

## BIOREMEDIATION OF MICROCYSTINS BY TWO NATIVE BACTERIA: *BACILLUS CEREUS* AND *RAHNELLA AQUATILIS*

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**Key words:** Biodegradation, Microcystin-LR, BIOLOG MT2 plates, *B. cereus*, *R. aquatilis*

**Abstract** – Microcystins (MCs) are a group of cyclic heptapeptide hepatotoxins produced by cyanobacteria. Among 70 analogues of MCs, Microcystin-LR (MC-LR) is the dominant type in Sri Lankan water bodies. Contamination of MC-LR in drinking water is a serious health issue. Thus, the present study was carried out to identify freshwater bacteria, which are capable of degrading MC-LR. BIOLOG MT2 plate assay was employed to confirm MC-LR biodegradation by the bacterium *Bacillus cereus* and *Rahnella aquatilis* which were isolated from Girandurukotte reservoir and form Ulhitiya reservoir respectively. Classic batch culture experiments showed that *B. cereus* and *R. aquatilis* removed 100% of MC-LR at 28°C within 8 and 10 days of incubation respectively. Optimized biodegradation conditions for MC-LR by *B. cereus* and *R. aquatilis* were recorded at 32°C when phosphates and nitrate concentrations were 0.01ppm and 0.4 ppm respectively in experimental medium. Moreover, *B. cereus* and *R. aquatilis* were capable of degrading other MCs analogues; Microcystin-LF (MC-LF), Microcystin –LW (MC-LW), Microcystin –RR (MC-RR) and nodularin (NOD) as well. Molecular studies confirmed that *B. cereus* and *R. aquatilis* harbored MC degrading *mlrA*, *mlrB*, *mlrC* and *mlrD* genes. This is the first record for degradation of MCs and NOD by *R. aquatilis*.

### INTRODUCTION

A diverse group of cyanobacteria belonging to genera *Microcystis*, *Planktothrix* (*Oscillatoria*), and *Anabaena* produces toxigenic cyanobacterial blooms (Idroos *et al.*, 2014). Microcystins (MCs) are toxic secondary metabolites produced by bloom forming cyanobacteria. MCs are the most frequently detected cyanobacterial toxins, which cause hepatotoxicity and tumor promotion in wild animals, livestock, and humans (Jochimsen *et al.*, 1998). MCs are chemically stable over a wide range of temperature and pH, possibly because of their stable cyclic structure (Lawton and Robertson, 1999). Conventional water treatment processes cannot eliminate these toxins (Hoffman, 1976). Activated carbon adsorption and ozone oxidation are successful in removal of MCs from drinking water (Jones *et al.*, 1994; Newcombe *et al.*, 2003; Rositano *et al.*, 2001). However, such technologies are not applicable for most part of the world due to high cost, and this makes that techniques inaccessible to developing countries. Overall, unaffordable water

treatment costs, water scarcity and the world's increasing population have set a global challenge to provide safe drinking water (Manage *et al.*, 2010). Thus, there is a need to have a simple, reliable and cost effective green solution to remove these contaminants from drinking water.

Biodegradation using heterotrophic bacteria is one of the promising approach to eliminate MCs from drinking water (Jones *et al.*, 1994., Manage *et al.* 2009). Biodegradation studies done so far, have discovered more than 30 different bacterial genera that are capable of successfully degrading MCs from potable waters. Jones *et al.*, (1994), successfully isolated the first MC degrading bacterium, which was characterized by Bourne *et al.* (1996), as *Sphingomonas* sp. ACM 3962. Molecular characterization, of *Sphingomonas* sp. ACM 3962 has reported that this bacterium poses a specific cluster of four genes (*mlrA*, *mlrB*, *mlrC* and *mlrD*) for the degradation of MC-LR. Furthermore, MC-LR degrading bacteria possessing the *mlrA* gene has been reported from family *Sphingomonadaceae* and family *Bacillaceae* (Harada *et al.*, 2004; Ho *et al.*,

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2007a; Saitou *et al.*, 2003). In contrast, Manage *et al.*, (2009) reported MC-degrading *Actinobacteria* isolates of *Arthrobacter* sp., *Brevibacterium* sp. and *Rhodococcus* sp. which did not harbor *mlr* genes. However, *mlr* genes mediated MC-degradation (Bourne *et al.*, 1996) remains the only characterized degradation pathway up to date. Hence, the diversity of MC degrading isolates and their degradation mechanism(s) remains marginal.

The present study reports the isolation and characterization of two MCs degrading bacteria, *B. cereus* (12GK) and *R. aquatilis* (13UL) from two different freshwater reservoirs; Girandurukotte (7°27'55"N 81°1'22"E) and Ulhitiya (7°27'26"N 81°4'2"E), Sri Lanka. During the study period, both the water bodies had severe *Microcystis* bloom condition. The BIOLOG MT2 plates were used as a rapid tool for screening MC-LR degrading ability of isolated bacterial strains (Manage *et al.*, 2010). Classic batch experiments were carried out to evaluate the degradation kinetics of MC-LR as well as its different analogues. Further experiments were performed to study the effect of temperature and some nutrients on the rate of MC-LR degradation. Identification and characterization of the bacterial strains were based on the 16sRNA analysis and a PCR assay was carried out to investigate if these isolates contain *mlr* gene cluster.

