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Occurrence, Biosynthesis and Consequences of Microcystin: A Potent Hepatotoxin of Cyanobacteria

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ABSTRACT

The production of toxin from bloom, scum or mat forming cyanobacteria in fresh, brackish and marine waters is becoming a global issue. The toxins produced comprise mainly hepatotoxins and neurotoxins. Of these toxins microcystin is a cyclic heptapeptide produced by *Microcystis, Planktothrix, Anabaena, Nostoc, Hapalosiphon* and *Anabaenopsis*. Microcystins (MCs) comprise more than 80 variants and are highly toxic and inhibit eukaryotic protein phosphatases 1 and 2A activity. Chronic exposure to microcystin causes tumor promotion in human. Microcystins are synthesized nonribosomally via thiotemplate mechanism by microcystin synthetase, encoded by microcystin synthetase gene cluster (*mcy*). The gene cluster (*mcyABCDEFGHIJ*) has been cloned and sequenced from *Microcystis, Planktothrix* and *Anabaena.* Microcystin is frequently released in water after autolysis of blooms and may impose a risk to human health, when contaminated water is used for drinking purpose. In this review an attempt has been made to assemble all the known information on microcystin.

Keywords: Cyanobacteria; *Microcystis*; hepatotoxin; microcystin; *mcy* gene

INTRODUCTION

Cyanobacteria (blue-green algae), a group of O_2-evolving gram-negative photosynthetic prokaryotes, are known to inhabit a wide spectrum of habitats including terrestrial, freshwater and marine ones (Tyagi et al., 1999). All the members of this group perform typical oxygenic photosynthesis and possess chlorophyll-a, and water soluble phycobilin proteins (Pearson and Neilan, 2008). Certain members are capable to grow in the absence of fixed inorganic nitrogen sources by fixing atmospheric dinitrogen gas. Environmental conditions such as higher temperature and pH values, low turbulence, and high nutrient inputs (eutrophication) enhance the development of planktonic cyanobacteria in lakes and reservoirs, leading to the formation of surface bloom that may accumulate as scum. The occurrence of these blooms are known to create serious water quality problem, as several species of cyanobacteria are known to produce a class of secondary metabolites which are toxic to many organisms, including human. These secondary metabolites include alkaloids, lipopolysaccharides, polyketides and peptides (Börner and Dittmann, 2005) and grouped together as cyanotoxin. Cyanotoxins are very diverse in their chemical structure and toxicity (Kaebernick and Neilan, 2001; Kumar et al., 2000; Kumar et al., 2011). Based on the toxic effects, they are classified as dermatotoxins (lipopolysaccharides, lyngbyatoxin-a, and aplysiatoxins), neurotoxins (anatoxin-a, homoanatoxin-a, anatoxin-a(s), and saxitoxins), and hepatotoxins (microcystins, nodularin, and cylindrospermopsin). These toxins generally present within cells, but can be released in high concentrations after cell lysis (Sivonen and Jones, 1999). Hepatotoxins are cyclic peptides such as microcystins (Tyagi et al., 2006) and nodularin or alkaloid such as cylindrospermopsin. On the other hand, neurotoxins are alkaloids in nature (anatoxin-a, saxitoxins etc). Hepatotoxins affect liver whereas neurotoxins act on nervous system. First report of toxic cyanobacterium (*Nodularia spumigena*) dates back to the late 19th century, now a vast array of toxins from various species of cyanobacteria have been reported from different parts of the world (Carmichael, 1994; Kurmayer and Christiansen, 2009).

Microcystins

Microcystins are the most common group of low molecular weight cyclic heptapeptides produced by cyanobacteria. They are water soluble and highly thermostable compounds. Their molecular weight ranges from 909 Da to 1115 Da (Kurmayer and Christiansen, 2009). They are potent inhibitors of eukaryotic protein phosphatases 1 and 2A and are linked to the deaths of wild animals and livestock worldwide (Sivonen and Jones, 1999). During the last two decades, microcystins have attracted attention of many researchers due to their ability to cause acute poisonings as well as tumor promotion potential following chronic exposure to humans even at low concentrations present in drinking water (Singh et al., 2001a; WHO, 1999). Microcystins have general structure as cyclo (D-Ala^1-X^2-D-MeAsp^3-Z^4-Adda^5-D-Glu^6-Mdha^7) where X and Z are variable L-amino acids (Figure 1). MeAsp stands for erythro-β-methyl aspartate and Mdha is *N*-methyl-dehydroalanine (Carmichael, 1994; Kurmayer and Christiansen, 2009). Adda [(2S, 3S, 8S, 9S)-3-amino-9-methoxy-2, 6, 8-trimethyl-10- phenyldeca-4, 6-dienoic acid] is considered responsible for causing hepatotoxicity (Dawson, 1998). Besides hepatotoxicity, Adda also provides a characteristic absorption property to microcystin at 238 nm due to the presence of a conjugated diene group in the long carbon chain. Microcystins are produced predominantly by planktonic strains of the distantly related cyanobacterial genera such as

