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Research Article

ISOLATION AND CHARACTERIZATION OF MICROCYSTIN DEGRADING BACTERIA FROM HOLY PONDS IN INDIA

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Abstract

Microcystins (MCs) are toxic cyclic heptapeptides produced by few toxic cyanobacteria and generally form blooms in eutrophic surface fresh water bodies. They cause acute to chronic poisoning and other health related problems mainly by irreversible inhibition of protein phosphatases (PP1 and PP2A) and increased formation of reactive oxygen species (ROS). Due to limitation of non-biological methods of water treatments the exploration of MCs degrading bacteria is emerging at a quite pace to address, through bioremediation, the problems posed by MCs in water and water-bodies. Report and study of MCs biodegrading bacteria from India were lacking. However it was evident, from our previous study, that microcystin degradation can be achieved by indigenous microcystin degrading bacterial population in its natural place where microcystin producing blooms occur. This study has presented isolation and characterization of indigenous microcystin degrading bacteria from holy ponds in Utter Pradesh of India. Overall 20 bacterial isolates were isolated from *Microcystis* infested different ponds. Out of these 13 isolates were *mlr*A positive by PCR and were found to be distinct isolates by amplified ribosomal DNA restriction analysis (ARDRA). However, ARDRA analysis revealed overall four bacterial groups. On the basis of 16S-rRNA gene sequence the Gram-positive-rod isolate PM1 was identified, with 99% identity, as *Bacillus licheniformis* which was shown earlier to cluster with microcystin degrading bacterium *B. subtilis*. Thus the present study revealed, for the first time, probable microcystin degrading bacteria in water-bodies from India. The potential and the metabolic pathway of PM1 and other *mlr*A positive isolates need to be further studied and validated to confirm their application in microcystin bioremediation.

Keywords: India; microcystin; biodegradation; *mlr*A; *Bacillus licheniformis;* Pishachmochan

Introduction

Microcystin is a well characterized cyclic heptapeptide hepatotoxin and it has been reported from various places of the world. (Carmichael, 1992; Carmichael *et al.*, 2001; Chaturvedi *et al.*, 2015; Harke *et al.* 2016; Herry *et al.*, 2008; Izaguirre *et al.*, 2007; Kumar and Verma, 2012; Kumar et al, 2011; Lalita *et al.* 2009; Lopez-Archilla *et al.*, 2004; Lehman, 2007; Prakash *et al.* 2009 and 2016; Tyagi *et al.*, 2006; Xiao *et al.*, 2009). Microcystins (MCs) are produced by few cyanobacterial species belonging to genera *Anabaena*, *Microcystis*, *Nostoc*, *Planktothrix* and *Nodularia* (Chaturvedi *et al.*, 2015; Christiansen *et al.*, 2003; Codd *et al.*, 1997; Keil *et al.*, 2002; Moffitt and Neilan, 2004; Rouhiainen *et al.*, 2004; Satyaprakash *et al.*, 2009 and 2016; Singh *et al.*, 2001; Tillett *et al.*, 2000; Tyagi *et al.*, 2006). MCs are non-ribosomal peptides and are synthesized by a large multifunctional enzyme complex consisting of non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) domains encoded on microcystin synthetase (*mcy*) gene cluster of variable composition and organization (Carmichael, 1992; Christiansen *et al.*, 2003; Imanishi and Harada, 2004; Moffitt and Neilan, 2004; Pearson *et al.*, 2010; Rouhiainen *et al.*, 2004; Tillett *et al.*, 2000). Owing to the variability of genes in the cluster, over 70 structural variants of microcystin are known (Welker and von Dohren, 2006). Microcystin-LR (MCLR) is the most common reported isoform with a structure cyclo-(D-AlaL^{1}-Leu^{2}-D-MeAsp^{3}-L-Arg^{4}-Adda^{5}-D-Glu^{6}-Mdha^{7}), (Figure-1) where D-MeAsp stands for D-erythro-β-methylaspartic acid, Mdha for N-methyl-dehydroalanine and Adda is (2S, 3S, 8S, 9S)-3-amino-9- methoxy-2, 6, 8-trimetyl-10-phenyldeca-4(E), 6(E)-dienoic acid (McElhiney and Lawton, 2005). Other structural isoforms of microcystin vary in amino acid composition at two position 2, 4 and various modification at various position of the structure (McElhiney and Lawton, 2005; Welker and von Dohren, 2006).

MCs have been studied for various activities and their existence in water bodies as well as in drinking water. It causes acute poisoning, tumor promotion and growth, loss of cytoskeleton integrity, cytolysis/apoptosis, cellular

damage and genotoxicity. MCs act by irreversible inhibition of serine/threonine protein phosphatases (PP1 and PP2A) **(**Fig. 1**)** and formation of reactive oxygen species (ROS) (Campos and Vasconcelos, 2010; Craig *et al.*, 1996; Falconer and Yeung, 1992; MacKintosh *et al.*, 1990; McElhiney and Lawton, 2005; Ohta *et al.*, 1992; Welker and von Dohren, 2006; Wickstrom *et al.*, 1995).

Its wide distribution, acute and chronic toxicity in animals and humans, exposed via various routes, has invited the interest of scientific community to find the ways either to remove or destroy the toxin. Though the impact of microcystin in natural water can be mitigated by dilution process (Harada *et al.*, 1996; Tsuji *et al.*, 1995), it cannot be the adequate and sustainable measure to the problem. Various approaches, traditional to advanced techniques, have been employed to evaluate their performance. MCs have relatively been reported to be stable to physicochemical and biological factors (that usually contribute to inactivation or degradation) such as pH, temperature, sunlight, and other common proteases (Alamri, 2012; Jones *et al.*, 1994; Lawton and Robertson, 1999; Schmidt *et al.*, 2014; Tsuji *et al.*, 1995). Most of physical and chemical methods of degradation employing chlorine, permanganate, chloride dioxide, ozone, photobleaching and advanced oxidation methods of water treatment are inadequate and un-predictable to the drinking purposes (de la Cruz *et al.*, 2011; Kormas and Lymperopoulou, 2013). Furthermore the chemical based methods of water treatment face limitation over their utilization due to probable production of carcinogenic and mutagenic substances during the process (Bellar *et al.*, 1974; Hemming *et al.*, 1986; Kornberg *et al.*, 1988; Rook, 1974;). Other advance chemical techniques using granular activated carbon, powdered activated carbon, and membrane filtration are good choice of methods of MCs removal. Reverse Osmosis (RO) is a suitable method of producing safe drinking water, but safe disposal of the toxin enriched retention water is of great concern. Toxins are not destroyed by this process rather it is retained in the purifier. It further requires other methods to destroy it for its safe disposal. Employing RO in treatment of natural water are expensive too (Kormas and Lymperopoulou, 2013).

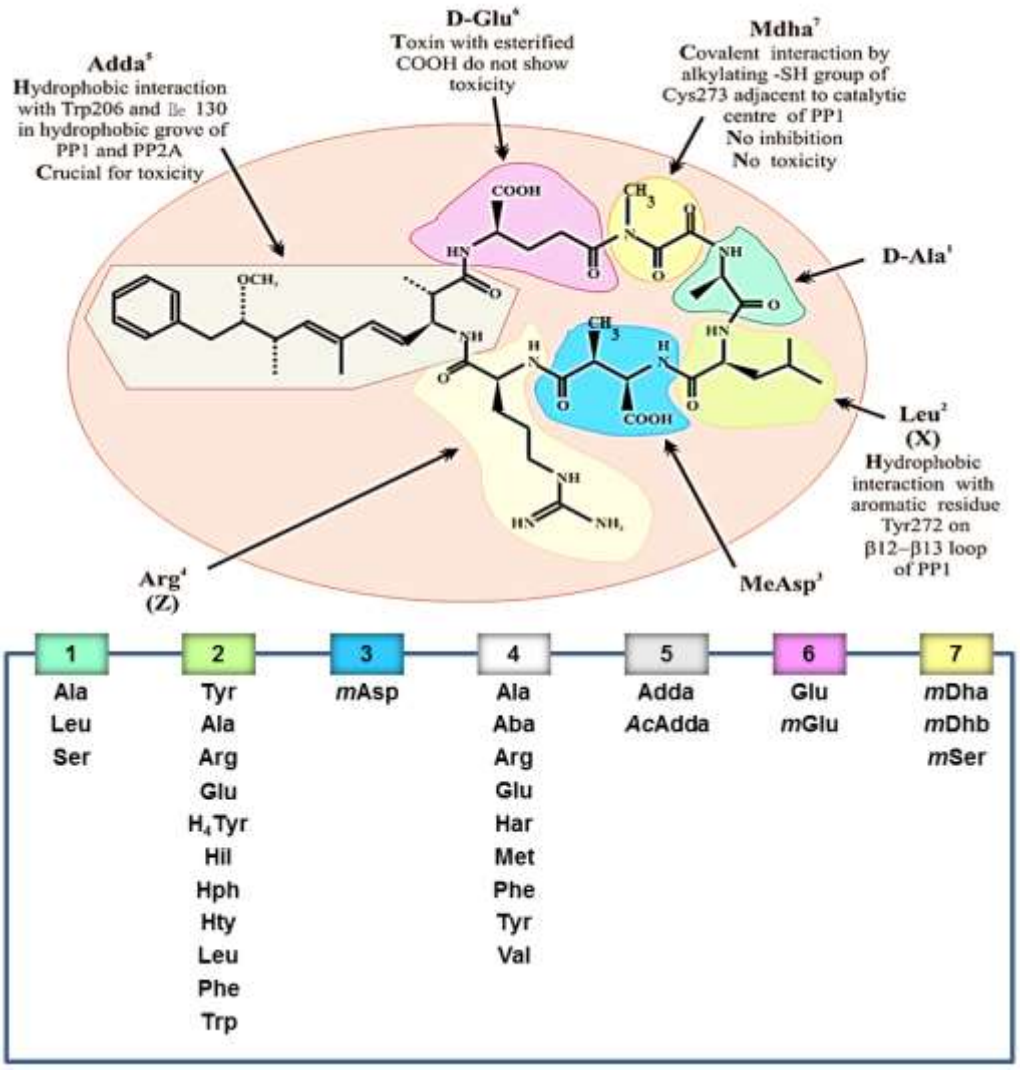

Fig. 1: Cyclic structure of MC-LR and schematic general structure of various isoforms of microcystin showing variations at positions 1-7 (McElhiney and Lawton 2005; Welker and von Dohren 2006).

Use of microbes has been shown to be the most sustainable and efficient method for their realization in water treatment for removal and degradation of MCs (Edwards and Lawton 2009). Though few microbes have been reported to degrade MCs, the list is increasing with newly reported microbes of different taxonomic ranks. Microorganisms such as *Sphingomonas* sp., *Sphingopyxis* sp., have been found to possess the gene cluster *mlr*A, *mlr*B, *mlr*C and *mlr*D encoding different enzymes responsible to degrade MCs (Fig. 2). These cluster and genes *mlr*A, *mlr*B, *mlr*C and *mlr*D encode microcystinase, serine protease, peptidase, tripeptidase respectively and others yet to characterize (Bourne *et al.*, 2001; Kormas and Lymperopoulou, 2013; Saito *et al.*, 2003; Shimizu, *et al.*, 2012). Enzyme encoded by *mlr*A gene cleaves the Adda-Arg peptide bond of MC-LR and convert it from cyclic structure, which render MCs stable against general protease and other factors, to linear form. Thus *mlr*A is considered to play the most important role in the microbial microcystin metabolism. Other enzymes encoded by *mlr*B, *mlr*C and *mlr*D genes lead to other biodegradation products of MCs which are nontoxic even at concentration 5 times higher than the lethal dose of parent MC-LR (Bourne *et al.*, 1996). The whole process being intracellular renders biodegradation advantageous over other methods.

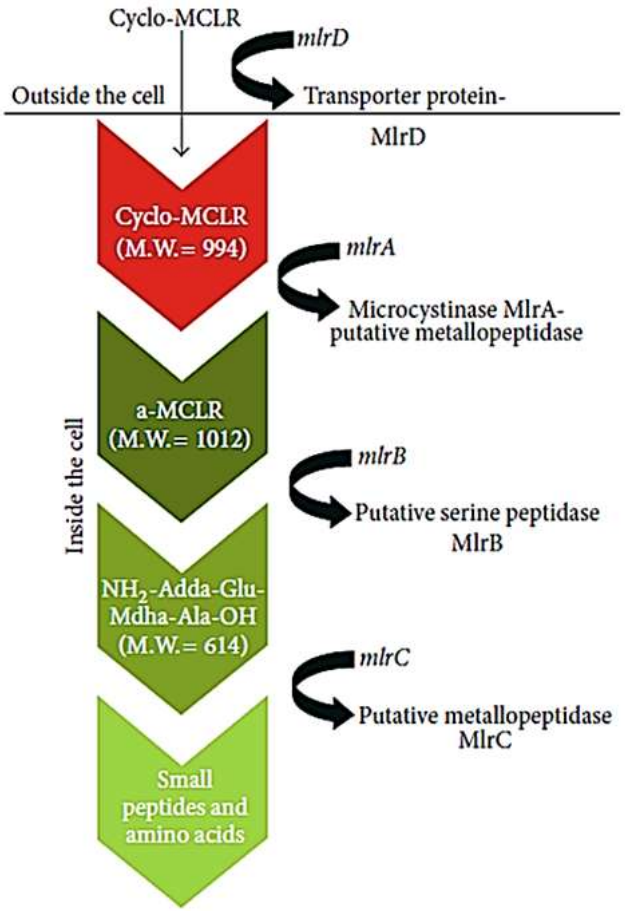

Fig. 2: The pathway of microcystin LR biodegradation by *Sphingomonas* sp. strain ACM-3962. MW: molecular weight (Bourne *et al.*, 2001; Kormas and Lymperopoulou., 2013).

Owing to the diversity of MCs and specificity of enzyme there may exist gene cluster of different organization and/or composition as is in the case of MC-LR, RR and YR (Alamri, 2010 and 2012; Saito *et al.*, 2003; Xu *et al.*, 2015). Hence the investigation of other microbes from sources of different geographical location will help to elucidate insights of the complete pathways of MC bio-degradation. In this present original article we have isolated bacteria from well-studied sites in India where the occurrence of toxic microcystis bloom have been reported for more than 10 years (Kumar *et al.*, 2011; Tyagi *et al.*, 2006). This is the first study which reports the isolation and identification of probable MC degrading bacteria from microcystin infested water bodies in India (Agrawal *et al.*, 2006; Chaturvedi *et al.*, 2015; Karke *et al.*, 2016; Kumar and Verma, 2012; Kumar *et al.*, 2011; Lalita *et al.*, 2009; Prakash *et al.*, 2009 and 2016; Prasath *et al.*, 2014; Tyagi *et al.*, 2006).

Materials and Methods

Sample Collection, Isolation and Culture Purification

Sampling was made from Durgakund (DK), Lakshmikund (LK), Pishachmochan (PM) in month of June, 2015 from Guradari (Gd) in the month of March, 2016. Water-samples from microcystin infested ponds namely DK, LK, PM and Gd were collected in sterile Flacon tubes (Kumar *et al.*, 2011; Tyagi *et al.*, 2006). All the ponds under study are situated adjacent to temples in Utter Pradesh of India. DK, LK and PM are located in Varanasi, Utter Pradesh, India, whereas Guradari is situated at Karha of district Mau-Nath Bhanjan, Uttar Pradesh, India. Geographical locations of these ponds were recorded contemporary to the sample collection by GPS app as: DK (23°26'22.0"N, 85°08'46.6"E), LK (23°26'22.0"N, 85°08'46.7"E), PM (25°19'21.1"N, 82°59'43.3"E) and Gd (25°58'02.8"N, 82°59'43.3"E). Standard microbiological pure culture techniques, Streak plate technique and spread plate technique, were employed to obtain pure cultures of bacteria from respective water samples (Willey *et al.*, 2008a). Bacteria were grown on solid Luria Bertani (LB) agar medium (HiMedia, India) at 37°C under aerobic condition in Microbiological incubator. Pure clonal cultures were obtained by repeated subculture from isolated single colony. Pure cultures of bacteria were routine cub-cultured every 30 days and were stored at 4°C.

Morphological and Biochemical Characterization of Bacteria

Bacteria were grown on solid LB-agar plate and morphological characteristics of the bacterial colonies were observed and recorded as per the guidelines of Willey *et al.* (2008b). The color, shape, size, form, margin, elevation and transparancy of the colonies were examined and recorded. Shape and cell wall characteristics of the bacteria were observed following gram staining by standard protocol using gram staining kit (HiMedia, India). Isolates were

further subjected to various other biochemical tests including catalase test, lactose fermentation test, and urease test as per the respective protocol (Cappuccino and Sherman, 2001; Shakoori *et al.*, 2010; Vergis *et al.*, 2013). All the isolates were compared, on the basis of morphological and biochemical characterization, to each other to differentiate and exclude, as much possible, repeating isolates. These distinct bacteria were further screened by *mlr*A specific PCR to find probable microcystin degraders.

Isolation of Genomic DNA

The bacterial isolates were grown aerobically overnight in Nutrient Broth (NB) under shaking at 130 rpm and cells were recovered by centrifugation at 10000×g for 5 minutes. The genomic DNAs were isolated from cells as per the method described by Yeates *et al.* (1998). Quality of the genomic DNA (purity and integrity) was assessed by UV-Visible Spectrophotometer (PerKin Elmer UV-Vis Spectro λ35, USA; pure DNA preparations have A_{260}/A_{280} value of 1.8) and agarose gel electrophoresis (BIORAD, USA). PCR-grade quality of DNA was also checked by 16S-rDNA amplification. PCR grade genomic DNA of isolates were used for *mlr*A specific PCR amplicon to find probable microcystin degraders.

