in India (Prakash et al. 2009). This might be due to the prevalence of less toxic variants like MC-RR, or in some cases, a lack of awareness and knowledge to correlate properly the toxicity with the prevailing cyanobacterial blooms (Sangolkar et al. 2009). Cyanobacterial blooms that produce MC-LR, MC-RR and its demethylated variant have been reported in India (Agrawal et al. 2006; Prakash et al. 2009), and adjacent tropical countries including Korea (Kim et al. 1999; Oh et al. 2001) and Thailand (Wang et al. 2002). In this study, we have reported the investigations into the occurrence of MC-producing *Microcystis* sp. in Theni District, Tamil Nadu, South India.

## Materials and Methods

### Bloom Sampling and Strain Culture Conditions

Cyanobacterial bloom sample was collected from Manjalar Dam (10°11′37.15″N 77°37′55.86″E), Theni District, Tamil Nadu, India (Fig. 1). The sample was identified as containing primarily of *Microcystis aeruginosa*. Generic assignment of the isolate was based on morphological criteria (Rippka et al. 1979). The bloom sample was initially grown in BG-11 medium with nitrate source. The culture was

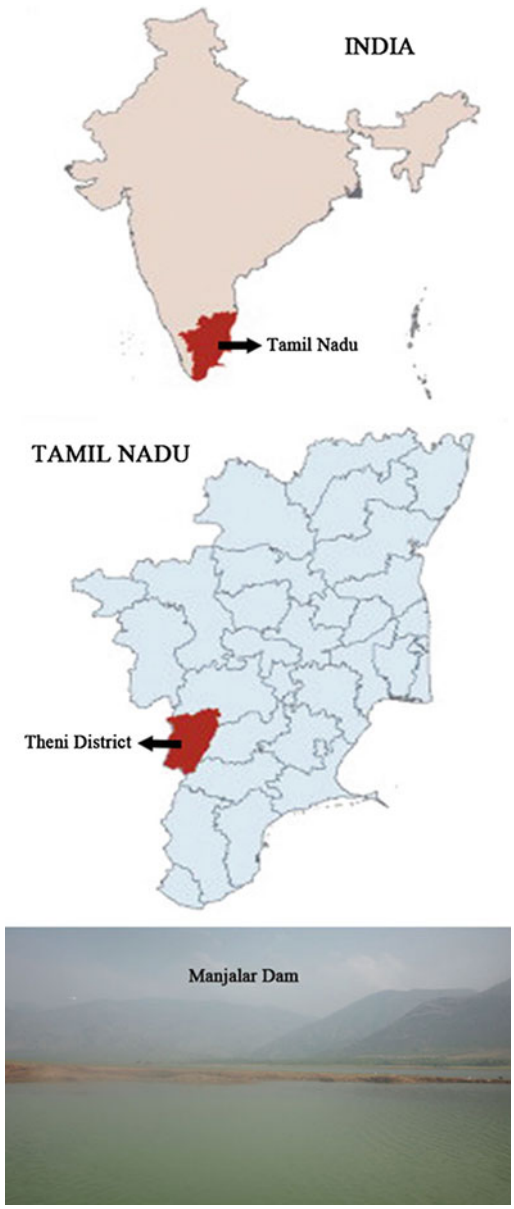


**Fig. 1** Map of Tamil Nadu, India, showing Theni District (in *red*) and the Manjalar Dam was shown in the picture (10°11′37.15″N 77°37′55.86″E), where the bloom sample was collected

incubated under constant light intensity (50 μE $m^{-2}$ $S^{-1}$) for up to 10 days at 25 °C. No bacterial contamination was detected during microscopic observation of the culture.

## Extraction and Analysis of Microcystins

Toxin was extracted as described previously (Frias et al. 2006). Briefly, late log phase culture (15 days old) of *Microcystis aeruginosa* MBDU 626 was centrifuged at 5,000 × g for 15 min at 4 °C (Remi, India), and the pellets were freeze dried and stored at −20 °C until further analysis. MC was extracted with MeOH/$H_2O$ (3:1, v/v) from frozen samples (∼1 g) submitted to sonic disruption for 25 min. Extract was centrifuged (10,000 × g for 15 min) and the supernatant collected. The pellet obtained was re-extracted. The supernatant was combined and evaporated to dryness in a rotary evaporator (40 °C). The dried material was resuspended in MeOH and partitioned with $CHCl_3$:MeOH:$H_2O$ (7:6:3, v/v/v) to remove hydrophobic compounds and pigments. The hydro-alcoholic phase was evaporated and dissolved in 1 ml of MeOH/$H_2O$ (7:3 v/v). The extract was filtered through 0.45 μm millipore membranes and injected into the HPLC system.

## HPLC Analysis

A high-performance liquid chromatography equipped with a constant flow pump (Shimadzu LC 8A, Japan) was used. Separation was accomplished under reversed phase isocratic conditions with (Shim-Pack CLC-Octa decyl silane) ODS-$C_{18}$ column (4.6 mm ID × 25 cm) and guard column (Shim-Pack G-ODS) (4 mm ID × 1 cm) and mobile phase of 100 % methanol. The flow rate was 1 ml/min for analysis, and UV absorbance at 254 nm was used as detector.

