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RESEARCH ARTICLE

Biodegradation of microcystin analogues by *Stenotrophomonas maltophilia* isolated from Beira Lake Sri Lanka

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Abstract: Bacterial strain 4B4 capable of degrading microcystin-LR (MC-LR) was isolated from the Beira Lake (N 6° 55' 46'', E 79° 51' 15'') in Sri Lanka. Based on phylogenetic analysis of the 16S rRNA gene sequence, the isolate 4B4 was identified as *Stenotrophomonas maltophilia*. The bacterium showed 100 % removal of MC-LR in 10 and 8 days of incubation at 28 °C and 32 °C, respectively. Optimisation studies confirmed that the addition of nitrates (0.4 ppm) and phosphates (0.02 ppm) to the medium can increase the microcystin removal rate of the bacterial strain. Further evaluation of *S. maltophilia* revealed that it is capable of degrading other microcystin analogues; microcystin-RR, -LW, -LF and nodularin (NOD) as well.

Keywords: 16S rRNA, microcystin, nitrates, optimisation, phosphates, *S. maltophilia.*

INTRODUCTION

Cyanotoxins and toxic cyanobacterial blooms in eutrophic lakes, rivers, and reservoirs have been reported during the last two decades all over the world (Skulberg *et al*., 1992). These toxic blooms are produced by a diverse group of cyanobacteria belonging to the genera *Microcystis, Planktothrix* (*Oscillatoria*), and *Anabaena* (Carmichael, 1994). Microcystins (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 monocyclic heptapeptides with the characteristic feature, unusual β-amino acid Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4E, 6E-dienoic acid). The general structure of microcystins is a cyclo (- D-alanine1 - L-X2-D-erythro-ß-methylaspartic acid3-L-Y4 -Adda5-D-glutamate6-N-methyldehydroalanine7). The molecular weight of MCs varies in the range of 909 to 1115 (Duy *et al.,* 2000). The main structural variations in MCs are observed in the L-amino acid residues X and Y. For example, microcystin - LR (MC-LR), the dominant type of MC in Sri Lanka, contains leucine (L) and arginine (R) in X and Y positions (Figure 1).

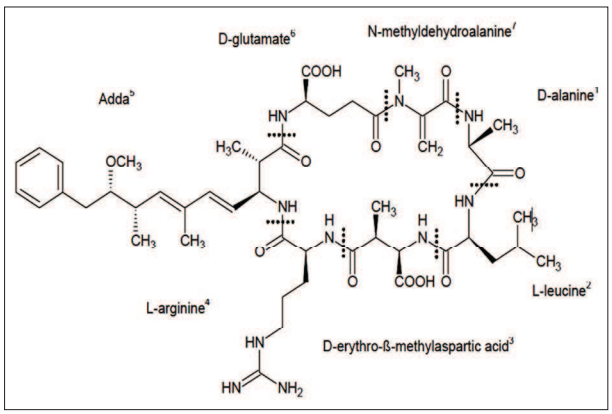

Figure 1: Cyclic structure of microcystin-LR (Chorus & Batrum, 1999)

MCs are chemically stable over a wide range of temperature and pH, possibly as a result of their cyclic structure (Lawton & Robertson, 1999). These toxins have also been documented to be recalcitrant to conventional water treatment processes (Hoffman, 1976). Activated carbon adsorption and ozone oxidation have been shown to be successful in their removal from drinking water (Jones *et al*., 1993; Rositano *et al*., 2001; Newcombe

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et al., 2003). However, these technologies require expensive facilities, a fact that makes these techniques difficult to implement and maintain (Eleuterio & Batista, 2010). Thus, there is a need for a simple, reliable and cost effective approach to remove these contaminants, ensuring safe drinking water.

Jones *et al*. (1994) recorded that naturally occurring MC-LR in water could be eliminated by a native heterotrophic bacteria, and this bacterium was later characterised by Bourne *et al*. (1996) as *Sphingomonas* sp. ACM 3962. In the quest of exploring the bacterial biodegradation of MCs, Bourne *et al*. (1996) identified and characterised a degradation pathway for MC-LR by *Sphingomonas* species. According to Bourne *et al*. (1996), MC degradation is carried out by four intracellular hydrolytic enzymes: microcystinase - a putative metalloprotease (Mlr A), a putative sereine peptidase 2 (Mlr B), a putative metalloprotease 3 (Mlr C) and a putative oligopeptide transporter, which takes part in the uptake of MCs into the cell (Mlr D). These enzymes are encoded by the 5.8 Kb gene cluster including *mlrA, mlr B, mlr C* and *mlrD* genes (Figure 2).

