Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

# BIODEGRADATION OF CYANOBACTERIAL HEPATOTOXINS [Dha$^7$]MC-LR AND MC-LR BY NATURAL AQUATIC BACTERIA

Theerasak Somdee

A thesis submitted for fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology

Institute of Food, Nutrition and Human Health
College of Sciences
Massey University at Wellington
New Zealand

May 2010

# Abstract

The aims of this doctoral study were to: isolate naturally occurring bacteria, able to degrade microcystins (MCs), from New Zealand waterbodies; to understand the biological processes of microcystin degradation by bacteria; and to develop small scale biofilm technology for testing the effectiveness of bacteria for microcystin degradation and/or remediation.

A significant amount of microcystins were required for biodegradation experiments. A modified method, using DEAE and Strata-X cartridge chromatography, was optimized for purifying microcystin variants from lyophilized bloom samples of the cyanobacterium *Microcystis aeruginosa*, collected en masse from Lake Horowhenua. Seven microcystin variants, MC-RR, MC-dMe-RR, MC-YR, MC-LR, [Dha$^7$]MC-LR, MC-FR, and MC-AR were purified by chromatography and then identified by reverse-phase High Performance Liquid Chromatography (HPLC) with UV detector (UVD) and Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). A mixture of [Dha$^7$]MC-LR and MC-LR, the main microcystin variants present, was used for examining biodegradation of microcystins by degrading bacteria.

Three isolates of bacteria—NV-1, NV-2 and NV-3—purified from Lake Rotoiti, New Zealand were capable of degrading [Dha$^7$]MC-LR and MC-LR. Among these isolates, NV-3 demonstrated the strongest degradative activity and was identified as a member of the genus *Sphingomonas.* On the basis of 16S rRNA sequencing, and 100% nucleotide sequence homology, it aligned most closely to strain MD-1. Based on the detection of two intermediate by-products (linearized peptides and a tetrapeptide) and the identification of four genes (*mlr*A, *mlr*B, *mlr*C and *mlr*D), that encode four putative proteins (enzymes) involved in microcystin degradation, it was suggested that the degradation of [Dha$^7$]MC-LR and MC-LR by the *Sphingomonas* isolate NV-3 occurred by a similar mechanism previously described for *Sphingomonas* strain MJ-PV (ACM-3962).

The bacterium *Sphingomonas* isolate NV-3 was examined for its ability to inhibit the growth of the cyanobacterium *Microcystis aeruginosa* strain SWCYNO4. It was found that the bacterium did not have any significant affect on the growth of the cyanobacterium, either by means of secretion of bacterial extracellular products or cell-to-cell contact between bacterial and cyanobacterial cells.

It was established that *Sphingomonas* isolate NV-3 was a moderate biofilm former, based on two types of biofilm formation assays, namely, microtiter plate assays and coupon biofilm assays. This was carried out in preparation for using the bacterium in a bioreactor for biodegradation of [Dha$^7$]MC-LR and MC-LR. The bacterium attached most effectively to ceramic, followed by PVC, polystyrene, stainless steel, and finally glass coupons. Biodegradation of MCs by the bacterium, in an internal airlift loop ceramic honeycomb support bioreactor (IAL-CHS bioreactor), was established in batch and continuous-flow experiments. In the batch experiment, NV-3 degraded a combination of [Dha$^7$]MC-LR and MC-LR at an initial concentration of 25 µg/ml at 30$^o$C in 30 hours, whereas in the continuous-flow experiment, NV-3 degraded the same concentration of [Dha$^7$]MC-LR and MC-LR in 36 hours with an hydraulic retention time (HRT) of 8 hours.

This study has demonstrated that microcystin-degrading bacteria are present in New Zealand waterbodies and that these bacteria could be used, potentially on a larger scale, for removing microcystins from water.

## Acknowledgements

I would like to express my deep appreciation and sincere gratitude to my supervisor Dr John Ruck for his dedication, thoughtful guidance, encouragement, and constant inspiration throughout the course of this study. I have learned so much from you about science, language, writing, and many other things. I am also deeply grateful to my co-supervisor Dr Rachel Page for her kind and invaluable advice and comments. Thank you my supervisors, for the proof-reading of several manuscripts and this thesis and for being very patient supervisors. I am very grateful for having been able to undertake this PhD study under your supervision. I hope I can continue learning from both of you in the future.