Microcystis, Planktothrix and *Anabaena* but planktonic, benthic and terrestrial strains of the genera *Nostoc, Anabaenopsis, Hapalosiphon* and *Phormidium* are also capable to produce microcystin although in small quantity (Fewer et al., 2007; Sivonen and Jones, 1999).

Microcystins include high number of isoforms, often associated with changes in the synthetases as a result of mutations in the *mcy* gene cluster (Mikalsen et al., 2003). Several independent investigations on *Microcystis* and *Planktothrix* strains have concluded that the natural variation in the gene cluster within each strain is often caused by recombination. The regions with highest site variation seem to be *mcyA* and *mcyB* (Mikalsen et al., 2003; Tanabe et al. 2004). More than 80 variants of microcystin have been identified (Feurstein et al., 2009) which probably arise mainly due to the variable amino acids present at position X and Z (Figure 1), the most frequent and studied variant is microcystin-LR (MC-LR) with the variable amino acids leucine (L) and arginine (R).

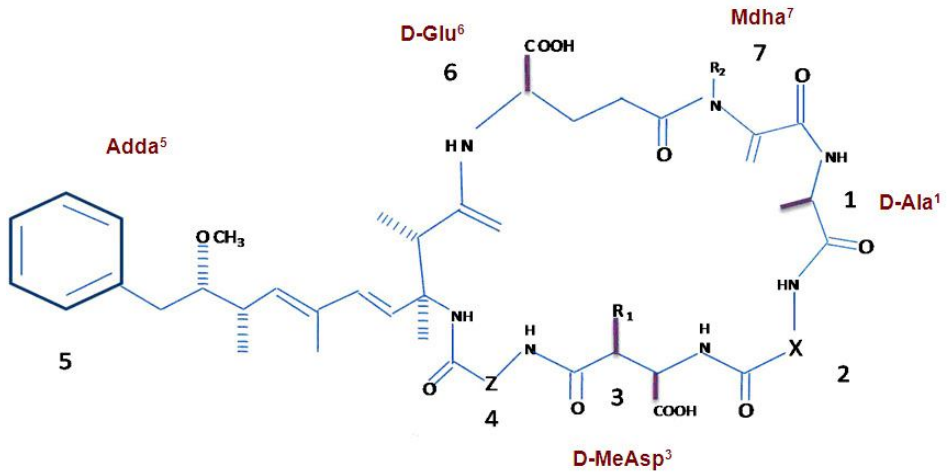

Figure 1 Typical structure of microcystin. Variable L-amino acid residues are found at positions X and Z.

Biosynthesis

Nonribosomal synthesis of microcystins is catalyzed by a multifunctional enzyme complex called microcystin synthetase, via thiotemplate mechanism. The enzyme complex (microcystin synthetase) includes non-ribosomal peptide synthetases (NRPS), polyketide synthases (PKS), and tailoring enzymes (Nishizawa et al., 2000; Tillett et al., 2000). NRPSs possess a highly conserved modular structure with each module being comprised of catalytic domains responsible for the adenylation, thioester formation and in most cases condensation of specific amino acids (Marahiel et al., 1997). The arrangement of these domains within the multifunctional enzymes determines the number and order of the amino acid constituents of the peptide product. Additional domains for the modification of amino acid residues such as epimerization, heterocyclization, oxidation, formylation, reduction or N-methylation may also be included in the module (Marahiel et al., 1997). Nonribosomal synthesis of peptides requires at least three proteins namely adenylation domain, peptidyl carrier protein and condensation domain where adenylation domain (~550 aa) is responsible for the selection of the cognate amino acids and their activation as amino acyl adenylate. The activated amino acid is then transferred to a peptidyl carrier protein (PCP, size ~80–100 aa), which represents the transport unit and assumes a function similar to the tRNA because it is the transport unit of the activated intermediate. The condensation-domain (~450 aa) is the central entity of nonribosomal peptide synthesis which finally catalyzes the formation of the peptide bond (Keating et al., 2002). It is assumed that this enzyme has an acceptor site for the nucleophile and a donor site for the electrophile (Finking and Marahiel, 2004).