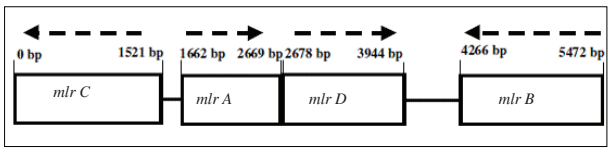

Figure 2: *mlr* gene clusters indicating the presence of *mlrA, mlrB, mlrC* and *mlrD* genes

Subsequently, many scientists have reported the removal of MCs by a diverse group of bacteria belonging to the class gammaproteobacteria, which composed of families Sphingomonadaceae, Burkholderiacea, Bacillacea, and Xanthomonadadea (Saitou *et al*., 2003; Harada *et al*., 2004; Ho *et al*., 2007; Okano *et al*., 2009; Chen *et al*., 2010; Idroos *et al*., 2014). However, Manage *et al*. (2009) reported three novel strains; *Arthrobacter* sp., *Brevibacterium* sp. and *Rhodococcus* sp., which belonged to the class alphaproteobacteria.

The present study reports the isolation and characterisation of the MC-degrading bacterium, *S. maltophilia* from the Beira Lake, Sri Lanka (N 60 55' 46'', E 790 51' 15''). During the study a severe *Microcystis* bloom condition was recorded in the Beira Lake. Classical batch experiments were carried out to evaluate the degradation kinetics of MC-LR as well as its different analogues. Further evaluation was carried out to study the effect of temperature and some nutrients on the rate of MC-LR degradation. Identification and characterisation of the bacterial strain was based on the 16s r RNA analysis, and a PCR assay was performed to screen the presence of *mlr* gene cluster.

METHODOLOGY

Chemicals

HPLC-grade methanol, Milli-Q water, acetonitrile and trifluoroacetic acid (TFA) for HPLC systems 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 the Robert Gordon University, UK.

Tryptone, yeast extract, sodium chloride, bacteriological agar and phosphate buffer saline needed for bacteriological studies were purchased from Hardy Diagnostics. Molecular grade chemicals needed for molecular biological studies were purchased from Promega, USA and Thermofischer Scientific, USA.

Sampling and storage

Water and fresh scum samples were collected from the Beira Lake during December 2012. Collected water samples were stored in ice boxes and transported to laboratory for analysis.

Extraction of microcystins

The extraction of MCs was carried out according to Lawton *et al.* (1994). Water and scum samples were filtered through 0.45 µm GF-C filters to retain cyanobacterial cells to extract intracellular MCs. Intracellular MCs were extracted repeatedly (×2) in 80 % methanol, rotary evaporated and the residue was reconstituted in 100 % HPLC grade methanol. The filtrates were subjected to extract dissolved MCs (extracellular MCs). Filtrates with extracellular MCs were loaded into a pre-conditioned C18 cartridge (10 × 26 mm; 50 µm particle size) attached to iST 121-2090 Vac Master vaccum system at 40 mL min^{-1} and eluted with an increasing gradient of HPLC grade methanol from 0 % to 100 %, in 10 % increments. The quality and quantity of eluted MCs in each fraction was determined by analysing the samples (3 mL) by PDA-HPLC according to the method described by Lawton *et al*. (1994). Extracted MC-LR was used for enrichment studies.

Enrichment studies

To enrich bacteria with the ability to degrade MC-LR, the extracted MC-LR was spiked aseptically at a final concentration of 5 μg mL^{-1} in triplicates of 50 mL of experimental samples (water from Beira Lake) and sterile controls (filtered and sterilised water from Beira Lake) (3 × 50 mL). The flasks were incubated at 28 °C and shaken at 100 rpm for 14 ds. Following 14 ds of incubation, 1 mL of sample aliquots were taken and plated on LB agar using a series of dilutions from 10^{-1} to 10^{-5}. Following 3 ds of incubation the bacterial colonies with different colony colour, shape, elevation and margin were picked and re-suspended in liquid LB medium. The liquid cultures were incubated at 28 °C for 3 ds at 50 rpm and they were used to prepare pure bacterial cultures by repeated streaking in LB agar plates. Subsequently, pure bacterial cultures were sub cultured and stored in LB-glycerol at -20 °C.