I wish to thank the following organizations/institutions:

- The Royal Thai Government, Thailand for the opportunity to pursue my doctoral study and for financial support throughout the study.
- Massey University, in particular the Institute of Food, Nutrition and Human Health (IFNHH), Massey University at Wellington for laboratory space, technical and financial support provided during my PhD study.
- Cawthron Institute, Nelson for material and technical assistance during two weeks training.
- New Zealand Freshwater Sciences Society for a travel grant and Student Prize at the 2006 Annual meeting at Rotorua.

Throughout this study, I have received lots of help and kindness from many people. I would like to thank the following individuals for their invaluable advice and assistance:

- Ms Margaret Allison, Science technician at IFNHH, Massey University at Wellington for her valuable support, suggestions, and discussion during our fortnightly meetings which were valuable for my study.
- Dr Isabelle Hoong, Lecturer at IFNHH, Massey University at Wellington for her technical skills in PCR and DNA sequencing.
- Dr Patrick Holland, Senior Scientist at Cawthron Institute, Nelson for his expert guidance in the field of analytical chemistry and LC-MS analysis.

- Ms Marilyn Mabon, Head Technician at IFNHH, Massey University at Wellington for her assistance in so many ways which I deeply appreciate.
- Dr Susan Allison, Margaret's daughter, for initial proof-reading of Chapter 4.
- Dr Susanna Wood, Freshwater Scientist at Cawthron Institute, Nelson, for providing water samples containing MC-degrading bacteria, and cultures of the cyanobacterium *Microcystis aeruginosa* strain SWCYNO4.
- Professor Richard Archer, Head of IFNHH, Massey University for his suggestion of applying biodegradation in large scale water treatment.
- Ms Wilma Tielemans, Mr Jim Clarke, Dr Karen Krauel-Goellner, Dr Robert Lau, Mr Stan Abbott, Dr Stuart McLaren, IFNHH staff (Senior Lecturers and Lecturers), Massey University at Wellington campus for their kind hospitality and all sorts of help.
- Ms Janet Langbein and Ms Lucretia Teki for their administrative support.
- Mr Doug Hopcroft, Manager of Manawatu Microscopy & Imaging Centre, Massey University at Palmerston North, for assisting with processing of samples for electron microscopy.
- Ms Fliss Jackson, Manager of the Nutrition Laboratory, IFNHH, Massey University at Palmerston North for supplying Milli-Q water during the study and processing freeze-dried samples.

Finally, I would like to thank my parents, Sawang and Hom Somdee and my sister Thidarat Somdee (Tai). Thank you for your support and unconditional love which always brings me strength and happiness. I am proud to be a part of our family.

Last, my special gratitude to my wife 'Aoy' (Anachana Somdee) for her love, encouragement, understanding and patience and my son, 'baby Atom' (Krittawattana Somdee), a special gift while I'm studying, for his smile always gives me happiness and refreshment. Without you, this thesis would not have been completed.

# Table of Contents


Page

Abstract …… i

Acknowledgements ……..... iii

Table of Contents …… v

List of Tables …….. xi

List of Figures ……. xii

**1. Introduction, chapter summary, and objectives ……. 1**

1.1 References ……. 5

**2. Literature review ……... 8**

2.1 Cyanobacteria …… 8
2.2 Cyanobacterial blooms …….. 10
2.3 Occurrence of cyanobacterial blooms ……... 12
2.4 Cyanobacterial blooms in New Zealand …… 13
2.5 Cyanotoxins ……... 14
2.5.1 Neurotoxic alkaloids …… 18
2.5.2 Cytotoxic alkaloids …….. 19
2.5.3 Hepatotoxic cyclic peptides ……. 19
2.5.3.1 Nodularins …….. 19
2.5.3.2 Microcystins (MCs) ……..... 20
2.5.3.2.1 Structure of MCs ……..... 21
2.5.3.2.2 Property and toxicity of MCs ……. 22
2.6 References ……. 25

**3. Extraction, purification and identification of microcystins from a cyanobacterial bloom in Lake Horowhenua, New Zealand …… 32**