### Materials and Methods

Chemicals for High performance liquid chromatography (HPLC) systems [HPLC-grade Methanol, Milli-Q water, Acetonitrile (ACN), Trifluoroacetic acid (TFA)] were purchased from Sigma, Aldrich. Microcystin-LR (MC-LR), Microcystin-LF (MC-LF), Microcystin-LW (MC-LW), Microcystin-RR (MC-RR) and Nodularin (NOD) standards were received from Robert Gordon University, UK. Chemicals needed for microbiological study were purchased from Hardy diagnostics while Molecular grade chemicals were purchased from Promega, USA and Thermofischer scientific, USA. Water and fresh scum samples were collected from Girandurukotte and Ulhitiya reservoirs during April 2012. Collected samples were transported to the laboratory under chilled conditions.

**Extraction Of Cell Bound And Dissolved Microcystin-LR (MC-LR) :** Extraction of MC-LR was carried out with some modifications of method described by Lawton *et al.*, (1994). Water and scum samples were filtered through 0.45 µm GF-C filters

to retain cyanobacterial cells to extract intracellular MC-LR. Filter discs were repeatedly extracted in 80% methanol. The methanol extract was then rotary evaporated until dryness. Then the residue was reconstituted in 100% HPLC grade methanol. Extracellular MC-LR was extracted by passing filtrates (excluding cyanobacterial cells) through a pre-conditioned (methanol and water) C18 cartridge (10mm I.D. x 26mm long; 50µm particle size) attached to iST 121-2090 Vac Master vacuum system. Filtrates with extracellular MC-LR were loaded at 40 ml min<sup>-1</sup> and eluted with an increasing gradient of HPLC grade methanol from 10% to 30%, in 10% increments. Trapped MC-LR in the cartridge were eluted with 80% HPLC grade methanol (3). The quality and quantity of the eluted MCs in each fraction was determined by analyzing samples (3ml) by HPLC.

**Quantification Of Microcystin-LR :** Quantification of MC-LR was carried out using the HPLC system consisting of Agilent 1200 series with certain modification of the method described by Lawton *et al.*, (1994). A Sample volume of 25 µL was injected into a 250 x 4.6 mm, C18 column at a flow rate of 1 mL min<sup>-1</sup>. Two mobile phases were used for the gradient run (35% ACN/0.05% TFA and 65% Milli-Q water /0.05% TFA). Concentrations of MC-LR were determined by calibration of the peak areas (at 238 nm) with that of an external standard. The HPLC method had a detection limit of 0.5 µg mL<sup>-1</sup>. MC-LR recoveries were greater than 95% with a relative precision of 5%.

**Isolation And Screening Of Microcystin-Degrading Bacteria:** Extracted MC-LR was aseptically spiked to water samples at a final concentration of 5 µg mL<sup>-1</sup> in triplicates to enrich samples with MC-LR degrading bacteria. Flasks were incubated at 28 °C with continuous shaking at 100 rpm for 14 days. Following 14 days of incubation, 1ml of sample aliquots were removed and pour plate method was employed to isolate colonies with different morphological features. Following three days of incubation, bacterial colonies with different colony color, shape, elevation and margin were picked up and re-suspended on liquid LB medium. The liquid cultures were incubated at 28 °C for 3 days at 50 rpm and they were used to prepare pure bacterial cultures by repeated streaking in LB agar slants. Subsequently, pure bacterial cultures were sub cultured and stored in LB-glycerol at (-20 °C). BIOLOG MT2 plate assay

described by Manage *et al.*, (2010). was employed with some modifications to screen MC-LR degrading bacterial strains. A total of 62 bacterial isolates were screened in BIOLOG MT2 (BIOLOG, US) assay to evaluate their capability to metabolize MC-LR. For the BIOLOG MT2 assay, a loop of each bacterial strain was transferred into 5 ml of liquid LB medium and incubated overnight at 28 °C. The exponentially growing cultures were centrifuged at 10,000 rpm for 20 minutes and the supernatant was discarded. Bacterial pellets were re-suspended in 0.9 % saline solution and incubated (28 °C, 100 rpm) overnight to remove residual carbon content. Then the bacterial pellets were washed three times using 0.9 % saline solution. Turbidity of all bacterial suspensions were equalized ( $A_{590}=0.35$ ) using spectrophotometer (SPECTRO UV-VIS double beam PC). Aliquots (10 µl) of MC-LR were added into bacterial suspensions (150 µl; triplicates) at three different concentrations of MC-LR (10 µg mL<sup>-1</sup>, 1 µg mL<sup>-1</sup> and 0.1 µg mL<sup>-1</sup>) in BIOLOG MT2 plates. MC-LR acted as the sole carbon source for bacteria in BIOLOG MT2 plates. The plates were incubated at 28 °C. The absorbance of the plate was read at 0, 3, 6, 15, 18, 24 and 48 hours interval at 595 nm using an ELISA plate reader (Thermo, Scientific, USA).