Degradation of MC-LR

Twelve bacterial isolates obtained from enrichment studies were selected for degradation studies. These bacteria were grown in liquid LB medium, carbon depleted and washed 3 times with PBS. Following equalising the optical density of all the bacterial strains (A_{590} = 0.35), 0.5 μL of each bacterial suspension was inoculated into filter-sterilised lake water containing MC-LR at a final concentration of 5 μg mL^{-1} (×3). All flasks were incubated at 28 °C with shaking at 100 rpm. Aliquots (1 mL) were removed from each flask under sterile conditions at 2 d intervals, transferred into 2 mL microcentrifuge vials, and frozen (-20 °C) immediately. Die-away kinetic of each bacterium was analysed for 14 ds. The frozen samples were freeze-dried, reconstituted in 200 μL of 50 % aqueous HPLC grade methanol, and centrifuged at 15,000 g for 10 min. The supernatant (100 μL) was removed for HPLC analysis. Control samples were prepared in triplicate without bacterial inoculation. The percentage of MC-LR removal by each bacterial strain was calculated using the following equation during 14 ds of incubation.

MC-LR removal percentage = [(a-b)/a] × 100

where,

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

Effect of phosphate, nitrate and temperature on MC-LR degradation

The 4B4 strain showed the highest MC-LR degradation rate among 12 selected strains. Thus, 4B4 strain was selected for optimisation studies. MC-LR degradation of 4B4 was optimised for different phosphate and nitrate concentrations, and temperature of the incubating medium. Previous studies have reported that the natural levels of phosphate in freshwater usually ranged from 0.005 to 0.05 mg L^{-1}, while the nitrate levels varied between 0 – 18 mg L^{-1} (James *et al*., 2005; Sethunge & Manage, 2010). To determine the effect of phosphate on MC-LR degradation, filter-sterilised lake water (0.001 ppm) was prepared and supplemented with K_2HPO_4 with varying final concentrations (0.005, 0.008, 0.01, 0.02, 0.03, 0.04, 0.05 ppm). Another study was carried out using KNO_3 enriched (< 0.1 ppm) lake water with varying final concentrations from 0.1 – 2.5 ppm. Nitrate and phosphate concentrations were varied based on the water quality parameters of the Beira Lake. To investigate the optimum temperature required for the biodegradation of MC-LR, isolated 4B4 strain was incubated at 18, 28 and 32 °C following the experimental parameters as described in the previous section.

Degradation of microcystin analogues and nodularin

The ability to degrade multiple variants of MCs (MC-LR,-LF,-LW,-RR) and NOD at a final concentration of 5.5 μg mL^{-1} of each toxin was studied for 4B4 bacterial strain following the procedure described earlier.

Sequencing of the isolated bacterium 4B4

Partial sequence of the 16S rRNA gene of 4B4 strain was amplified and sequenced using the primer pair 16F27 (5′-AGAGTTTGATCMTGGCTCAG-3′) and 16R1541 (5′- AAGGAGGT GATCCAGCCGCA-3′). The reaction mixture (50 μL) contained 20 ng genomic DNA, 0.5 μM of each primer (IDT), 1× Go Taq Green Master Mix (Promega, USA), and 1 mM $MgCl_2$ (Promega, USA). Thermal cycling was performed using a PTC-200 Peltier Thermal Cycler (MJ Research Inc., USA). The initial denaturation step at 94 °C for 2 min was followed by 30 cycles of DNA denaturation at 94 °C for 10 s, primer annealing at 55 °C for 20 s, strand extension at 72 °C for 1 min and final extension at 72 °C for 7 min. DNA sequencing was performed through the commercially available service by Macrogen, Korea. The gene

sequences were deposited in the GenBank. Neighbour-joining phylogenetic analysis using 16S rRNA sequences of the isolated bacterium and previously recorded MC-degrading bacteria was carried out and analysis of close relatives was performed using Bio-edit software.

Identification of *mlrA, mlrB, mlrC* and *mlrD* genes

Specific oligonucleotide primer sets were used in PCR to screen 4B4 strain for the presence of *mlrA, mlrB, mlrC* and *mlrD* genes. Amplifications were conducted on a Peltier Thermal Cycler (MG 25+, 001-31085). PCR products were separated by electrophoresis on a 1 % agarose gel containing ethidium bromide. DNA bands were visualised on a Dark Readers Transilluminator (Alphalmager, MINI, France). mlrBf1-mlrBr1, mlrCf1-mlrCr1 and mlrDf1-mlrDr1 (Table 1) primer sets designed by Ho *et al*. (2007) were used to amplify *mlrB, mlrC* and *mlrD* gene sequences of the 4B4 strain. Amplifications were performed in 50 µL volumes, containing 1 mmol of each primer, 10× Dream Taq PCR buffer II (Thermofischer Scientific, USA), 1 mmol of deoxynucleoside triphosphates (Thermofischer Scientific, USA), 1 unit of Dream Taq DNA polymerase (Thermofischer Scientific, USA) and 5 µL of genomic DNA template. A GeneAmps 2400 PCR system (Perkin Elmer, USA) was utilised for the amplifications under the following conditions: 94 °C for 3 min; 40 cycles of 94 °C for 30 s, 51 °C for 30 s and 72 °C for 1 minute; and 72 °C for 10 mins. PCR products were analysed by gel electrophoresis as described above. DNA sequence for each of the PCR fragments of expected size was subsequently determined as described previously (Hoefel *et al*., 2005).

Table 1: Forward and reverse primers used for the detection of *mlr* gene clusters

Gene	Primer	Sequence (5´-3´)
mlrA	mlrAf1	GACCCGATGTTCAAGATACT
	mlrAr1	CTCCTCCCACAAATCAGGAC
mlrB	mlrBf1	CGACGATGAGATACTGTCC
	mlrBr1	CGTGCGGACTACTGTTGG
mlrC	mlrCf1	TCCCCGAAACCGATTCTCCA
	mlrCr1	CCGGCTCACTGATCCAAGGCT
mlrD	mlrDf1	GCTGGCTGCGACGGAAATG
	mlrDr1	ACAGTGTTGCCGAGCTGCTCA

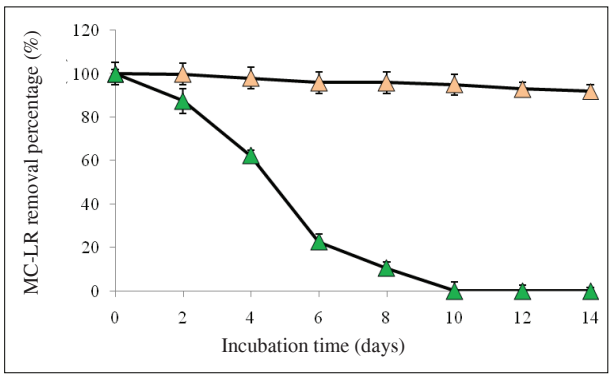

Figure 3: Degradation kinetics of selected bacterial strain 4B4 at 28 °C (△ Control; ▲ Experiment)

RESULTS AND DISCUSSION

Bacterial degradation of MC-LR

Among the twelve strains used for degradation experiment, 4B4 strain showed 100 % removal of MC-LR within 10 days of incubation (Figure 3), whereas the remaining strains did not show any effect on MC-LR degradation within 14 days. Moreover, progressive reduction of the peak areas relevant to MC-LR in HPLC chromatogram confirmed the removal of MC-LR in the sample (Figure 4).