3.1 Abstract ……. 32

3.2 Keywords ……... 32

3.3 Introduction ……... 32
3.3.1 Extraction and purification of MCs ……. 33
3.3.1.1 Extraction of MCs …….. 34
3.3.1.2 Sample concentration of MCs …… 34
3.3.1.3 Separation of the toxins …….. 35
3.3.2 Identification of MCs …….. 37
3.3.2.1 High performance liquid chromatography (HPLC) ……... 38
3.3.2.1.1 Separation ……... 38
3.3.2.1.2 Detection ……. 39

3.3.3 Rationale for choice of methods used in this study ……. 43
3.4 Objectives of the chapter ……... 43
3.5 Methods ……. 44
3.5.1 History of cyanobacterial blooms and sampling collection …… 44
3.5.2 Extraction of the lyophilized material ……. 44
3.5.3 Packing and equilibrating Toyopearl DEAE-650M column ……... 45
3.5.4 First purification with DEAE anion exchange chromatography ……. 45
3.5.5 Secondary purification with solid phase extraction (SPE) cartridges …. 45
3.5.5.1 Optimization of toxin elution ……. 45
3.5.5.2 The clean-up of the purified toxins with the SPE cartridges …….. 46
3.5.6 Identification of the purified toxins ……. 46
3.5.6.1 Identification of MCs with HPLC-UV detector ……. 46
3.5.6.2 Identification and characterization with LC-MS/MS ……. 46
3.5.7 The yield and purity of the purified toxins in the pooled fractions …… 47
3.5.7.1 The yield of the purified toxins …….. 47
3.5.7.2 Purity of the purified toxins …… 47
3.5.7.3 The concentration of the purified toxins …… 48
3.6 Results ……... 48
3.6.1 First identification of MCs from crude extracts with LC-MS/MS (before purification) ……. 48
3.6.2 Extraction and first purification with anion exchange chromatography.. 50
3.6.3 Optimization of toxin elution using Strata-X SPE cartridges ……. 50
3.6.4 Identification of purified MCs with LC-MS/MS ……. 51
3.6.5 The yields and purity of MCs …….. 56
3.7 Discussion …….. 56
3.8 References ……. 59

**4. Isolation and characterization of microcystin-degrading bacteria from New Zealand lakes …… 68**

4.1 Abstract ……. 68
4.2 Keywords …….. 68
4.3 Introduction ……... 69
4.3.1 Stability of MCs ……. 69
4.3.2 Natural processes of MC degradation …… 70
4.3.2.1 Degradation of MCs by water-dilution, photodegradation and adsorption on suspended particles and sediments ……. 71
4.3.2.2 Biological degradation of MCs …….. 72
4.3.2.2.1 MC-degrading bacterial strains …….. 72
4.3.2.2.2 Bacterial process and intermediate products of MC degradation …….. 77
4.3.2.2.3 Characterization of genes that encode the enzymes responsible for degradation of MC ……..... 79
4.3.3 Bacterial identification …….. 82

4.4 Objectives of the chapter ……... 85

4.5 Methods ……. 85
4.5.1 Isolation of MC-degrading bacteria and their biodegradation …… 85
4.5.2 Preservation of bacterial culture …….. 86
4.5.3 Identification and characterization of the MC-degrading bacteria ……. 86
4.5.3.1 Preliminary characterization of the bacteria …….. 86
4.5.3.2 Bacterial morphology under scanning and transmission electron microscopy …… 86
4.5.3.3 Biochemical and nutritional characteristics …….. 87
4.5.3.4 16s rRNA sequencing …… 88
4.5.4 Optimum temperatures of bacterial growth …… 88
4.5.5 Bacterial growth curve experiments ……... 88
4.5.6 Effect of temperature, bacterial and MC concentration on MC degradation by the bacterium isolate NV-3 ……..... 89
4.5.6.1 Temperature …….. 89
4.5.6.2 Bacterial concentration ……. 89
4.5.6.3 MC concentration ……. 90
4.5.7 Detection of MC degradation by-products with LC/MS-MS ……. 90
4.5.8 Detection of genes that encode MC-degrading enzymes ……... 91
4.5.8.1 Genomic DNA isolation and purification …….. 91
4.5.8.2 Determination of DNA purity and concentration …….. 91
4.5.8.3 Amplification of fragments of *mlr*A, *mlr*B, *mlr*C and *mlr*D genes using PCR …… 92
4.5.8.3.1 Oligonucleotide primers …… 92
4.5.8.3.2 Reagents and cycling conditions for amplification of the *mlr*A gene……. 92
4.5.8.3.3 Reagents and cycling conditions for amplification of the *mlr*B, C and D genes ……... 93
4.5.8.3.4 Agarose gel electrophoresis of PCR products …….. 93
4.5.8.3.5 Purification of PCR products …… 93
4.5.8.3.6 DNA sequencing ……... 94
4.5.8.4 Bioinformatic analysis …….. 94

4.6 Results ……... 95
4.6.1 Isolation of MC-degrading bacteria …… 95
4.6.2 MC-degradation by isolated bacteria ……. 96
4.6.3 Characterization of MC-degrading bacteria …….. 97
4.6.3.1 Characterization of bacterial isolate NV-3 ……... 97
4.6.3.2 Characterization of bacterial isolate NV-1 …….. 101
4.6.4 Optimum temperatures for NV-3 growth …….. 102
4.6.5 Bacterial growth curve assays of the bacterial isolate NV-3 ……. 103
4.6.6 Effect of temperature, bacterial and MC concentration on degradative activity of the isolate NV-3 ……... 103
4.6.7 Detection of MC-degraded by-products from the isolate NV-3 …… 106
4.6.8 Detection of *mlr*A, *mlr*B, *mlr*C and *mlr*D genes of NV-3 and NV-1 … 111
4.6.8.1 Detection of *mlr*A ……..... 112
4.6.8.2 Detection of *mlr*A, *mlr*B, *mlr*C and *mlr*D …… 113

4.6.8.3 Homology of MC-degrading genes of the isolates NV-1 and NV-3 ……..... 114
4.6.8.4 Analysis of *mlr*A, *mlr*B, *mlr*C and *mlr*D genes of NV-1 and NV-3 ……. 115
4.6.8.4.1 Nucleotide analysis of *mlr*A, *mlr*B, *mlr*C and *mlr*D genes ……..... 115
4.6.8.4.2 Protein analysis of translated polypeptide sequences from *mlr*A, *mlr*B, *mlr*C and *mlr*D …… 116
4.7 Discussion …….. 129
4.8 References ……. 139

**5. Effect of the bacterium *Sphingomonas* isolate NV-3 on the cyanobacterium *Microcystis aeruginosa* strain SWCYNO4 …….. 149**

5.1 Abstract ……. 149
5.2 Keywords …….. 149
5.3 Introduction …….. 149
5.3.1 *Microcystis* ……... 150
5.3.2 Control of *Microcystis* in freshwater bodies …… 151
5.3.2.1 Nutrient limitation …….. 151
5.3.2.2 Direct eradication ……... 152
5.3.2.3 Biological control …… 153
5.3.2.3.1 *Microcystis* control with bacteria …… 153
5.3.2.3.2 *Microcystis* control with fungi ……. 157
5.3.2.3.3 *Microcystis* control with virus ……. 157
5.3.2.3.4 *Microcystis* control with zooplankton ……. 158
5.4 Objective of the chapter ……. 159
5.5 Methods ……. 159
5.5.1 Microorganisms and culture conditions …….. 159
5.5.2 Cyanobacterial growth curve …….. 159
5.5.3 Preparation of bacterial cells for growth inhibition assays ……..... 160
5.5.4 Preparation of cyanobacterial cells for growth inhibition assays ……..... 161
5.5.5 Growth inhibition effect of the bacterium *Sphingomonas* isolate NV-3 on *M. aeruginosa* strain SWCYNO4 …… 161
5.5.5.1 Growth inhibition effect by ‘bacterial culture’ (bacterial cells, extracellular products and bacterial medium) of the bacterium *Sphingomonas* isolate NV-3 ……... 162
5.5.5.2 Growth inhibition effect by ‘bacterial supernatant’ (extracellular products and bacterial medium) ……..... 162
5.5.5.3 Growth inhibition effect by ‘bacterial cells’ ……..... 162
5.5.6 Effect of different bacterial concentrations, and different volumes of ‘bacterial culture’ and ‘bacterial supernatant’ of the bacterium *Sphingomonas* isolate NV-3 on growth inhibition of *M. aeruginosa* strain SWCYNO4 ……. 163
5.5.6.1 Effect of different volumes of ‘bacterial culture’ of the bacterium *Sphingomonas* isolate NV-3 on growth inhibition …. 163

5.5.6.2 Effect of different volumes of 'bacterial supernatant' of *Sphingomonas* isolate NV-3 on growth inhibition ……... 164
5.5.6.3 Effect of different 'bacterial cells' concentration of *Sphingomonas* isolate NV-3 on growth inhibition …….. 164

5.6 Results ……... 165
5.6.1 Cyanobacterial growth curve assays of *M. aeruginosa* strain SWCYNO4 ……... 165
5.6.2 Growth inhibition effect by bacterial cells, extracellular products and bacterial media of *Sphingomonas* isolate NV-3 ……. 165
5.6.3 Effect of the volume of 'bacterial culture' and 'bacterial supernatant' of *Sphingomonas* isolate NV-3 on cyanobacterial growth …….. 167
5.6.4 Effect of 'bacterial cells' concentration on growth inhibition effect ..... 169

5.7 Discussion ……. 170

5.8 References ……. 173

**6. Biodegradation of [Dha$^7$]MC-LR and MC-LR by a microcystin-degrading bacterium *Sphingomonas* isolate NV-3 in an internal airlift loop ceramic honeycomb support bioreactor …… 181**

6.1 Abstract ……. 181

6.2 Keywords ……... 181

6.3 Introduction …… 181
6.3.1 Water treatment processes …….. 183
6.3.2 Water treatment processes for removal of MCs ……. 184
6.3.2.1 Water treatment processes for cyanobacterial cell removal …….. 184
6.3.2.2 Water treatment processes for dissolved MC removal ……. 185
6.3.2.2.1 Photolysis …….. 186
6.3.2.2.2 Ozonation …….. 186
6.3.2.2.3 Chlorination …….. 187
6.3.2.2.4 Activated carbon filtration ……..... 188
6.3.2.2.5 Slow sand filtration ……... 191
6.3.3 Airlift bioreactor …… 193

6.4 Objectives of the chapter ……... 197

6.5 Methods ……. 197
6.5.1 Bacterial strains and culture media ……. 197
6.5.2 Biofilm formation characteristics …… 197
6.5.2.1 Microtiter plate biofilm formation assay …… 197
6.5.2.2 Coupon biofilm formation assay …… 198
6.5.3 Scanning electron microscopy ……. 199
6.5.4 Degradation of MCs in the bioreactor ……. 200
6.5.4.1 Biofilm reactor …… 200
6.5.4.2 Abiotic loss of the MCs through the CHS……. ……. 201
6.5.4.3 Cell immobilization on the CHS of the bioreactor…….. 202

6.5.4.4 Batch experiment of MC-degradation …… 202
6.5.4.5 Continuous-flow experiment of MC-degradation ……. 203

6.6 Results ……... 204
6.6.1 Microtiter plate biofilm formation assay ……. 204
6.6.2 Coupon biofilm formation assay ……. 205
6.6.3 Abiotic loss of the toxins adsorbed to surface of ceramic support …….. 206
6.6.4 Biofilm formation on the ceramic coupon …… 207
6.6.5 Batch experiment of MC-degradation ……. 208
6.6.6 Continuous-flow experiment of MC-degradation ……..... 209

6.7 Discussion …….. 210

6.8 References ……. 215

**7. General discussion and conclusions ……. 227**

7.1 References ……. 236

## Appendices

Appendix 1. Chemicals, materials, and instruments of Chapter 3 …….. 241
Appendix 2. Identification and characterization of MCs with LC-MS/MS ……. 244
Appendix 3. Calibration plot of microcystin-LR concentration ……. 245
Appendix 4. HPLC chromatograms of microcystin elution from the Strata-X SPE cartridges ……... 246
Appendix 5. Chemicals, materials, and instruments of Chapter 4 …….. 252
Appendix 6. Reading and interpretation of API 20 NE identification kit ……. 256
Appendix 7. 16s rRNA sequencing method..... 258
Appendix 8. Taxonomic identification reports of bacterium isolate NV-3 …….. 260
Appendix 9. Taxonomic identification reports of bacterium isolate NV-1 …….. 262
Appendix 10. Partial nucleotides of *mlr*A, *mlr*B, *mlr*C and *mlr*D genes and predicted amino acids of putative MlrA, MlrB, MlrC and MlrD proteins of the bacterial isolate NV-1 …… 264
Appendix 11. Partial nucleotides of *mlr*A, *mlr*B, *mlr*C and *mlr*D genes and predicted amino acids of putative MlrA, MlrB, MlrC and MlrD proteins of the bacterial isolate NV-3 ……... 268
Appendix 12. Chemicals, materials, and instruments of Chapter 5 …….. 272
Appendix 13. Chemicals, materials, and instruments of Chapter 6 …….. 275

# List of Tables


| Title | | Page |
|---|---|---|
| Table 2.1 | Known occurrences of toxic cyanobacterial blooms, scum, or mats ……. | 12 |
| Table 2.2 | Confirmed toxin-producing species of cyanobacteria … | 15 |
| Table 2.3 | Cyanotoxins: general features and producer organisms ……. | 17 |
| Table 3.1 | LC-MS/MS parameters and concentration of MCs from the lyophilized material before purification ……... | 48 |
| Table 3.2 | Predicted fragment ion observed in mass spectra of [Dha$^{7}$]MC-LR ……. | 54 |
| Table 3.3 | The yield and purity of MCs from DEAE and Strata-X cartridges chromatography …… | 56 |
| Table 4.1 | MC-degrading bacteria ……..... | 73 |
| Table 4.2 | Oligonucleotide primers used for amplification of *mlr*A, *mlr*B, *mlr*C and *mlr*D genes …….. | 92 |
| Table 4.3 | Bacterial isolates from Lake Horowhenua, Lake Rotoehu, Lake Rotoiti and Lake Rotorua…… | 96 |
| Table 4.4 | Morphological and biochemical characteristics of the isolates NV-3 and NV-1 from ESR ……... | 99 |
| Table 4.5 | Biochemical characteristics of the bacterium strain NV-3 and NV-1 using API 20 NE …… | 100 |
| Table 4.6 | Predicted fragment ion observed in mass spectra of [Dha$^{7}$]MC-LR ……. | 108 |
| Table 4.7 | Predicted fragment ion observed in mass spectra of the by-product A ……. | 110 |
| Table 4.8 | Predicted fragment ion observed in mass spectra of the by-product B ……. | 111 |
| Table 4.9 | Homology of MC-degrading genes of the isolates NV-1 and NV-3 …… | 114 |
| Table 4.10 | BLASTN analysis of the *mlr* nucleotide sequences from NV-1 ……. | 115 |
| Table 4.11 | BLASTN analysis of the *mlr* nucleotide sequences from NV-3 ……. | 116 |
| Table 4.12 | BLASTP analyses of translated polypeptide sequences of *mlr*A, B, C and D genes from the isolate NV-1……... | 117 |
| Table 4.13 | BLASTP analyses of translated polypeptide sequences of *mlr*A, B, C and D genes from the isolate NV-3 …….. | 118 |
| Table 4.14 | Protein analysis of translated polypeptide sequences of *mlr*A, B, C and D genes from the bacterial isolate NV-1 and NV-3 …… | 119 |
| Table 5.1 | Treatment conditions for growth inhibition effects of *Sphingomonas* isolate NV-3 on *M. aeruginosa* strain SWCYNO4 …… | 161 |

# List of Figures


| Title | | Page |
|---|---|---|
| Figure 2.1 | *Microcystis* bloom and *Microcystis* cells embedded in mucilage …… | 9 |
| Figure 2.2 | Chemical structure of nodularins ……..… | 20 |
| Figure 2.3 | Chemical structure of MCs …….. | 21 |
| Figure 3.1 | Base peak intensity and reconstructed ion chromatograms of MCs in the lyophilized material before purification ……. | 49 |
| