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REMOVAL OF MICROCYSTIN-LR USING CELLULAR EXTRACTS OF *Bacillus cereus*

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ABSTRACT

Microcystin –LR (MC-LR) is considered to be the most dominant type of cyanobacterial toxins present in water bodies. The present study focuses on using cellular extracts of Bacillus cereus in removal of MC-LR in water. Bacterial cell extracts were prepared using overnight grown fresh cultures of B. cereus which was previously recorded as a potent MC-LR degrading bacterium. Bacterial cell disruption was performed by bead beating on a micro-mini bead beater. Cell debris was removed by centrifugation at 13000 rpm, 20 min. Subsequently, a series of concentrations of cellular extract (100%, 75%, 50% and 25%) was prepared. These cell extracts were separately incubated at 280C with 100µg ml-1 of MC-LR for a period of 4 days. 1ml aliquots were removed at 24 hour intervals for four days and frozen at (-20) 0C. Then the frozen samples were freeze-dried and subjected to Photo diode array- High Performance Liquid Chromatography (PDA-HPLC) analysis to detect the remaining MC-LR concentrations of the samples. At the end of fourth day, 81.1 µgml-1 of MC-LR was removed when 100% of cell extract was used. When 75 % of cell extract was used, 77.6 µgml-1 of MC-LR removal was evident at the end of fourth day, whereas when 50 % and 25% of cell extract were used only 40.7 µgml-1 and 25.7 µgml-1 of MC-LR removal was detected respectively. The results of the present study indicate that bacterial cell extracts of B. cereus has the ability to remove MC-LR by an enzyme mediated mechanism.

KEYWORDS: *Microcystin-LR, Bacillus cereus, cellular extracts, PDA-HPLC*

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1 INTRODUCTION

Microcystins (MCs) are a family of monocyclic heptapeptide hepatotoxins produced by different genera of freshwater cyanobacteria including, *Microcystis, Anabaena, Oscillatoria (Planktothrix), Nostoc*, and *Anabaenopsis* (Sivonen and Jones, 1999, Chorus, 2001a). 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, which are indicated by a two-letter suffix in the name. There are more than 70 structural variants (congeners) of MCs identified so far (Codd et al. 2005a). Among, these variants Microcystin-LR (MC-LR) is considered as the most toxic variant (Fawell et al. 1993). Consumption of MC-LR contaminated water has resulted in hepatotoxic effects, renal damages, deformities in renal cell lines tumor promoting activities and DNA damage (Falconer *et al*. 1986., Ito *et al*. 1997, Dietrich and Hoeger, 2005). Therefore, the WHO has established a provisional guideline value for the concentration in drinking water of MC-LR as 1 µg l-1.

MCs are stable and recalcitrant to conventional water treatment (Lawton and Robertson, 1999). Ozonation, chlorination, reverse osmosis and photocatalytic degradation by TiO2 are used in many countries to treat MC-LR contaminated water. However, Jones and Orr (1994) recorded bioremediation of MC-LR by natural heterotrophic bacteria in environment by isolating a *Sphingomonas sp* which was capable of degrading MC-LR. To date over 30 bacterial strains are reported for efficient degradation of MCs and its variants (Idroos *et al* 2017, Manage *et al*. 2009a, 2009b) . Table 1 presents some of MC and Nodularin (NOD) degrading bacterial strains recorded in the world.