Effect of phosphate, nitrate and temperature on MC-LR degradation

Park *et al*. (2001) reported that the MC-LR degradation time in the organic-free medium, which contained only MC and inorganic nutrients was four times faster than the organic nutrient medium. Moreover, the studies carried out by Zaccone *et al*. (2002) have clearly described the effect of seasonal variance of inorganic nutrients in the water that increases the metabolic activities in heterotrophic bacteria. Therefore, the effect of nutrients (phosphates and nitrates) on bacterial degradation was evaluated in the present study based on the available water quality data of the source (Beira Lake) of bacterial isolation. To evaluate the possible influence of nutrients, especially nitrates and phosphates on bacterial degradation of MCs, the bacterial culture was incubated separately with MC-LR and different concentrations of nitrate and phosphate. 4B4 showed the highest MC-LR degradation rate of 0.38 ± 0.01 µg day^{-1} when the total phosphate concentration of the medium was increased up to 0.02 ppm, and higher levels of phosphate showed a decrease in the degradation of MC-LR (Figure 5). In contrast, a rapid increase in degradation rate of MC-LR from 1.98 ± 0.17 to 3.55 ± 0.18 µg day^{-1} was recorded with the increase of nitrate concentration in the medium from 0.1 to 0.4 ppm. MC-LR degradation rate for 4B4 decreased in nitrate concentrations > 0.4 ppm (Figure 6).

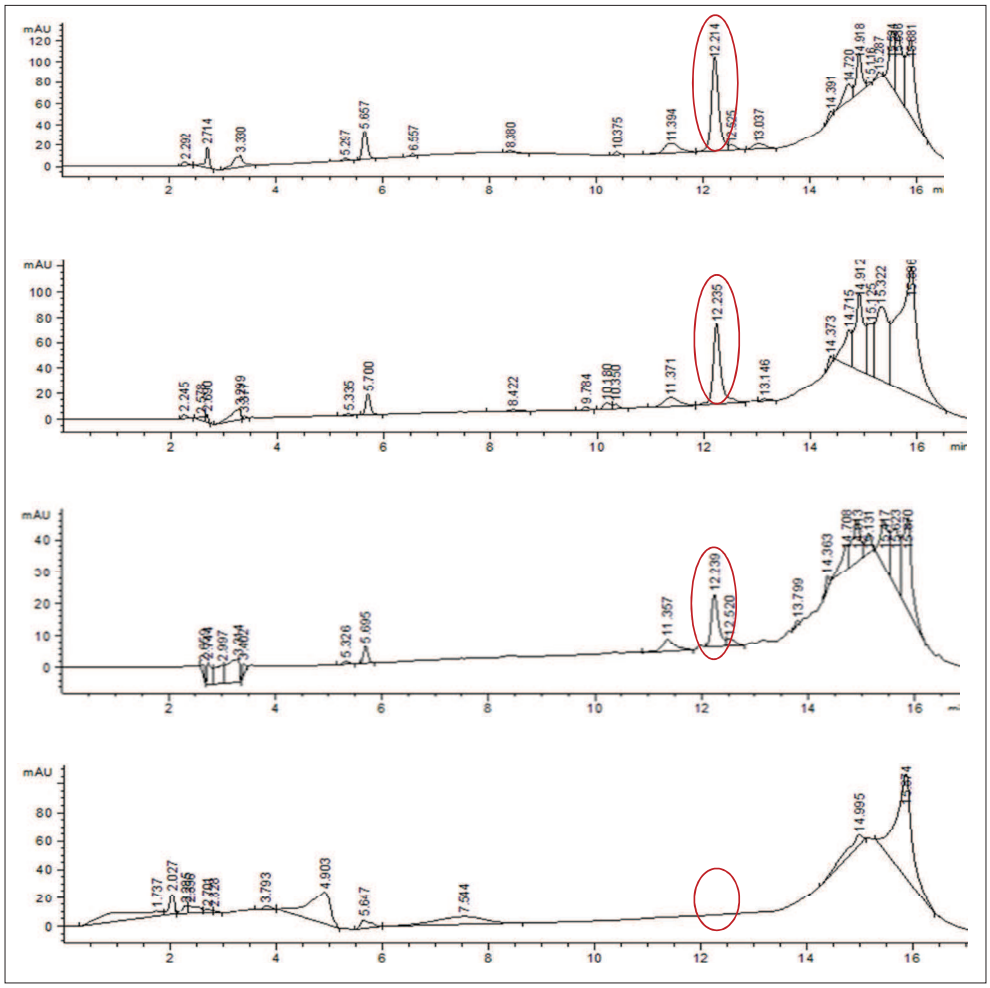

Figure 4: Progressive reduction peaks relevant to MC-LR in HPLC chromatograms

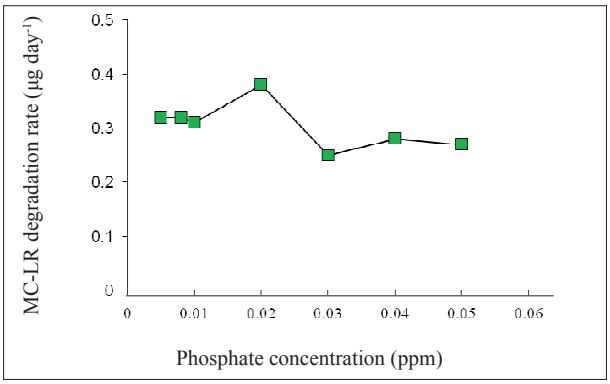

Figure 5: Degradation of MC-LR by 4B4 in the presence of phosphate (when error bars are not shown, values are within the symbols)

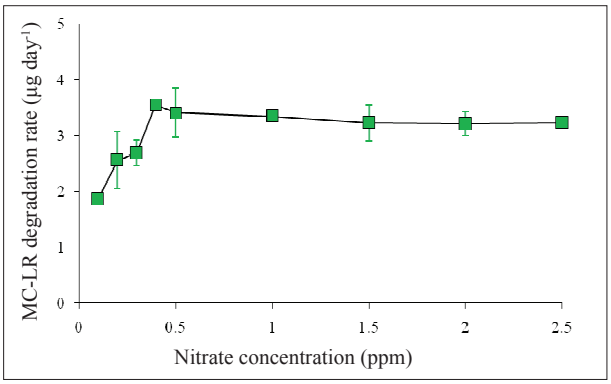

Figure 6: Degradation of MC-LR by 4B4 in the presence of nitrates

Li *et al*. (2012) also reported that MC-LR biodegradation was inhibited in the presence of phosphate in incubation medium. However in the present study, the bacterial isolate responded only to a limited range of phosphates (< 0.02 ppm) in water during their biodegradation of MC-LR. On the other hand, increase of nitrate concentrations from 0.1 to 0.4 ppm resulted in a pronounced increase in MC-LR degradation rate (Figure 6). However concentrations above 0.4 ppm has resulted in a reduction in bacterial degradation of MC-LR. According to Jieming *et al*. (2011) nitrates in the medium can induce bacterial degradation of MC-LR, while higher concentrations can result in inhibitory effects due to toxic effects. Therefore, optimum degradation of MC-LR could be achieved by the 4B4 strain at 0.02 ppm of phosphate and 0.4 ppm of nitrates.

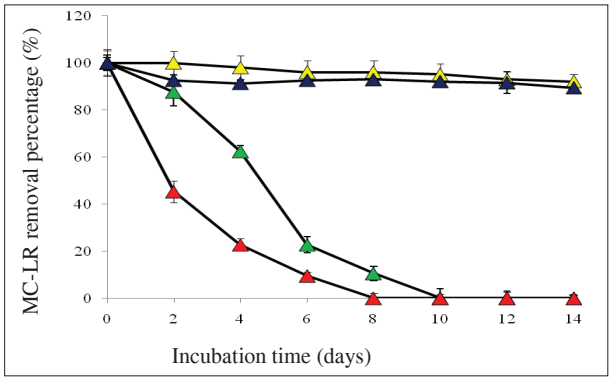

Figure 7: Degradation of MC-LR by 4B4 strain at different temperatures (△ - control at 28 °C; ▲ - sample at 18 °C; ▲ - sample at 28 °C; ▲ - sample at 32 °C)

The reduction of MC-LR degradation by bacteria in higher phosphate and nitrate levels are related to the *mlrA* gene in the bacterial strain. According to Li *et al*. (2011) the expression of *mlrA* gene, which encodes a metallopeptidase to cleave the complex structure of MC-LR is suppressed at higher phosphate and nitrate concentrations. Hence, MC-LR degradation is not initiated at high water phosphate and nitrate levels. Therefore, when studying about bacterial degradation of MC-LR it is obligatory to focus on water quality parameters of the particular environment.