## Acid Hydrolysis and Derivatization of the Toxin

The isolated compound was mixed with 6 N HCl (100/900 μl) and heated at 110 °C for 22 h. The reaction mixture was cooled to room temperature and evaporated in a stream of $N_2$, then

equal volume (200 µl) of dichloromethane ($CH_2Cl_2$) and trifluoroacetic anhydride (TFAA) was added, and again the mixture was heated at 110 °C for 5 min, then evaporated by $N_2$ (Namikoshi et al. 1992b). The residue was dissolved in MeOH for GC/MS analysis.

### GC/MS Analysis

GC/MS was performed with an Agilent gas chromatograph coupled to a JEOL GC/MS II MATE ion trap mass spectrometer. HP5 fused silica capillary column (30 m × 0.25 mm × 0.25 µm) was operated in a split less mode, and the injector temperature was 220 °C. The carrier gas (He) flow was adjusted to 1 ml $min^{-1}$. Samples of 1 µl in MeOH were injected into the GC/MS. The program rate for the analysis of amino acid derivatives was 80–250 °C at 20 °C/min.

### Molecular Analysis

Total genomic DNA was isolated from the tested cyanobacterial strain following the previously described method (Neilan 1995) and was used as a template in PCR; 16S rDNA was amplified from the genomic DNA using the cyanobacterial specific primers (Wilmotte et al. 1993; Nelissen et al. 1994). Purified PCR product was sequenced using the BigDye Terminator Cycle Sequencing v2.0 kit on an ABI 310 automatic DNA sequencer (Applied Biosystems, CA, USA). The 16S rDNA gene sequence determined in this study was deposited in the GenBank database under the accession number JN542384.

## Results

### Strain Characterization

The results of the present study revealed the occurrence of *M. aeruginosa* in Manjalar dam bloom samples. The isolated strain was characterized by both morphological and 16S rDNA sequence analysis. Figure 2 shows the typical morphology of *M. aeruginosa* strain isolated from the sampling site. 16S rDNA sequence analysis revealed that the isolated strain was having 95 % similarity to *M. aeruginosa* LMECYA 106 (EU078498) and *M. aeruginosa* UWOCC 019 (AF139295), confirming its identity.

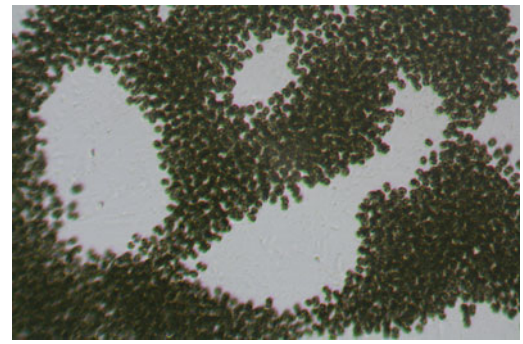

**Fig. 2** Photomicrograph illustrating the morphological features of *Microcystis aeruginosa* MBDU 626 isolated from water bloom sample

### GC/MS Analysis of Microcystin

MCs were generally detected as singly protonated molecular ions. GC/MS analysis revealed the presence of two different MC isoforms. Both isoforms showed the characteristic fragment ion peak 135 [M + $H^+$] (Tables 1 and 2), the Adda side-chain [$PhCH_2CH(OMe)^+$], which is a key indicator for the presence of MCs. Further investigation into the fragment ion peaks enabled the identification of the isoforms as MC-LR and [D-$Asp^3$] MC-LR.

Product assignation of the fragment at *m/z* 994.5[M + $H^+$] in GC/MS spectrum revealed the presence of MC-LR. The detected fragment ions at *m/z* 86 and 112 show the presence of immonium ions. These *m/z* values indicate the presence of Leu and Arg residues, and this result is also corroborated by the molecular mass of 994 Da. The ion fragment at *m/z* 553.4 corresponds to [Mdha-Ala-Leu-MeAsp-Arg + H]$^+$ evidencing the presence of other amino acid residue characteristic of MCs, Mdha and also

**Table 1** Characteristic fragment ions in GC/MS analysis of $[M + H]^+$ ions at *m/z* 994 corresponding to MC-LR obtained from *M. aeruginosa* MBDU 626 strain

| Fragment ions | MC-LR (*m/z*) |
|---|---|
| [Immonium of Arg]$^+$ | 70.2 |
| [Leu]$^+$ | 86.2 |
| [Immonium of Arg]$^+$ | 112.2 |
| [$PhCH_2CH(OMe)$]$^+$ | 135.1 |
| [Glu-Mdha + H]$^+$ | 213.3 |
| [Mdha-Ala-Leu + H]$^+$ | 268.1 |
| [Arg-MeAsp + H]$^+$ | 286.2 |
| [$C_{11}H_{14}O$-Glu-Mdha + H]$^+$ | 375.2 |
| [Arg-MAsp-Leu-Ala + H]$^+$ | 470.1 |
| [$C_{11}H_{14}O$-Glu-Mdha-Ala + H]$^+$ | 466.2 |
| [Adda-Arg-Masp + H]$^+$ | 599.8 |
| [Arg-Masp-Leu-Ala-Mdha + H]$^+$ | 553.4 |
| Loss of PhCH = CH(OMe) | 861.5 |
| $[M + H]^+$ | 994.5 |