Amplification of Target DNA and DNA Sequencing

Molecular biology grade Milli Q water (HiMedia, India); 10 X *Taq* DNA polymerase assay buffer, 25 mM $MgCl_2$, mM dNTPs, Primers and *Taq* DNA polymerase (New England Biolabs) were purchased and used for all PCR in S1000 thermal cycler (BIORAD, USA).

mlrA Specific PCR-Screening of Probable Microcystin Degrader

*mlr*A gene specific primer sets *mlr*A-MF:5'-GACCCGATGTTCAAGATACT-3 (Tm as provided by manufacturer-51.6 °C) and *mlr*A-MR:5'-CTCCTCCCACAACTCAGGAC-3' (Tm as provided by manufacturer- 55.1 °C), adopted from Saito *et al.* (2003), were used for PCR based screening. Reaction was performed in a final volume of 25 µl which contained bacterial genomic DNA (50 ng), 1X *Taq* buffer, 1.0 U *Taq* polymerase, 5 mM $MgCl_2$, 25 µM dNTPs, and 10 µM of each primer. PCR was performed in a S1000 thermal cycler. Thermal profile for *mlr*A was adopted from Saito *et al.*, (2003) with suitable modification as described. Touch-up-PCR: Initial denaturation at 94°C for 5 minutes, followed by 10 cycles of 94°C for 30 seconds, 42°C+1°C/cycle for 30 seconds, 72°C for 1 minute, followed by 20 cycles of 94°C for 30 seconds, 52°C for 30 seconds, 72°C for 1 minute, followed by final extension at 72°C for 8 minutes and infinitely held at 4°C. Gradient PCR: Initial denaturation at 94°C for 5 minutes, followed by 30 cycles of 94°C for 30 seconds, 50°C to 65°C for 30 seconds, 72°C for 1 minute, followed by final extension 72°C for 8 minutes and infinitely held at 4°C. Standard PCR: Initial denaturation at 94°C for 5 minutes, followed by 30 cycles of 94°C for 30 seconds, 53°C for 30 seconds, 72°C for 1 minute, followed by final extension 72°C for 8 minutes and infinitely held at 4°C. 10 µl of each amplified target DNA fragments were mixed with 6X DNA loading dye (6X: Glycerol-30% v/v, Bromophenol Blue- 0.25% w/v, Xylene cynol- 0.25% w/v) and electrophoresed in agarose gel (1.5% w/v) in TAE buffer (40mM Tris, 20 mM Acetic acid, 1 mM EDTA; pH 8.4) at 5 V/cm. DNA specific bands were visualized under trans-UV after staining the gel with ethidium bromide (0.5 µg/ml), and photographs were taken in a AlphaImager MINI gel-documentation system (Cell Biosciences Inc., USA). Sample of *mlr*A gene specific amplicons from PM1, derived by standard PCR, was sent for DNA sequencing of both strands.

16S PCR

Universal primers set 41F-5'-GCTCAGATTGAACGCTGGCG-3' and 1488R-5'-CGGTTACCTTGTTACGACTTCACC-3' were used for amplification 16S-rRNA specific DNA target. Reaction was performed in a final volume of 25 µl which contained bacterial genomic DNA (50 ng), 1X *Taq* buffer, 1.0 U *Taq* polymerase, 5 mM $MgCl_2$, 25 µM dNTPs, and 10 µM of each primer. PCR was performed in a S1000 thermal cycler. Thermal cycle for 16S-PCR was adopted from Estrella *et al.* (2009) with suitable modification as described: Initial denaturation at 94°C for 5 minutes, followed by 35 cycles of 94°C for 1 minute, 55°C for 1 minute, 72°C for 1:30 minute, followed by final extension at 72°C for 8 minutes and infinitely held at 4°C. 10 µl of each amplified target DNA fragments were mixed with gel loading dye (composition) and electrophoresed in agarose gel (1.5% w/v) in TAE buffer at 5 V/cm. DNA specific bands were visualized under trans-UV after staining the gel with ethidium bromide (0.5 µg/ml), and photographs were taken in AlphaImager MINI gel-documentation system (Cell Biosciences Inc., USA). Sample of 16S-rRNA gene specific ~1.5 kb amplicons from PM1, derived by 16S PCR, was sent for DNA sequencing for further analysis.

DNA Sequencing and Sequence Analysis

DNA sequence of 16S-rRNA gene and mlrA was analyzed by Banaras Lab as per the details given below. Genomic DNA and PCR products were sent for analysis of DNA sequence of 16S and mlrA respectively. Genomic DNA was used to amplify the specific region 16S-rRNA in 25 µl PCR mix containing 5 µl of 10x Pfu reaction buffer with $MgSO_4$, 1 µl of dNTPs, 1µl of 10 µM forward primer 16S-F, 1 µl of 10 µM reverse primer 16S-R, 0.5 µl Pfu enzyme (ThermoFisher Scientific, USA), 2 µl of 50 ng/µl Genomic DNA and 14.5 µl nuclease free water. 16S and mlrA PCR products following electrophoresis were purified from agarose gel using Wizard® SV Gel and PCR Clean-Up System (Promega, USA) following manufacturer's instructions. Following gel purification, the DNA

concentration of PCR products was estimated by Quantus fluorometer (Promega, USA), and the integrity was checked on EtBr stained agarose gel (1%), and diluted to 10 ng/µl. The cycle sequencing reaction was carried out in 5 µl reaction volume including respective sequencing primers (16S-F or 16S-R for 16S rRNA; *mlr*A-MF or *mlr*A-MR for mlrA) in a heated lid thermal cycler (ABI 2720) and the products were cleaned and air dried to remove unincorporated Big-Dye-dNTPs and traces of ethanol. Dried pellet was resuspended in 15 µl of Hi-Di formamide (ThermoFisher Scientific, USA), an aliquot transferred to autosampler tubes (ThermoFisher Scientific, USA) and processed for sequencing. Nucletide sequences of 16S and *mlr*A were determined by an automated DNA sequencer Genetic Analyzer (Thermo 3500, ThermoFisher Scientific, USA). Sequence chromatograms (electropherograms) were viewed and corrected manually using Chromas software. Regions of overcrowded/overlapping/poor peaks at start and end of the read and 'N' in the internal positions of the read were removed and corrected respectively. Complete aligned sequence was obtained by using CAP3 online contigs alignment tool (Huang and Madan, 1999). The sequences of 16S and *mlr*A specific amplicons, thus obtained, were used for sequence analysis using different tools and softwares.

mlrA Sequence Homology Study

Multiple sequence alignment (MSA) was done to assess homology of the *mlrA* specific sequence of PM1 (*Bacillus licheniformis* PM1) with different *mlrA* genes which are already published in NCBI database. Accession number (AF411068.2) of *mlr*A sequence from a known microcystin degrader (*Sphingomonas* sp. ACM-3962) was used for retrieval of related sequences. BLAST search was done using AF411068.2 and the *mlr*A related sequences, in FASTA format, were retrieved from the NCBI database. mlrA.txt file of these retrieved sequences was generated. Our sequence specific to *mlr*A from PM1 (*Bacillus licheniformis* PM1) in FASTA format was included into the mlrA.txt file. The mlrA.txt file was subjected to multiple sequence alignment by offline multiple sequence alignment software ClastalX2.1 which resulted in output files mlrA.aln and mlrA.dnd file. Flanking extra length of reference sequences compared to our sequence was removed and further subjected to the above MSA. Using mlrA.dnd file a guide tree (1000 bootstrap, 5 times randomized sequence order, all gaps site ignored, 10000 equally best trees retained) was estimated by parsimony method in Seaview software. The tree was exported in pdf file. *mlr*A sequence of PM1 was submitted to NCBI database using online submission tool Banklt.

Phylogenetic Analysis of PM1

Phylogenetic analysis of 16S-rDNA sequence of isolate PM1 was done online at NCBI. 1279 nucleotide DNA sequence specific to 16S rRNA gene of PM1 isolate was run into BLAST (Program optimized for- megablast, search set database-16S ribosomal sequences of Bacteria and Archaea, Algorithm parameter: Default) at NCBI. 28 (Unique or 2 repeats of different species) sequences producing significant alignments with 100% query coverage were selected and subjected to construction of distance tree by Neighbor Joining method at maximum sequence difference of 0.75. The resultant tree was viewed in NCBI Tree Viewer (Blast Tree View). The sequence of PM1 was submitted to NCBI database using online submission tool Banklt.

Amplified Ribosomal DNA Restriction Analysis (ARDRA)

Amplified ribosomal DNA restriction analysis (ARDRA) is a commonly used tool to study microbial diversity that relies on r-DNA restriction fragment length polymorphism (Deng *et al.*, 2008). 16 S rDNA sequence of PM1 isolate was subjected to *In-silico* restriction digestion by NEB cutter and three restriction endonucleases namely *Eco*RI, *Bam*HI and *Hpa*II, readily available in our stock, were selected for ARDRA. 16S rRNA genes specific PCR products of isolates DK1, DK2, DK4, DK6, DK8, DK9, PM1, LK1, LK3, LK4, Gd1, Gd2 and Gd3 were digested with *Eco*RI, *Bam*HI and *Hpa*II for 1 hour at 37°C as per the manufacturer's instructions (Fermentas, Life Sciences) followed by enzyme inactivation as per the manufacturer's instructions (Fermentas, Life Sciences). Digested products and molecular weight marker were resolved at 5 V/cm in 2% agarose gel prepared in TAE buffer. Digestion products (DNA bands) were visualized under trans-UV after staining the gel with ethidium bromide and photographs were taken in AlphaImager Mini gel-documentation system (Cell Biosciences Inc., USA). The size of the fragments was determined with the help of DNA molecular weight markers included in the gel. Polymorphism (ARDRA) was analyzed by Alpha View Fragment Analysis software (AlphaImager Mini gel-documentation system, Cell Biosciences Inc., USA) and dendogram was constructed using UPGMA method and Jacard coefficient matrix.

Results and Discussion

Sample Collection, Isolation and Biochemical Characterization of Bacteria

In order to obtain native bacteria with high MC-degradation efficiency, samples were collected from four ponds which were reported earlier/infested with cyanobacterial blooms. Geographical location of these pond sites were recorded as DK (23°26'22.0"N, 85°08'46.6"E), LK (23°26'22.0"N, 85°08'46.7"E), PM (25°19'21.1"N, 82°59'43.3"E) and Gd (25°58'02.8"N, 82°59'43.3"E). DK and PM ponds showed visible bloom of *Microcystis* whereas other ponds did not show the detectable population of *Microcystis* by microscopy. Bacteria were grown on LB agar and were isolated to pure cultures employing standard pure culture techniques. On the basis of morphological, biochemical and

staining characteristics 20 bacterial isolates could be distinguished (Table 1). Among these isolated strains, only the PM1 strain showed remarkable difference in broth culture. PM1 grew as small clumps whereas others grew as homogeneous suspension. This strain was found to be aerobic, gram-positive and rod-shaped bacterium.

Screening of probable microcystin degrading bacteria

Products *of mlr*A gene specific PCR assay using genomic DNA of all the 20 isolates were resolved by gel electrophoresis and visualized by ethidium bromide under UV. Touch up PCR of selected isolates resulted in fragment of more than one size (particularly in LK3 and PM1) on agarose gel. Using genomic DNA of PM1 the specificity and yield of *mlrA* specific PCR was optimized by gradient PCR to get single and specific band. Annealing temperature (Ta) 53.4°C resulted in single specific but low intensity (yield) band. This annealing temperature complied well with the Tm of the primer sets used. Earlier studies had used annealing temperature of 58 and 60°C however we could not detect bands at/above Ta 55.9°C. Approximately 550 bp fluorescent DNA bands were detected in DK1, DK2, DK4, DK6, DK8, DK9, LK1, LK3, LK4, PM1, Gd1, Gd2 and Gd3 isolates by standard *mlr*A gene specific PCR performed at Ta 53°C (Fig. 3). This band was detected as prominent band in all these isolates except LK3, LK4 and Gd2. An additional band of 700 bp was seen in isolates LK3 and LK4 but its intensity was similar or low respectively. The size of both the band do not exactly comply with earlier reported size in the cases of microcystin degrading bacteria (Alamri, 2010 and 2012; Ho *et al.*, 2007; Saito *et al.*, 2003).

Table 1: Morphological and biochemical characters of the various isolated bacterial cultures

Isolates	Color	Form/ Margin	Size (mm)	Elevation	Optical property	Texture	Tests (U, L, C, G)*			
DK1	Off white	C/E	<1	C	O	Soft	U^+	L^+	C^+	G^+
DK2	Yellow	C/E	1-2	C	Tl	Soft	U^-	L^+	C^-	G^+
DK4	Dirty white	C/E	1-2	C	Tl	Soft	U^-	L^-	C^-	G^+
DK6	Dirty white	C/E	1-2	C	Tl	Soft	U^+	L^+	C^-	G^+
DK8	Yellow	C/E	1-2	C	Tl	Soft	U^+	L^-	C^+	G^+
DK9	Dirty orange	C/E	1-2	C	Tl	Soft	U^-	L^+	C^+	G^+
LK1	Dirty pink	C/E	1-2	C	Tl	Soft	U^-	L^-	C^+	G^+
LK3	Dirty pink	C/E	1-2	C	Tl	Soft	U^+	L^+	C^+	G^+
LK4	Dirty pink	C/E	1-2	C	Tl	Soft	U^+	L^-	C^+	G^+
PM1	Dirty white	C/E	1-2	C	Tl	Hard	U^+	L^+	C^+	G^+
Gd1	Dirty yellow	P	<1	C	Tl	Soft	U^+	L^-	C^+	G^+
Gd2	Milky white	C/E	1-2	C	Tp	Soft	U^-	L^-	C^-	G^+
Gd3	Dirty white	C/E	1-2	C	Tl	Soft	U^-	L^-	C^-	G^+

C/E: circular/entire; P: punctiform; C: convex; O: opaque; Tl: translucent; Tp: transparent. *- U: urease test; L: lactose fermentation test on MacConkey agar; C: catalase test; G: gram's staining; '+' : positive test; '-': negative test

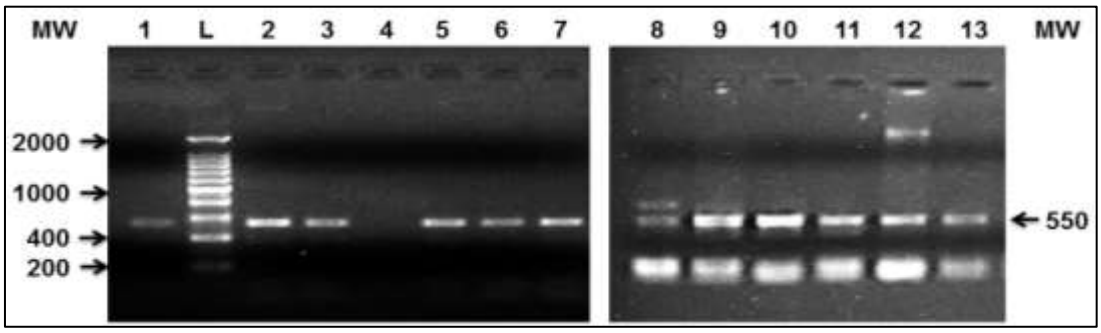

Fig. 3: 1.5% Agarose Gel Electrophoresis of amplified *mlr*A specific target of genomic DNA of bacterial isolates. Lane L is 200 bp DNA Ladder: 200; 400; 600; 800; 1000; 1200; 1400; 1800; 2000 bps respectively. Lane numbers 1-13 are amplicons: Lanes 1: DK1; 2: DK2; 3: DK4; 4: DK6; 5: DK8; 6: DK9; 7: LK1; 8: LK3; 9: LK4; 10: PM1; 11: Gd1; 12: Gd2; 13: Gd3. All the molecular weights are written in base pairs (bp).

513 bp *mlr*A specific nucleotide sequence of PM1 isolate was derived by DNA sequencing. BLAST search of this nucleotide stretch showed 99% identity (98% query coverage, 0.0 E value and 1 Gap) to 514382-51488 region encoding superoxide dismutase (ACQ69498.1) of *Exiguobacterium* sp. AT1b complete genome (CP001615.1) of the database at NCBI. It showed 79% or less identity to others with less query coverage (91% or less) and poor expect value. Guide tree generated by MSA of *mlr*A specific sequence of PM1 with other 10 *mlr*A nucleotide sequences of known microcystin degraders retrieved from NCBI databank revealed strong clustering of PM1with bacterium *Sphingomonas* Y2 (96% bootstrap support) (Fig. 4). Pairwise alignment of *mlr*A specific fragment of PM1 with *mlr*A nucleotide sequence of *Sphingomonas* Y2 and, *mlr*A nucleotide sequence of *Sphingomonas* Y2 with *mlr*A nucleotide sequence of *Sphingomonas* MD-1 showed 42.3% and 75% identity respectively. Since the primer is specific to *mlr*A, less similarity of the *mlr*A specific fragment of PM1 (with respect to band size and DNA sequence) may be attributed to the following factors: 1- the primer set has been designed against *Sphingomonas*, 2- sequence information of *mlr*A gene is limited to only few genus so far, 3- influence of geographical separation on diversity of *mlr*A sequence, etc. Alamri (2010 and 2012) has amplified *mlr*A and reported microcystin degrading bacterium from genus *Bacillus* but information about *mlr*A nucleotide sequence is lacking in the databank. At present these isolates deserves their characterization as probable microcystin degraders in their country of origin (India). Biological degradation of microcystin in natural pond water system was evident from our earlier studies (Unpublished data-Thesis: Physiological and molecular basis of toxin production by the blooms of *Microcystis* spp., awarded to Anil Kumar in Year 2009) conducted during year 2006 where content of microcystin (quantified by Quantiplate Kit, Envirologix Inc., Portland, ME, USA) reduced considerably in natural pond water incubated under laboratory condition as compared to incubation of filter sterilized (0.45 micron) pond water. Enzymatic assay of microcystin degradation is, however, inevitable to confirm microcystin degrading character of PM1 and other isolates in this article. *mlr*A specific sequence of PM1 has been submitted to NCBI GenBank database under accession nnumber KX756456.

Phylogenetic and ARDRA analysis

Based on BLAST search with 1279-bp 16S rRNA gene nucleotide sequence, PM1 was found to be 99% similar to *Bacillus licheniformis* (NR118996.1). PM1 was tentatively identified as *Bacillus licheniformis* and its 16S rRNA gene nucleotide sequence has been submitted to GenBank under accession number KX604089. 16S rRNA gene nucleotide sequence based neighbor-joining phylogenetic tree shows close clustering of PM1 with *Bacillus licheniformis* followed by *B. sonorensis* and *B. aerius* (Fig. 5). *B.licheniformis* was earlier shown to cluster with microcystin degrading bacteria *Bacillus subtilis* JSEM1/EMB however; sequencing of *mlr*A specific PCR fragment was neither performed nor reported by them (Alamri, 2010 and 2012; Kormas and Lymperopoulou, 2013). Identity of isolate PM1 to *B.licheniformis* which clustered with microcystin degrading bacteria further corroborate our finding that PM1 may be the probable microcystin degrader. However further assay is inevitable to functionally validate microcystin degradation and its metabolic pathway.

Restriction digestion of amplified ~1.5kb 16S rRNA gene of our *mlr*A positive isolates using *Hpa*II showed polymorphisms among the various isolates and revealed these isolates to be distinct, though they broadly clustered into four groups: (DK9, DK6, Gd3), (DK8, DK4, DK2, DK1), (Gd2, Gd1), (LK1, LK3, LK4, PM1) (Figure 6A and B). Different isolates from one pond clustered together to their respective ponds except Gd3, DK6, DK9 and PM1. The finding indicated existence of different types of indigenous bacteria and their role in naturally preventing prolonged accumulation of high level of microcystin which, generally, is observed during sudden lysis and disappearance of toxic bloom scum in infested ponds.

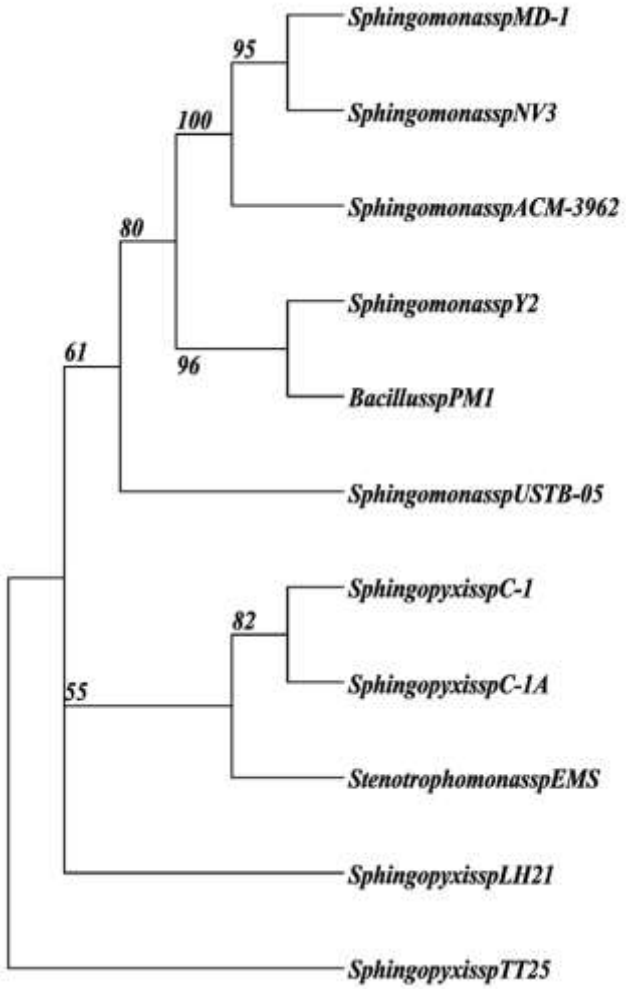

Fig. 4: A guide tree of MSA of *mlr*A specific sequence of PM1 with known reference *mlr*A target sequences retrieved from GenBank. Tree was constructed by ClustalX and visualized by Seaview [Dnapars, bootstrap with 1000 replicates, 350 steps, 464 sites (59 informative)]. Accession numbers of reference sequences are: AF411068.2 (*Sphingomonas* sp. ACM-3962); JN256930.1 (*Sphingomonas* sp. NV3); HM245411.1 (*Sphingomonas* sp. USTB-05); AB468058.1 (*Sphingopyxis* sp. C-1); GU224277.1 (*Stenotrophomonas* sp. EMS); AB161685.1 (*Sphingopyxis* sp. C-1A); DQ112243.1 (*Sphingopyxis* sp- LH21); JQ398615.1 (*Sphingopyxis* sp- TT25); AB114203.1 (*Sphingopyxis* sp Y2); AB114202.1 (*Sphingomonas* sp. MD-1); KX756456 (*B. licheniformis* PM1).

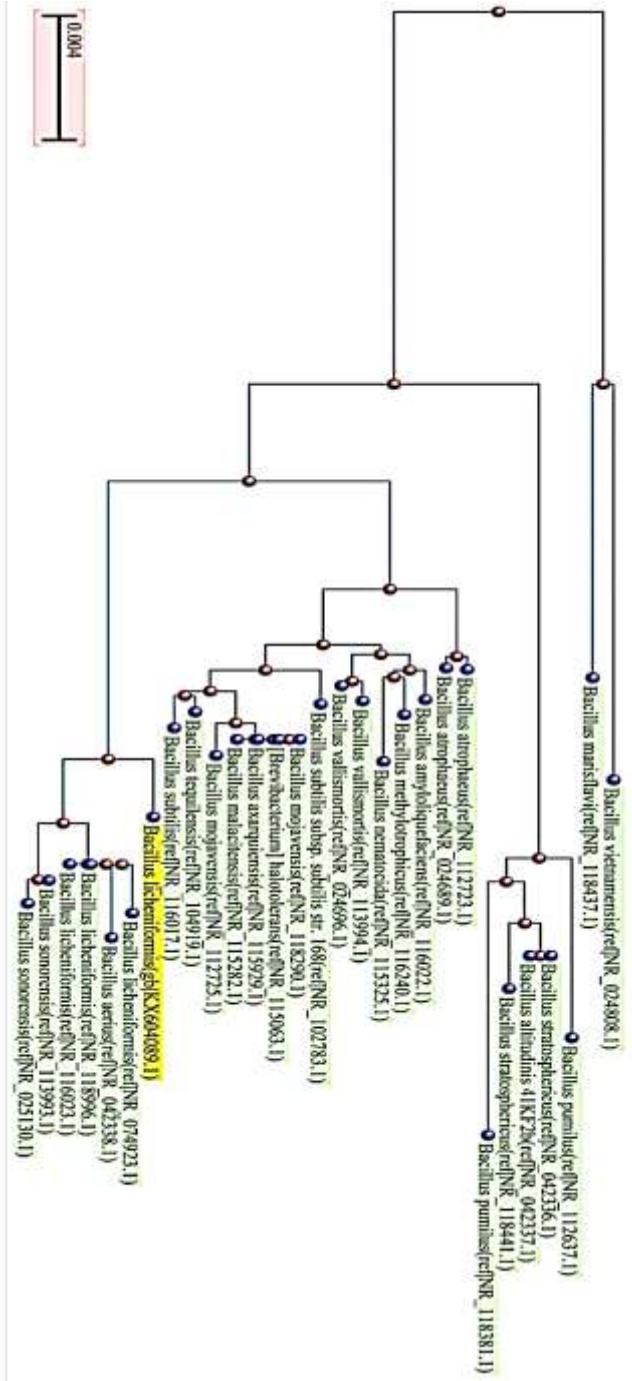

Fig. 5: 16S-rRNA based Phylogenetic tree (neighbor-joining method) of isolate PM1 [Bacillus licheniformis(gb|KX604089.1)] and closely related reference strains retrieved from BLAST result. Sequence labels in the tree are represented by Taxonomic Name (Sequence ID).