The rate of biodegradation of MC-LR by 4B4 strain was strongly dependant on temperature (Figure 7). MC-LR degradation at 18 °C remained less than 20 % following 14 days of incubation. The highest MC-LR removal percentage was recorded at 32 °C. This was different from the removal percentages obtained at 28 °C and 18 °C. The 4B4 strain showed 100 % removal of MC-LR within 8 days at 32 °C. The control samples showed no degradation of MCs at the end of incubation.

Similar conditions were reported by Park *et al*. (2001) stating that the MC-LR degradation rate of bacteria strongly depends on the temperature as *Sphingomonas* strain Y2 isolated from lake Suwa, Japan showed maximum degradation at 30 °C. According to Phujomjai and Somdee (2013), MC-LR degradation by KKU-12 bacteria was lowest at 15 °C and highest at 30 °C, and it was established that the optimum temperature for biodegradation was 30 °C. Therefore, results of the present study could be explained by the fact that at 18 °C, the bacterial cells are metabolically inactive. Thus, it results in a lag period of MC-LR degradation. However, at 28 °C and 32 °C, the bacteria become metabolically active, enhancing enzymatic activity and bacterial metabolism of MC-LR.

However the mean water temperature of the Beira Lake was 29.4 ± 1.58 °C. Therefore the MC-LR degradation rate by *S. maltophilia* will not be maximum in the natural environment.

Bacterial degradation of microcystin analogues and nodularin

The degradation capability of 4B4 of a microcystin cocktail with MC-LR, MC-LF, MC-LW, MC-RR and NOD was analysed. 4B4 showed complete removal of MC-LR, MC-RR and MC-LF within 10, 12 and 14 days of incubation, respectively (Figure 8). MC-LR was found to be degraded easily compared to the remaining MC analogues and NOD. It is also possible that the specific peptide arrangement or peptide bond(s) specific for MC-

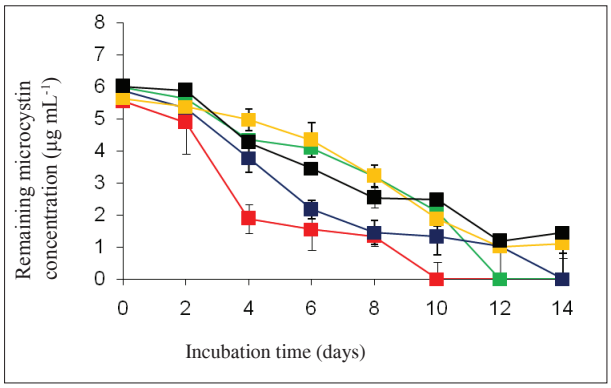

Figure 8: Degradation of a cocktail mixture of microcystins by 4B4-*S. maltophilia* (■ - MC-LR; ■ - MC-RR; ■ - MC-LW; ■ - MC-LF; ■ - Nodularin). Error bars represent standard deviation (n = 3).