**Table 2** Characteristic fragment ions in GC/MS analysis of $[M + H]^+$ ions at *m/z* 981 corresponding to [D-$Asp^3$] MC-LR were obtained from *M. aeruginosa* MBDU 626 strain

| Fragment ions | [D-$Asp^3$]MC-LR (*m/z*) |
|---|---|
| [$PhCH_2CH(OMe)$]$^+$ | 135.2 |
| [Mdh-Ala + H]$^+$ | 155.1 |
| [$C_{11}H_{14}O$ + H]$^+$ | 163.3 |
| [(Arg + $NH_2$) + 2H]+ | 174.4 |
| [Glu-Mdha + H]$^+$ | 213.2 |
| [MeAsp-Arg + H]$^+$ | 272.1 |
| [(Arg + $NH_2$)-MeAsp + 2H]+ | 289.1 |
| [$C_{11}H_{14}O$-Glu-Mdha + H]$^+$ | 375.3 |
| [$C_{11}H_{14}O$-Glu-Mdha-Ala + H]$^+$ | 466.3 |
| [Mdha-Ala-Leu-MeAsp-Arg + H]$^+$ | 539.2 |
| [MeAsp-Arg-(Adda-MeOH) + H]$^+$ | 553.3 |
| [MeAsp-Arg-(Adda-MeOH)-Glu + H]$^+$ | 682.4 |
| [(M-$PhCH_2CH(OMe)$) + H]$^+$ | 847.4 |
| $[M + H]^+$ | 981.5 |

indicating the presence of the residues Leu and Arg at positions 2 and 4, respectively. Additionally, the following molecular ion species have provided full confirmation of MC-LR identity: [Glu-Mdha + H]$^+$ at *m/z* 213.1, [M + H-Adda]$^+$ at *m/z* 861.5, [Arg-Adda-Glu + H]$^+$ at *m/z* 599.8, [M + H-Glu]$^+$ at *m/z* 866.6 and [$C_{11}H_{14}O$-Glu-Mdha]$^+$ at *m/z* 375.2. A complete list of the detected fragment ion peaks for MC-LR is shown in Table 1.

The second MC isoform identified in the strain tested was [D-$Asp^3$] MC-LR at *m/z* 981.5[M + $H^+$]. This MC isoform has a molecular weight of 981 Da. The detected fragment ion peak at *m/z* 539[Arg-Asp-Leu-Ala-Mdha + H]$^+$ is characteristic for this demethylated MC-LR isoform (Table 2). Indeed, the fragmentation pattern of the *m/z* ion completely matched with that expected from [D-$Asp^3$] MC-LR. Table 2 shows the product assignation of the fragment produced in GC/MS. The *m/z* ions at 213.2[Glu-Mdha + H]$^+$, 155.2[Mdha-Ala + H]$^+$ and 446.3[$C_{11}H_{14}O$-Glu-Mdha-Ala + H]$^+$ indicated the presence of Mdha and