Genetic basis of biosynthesis

Biosynthesis of microcystins is encoded by microcystin synthetase genes cluster (*mcy*) including genes for peptide synthetases, polyketide synthases, mixed peptide/polyketide synthases and tailoring enzymes. Gene cluster has been characterized in detail for *Microcystis* (Nishizawa et al., 1999, 2000; Tillett et al., 2000), *Planktothrix* (Christiansen et al., 2003) and *Anabaena* (Rouhiainen et al., 2004). The gene cluster (*mcyABCDEFGHIJ*) encoding microcystin synthetase has been cloned and sequenced from *Microcystis, Planktothrix* and *Anabaena* (Christiansen et al., 2003; Rouhiainen et al., 2004; Tillett et al., 2000). Size of gene cluster encoding microcystin synthetase varies in different toxin- producing cyanobacterial genus. It is 55 kb long in *Microcystis*, 55.6 kb in *Planktothrix*, and 55.4 kb in the case of *Anabaena* sp. 90.

In *Microcystis*, and *Anabaena* the genes are transcribed from a central bidirectional promoter region (Kaebernick et al., 2002), whereas in *Planktothrix* all *mcy* genes, except *mcyT*, seem to be transcribed unidirectionally from a promoter located upstream of *mcyD*. Insertional gene knockout experiments have demonstrated that all the microcystin variants produced by a strain are

synthesized by a single enzyme complex (Christiansen et al., 2003; Nishizawa et al., 1999). Gene cluster of the genus *Microcystis* comprises 10 bidirectionally transcribed open reading frames (*mcyABCDEFGHIJ*) arranged in two putative operons (*mcyA-C* and *mcyD-J*). Altogether 48 sequential catalytic reactions are involved in microcystin synthesis, in which 45 have been assigned to catalytic domains within six large multienzyme synthases/synthetases (*mcyA-E,G*), which incorporate the precursors namely phenylacetate, malonyl-CoA, S-adenosyl-L-methionine, glutamate, serine, alanine, leucine, D-methyl-isoaspartate, and arginine. The additional four monofunctional proteins are putatively involved in O-methylation (McyJ), epimerization (McyF), dehydration (McyI), and localization (McyH) (Tillett et al., 2000).

Tillett et al. (2000) for the first time reported the complete description of the biosynthetic pathway of microcystin from *M. aeruginosa* PCC 7806. It was reported that the multi-enzyme components of the Mcy complex comprise the minimal set of domains needed for the assembly of seven amino acids and four acetate units plus additional integrated domains with epimerase, methyltransferase, and aminotransferase activities. During the course of microcystin biosynthesis, M*cy*G presumably starts microcystin synthesis by activation of phenylacetate followed by transfer to the 4-phosphopantetheine of the first carrier domain. It is the first step in Adda biosynthesis (Pearson and Neilan, 2008). Subsequently, Adda is formed by the four PKS modules of McyG, D and E. McyJ is needed for an O-methylation step. An integrated domain with similarity to glutamate semialdehyde aminotransferases most likely provides the amino group of the Adda moiety. Finally, the first condensation domain of McyE condenses Adda with the activated glutamate thereby linking the PKS with the NRPS part of microcystin biosynthesis. The NRPS module of McyA, B and C activates the remaining five amino acids and incorporate them into the growing peptide structure. It has been proposed that the thioesterase domain of McyC is responsible for cyclization and release of microcystin from the synthetase complex (Christiansen et al., 2003). McyF is a racemase and provides D-aspartate and most likely also D-methyl-aspartate. McyI is putatively involved in the production of D-methylaspartate at position three within the micocystin structure. Since McyH shows significant similarity to exporters of the ABC transporter family it might play a role in microcystin export (Pearson et al., 2004).