**Bacterial Degradation Of MC-LR :** Ten bacterial isolates which showed rapid metabolism in the BIOLOG MT2 plate assay were selected for the batch degradation experiment. 0.5 µL of carbon depleted and turbidity equalized bacterial suspension was inoculated into filter-sterile lake water (Girandurukotte and Ulhitiya reservoir) containing MC-LR at a final concentration of 5µg mL<sup>-1</sup> in triplicates. All flasks were incubated at 28°C with shaking at 100 rpm for 14 days. Aliquots (1 mL) were removed from each flask under sterile conditions at 2 days intervals, transferred into 2 mL microcentrifuge vials, and freeze dried. Remaining MC-LR concentrations of the collected samples were analyzed by freeze drying frozen samples and reconstituting in 200 µL of 50% aqueous HPLC grade methanol. The supernatant (100 µL) was removed for HPLC analysis. Control samples were prepared in triplicates without bacterial inocula. MC-LR removal percentage of each bacterial strain was calculated using the equation given below during 14 days of incubation.

$$\text{MC-LR removal percentage} = [(a-b)/a] \times 100$$

Where,

a- Initial MC-LR concentration                      b- MC-LR concentration on sampling day

Optical density (OD) at 590nm of the experimental flasks were measured at every two days interval. Moreover, MC-LR consumption rate and bacterial growth rate for 12GK and 13UL strains were calculated using the Gompertz degradation model and Gompertz growth model as described by Fan *et al.*, (2004).

$$S_0 - S = A/Y \exp \{-\exp [(\mu_{m/Y}) e / (A/Y) (\lambda-t) + 1]\} \\ \rightarrow \text{Gompertz degradation model}$$

$$X = A \exp \{-\exp [\mu_m e / A (\lambda-t) + 1]\} \rightarrow \text{Gompertz growth model}$$

$$R_m = \mu_{m/Y}$$

#### Nomenclature

A - asymptotic phase

X- biomass concentration

e -constant (2.718281828)

Y- yield coefficient (l mg<sup>-1</sup>)

S -substrate concentration (mg L<sup>-1</sup>) λ - lag phase time (d)

S<sub>0</sub> - initial substrate concentration (mg L<sup>-1</sup>) μ<sub>m</sub> - maximum biomass growth rate (d<sup>-1</sup>)

t- Incubation time (d)

R<sub>m</sub> - Maximum substrate utilization rate (mg L<sup>-1</sup> d<sup>-1</sup>)

**Optimization of Nutrients To Enhance Bioremediation of MC-LR:** Degradation studies in 2.4 confirmed that 12GK strain from Girandurukotte and 13UL strain from Ulhitiya showed highest MC-LR removal percentages. Therefore 12GK and 13UL strains were selected for optimization studies. MC-LR degrading ability of 12GK and 13UL were optimized for temperature, phosphate and nitrate concentrations. To investigate the optimum temperature required for the biodegradation of MC-LR, isolates 12GK and 13 UL were incubated at 18°C, 28°C and 32°C following the other experimental parameters as described in section 2.4.

To investigate the optimum phosphate concentration on MC-LR bioremediation, filter sterile lake water was prepared and supplemented with K<sub>2</sub>HPO<sub>4</sub> with varying concentrations (0.005, 0.008, 0.01, 0.02, 0.03, 0.04, 0.05 ppm). A parallel study was carried out using KNO<sub>3</sub> enriched lake waters with varying concentration from 0.1 ppm- 2.5 ppm to find the optimum nitrate concentration for bioremediation. These nutrient concentrations required for the experiment was decided based on water quality parameters of Girandurukotte and Ulhitiya reservoirs where bacteria strains were isolated (Data not shown).



**Bacterial Degradation Of Microcystin Variants And Nodularin** : Bioremediation of a toxin cocktail containing MC-LR, MC-LF, MC-LW, MC-RR and NOD at a final concentration of  $5 \mu\text{g mL}^{-1}$  of each toxin was studied for both 12GK and 13UL bacterial strains following the procedure described in section 2.4.

**Genotypic Identification Of Microcystin-LR Degrading Bacteria:** Genotypic identification of MC-LR degrading bacteria was carried out by amplifying and partial sequencing of the 16S rRNA region of 12GK and 13UL bacterial strains. 16F27 (5'-AGAGTTTGATCMTGGCTCAG-3') and 16R1541 (5'-AAGGAGGT GATCCAGCCGCA-3') primers were employed for the amplification of 16S rRNA region. The PCR reaction mixture (50  $\mu\text{L}$ ) contained 20 ng genomic DNA, 0.5  $\mu\text{M}$  of each primer (IDT), 1x Go Taq Green Master Mix (Promega, USA), and 1 mM  $\text{MgCl}_2$  (Promega, USA). Thermal cycling was performed using a Peltier thermal cycler (MG 25+, 001-31085). The initial denaturation step at  $94^\circ\text{C}$  for 2 min was followed by 30 cycles of DNA denaturation at  $94^\circ\text{C}$  for 10 s, primer annealing at  $55^\circ\text{C}$  for 20 s, strand extension at  $72^\circ\text{C}$  for 1 min and final extension at  $72^\circ\text{C}$  for 7 min. DNA sequencing was performed through commercially available service by Macrogen, Korea. The gene sequences were deposited in the Gen Bank.