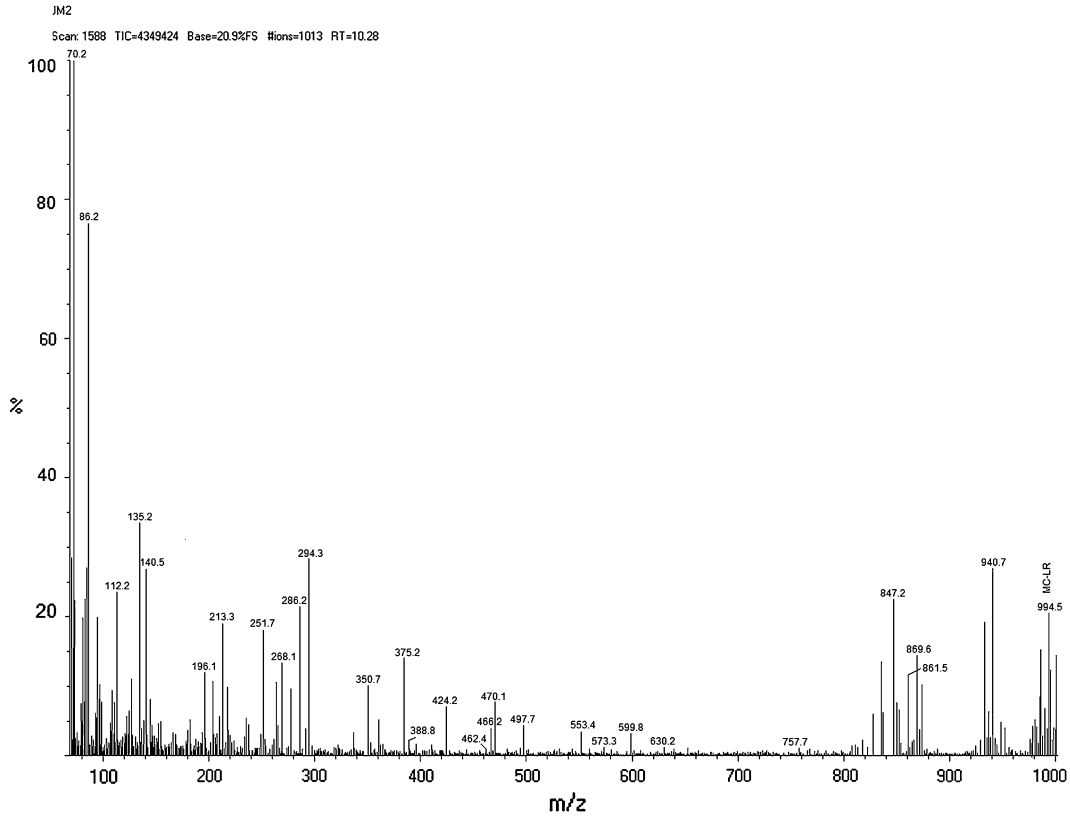


**Fig. 3** GC/MS spectrum of MC-LR detected from *Microcystis aeruginosa* MBDU 626. (See Table 1 for fragment ion identifications)

Ala in position 7 and 1, respectively. On the other hand, the *m/z* ions at 289.1[(Arg + $NH_2$)-Asp + 2H]$^+$, 272.1[Asp-Arg + H]$^+$, 553.3[Asp-Arg-(Adda-MeOH) + H]$^+$ and 682.4[Asp-Arg-(Adda-MeOH)-Glu + H]$^+$ strongly indicated the presence of Asp instead of MeAsp in position 4, proving the demethylated MC to be [D-Asp$^3$] MC-LR. The mass spectrum of both MC-LR and [D-Asp$^3$] MC-LR MC isoforms detected in this study shown in Figs. 3 and 4, respectively.

It is interesting to note that the tested *M. aeruginosa* MDBU 626 had shown five peptides identical to microginin, aeruginosin 602, aeruginosin 101, anabaenopeptin and kasumigamide at 698.3 *m/z*, 603.3 *m/z*, 645.6 *m/z*, 851.5 *m/z* and 788.6 *m/z*, respectively. Microginin are linear peptides with a characteristic N-terminal 3-amino-2 hydroxydecanoic acid (Ahda). The fragment ions at *m/z* 698.3 (Ahda-Thr-Pro-Tyr-Trp) from the side chain of Ahda were observed with the same ions in the mass spectrum (Fig. 6).

The other peptide aeruginosin is linear tetrapeptides with the unique moiety 2-carboxy-6-hydroxy-octahydroindole (Choi) and a C-terminal Arg derivative. Fragment spectra of two peptides characteristically show an intense mass signal detected at *m/z* 140 Da, the Choi-immonium ion which is indicative of aeruginosins. A peptide with [M + H]$^+$ at *m/z* 645.36 could also be identified as an aeruginosin (aeruginosin 644) with a number of fragments identical to fragments of aeruginosin 602: *m/z* 86, 140, 250, 266 and 350 Da. The fragment ions at *m/z* 86.2 (Leu- or Ile-immonium ions), *m/z* 140.1 (Choi-immonium ions), *m/z* 250.1 (Hpla-Leu-Choi-Argal), *m/z* 266.2 (Choi-Arginal-$CH_3N_2$–$H_2O$ + H) and *m/z* 350.4 (Choi-Ac Argininal −$NH_2$ +H) were observed together with the same ions in the mass spectrum (Fig. 5). The predominant fragment