Toxicology of microcystin

Microcystins show toxic effects on multi-cellular organisms ranging from planktonic crustaceans to vertebrates such as fish and mammals (Singh et al., 2001b; 2003). Microcystin-intoxication in vertebrates mainly affects the intestine and liver although liver is the maximum affected organ, with abnormal function, and rapid and fatal failure. The toxic effects are largely linked to the inhibition of protein serine-threonine phosphatases (PP) in the affected organism (Carmichael, 1994; Chen et al., 2005). Non-covalent interactions between different amino acids of protein phosphatases and microcystin play a central role in the inhibitory mechanisms of PP by microcystin. The toxicity of microcystins is attributed to the presence of the unusual amino acid Adda. Hydrophobic interaction between the residues Trp206 and Ile130 in the hydrophobic groove of PP1 and the unusual amino acid Adda side-chain are crucial for the microcystin toxicity (Holmes et al., 2002). The Adda moiety is also required for the binding of the toxin to protein phosphatases which occurs via covalent bonds and is highly specific. High concentrations of microcystin promote liver cell death and are induced by ROS signaling without the involvement of typical apoptotic proteins while its intermediate doses activate classical apoptotic pathways. Low concentrations however, increase liver cell survival and proliferation, and can cause primary liver cancer. When chronically administered microcystins may promote liver cancer in mammals (Ito et al., 1997) by inducing oxidative DNA damage (Zegura et al., 2006). Mice exposed to a sub lethal dose of MC-LR by intraperitoneal (i.p.) injections developed multiple neoplastic nodules in the liver (Ito et al., 1997).

In mice, the intraperitoneal (i.p.) LD_{50} value for MC-LR is usually close to 50 µg/ kg body weight (Dow and Swoboda, 2000) but it can range from 25 to 125 µg/ kg body weight (Dawson, 1998; WHO, 1999). Although the inhalation toxicity of MC-LR is also high (Dawson, 1998), this toxin is much less toxic by oral ingestion (Ito et al., 1997), with LD_{50} of 5 mg/ kg body weight in mice (WHO, 1999). Estimation of the toxic threshold value of dietary microcystin for male and female fish has recently been reported by Deng et al. (2010). Due to its toxic nature WHO has established the guideline of 1 µg/L for MC-LR in drinking water as the safe consumption level and many countries have followed (with slight changes) this guideline for drinking water and food as well.

Symptoms of microcystin poisoning

Signs of microcystin poisoning in mice include weakness, anorexia, recumbency, pallor, cold extremities, laboured breathing, apathy, respiratory problems, vomiting and diarrhoea and necrosis of liver (Dow and Swoboda, 2000). Death due to respiratory arrest is preceded by disruption of liver structure and function and haemorrhage into this organ after some hours or days (Carmichael, 1994) depending upon the dose of toxins. In mice, prior to death, signs of restlessness, sedation, movement disorders, spasms and spasmodic leaping, loss of coordination and spreading of limbs, and severe cramps are observed (Tyagi et al., 1999). It has been reported that the liver weight may increase up to 2 times as compared to control mice. Liver is the main target of microcystin poisoning where due to disintegration of intermediate filament, microfilament and microtubules, shrinkage of endothelial cell lining occurs and many associated functions are affected. As a consequence blood capillaries may rupture and release of blood occurs.

Mode of action

Since microcystin is hydrophilic and thus unable to penetrate the plasma membrane, it requires active uptake mechanisms to enter cells. Such carriers are found only in the liver and demonstrated to be specific bile acid transport mechanisms. It acts by

inhibiting protein phosphatases which are essential for cell regulatory processes such as growth, protein synthesis, glycogen metabolism and muscle contraction (Falconer, 2005). In mammals, microcystins are selective for hepatic cells, irreversibly inhibiting serine/threonine protein phosphatases PP1 and PP2A (Dawson, 1998) and causing disintegration of hepatocyte structure, apoptosis, liver necrosis, and internal hemorrhage that may lead to death by hemorrhagic shock (Dow and Swoboda, 2000). In the hepatocytes, they form adducts with PP1 and PP2A thereby affecting the normal activity of PP2A which plays an important role in cell-cycle regulation, cell growth control, and regulation of multiple signal transduction pathways, cytoskeleton dynamics, cell mobility and also as tumor-suppressor protein. MC-LR is also reported to bind to ATP synthetase, potentially leading to cell apoptosis. Orally ingested microcystins are transported across the ileum into the bloodstream via a bile-acid transporter that exists in hepatocytes and cells lining the small intestine. Structurally cytoskeleton consists of three major structural elements i.e., microtubules, microfilaments and intermediary filaments and play an important role in maintaining cellular architecture and internal organization, cell shape, motility, cell division, and many other processes within the cell. Their disintegration would lead to the cytoskeleton shrinkage of hepatocytes as well as halt of many associated functions. As a consequence fingerlike projections develop through which hepatocytes interact with each other as well as with cells lining of sinusoidal capillaries, are withdrawn and intercellular contacts are broken. The structure and function of cytoskeleton are influenced by regulated phenomena of phophorylation and dephosphorylation by kinases and phosphatases respectively (Carmichael, 1994). The hyperphosphorylation of intermediate filaments and microfilaments or proteins that act on them, lead to the disintegration of intermediate filaments, disruption of microfilaments (Carmichael, 1994) and microtubules due to subunit loss and dissociation.