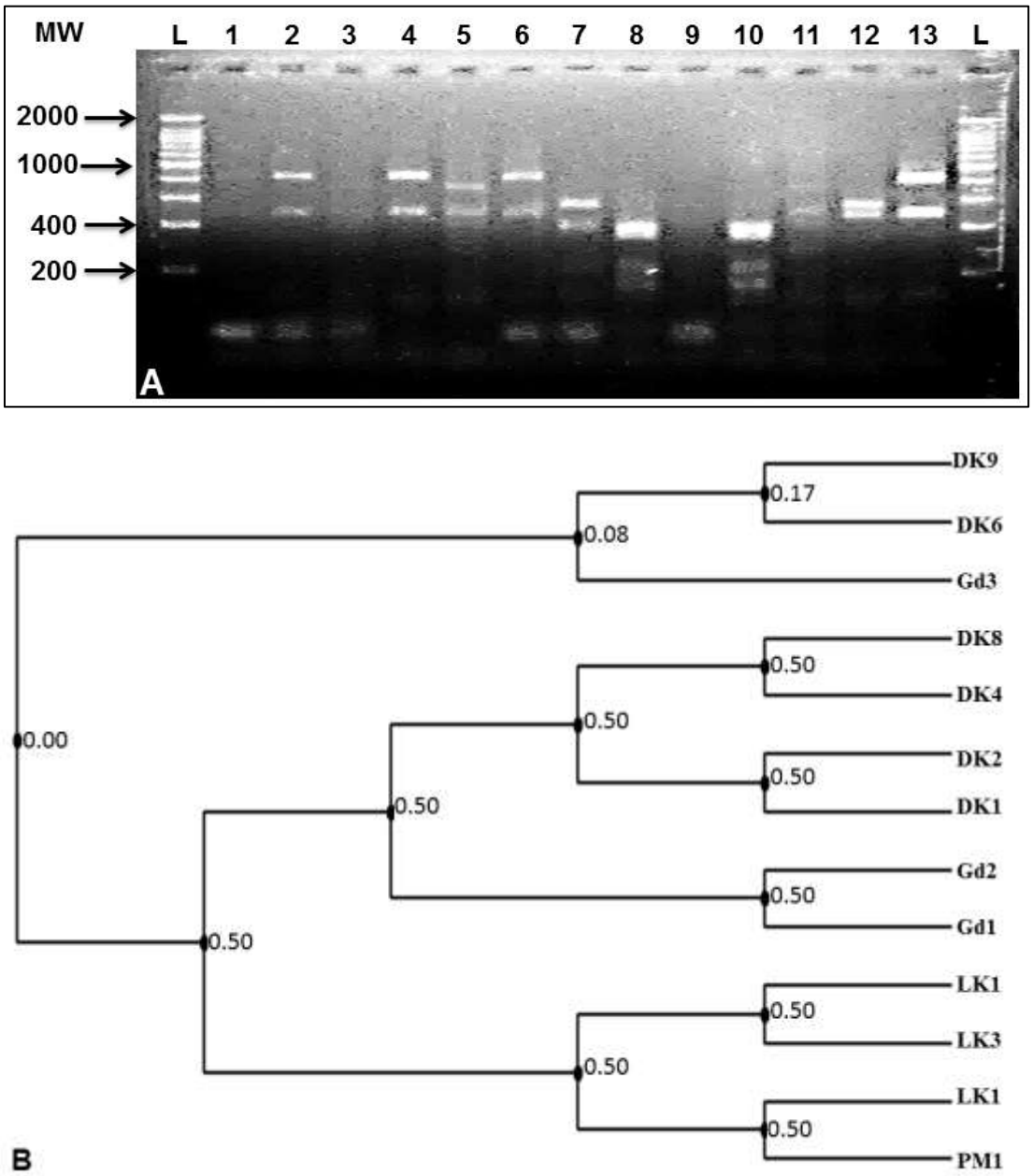

Fig. 6 (A and B): 6A: 2% Agrose Gel Electrophoresis of *Hpa*II digested products of 16S rRNA gene amplicons of various bacterial isolates. Lane L is 200 bp DNA Ladder: 200; 400; 600; 800; 1000; 1200; 1400; 1800; 2000 bps respectively. Lane numbers 1-13 are *Hpa*II digested products: Lanes 1: DK1; 2: DK2; 3: DK4; 4: DK6; 5: DK8; 6: DK9; 7: LK1; 8: LK3; 9: LK4; 10: PM1; 11: Gd1; 12: Gd2; 13: Gd3. All the molecular weights are written in base pairs (bp). 6B: ARDRA based dendrogram showing polymorphism. Dendrogram was constructed by AlphaView Fragment Analysis Software (using Jacard Coefficient matrix and UGPMA method) on the basis of restriction fragment's banding pattern of different bacterial isolates

Conclusion

It was evident, from our previous study, that microcystin degradation could be achieved by indigenous microcystin degrading microbial population in its natural place where microcystin producing blooms occur. 13 bacterial isolates could be isolated which showed *mlr*A amplification and evidenced the probable potential to degrade microcystin. Though all the isolates were distinct, ARDRA analysis revealed overall four bacterial groups. BLAST and phylogenetic analysis of 16S rRNA gene from PM1 revealed that isolate PM1 was strongly identical to *B. licheniformis* which was shown earlier to cluster with microcystin degrading bacterium *B. subtilis*. Thus this study has presented primary investigation of isolation and characterization of probable microcystin degrading bacteria from water-bodies in India. Furthermore the present study is the first to report probable microcystin degrading bacterium, *Bacillus licheniformis*, from India. Coexistence of such bacteria in MCs infested sites can contribute to natural means of maintaining low level of such toxins. The

potential and the metabolic pathway of these isolates need to be further validated to confirm their application in microcystin bioremediation.

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Conflict of Interest

The authors have no conflicts of interest

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