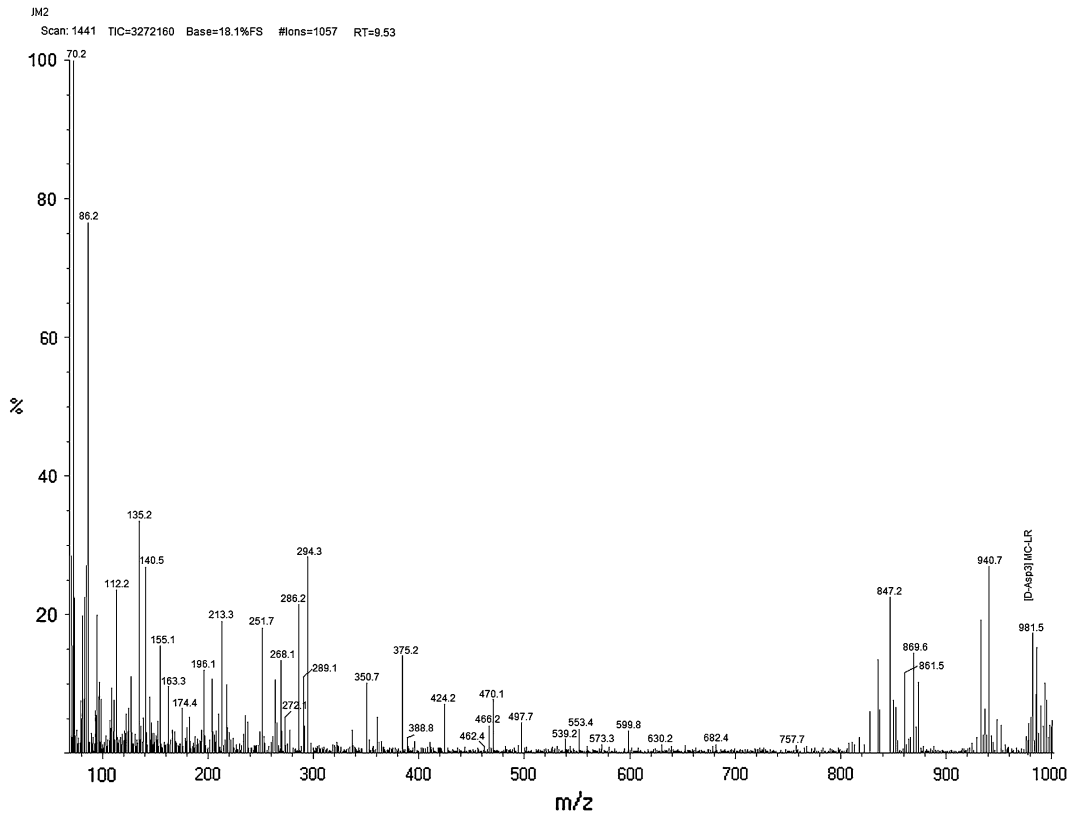


**Fig. 4** GC/MS spectrum of [D-Asp$^3$] MC-LR detected from *Microcystis aeruginosa* MBDU 626. (See Table 2 for fragment ion identifications)

ions were observed in the MS spectra of the related aeruginosin, which are summarized in the Table 3.

Kasumigamide, a linear tetrapeptide containing an N-terminal α-hydroxyl acid with *m/z* 787.38 (Pla-*β*Ala-Ahipa-Arg-phSer) having the C-terminal moiety, that is, hydroxy-group of phenyl-serine was observed in the mass spectrum (Fig. 5).

Anabaenopeptins, a group of cyclic hexapeptides are characterized by a 19-membered peptide ring that is formed by cyclization between the C-terminal amino acid and the ε-amine of a lysine residue. The α-amine of the lysine is further linked through an ureido group to a side-chain amino acid. Two anabaenopeptin variants with similar mass have been identified in this study. Fragmentation spectrum by GC/MS was indicated that the peak at m/z 851[M + H$^+$] corresponded to two isobaric anabaenopeptin variants, that is, anabaenopeptin B1 and F. The fragment ions at *m/z* 57 (MAla-Immonium ion) *m/z* 70 (Arg-/Lys-related ions), *m/z* 84 (Lys-Immonium ion), *m/z* 112 and 129 (Arg-Immonium ion), *m/z* 201 (CO + Arg) (side chain) *m/z* 233 (MAla + Phe + H), *m/z* 286 (Lys + CO + Arg-$CN_2H_2$), *m/z* 291(HTry + Ile + H), *m/z* 376 (MAla + HTyr + Ile + H), *m/z* 417 (HTyr + Ile + H), *m/z* 538 (Lys + Phe + MAla + HTyr + 2H) and *m/z* 651 (Lys + Phe + MAla + HTyr + Ile + 2H) were observed together with the same ions in the mass spectrum (Fig. 6 and Table 4).

## Discussion

Occurrence of cyanobacterial blooms and associated animal and human poisoning has been documented from over sixty-five countries