Detection, separation and characterization

The mouse bioassay is frequently used for toxicity assessment and also for the determination of lethal dose (LD_{50}) of toxin. For toxin purification, TLC was routinely used in spite of low sensitivity and specificity. However, high pressure liquid chromatography (HPLC) (Spoof et al., 2001) is now widely used in view of sensitivity of the detection of microcystins and other cyanotoxins. Furthermore this method allows investigators to make distinction between microcystin variants. Unfortunately, the method is expensive and time consuming, and requires a considerable sample volume and certified standards for the identification of MC variants.

The recently developed method MALDI-TOF-MS (matrix assisted laser desorption/ ionization-time of flight mass spectrometry) has been used for the analysis of many peptides, including cyanobacterial secondary metabolites (e.g., antibiotics or toxins such as microcystins) (Fastner et al., 2001; Welker et al., 2002). It requires only microgram quantities (not milligram quantities like HPLC or bioassays) of cell material and the detection is rapidly made, without the need for time-consuming extraction or purification processes, thereby allowing the identification of known microcystin variants and other unknown metabolites simultaneously (Fastner et al., 2001; Welker et al., 2002). Enzyme-linked immunosorbent assays (ELISAs) based on monoclonal (Zeck et al., 2001) and polyclonal antibody actions against microcystin structure have also been used by several workers. This assay method has low equipment requirement and allows rapid, easy, effective and sensitive detection of microcystins (particularly MC-LR) in water samples although toxicity assessment is not possible. In an attempt to improve the methodology, the problem of cross-reactivity with nontoxic compounds (leading to false positives) has been minimized with competitive ELISA method which may have detection limits of 0.07 µg/L (Zeck et al., 2001). This refinement has resulted in adopting ELISA as a suitable method for assessing microcystin concentrations below the WHO guideline of 1 µg/L in drinking water.

Another method frequently applied includes protein phosphatase inhibition assay (PPIA) which is based on immunodetection. The colorimetric PPIA assay is a rapid, easy and sensitive screening method (Metcalf et al., 2001) that does not require much equipment and is less expensive than ELISA or radio-labeled PPIA (which uses both PP1 and PP2A). As such this assay correlates positively with HPLC and there are recent options for detecting MC-LR in drinking water with detection limits below the WHO guideline of 1 µg/L (WHO, 1999). Competitive binding assays based on blockage of the active site of PP2A have also been developed for microcystins and there are immunoblotting procedures based on anti-microcystin-LR monoclonal antibodies to monitor the formation of microcystin-PP1 adducts in *vitro* and *in vivo*.

After the elucidation of *mcy* gene cluster, several studies have applied molecular methods for monitoring the presence of harmful toxic cyanobacteria and the genes involved in the biosynthesis of cyanotoxins (Hotto et al., 2007; Pearson and Neilan, 2008; Saker et al., 2007). Molecular approaches employing PCR have been frequently used for the screening of microcystin-producing strains belonging to different genera *viz*., *Anabaena, Microcystis*, and *Planktothrix*. Primers specific to various genes of *mcy* gene cluster have been designed and amplification of desired genes from the toxic strains has been reported (Hisbergues et al., 2003; Saker et al., 2007). Although certain studies have indicated that all the strains of the reported genera (*Microcystis*, *Planktothrix*, *Anabaena*, *Nostoc* and *Anabaenopsis*) do not make microcystins, and both toxic and nontoxic strains occur in the same species (Rantala et al., 2006; Rinta-Kanto and Wilhelm, 2006). The reasons why certain strains of these species do not synthesize microcystins are not known, it is presumed that mutations within the *mcy* gene cluster might have occurred during growth under laboratory conditions (Pearson and Neilan, 2008). Presence of inactive microcystin (*mcy*) genotypes in natural populations of *Planktothrix* has been reported by Kurmayer et al. (2004).