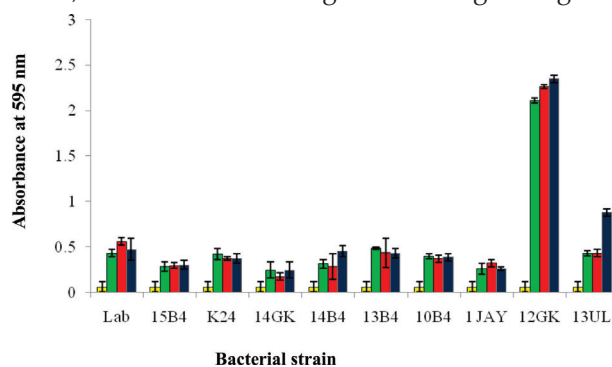
**Identification of Microcystin Degrading Genes in Bacteria:** Specific oligonucleotide primer sets were used in the PCR to screen bacterial isolates for the presence of *mlrA*, *mlrB*, *mlrC* and *mlrD* genes which are responsible for MC degradation. Details of the primers used for the study are given in Table 1. Amplifications were performed in 50  $\mu\text{L}$  volumes, containing 1 mmol of each primer, 10x Dream Taq PCR Buffer II (Thermofischer Scientific, USA), 1mmol dNTPs (Thermofischer Scientific, USA), 1 unit of Dream Taq DNA Polymerase (Thermofischer Scientific, USA) and 5  $\mu\text{L}$  of genomic DNA template. A GeneAmps 2400 PCR System (Perkin Elmer, USA) was utilized for the amplifications under the following conditions:  $94^\circ\text{C}$  for 3 min; 40 cycles of  $94^\circ\text{C}$  for 30 s,  $51^\circ\text{C}$  for 30 s and  $72^\circ\text{C}$  for 1 minute; and

$72^\circ\text{C}$  for 10 mins. PCR products were analyzed by gel electrophoresis. DNA sequence for each of the PCR fragments of expected size was subsequently determined as described previously by Hoefel *et al.*, 2005.

## RESULTS AND DISCUSSION

The BIOLOG MT2 assay confirmed that among the 62 bacterial isolates, that were employed for the initial screening of MC-LR degrading bacteria, 10 isolates (Lab, 15B4, K24, 14GK, 14B4, 13B4, 10B4, 1JAY, 12GK and 13UL) have shown pronounced metabolism of MC-LR (Figure 1).

When MC-LR is added as the sole carbon source for bacteria in BIOLOG MT2 plates, they produce carbon dioxide following utilization of MC-LR. The produced carbon dioxide could involve in a redox reaction and result in formation of formazan dye, producing color change of the colorless tetrazolium dye, which is coated in the BIOLOG MT2 plate (Manage *et al.*, 2010). The color change is measured spectrophotometrically and the intensity of color is proportionate to bacterial metabolism of MC-LR. Thus, efficient MC-LR degraders can give higher



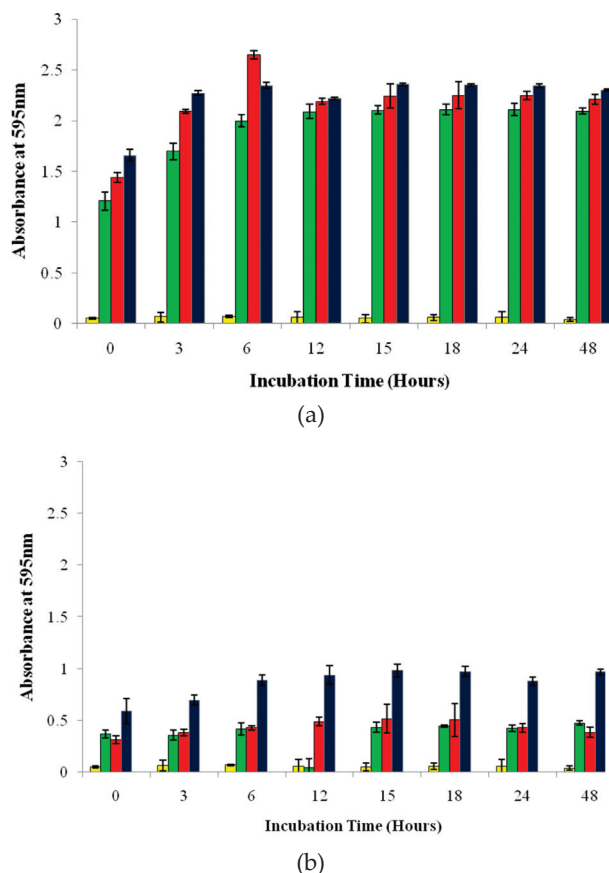
**Fig. 1.** BIOLOG MT2 plate screen for MC-LR metabolism by bacterial strains LAB, 15B4, K24, 14GK, 14B4, 13B4, 10B4, 1JAY, 12GK and 13UL after 24 h of incubation. Controlsamples (Yellow bars) contained no additional carbon source. MC-LR was added as the carbon source at 0.1  $\mu\text{g/ml}$  (Green bars), 1  $\mu\text{g/ml}$  (Red bars), and 10  $\mu\text{g/ml}$  (Blue bars). Error bars represent standard deviation ( $n=3$ )

**Table 1.** Forward and reverse Primers used for the detection of *mlr* gene clusters

Gene	Primer	Sequence (5'-3')
<i>mlr A</i>	mlrAf1mlrAr1	GACCCGATGTTCAAGATACTCTCCTCCACAAATCAGGAC
<i>mlrB</i>	mlrBf1mlrBr1	CGACGATGAGATACTGTCCCGTGCGGACTACTGTTGG
<i>mlrC</i>	mlrCf1mlrCr1	TCCCCGAAACCGATTCTCCACCGGCTCACTGATCCAAGGCT
<i>mlrD</i>	mlrDf1mlrDr1	GCTGGCTGCGACGGAAATGACAGTGTTGCCGAGCTGCTCA

absorbance values. The absorbance of ten isolated strains after 24 hours of incubation is shown in Figure 1. Among all ten strains 12GK and 13UL showed maximum absorption for the utilization of MC-LR (Fig.1). 12GK showed a highest value for absorption after 6 hours while 13UL showed a highest absorbance between 15-18 hours of incubation (Figure 2a and 2b).