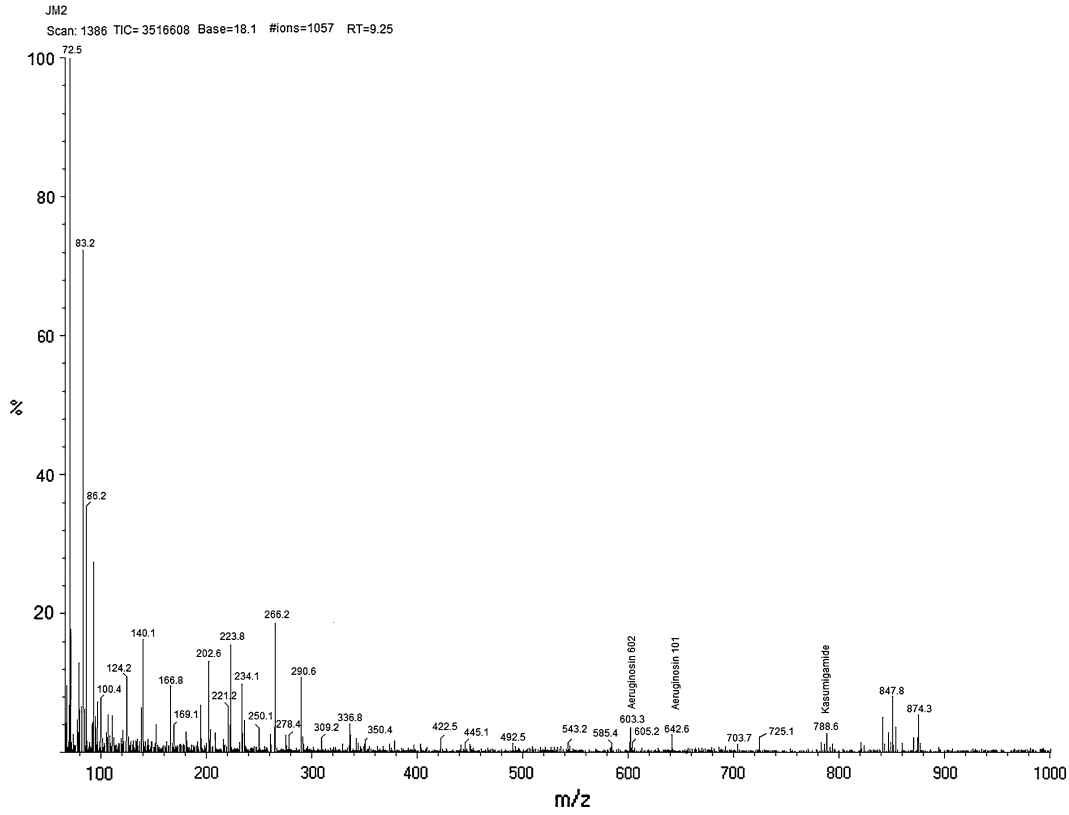


**Fig. 5** GC/MS spectrum of aeruginosin peptide detected from *Microcystis aeruginosa* MBDU 626; peak at *m/z* 603 and *m/z* 642 corresponding to aeruginosin 602 and aeruginosin 101; peak at *m/z* 788 identified as kasumigamide. (See Table 3 for fragment ion identifications)

**Table 3** Characteristic fragment ions in GC/MS analysis of $[M + H]^+$ ions at *m/z* 603 corresponding to aeruginosin peptide obtained from *M. aeruginosa* MBDU 626 strain

| Fragment ions | Aeruginosin (*m/z*) |
|---|---|
| Leu-immonium ion | 86.2 |
| Argal-fragment | 100.4 |
| Choi-immonium fragment | 140.1 |
| Choi + H | 169.1 |
| (Leu-Choi)fragment | 221.2 |
| $R_{1,2}$-Hpla-Leu-CO + H[b] | 250.1 |
| Hpla-Leu-H | 266.2 |
| Choi-Argininal-$CH_3N_2$-$H_2O$ + H[a] | 278.4 |
| Choi-Argininal-$NH_2$-$H_2O$ + H | 290.6 |
| Choi-Argininal-$NH_2$ + H | 309.2 |
| Choi-AcArgininal-$NH_2$ + H | 350.4 |
| M-Argal | 445.1 |
| M-$CH_3N_2$-$H_2O$ + Ha | 543.2 |
| M-$H_2O$ + H | 585.4 |
| M + H | 603.3 |

[a] $CH_3N_2$ is the ureido group of Argininal; [b] $R_1$ and $R_2$ are either a hydrogen or a chlorine in the non-, mono- or di-chlorinated variant, respectively

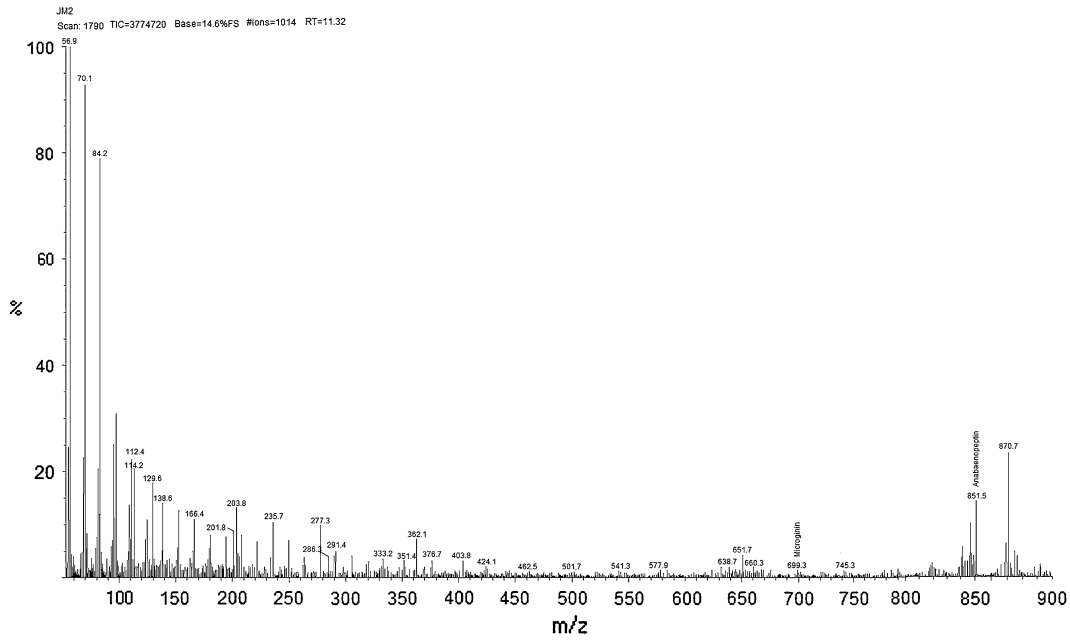


**Fig. 6** GC/MS spectrum of anabaenopeptin peptide detected from *Microcystis aeruginosa* MBDU 626; peak at *m/z* 851 corresponding to a mixture of anabaenopeptin F and [HArg$^6$]-anabaenopeptin B; peak at *m/z* 699 identified as microginin. (See Table 4 for fragment ion identifications)