Biotransformation/ degradation and removal

Kondo et al. (1992) were the first to demonstrate that microcystin (MC) could be synthetically converted into glutathione (GSH) and cysteine (Cys) conjugate. These conjugates showed reduced toxicity as compared to the native microcystin upon intravenous examination for determining its LD_{50} value. The conjugation of GSH with MCs clearly indicates its role in the metabolic pathway leading to the detoxification of MCs. In addition to the above report, Zegura et al. (2006) verified an increase of the levels of reduced glutathione (GSH) during the exposure of HepG2 cells to MC-LR, after an initial decay. Concomitant with this increase was the expression of glutamate-cysteine ligase (GCL), the rate-limiting enzyme of GSH synthesis indicating an increased rate of the *de novo* synthesis of GSH (Zegura et al., 2006). This result indicated the importance of GSH in the betterment of MC-LR genotoxicity which can be explained by its capacity to inhibit the activity of the toxin by forming MC-GSH conjugates or by its antioxidant activity.

Despite all the new technology, flocculation or slow sand filtration steps are effective for the removal of cell-bound microcystins (by removing the cells from the water without their lysis) but for the dissolved microcystins, ozonation and activated carbon adsorption are efficient methods to eliminate these hepatotoxins from the water.

Microcystins are readily biodegraded in water by certain bacteria (Eleuterio and Batista, 2010). Most common microcystin-degrading bacteria have been identified as *Sphingomonas* or its related genera (Bourne et al., 2001; Zhang et al., 2010). Bourne et al. (2001) first reported that *Sphingomonas* spp. capable of microcystin degradation have four genes arranged linearly on a 5.8 kb genomic fragment in the order *mlrC*, *mlrA*, *mlrD* and *mlrB*. These genes encode the three hydrolytic enzymes involved in degradation pathway of microcystin, where *mlrC*, *mlrA* and *mlrD* are transcribed in same direction while *mlrB* is transcribed in opposite direction (Bourne et al., 2001). *Sphingomonas* spp. may prove very useful in developing strategies for biological treatment and effective removal of the microcystins from water samples. Attempt should be made to screen and isolate other novel microbes which are capable to degrade cyanotoxins more effectively.

CONCLUSION

Our understandings of cyanotoxins particularly hepatotoxins have greatly advanced during past three decades. Of all the cyanotoxins, extensive works on microcystins (hepatotoxin) have been carried out and vast array of information pertaining to their occurrence, structure, mode of action etc have been gathered. The relationship between the worldwide increasing eutrophication of water bodies and the intensification of the worldwide occurrence of microcystin-producing blooms mainly dominated by *M. aeruginosa* strains have been well established (Carmichael, 1994). Microcystins, once thought to be produced solely by certain species of *Microcystis*, are now known to be produced by species of other genera such as *Anabaena*, *Planktothrix*, *Nosto*c, *Anabaenopsis* and *Hapalosiphon* (Fewer et al., 2007; Kaebernick et al., 2002). More than 80 structural variants are known and a single species may produce several microcystin variants (Feurstein et al., 2009), but only one or two isoforms dominate in any individual species. Synthesis occurs nonribosomally by the microcystin synthetase enzyme complex via a thio-template mechanism (Pearson and Neilan, 2008; Tillett et al., 2000). The gene cluster encoding microcystin synthetase enzyme complex, *mcy* has been sequenced and characterized in detail from *Microcystis*, *Planktothrix* and *Anabaena* (Christiansen et al., 2003; Kurmayer and Christiansen, 2009; Nishizawa et al., 2000; Rouhiainen et al., 2004; Tillett et al., 2000). Their occurrence in drinking water and or food is of major concern since chronic exposure to microcystin causes tumor promotion in human. Microcystins are structurally stable against abiotic and biotic factors such as temperature, sunlight, enzymes etc, under natural conditions. Although several chemical treatments of water for the removal of cyanotoxins have been proposed, there is apprehension that chemical treatment may produce carcinogenic substances and other mutagens. Recently, the bacterium, *Sphigomonas* species has been reported to degrade microcystins from surface water of various lakes (Bourne et al., 2001). Isolation of other novel bacteria efficient in MCs degradation requires immediate attention so as to avoid the risk of health hazard arising out due to the increasing abundance of bloom-forming toxic cyanobacteria. Besides the negative effects, pharmaceutical applications of microcystins are also being tested and if success attained, it would be find wide applications in drug and agro-industry.

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