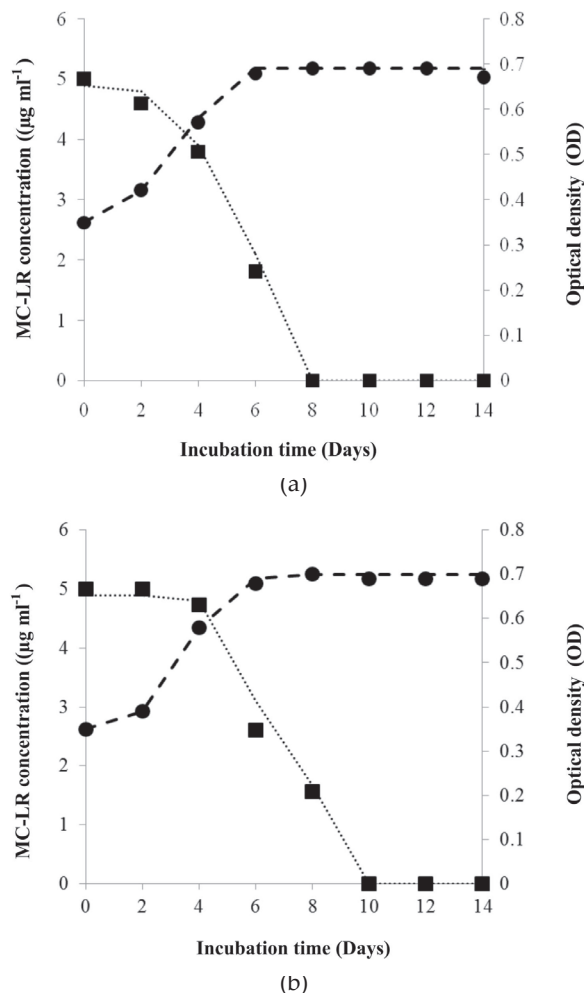
Degradation studies carried out using ten bacterial strains that were positive in BIOLOG MT2 plate study confirmed that 12GK and 13UL showed a pronounced degradation for MC-LR. 12GK showed 100 % removal of MC-LR within 8 days of incubation and OD for 12GK was at an optimum of 0.69 on 6<sup>th</sup> day of incubation (Figure 3a). 13UL required 10 days for complete removal of MC-LR



**Fig. 2.** Bacterial metabolism of MC-LR as screened by BIOLOG MT2 plates for bacterial strain 12 GK (a) and 13 UL (b) after 0, 3, 6, 15, 18, 24 and 48 h of incubation. Control samples (Yellow bars) contained no additional carbon source. MC-LR was added as the carbon source at 0.1 µg/ml (Green bars), 1 µg/ml (Red bars), and 10 µg/ml (Blue bars). Error bars represent standard deviation ( $n=3$ )

and showed a highest OD of 0.7 on 8<sup>th</sup> day of incubation (Figure 3b).

MC-LR degradation rate plot resulted from Gompertz degradation model and bacterial growth



**Fig. 3.** Degradation of MC-LR by (a) 12GK strain, (b) 13UL strain at 28°C. (Squares-MC-LR concentration in the sample, Circles- Optical densities at 590nm during the sampling day, Dotted lines- Gompertz degradation model, Dashed lines-Gompertz growth model)

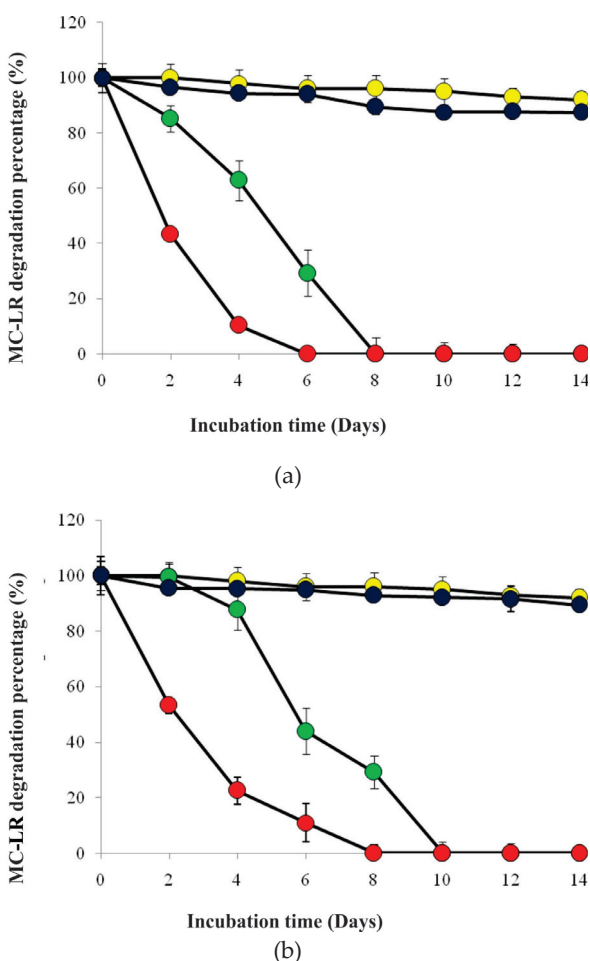
plot resulted from Gompertz growth model are illustrated in dotted and dashed lines respectively in figure 3a and 3b. Maximum substrate utilization rate and maximum bacterial growth rate for 12GK and 13UL calculated using Gompertz degradation model and Gompertz growth model are given in table 2.