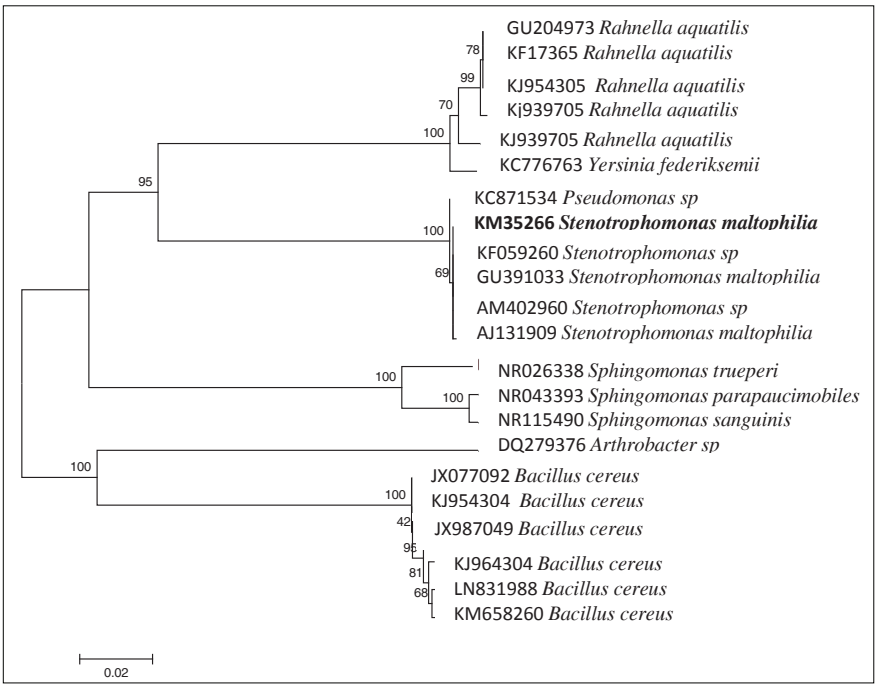

Figure 9: Evolutionary relationships of *Stentrophomonas maltophilia*

LR facilitated the degradation process (Lawton *et al*., 2011). MC-RR was also completely degraded by 4B4. Interestingly, out of hydrophobic MC-LF and MC-LW, only MC-LF was rapidly degraded by 4B4. This may be due to the permeability through cell membrane. Moreover, phenylalanine in the variable position of MC-LW may be preventing the enzymatic degradation due to the hydrophobic nature of benzyl sidechain, whereas in MC-LF the presence of –NH group has resulted in a hydrophilic condition favouring enzymatic degradation (Lawton *et al*., 2011). NOD is much smaller and has a tighter ring structure containing only five amino acids compared to MC analogues. Thus, it has been suggested that this makes it harder to open the ring structure of NOD and does not easily degrade (Lawton *et al*., 2011). A study carried out by Edwards *et al*. (2008) has also reported the stability of NOD compared to MCs in a mixed population of bacteria naturally present in water from the Carron River and Forfar Lake.

Phylogenetic analysis

The bacterial strain isolated from the Beira Lake (4B4) was a Gram negative, rod shaped, aerobic bacteria forming cream/ white coloured colonies in LB agar medium. Analysis of the 16S rRNA sequence confirmed that the 4B4 strain was 99 % similar to that of *Stenotrophomonas maltophilia* (Figure 9). The DNA sequence of 4B4 was deposited to GenBank under accession number KM35266. The phylogenetic tree was built using previously recorded MC-LR degrading bacterial strains and it was confirmed that the bacterial strain isolated in the present study is closely related to *Sphingomonas* strains.

Identification of *mlrA, mlrB, mlrC* and *mlrD* genes

The present study utilised primers specific for *mlr* gene cluster reported by Saitou *et al*. (2003). The PCR assay generated approximately 800 bp for *mlr A*, 400 bp for *mlrB*, 600 bp for *mlr C* and 674 bp for *mlrD* (Figure 10).

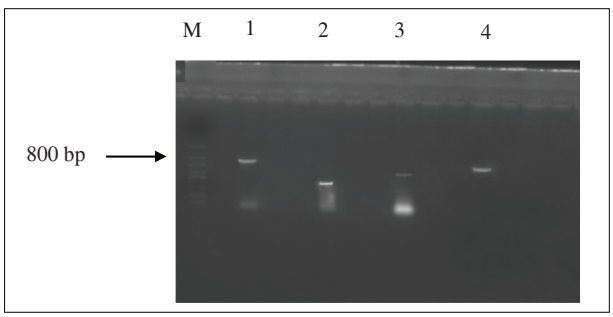

Figure 10: PCR bands obtained for the presence of *mlr* gene cluster in 4B4. M indicates the molecular marker. 1 - *mlr A* gene; 2 - *mlr B* gene; 3 - *mlr C* gene; 4 - *mlr D* gene

Somdee *et al*. (2013) have previously reported on MC-LR degradation by *Stenotrophomonas* sp. However this is the first report on MC-LR degradation by KM35266 *S. maltophilia*. The present study has recorded the degradation of MCs and NOD by *S. maltophilia* and have further optimised the optimum degradation conditions. Furthermore, through molecular studies, we have proved that *S. maltophilia* harbours the *mlr* gene cluster. Thus, *S. maltophilia* degrades MCs in a similar mechanism described by Bourne *et al*. (1996).

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