Table 1. Microcystin and nodularin degrading bacteria recorded in the world

Bacteria	Degradable analogous	Gene cluster	Gram Iden.	Reference
Sphingomonas sp. ACM-3962	MC-LR and –RR	*mlr*[a]	-ve	Bourne et al.(1996)
Novosphingobium sp. MD-1	MC-LR, -YR, and -RR	*mlrA*	-ve	Saitou et al. (2003)
Sphingosinicella microcystinivorans Y2	MC-LR, -RR, -YR, 6(Z)-Adda-LR	*mlrA*	-ve	Maruyama et al. (2003, 2006)
Sphingomonas sp. B9	MC-LR, -RR, dh-LR, LR-Cys, NOD	*mlrA*	-ve	Harada et al. (2004)
Sphingomonas sp. 7CY	MC-LR, -RR, -LY, -LW, -LF	NI	-ve	Ishii et al. (2004)
Paucibacter toxinivorans	MC-LR, MC-YR, NOD	NI	-ve	Rapala et al.(2005)
Sphingosinicella microcystinivorans B9	MC-LR, -RR	NI	-ve	Tsuji et al., (2006)
Sphingomonas sp. CBA4	MC-RR	NI	-ve	Valeria et al. (2006)
Sphingopyxis witflariensis LH21	MC-LA, MC-LR	*mlr*[a]	-ve	Ho et al. (2007)
Burkholderia sp.	MC-LR, [D-leu1]LR	*mlr*[a]	-ve	Lemes et al. (2008)
Sphingopyxis sp. C-1	MC-LR	*mlr*[a]	-ve	Okano et al. (2009)
Methylobacillus sp. J10	MC-LR, -RR	NI	-ve	Hu et al.(2009)
Arthrobacter sp. C6, F10,R1,R4,R9,R6,F7	MC-LR	NA	+ve	Manage et al. (2009b)
Brevibacterium sp. F3	MC-LR	NA	+ve	Manage et al. (2009b)
Rhodocoous sp. C1	MC-LR	NA	+ve	Manage et al. (2009b)
Stenotrophomonas sp. EMS	MC-LR, -RR	*mlrA*	-ve	Chen et al. (2010)
Sphingopyxris sp. USTB-05	MC-RR	NI	-ve	Zhang et al. (2010)
Bacillus sp. EMB	MC-LR, -RR	*mlrA*	+ve	Hu et al. (2012)
Bacillus sp.12GK	MC-LR,MC-RR,MC-LF,MC-LW,NOD	*mlr*[a]	+ve	Idroos et al. (2014)

Bacteria	Degradable analogous	Gene cluster	Gram Iden.	Reference
Stenotrphomonas maltophilia 4B4	MC-LR,MC-RR,MC-LF,MC-LW,NOD	*mlr*[a]	-ve	Idroos et al. (2014)
Rahnella aquatilis 13UL	MC-LR,MC-RR,MC-LF,MC-LW,NOD	*mlr*[a]	-ve	Idroos et al. (2014)

In the quest of exploring the potential of bacterial biodegradation of MCs, Bourne et al. (1996) identified and characterized a degradation pathway for MC-LR by Sphingomonas sp (Fig 1).

This bacterium was previously identified by Jones and Orr (1994) and shown to harbour specific MC degrading enzyme producing genes, namely, mlrA, mlrB and mlrC along with mlrD (oligopeptide transporter) in mlr gene cluster (Fig 2).

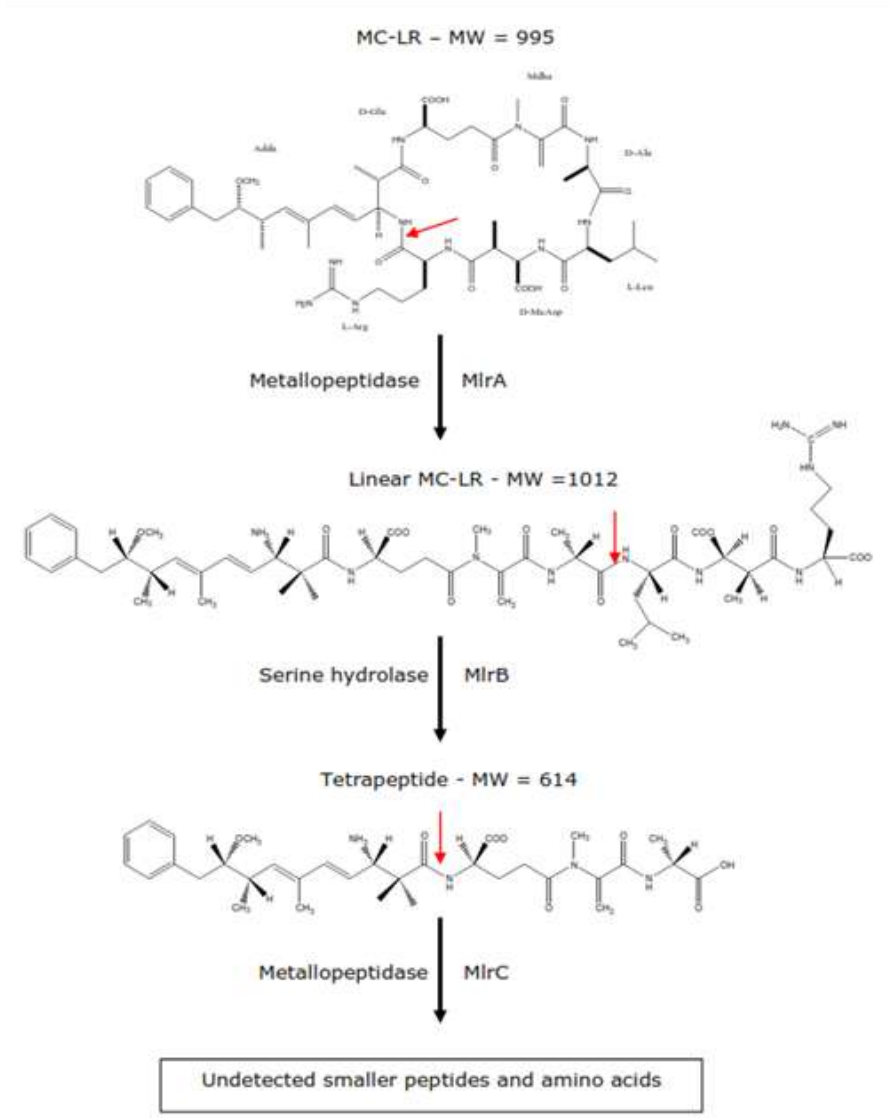


Figure 1: Proposed MC-LR degradation pathways by Bourne et al.1996

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).

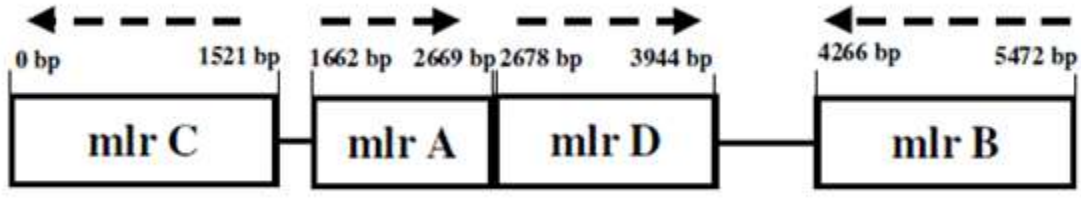


Figure 2: *mlr* gene clusters indicating the presence of *mlr*A, *mlr*B, *mlr*C and *mlr*D genes

Manage et al. (2010) and Nybome et al. (2012) suggested that if MCs are degraded enzymatically in the presence of probiotic bacteria, it is likely that proteolytic enzymes are involved in this process. Findings of Nybome et al. (2012) confirmed that extracellular proteinases of *Lactobacillus rhamnosus* are involved in the process of MC degradation. However, there is limited work done on utilizing of bacterial enzyme in degradation of MC-LR. Moreover, use of bacterial enzymes in bioremediation of MC-LR will help to overcome the inconvenience of maintaining bacterial biofilms in water treatment facilities.

KJ 954304 Bacillus cereus 12GK was isolated previously by authors from Girandurukotte reservoir, Sri Lanka as an efficient degrader of MC-LR (Idroos et al. 2014). However, there were no studies done on the MC-LR degrading mechanism of the bacterial strain. Therefore, the present study focuses elucidation of MC-LR removal mechanism of *B. cereus* and to utilize cellular extracts of the same strain in removing MC-LR in water.

2 MATERIALS AND METHODS

2.1 Chemicals

HPLC-grade Methanol, Milli-Q water, Acetonitrile (ACN), Trifluoroacetic acidacid (TFA) for HPLC analysis were purchased from Sigma, Aldrich. Microcystin-LR (MC-LR) standards were provided from Robert Gordon University, UK. Tryptone, Yeast extract, Sodium chloride, Bacteriological agar, phosphate buffer saline needed for bacteriological studies were purchased from Hardy diagnostics.

2.2 Extraction of MC-LR for studies

Surface scum samples collected from Beira Lake, Sri Lanka were filtered through 0.45 µm GF-C filters to retain cyanobacteria cells. The filter disk was extracted repeatedly (x2) in 80% HPLC grade methanol, rotary evaporated and the residue was reconstituted in 80% of HPLC grade methanol and transferred to HPLC vials.

2.3 Quantification of MC-LR and development of standard plots

Quantification of MC-LR was carried out using the PDA-HPLC system consisting of Agilent 1200 series. Sample volumes of 25 µL were injected into a 250 x 4.6 mm, C18 column at a flow rate of 1 mlmin-1. Two mobile phases were used for the gradient run (35% ACN/0.05% TFA and 65% 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 µgml-1. MC recoveries were greater than 95% with a relative precision of 10%.

A series of dilution ranging from 0.5-200 µg/ml of MC-LR was prepared using pure standards provided from the Cyano Solu lab in the Robert Gordon University, UK. These series were subjected to HPLC and peak areas were determined. Standard plots were developed by using the concentration of each toxin and the peak areas received (Fig 3).

2.4 Preparation of bacterial innocula

A loop of *B. cereus* pure culture was transferred into 5ml of liquid LB medium and incubated in a shaker (at 28 0C, 24 h, 150rpm). The exponentially growing bacterial culture was washed three times (x3) with an equal volume of 0.01 M Phosphate buffered Saline (PBS) by centrifugation at 3000 rpm for 15 min with re-suspension of the pellet in sterile 0.01 M PBS. The resulted pellet was re-suspended in 0.01M phosphate buffer saline solution (PBS) and kept overnight to let out residual carbon content. Then the suspension was centrifuged at 1000 rpm for 15 minutes and the pellet was washed three times using PBS. Optical density (OD) of bacterial suspension was equalized (A 590=0.35) using a spectrophotometer (SPECTRO UV-VIS double beam PC) by adding overnight grown bacterial cultures or by diluting with sterile 0.01 M PBS.

2.5 Elucidation of MC-LR degradation mechanism

Following equalizing the turbidities of bacterial strain at A 590=0.35, 0.5 µl of the bacterial suspension was inoculated into 100ml of filter sterile (0.2 µm) lake water (Beira lake) containing MC-LR at a final concentration of 5µg ml-1. Control sample was prepared without bacterial inoculation. All flasks were incubated at 28 °C and shaken at 100 rpm for 3 days.

Following three days of incubation 0.5ml sample aliquot was removed from both experimental and control flask and frozen at (-20 °C). Then 75ml of experimental sample was filtered under sterile conditions using 0.2 µm filter to remove bacterial cells. Then the filtrate was placed immediately in ice to prevent denature of enzymes.

10 ml of original sample was maintained as the positive control. A series of dilutions was prepared using the filtrate including:100%, 75%, 50% and 25% of MC-LR was spiked to positive control sample and each diluted medium at a final concentration of 5µgml-1and maintained at 28ºC, 100 rpm for 3 days. 1ml aliquot was removed from initial control sample and 100%, 75%, 50% and 25% of filtrate dilutions for 0-3 days of incubation. These samples were frozen and processed for HPLC analysis.

2.6 MC-LR removal by bacterial cellular extracts

Bacterial cell extracts were prepared using fresh cultures of *B. cereus*. Bacterial cell disruption was performed by bead beating on micro-mini bead beater (BIOSPEC, TP308, USA). Cell debris was removed by centrifugation (Biofuge A, 162816, Germany) at 13000 rpm, 20 min and the cell extract supernatant was diluted to prepare cell extracts of 100%, 75%, 50% and 25%.

These extracts were used for the MC-LR removal assays. MC-LR at a final concentration of 100 µgml-1 was introduced to 100%, 75%, 50% and 25% bacteria cell extracts. 1ml sample aliquots were removed continuously for four days and frozen at (-20) 0C. Then frozen samples were freeze-dried and subjected to High Performance Liquid Chromatography (HPLC) to detect the remaining MC-LR concentrations of the samples.

3 RESULTS AND DISCUSSION

MC-LR quantification was validated by the determination of the linearity of the calibration plot. Fig. 3 presents the calibration plots developed for MC-LR. Equation 1 was derived from the calibration plots and it was used in calculation of MC-LR concentrations in the degradation experiment. The regression value for calibration plot was 0.967.

MC-LR concentrations in samples were calculated using the following equation

$$C_{MC\text{-}LR} = (A - 51.12)/ 140.9 \quad (1)$$

Where,

A- Peak area (mAU)

$C_{MC\text{-}LR}$ - MC-LR concentration (ppm)

The UV chromatogram and spectrum of standard MC-LR is given below (Fig 4). The chromatograms and spectrums of experimented samples were compared with the standard given in fig. 4 to identify and quantify MC-LR.

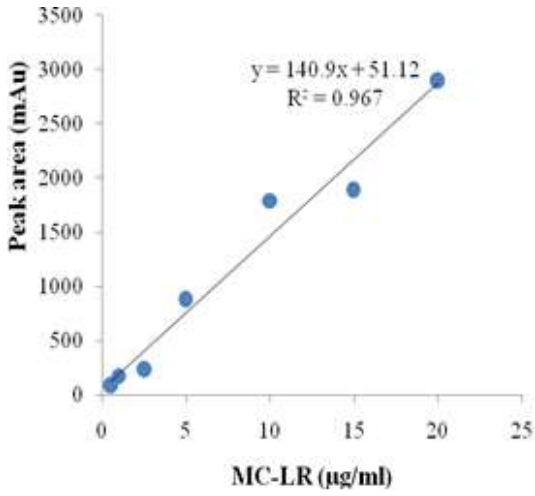
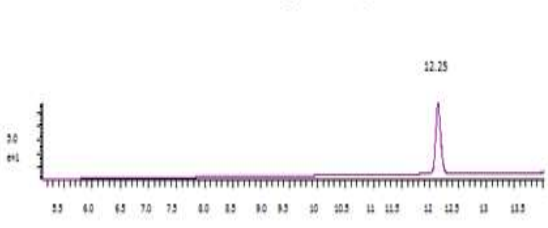
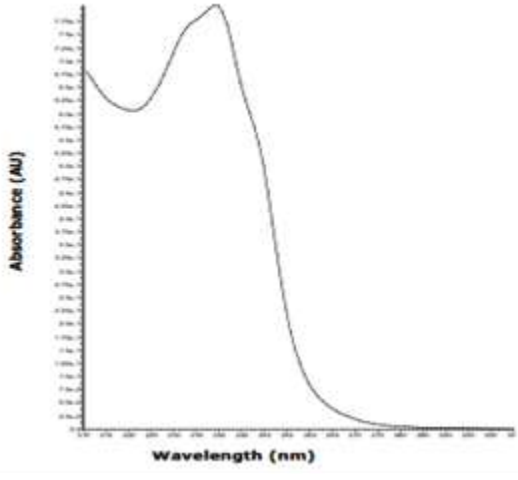


Figure 3: Standard calibration plot for MC-LR



(a)



(b)

Figure 4: (a) UV chromatogram at (200-300 nm), (b) UV spectrum 238 nm with relative purity > 95% of MC-LR.

Fig.5 represents the chromatogram for MC-LR detected in Beira Lake. The standard peak for MC-LR was received at 12.25 minutes and the area was 2896.35 mAu. Therefore, the MC-LR concentration was derived using the equation given in below.

Derivation : $C_{MC\text{-}LR}$ = (A- 51.12)/ 140.9

= (2896.35- 51.12)/140.9

MC-LR concentration in Beira lake =20.19 mg/l

Fig 6. indicates MC-LR removal by bacteria free filtrates. According to the results of the experiment the positive control which contained bacteria had reduced the MC-LR concentration from 5 µgml-1 to 1.8 µgml-1 following four days of incubation. However, bacteria free filtrate samples did not show considerable reduction of initial MC-LR concentration. Thus, the results convince that *B. cereus* degrades MC-LR as an intracellular metabolic activity. Thus, enzymes, which are, encoded by MC-LR degrading genes remain within the bacterial cell. The bacterium uptakes MC-LR through the cell membrane and proceeds with degradation.

Fig. 7. indicates MC-LR removal by cell extracts of *B. cereus*. At the end of fourth day, 81.1 µgml-1 of MC-LR was removed when 100% of cell extract was used. When 75 % of cell extract was used, 77.6 µgml-1 of MC-LR removal was evident at the end of the fourth day whereas when 50 % and 25% of cell extract were used only 40.7 µgml-1 and 25.7 µgml-1 of MC-LR removal was observed respectively. Thus, MC-LR removal by cell extracts of *B. cereus* strongly depends on concentration of cell extract.

Studies done by Wang et al. (2012) indicated that the intracellular extracts of M6 bacterial strain were able to degrade MC-LR. Wang et al. (2012) further confirmed that MC-LR degradation was optimum when 404.9 mgl-1 of intracellular extracts and 10 mgl-1 of the initial concentration of MC-LR were used. However, the present study has convinced that concentrated cell extracts of *B. cereus* can be used to remove high initial MC-LR concentrations like 100 µgml-1.

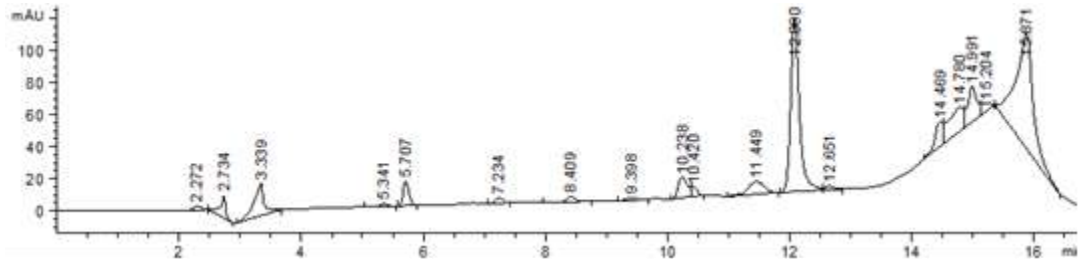


Figure 5: Chromatogram for MC-LR extraction from Beira Lake water

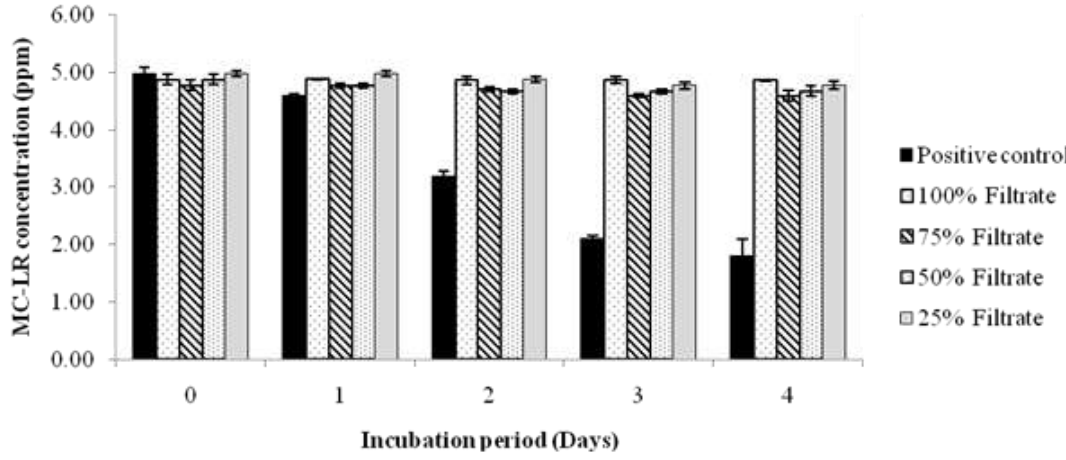


Figure 6: MC-LR removal by bacteria free filtrates . Error bars represent standard deviation.

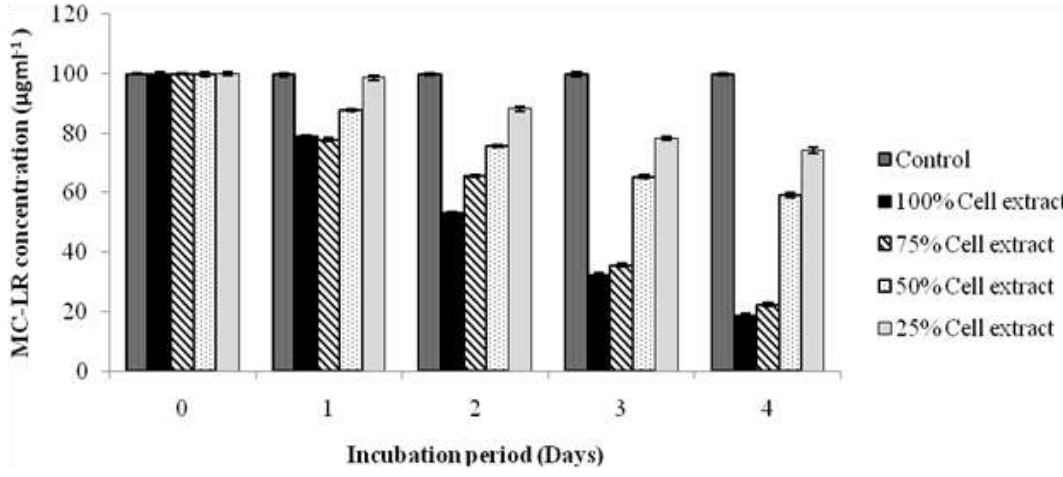


Figure 7. MC-LR removal by cell extracts of *B. cereus*.(Error bars represent standard deviation

4 CONCLUSION

The use of concentrated bacterial cell extracts is effective in removing MC-LR from water. Thus, this method has proposed a new perspective of using bacteria in bioremediation of MC-LR.

5 ACKNOWLEDGMENTS

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