The rate of MC-LR degradation by any bacterial isolate relies mainly on the incubation temperature (Somdee et al. 2013). Similarly both 12GK and 13UL

**Table 2.** Calculated parameter values for the Gompertz degradation model and Gompertz bacterial growth model

Bacterial strain	$R_m$ ( $\text{mg l}^{-1} \text{d}^{-1}$ )	$\mu_m$ ( $\text{d}^{-1}$ )	$R_m/\mu_m$
12GK	0.53	0.024	21.96
13UL	0.43	0.025	17.21

strains can change their MC-LR degradation rates depending on the temperature of the incubating medium. Figure 4 (a) and (b) describe the degradation of MC-LR by 12GK and 13UL strains at 18°C, 28°C and 32°C. Both strains do not exhibit MC-LR degradation at 18°C. Similarly, Ho *et al.*, (2007a) reported that *Sphingopyxis* strain LH21 did not show MC-LR degradation at 4°C but expressed a pronounced degradation at 22°C. Interestingly, in



**Fig. 4.** Degradation kinetics of MC-LR by isolated bacterial strains at three different temperatures. (a) 12 GK strain, (b) 13 UL strain, (Yellow circles-Control at 28°C, Blue circles-Sample at 18°C, Green circle-sample at 28°C, Red circles-Sample at 32°C) Error bars represent standard deviation ( $n=3$ )

the present study, both strains exhibited pronounced MC-LR degradation without a lag phase at 28°C and 32°C. 12GK achieved complete degradation of MC-LR after 8 days at 28 °C while 13UL strain required 10 days for the complete degradation of MC-LR. When temperature was raised up to 32 °C, 12GK showed a complete degradation of MC-LR, at the end of 6 days, while 13UL required 8 days. Similar results were reported by Park *et al.*, (2001) to support that the degradation rate strongly depends on temperature. According to Park *et al.*, (2001). *Sphingomonas* Y2 strain isolated from lake Suwa, Japan showed maximum degradation at 30°C. Similarly in the present study, *B. cereus* and *R. aquatilis* show efficient removal of MC-LR at a comparatively high temperature (32°C) due to increase in enzyme mediated reaction of MC-LR breakdown.

Figure 5a shows the effect of phosphate concentration in the medium on bioremediation of MC-LR by both studied microbes. According to Figure 5a, MC-LR degradation rate of 12GK and 13UL increased from  $0.43 \pm 0.05 \mu\text{g d}^{-1}$  to  $0.94 \pm 0.05 \mu\text{g d}^{-1}$  and from  $0.38 \pm 0.0105 \mu\text{g d}^{-1}$  to  $0.56 \pm 0.17 \mu\text{g d}^{-1}$ , respectively when phosphate levels in the incubating medium was increased from 0.005 ppm to 0.01 ppm. Phosphate concentrations higher than 0.02 ppm resulted a decrease in MC-LR degradation rate of 12GK and 13UL. Thus, high phosphate concentrations can inhibit MC-LR degradation ability of both strains. Li *et al.*, (2012). also reported that MC-LR degradation was inhibited in the presence of higher concentrations of phosphate in the incubation medium. In contrast, a rapid degradation of MC-LR was recorded by both strains, with increase of nitrate concentration in the medium from 0.1 ppm to 0.4 ppm (Figure 5b). MC-LR degradation rate for 12GK increased from  $1.76 \pm 0.0505 \mu\text{g d}^{-1}$  to  $3.98 \pm 0.1505 \mu\text{g d}^{-1}$  while MC-LR degradation rate of 13UL increased from  $1.86 \pm 0.0505 \mu\text{g d}^{-1}$  to  $3.55 \pm 0.11 \mu\text{g d}^{-1}$ . Nitrate concentrations higher than 0.4 ppm inhibited MC-LR degradation of all strains. According to Li *et al.*, (2011). nitrates in the medium can induce bacterial degradation of MC-LR to a certain extent, while high concentrations of nitrate can inhibit MC-LR degradation. Therefore elevated nitrate levels can result in toxic conditions subsequently reducing the MC-LR degradation rates of the bacterial strains. This is related to suppression of *mtrA* gene expression at higher nitrate concentrations (Gagala and Mankiewicz-Boczek, 2012).