**Table 4** Characteristic fragment ions in GC/MS analysis of $[M + H]^+$ ions at *m/z* 851 corresponding to [HArg$^6$]-anabaenopeptin B and anabaenopeptin F peptides obtained from *M. aeruginosa* MBDU 626 strain

| Fragment ions | [HArg$^6$]-Anabaenopeptin B *m/z* | Anabaenopeptin F *m/z* |
|---|---|---|
| MAla-Immonium ion | 57 | 57 |
| Arg/Lys-related ions | 70 | 70 |
| Lys-Immonium ions | 84 | 84 |
| Arg-Immonium ions | 112 | 112 |
| [MAla + CO + H] | 114 | 114 |
| Arg-Immonium ions | 129 | 129 |
| [CO + Arg](side chain) | 201 | 201 |
| [HTyr + Val + H] | 277 | – |
| Lys + CO + Arg-$CN_2H_2$ | 286 | 286 |
| [HTyr + Ile + H] | – | 291 |
| [MAla + HTyr + Val + H] | 362 | – |
| [MAla + HTyr + Ile + H] | – | 376 |
| [HTyr + Val + Lys] | 403 | – |
| [HTyr + Ile + Lys] | – | 417 |
| [Lys + Phe + MAla + HTyr + 2H] | – | 538 |
| [Lys + Phe + MAla + HTyr + Val + 2H] | 637 | – |
| [Lys + Phe + MAla + HTyr + Ile + 2H] | – | 651 |
| [M + H]+ | 851 | 851 |

(Codd et al. 2005), including India (Agrawal et al. 2006), Sri Lanka (Jayatissa et al. 2006) and Bangladesh (Welker et al. 2005). The warm water temperature in India promotes dense *Microcystis* growth almost throughout the year (Parker et al. 1997; Agarwal et al. 2001). There have been few reports of MC occurrence in India (Sangolkar et al. 2009), and information about the evidence of bloom formation and toxicity in South Indian water bodies is particularly scarce. During the biodiversity survey of different freshwater ponds of Thanjavur District, Tamil Nadu, it is reported that potentially toxic cyanobacterial blooms are common in the freshwater ponds of Tamil Nadu region (Muthukumar et al. 2007). Out of the five ponds investigated, Dabeerkulam pond showed low diversity of cyanobacteria which was attributed to a massive bloom of *Microcystis aeruginosa* (Muthukumar et al. 2007). Similarly, this study indicates the presence of toxigenic *M. aeruginosa* MBDU 626 in the fresh water of Manjalar Dam in Periyakulam, Theni District. The freshwater bodies of South India in general, Tamil Nadu in particular, have so far been given less attention. This work was an extension of our earlier report on the presence of MC-LA-producing *Microcystis aeruginosa* MBDU 013 in Kuttappar Lake at Tiruchirappalli District, Tamil Nadu (communicated data).

GC/MS method has been developed for screening MCs, in complex samples such as sediments. It is based on the detection of 2-methyl-3-methoxy-4-phenylbutyricacid (MMPB), which is formed when the Adda residue is split following oxidation of MCs (Harada et al. 1996; Kaya and Sano 1999; Tsuji et al. 2001). Mass spectrometry (MS) has proved to be a valuable technique for providing structural information on MCs (Harada 1995; Kondo and Harada 1996; Meriluoto et al. 2000), without need for toxin standards or specific retention times that are required for HPLC analyses (Jungblut et al. 2006).

The [D-Asp$^3$] MC-LR and MC-LR have been shown to form [M + H] $^+$ ion of *m/z* 981 and 995 (Diehnelt et al. 2005; Jungblut et al. 2006; Del Campo and Ouahid 2010). Similar fragment ions for [D-Asp$^3$] MC-LR and MC-LR were reported from an Antarctic cyanobacterial mat community by Q-Star quadrupole-TOF hybrid mass spectrometer (Jungblut et al. 2006). The characteristic fragment ion for MC-LR has also been reported by Diehnelt et al. (2005). In Uttar Pradesh, India, five eutrophic temple ponds in the vicinity of Varanasi city were reported for MC-LR-producing *Microcystis* blooms (Prakash et al. 2009). In addition to *Microcystis,* MC-LR forming *Nostoc* sp. BHU001 was reported from the agricultural pond of Banaras Hindu University, Varanasi, India (Bajpai et al. 2009). Frias et al. (2006) have reported that the occurrence of MC-LR in a bloom in the eutrophic reservoir Billings, Sao Paulo City, Brazil, by ESI–MS/MS analysis. In a similar study, ten out of 12 MCs, including [D-Asp$^3$] MC-LR and MC-LR, were detected from International Culture Collections strains of *Microcystis* (Del Campo and Ouahid 2010) and reported the fragment ions for [D-Asp$^3$] MC-LR at *m/z* 155.2, 213.2, 289.1, 553.3 and 682.4. Similar fragment ions were obtained from our experiment (Figs. 4 and 5). [D-Asp$^3$] MC-LR also has been detected in bloom samples from Morocco (Oudra et al. 2001) and the Philippines (Baldia et al. 2003). The characteristic Adda fragment for MCs was seen at 135.2 *m/z* (Figs. 3 and 4), possibly generated by the $\alpha$-cleavage at the methoxy group of the Adda $\beta$-amino acid moiety (Ortea et al. 2004).

Five peptides were identified as to aeruginosin, microginin, anabaenopeptin and kasumigamide (Figs. 5 and 6), and these were also identified from the *m/z* of GC/MS analysis. The MS approach was successful in detecting a multitude of known and new peptides from very small samples of cyanobacterial cells. Detectability of individual peptides depends partly on the efficiency with which they can be protonated (Karas et al. 2000). Further information on the identity of oligopeptides was gained from the comparison with published fragmentation data from pure substances and from a fragment database (Haande et al. 2007).

The co-occurrence of MCs and cyanopeptolins in *Microcystis* spp. dominated field samples was reported previously (Jacobi et al. 1996; Neumann et al. 2000). Many of the substances detected

belong to well-known groups of cyanobacterial peptides like MCs, anabaenopeptins, microginins, cyanopeptolins and aeruginosins, of which many have been discovered in *Microcystis* spp. (Namikoshi et al. 1996). With respect to known peptides, combinations of anabaenopeptins, microginins and aeruginosins were observed, while MCs were found along with aeruginosins. This correlates to the detection of aeruginosins as well as cyanopeptolins in both toxic and nontoxic *Microcystis* culture strains (Namikoshi et al. 1996; Dittmann et al. 1997).

A fragment ion *m/z* at 698.3$[M + H]^+$, characteristics of microginin, was reported from bloom material of lake Tegamura, Japan (Kodani et al. 1999). Our experiments identified similar fragment ions from the tested organism. A peptide with a molecular mass of *m/z* 603$[M + H]^+$ is probably a new variant of an aeruginosin-type peptide, as suggested by the fragment ion of *m/z* 140, indicating the presence of the unusual amino acid Choi, which is unique to aeruginosin-type molecules (Murakami et al. 1995; Matsuda et al. 1997; Erhard 1999). Ishida et al. (1999) have reported that aeruginosin 101 was originally isolated from *Microcystis aeruginosa* (NIES 101). Agarwal et al. (2006) have reported the presence of aeruginosin by MALDI-TOF/MS analysis, in the *Microcystis* blooms from Gosalpur Lake of Jabalpur in Central India. Kasumigamide, a novel antialgal peptide which shows a characteristic fragment *m/z* at 787.3$[M + H]^+$ was originally isolated from freshwater cyanobacterium, *Microcystis aeruginosa* (NIES-87) (Ishida and Murakami 2000). *Microcystis* colonies isolated from lakes Müggelsee, Pehlitzsee and Parsteiner See in and around Berlin, Germany, were shown to possess mainly of kasumigamide linear peptide (Welker et al. 2004a).

The co-occurrence of both MCs and anabaenopeptins in natural populations has been well documented (Kodani et al. 1999; Fastner et al. 2001; Grach-Pogrebinsky et al. 2003). In the samples dominated by *Microcystis* spp., anabaenopeptins were found only when MCs also were present (Gkelis et al. 2005) and similar results have been reported from natural population samples studied (Kodani et al. 1999; Fastner et al. 2001; Grach-Pogrebinsky et al. 2003). However, it is still unclear whether cyanobacterial strains produce both types of peptides simultaneously or produce only MCs. Our results support the hypothesis of the coexistence of toxic MC with nontoxic peptides.

This study reinforces the earlier investigations into cyanobacterial blooms in Central India on the occurrence of toxigenic species in freshwater bodies of Indian ecosystem and states that major concern should be given for the screening program at least for those freshwater bodies used for animal or human consumption.

For a variety of reasons, the harmful impact of cyanobacteria on human health was always been a topic of interest (Falconer 1996, 1997). Concern about the MCs health risk to humans through drinking water, led the WHO to develop and suggest a provisional guideline level of MC-LR at 1 $\mu g/L^{-1}$. Up to now, this value has been considered as a safe level in drinking water (Falconer et al. 1999). Further research and data analysis are needed to generate the information on MC occurrence, diversity and distribution with reference to climatic zones, namely temperate, tropical and subtropical regions (Sangolkar et al. 2009). This study clearly revealed that toxigenic *Microcystis* strains are present in the freshwater bodies of Southern Indian region and major attention should be given for the effective screening and mitigation strategies.

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