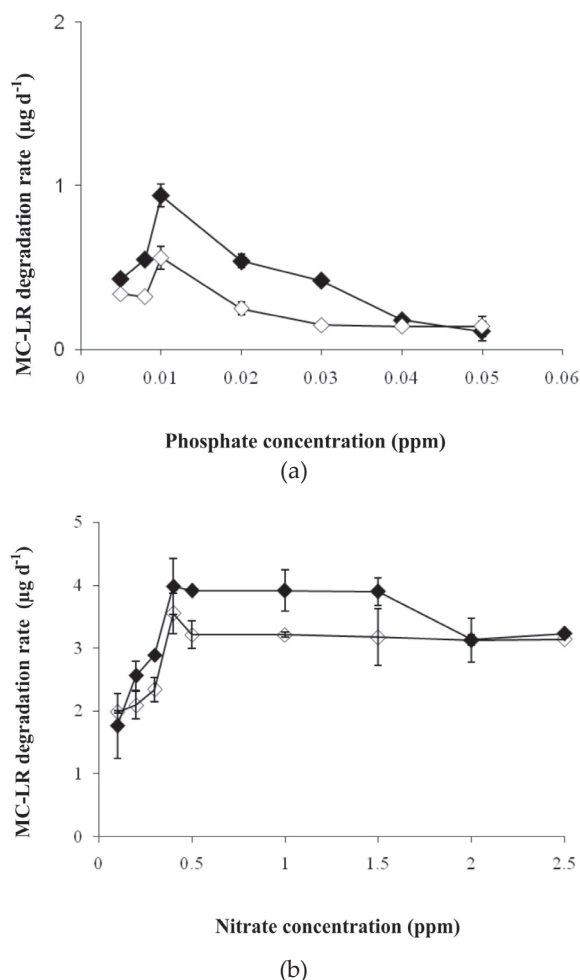


Fig. 5. Effect of phosphate concentration (a) and nitrate concentration (b) on degradation rate of Microcystin by bacteria 12 GK and 13 UL strains (Closed diamonds – Degradation by *B. cereus*, Open diamonds- degradation by *R. aquatilis*) Error bars represent standard deviation ( $n=3$ )

The degradation capability of 12GK and 13UL was assessed for a MC cocktail containing MC-LR, MC-LF, MC-LW, MC-RR and NOD. Both bacterial strains showed different rates of degradation for MC variants. 12GK completely degraded MC-LR and MC-LW within 8 and 10 days of incubation accordingly.  $1.09 \pm 0.01 \mu\text{g mL}^{-1}$  of MC-RR,  $1.11 \pm 0.04 \mu\text{g mL}^{-1}$  of MC-LF and  $1.45 \pm 0.31 \mu\text{g mL}^{-1}$  of NOD were remaining in the mixture following 14 days of incubation (Fig. 6a). The selective degradation of 12GK could be explained by looking into the structures of partially degraded MC analogues. MC-RR contains two arginines in the variable positions which increases its polarity and may cause steric

hindrance, reducing the rate of enzyme attack. Moreover, phenylalanine present in the variable position of MC-LF is recalcitrant to enzymatic degradation due to hydrophobic nature of benzyl side chain whereas in MC-LW the presence of  $-\text{NH}$  group has resulted in a hydrophilic condition favoring enzymatic degradation. In the case of NOD, it is a much smaller and tighter ring structure containing only five amino acids compared to seven in the MCs and it has been suggested that this makes it harder to open the ring structure to facilitate degradation (Lawton *et al.*, 2011).

Moreover, 13UL was capable of degrading all the MC analogues added into the cocktail, but only MC-LR was completely degraded at the end of incubation period.  $0.89 \pm 0.01 \mu\text{g mL}^{-1}$  of MC-RR,  $0.93 \pm 0.008 \mu\text{g mL}^{-1}$  of MC-LF,  $1.32 \pm 0.01 \mu\text{g mL}^{-1}$  of MC-LW and  $1.89 \pm 0.34 \mu\text{g mL}^{-1}$  of NOD were remaining in the mixture following 14 days of incubation (Fig. 6b). The results suggest the

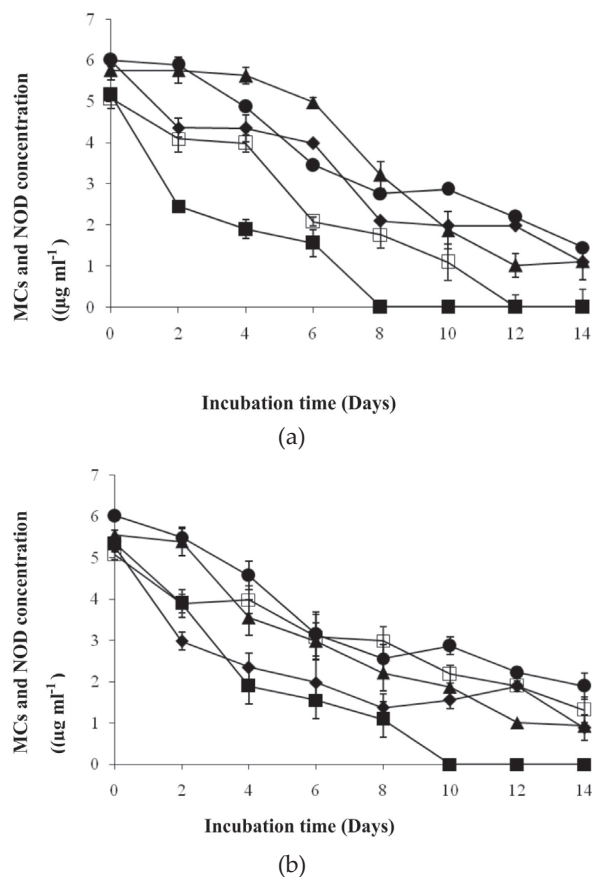


Fig. 6. Degradation of a cocktail mixture (a) 12GK strain, (b) 13UL (Closed squares-MC-LR, Closed diamonds-MC-RR, Open squares-MC-LW, Closed triangles- MC-LF, Black circles- Nodularin) Error bars represent standard deviation ( $n=3$ ).



substrate specificity of 13UL strain, especially for leucine and arginine as it could not completely degrade remaining analogues which had variable amino acids.

The phylogenetic analysis confirmed that the 12GK strain was a gram-positive, rod-shaped, facultative anaerobic and forms dull white colored colony with undulated margins on LB agar plates. Analysis of the 16S rRNA sequence confirmed that 12GK strain was 98% similar to that of *Bacillus cereus*. The 13UL strain was a gram negative rod shaped, facultative anaerobic and forms cream colored colonies in LB agar medium. 16S rRNA analysis of 13UL sequence confirmed that this strain was 99% similar to that of *Rahnella aquatilis*. Interestingly, this is the first world record on Microcystin degradation by *Rahnella aquatilis*. The DNA sequences of 12GK and 13UL were deposited to GenBank under accession number KJ954304 and KJ954305 accordingly.

The present study utilized primers specific for *mlr* gene cluster as recorded by Saitou et al. (2003) to screen the presence of MC-LR degrading genes in both bacterial strains. The PCR assay generated approximately 800 bp for *mlr A*, 400 bp for *mlrB*, 600 bp for *mlr C* and 674bp for *mlrD* (Fig. 7). Therefore, MC degradation by *B. cereus* and *R. aquatilis* followed the degradation path way, described by Bourne et al. (1996). The first enzyme involved in MC-LR degradation is metalloprotease encoded by *mlrA* gene. This enzyme cleaves the aromatic ring of MC-LR at the Arg-Adda peptide bond. This step results in a linearized MC-LR, which has a 160 fold reduction in toxic activity compared with the parent MC-LR. Subsequently, a serine peptidase, encoded by *mlr B* gene, catalyzes the linearized MC-LR at the Ala-Leu peptide bond, producing a tetrapeptide. Finally, the third enzyme, another metalloprotease, encoded by *mlrC*, breaks the peptide bonds randomly resulting in undetectable peptide fragments and amino acids. The fourth enzyme encoded by *mlrD* moves these amino acids out of the cell.

Therefore, *B. cereus* and *R. aquatilis* are able to breakdown toxic MC-LR into non- harmful products through biodegradation. Furthermore, the bacterial isolates of the present study were recorded from water bodies during *Microcystis* bloom condition. During the sampling period 4.8  $\mu\text{g mL}^{-1}$  of MC-LR was detected in Girandurukotte reservoir whereas 3.6  $\mu\text{g mL}^{-1}$  of MC-LR was recorded in Ulhitiya reservoir. Therefore, prior exposure for MC-LR may

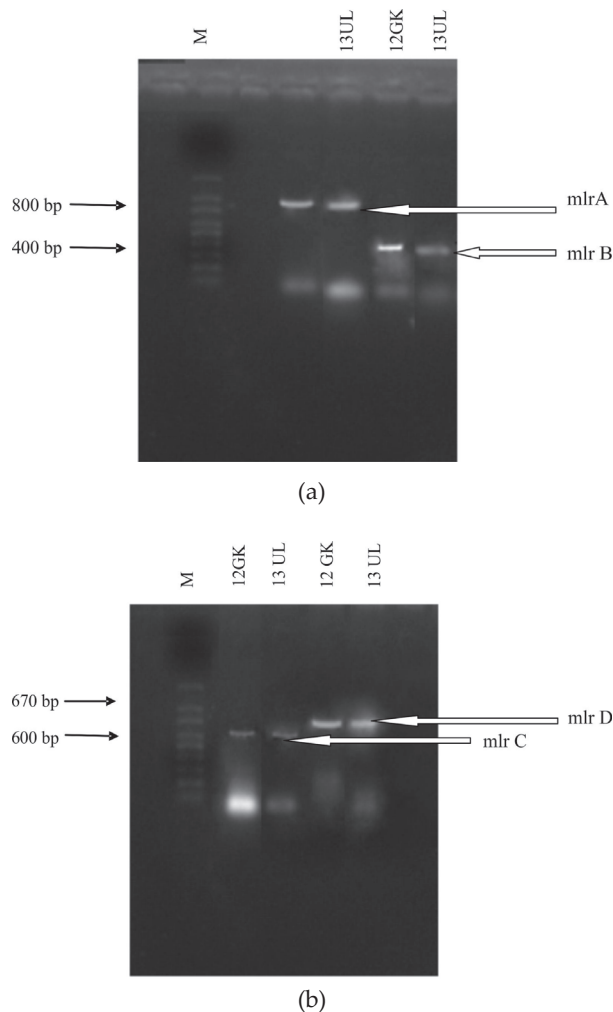


Fig. 7. *mlrA*, *mlrB*, *mlrC* and *mlrD* genes in 12GK and 13 UL strains. M indicates the 100bp Molecular marker

have enhanced the MC degrading abilities of both isolated strains.

## CONCLUSIONS

Following the completion of the study two MCs and NOD degrading bacterial strains namely KJ954304 *Bacillus cereus* 12GK and KJ954305 *Rahnella aquatilis* 13UL were isolated. Interestingly, this is the first report on MC-LR and NOD degradation by *R. aquatilis*. The optimum conditions for MC-LR degradation by both bacterial strains were 32°C temperature, 0.01ppm phosphates and 0.4 ppm nitrates in experimental medium. Both bacterial strains expressed biodegradation of other MC analogues; MC-LF, MC-LW, MC-RR and NOD as well showing their broad range of substrate



utilization ability. Moreover, molecular studies confirmed that *B. cereus* and *R. aquatilis* strains harbored *mlrA*, *mlrB*, *mlrC* and *mlrD* genes convincing that MC-LR is biodegraded by both bacterial strains into harmless products. Thus, the present study has provided a green solution to treat MCs contaminated drinking water.

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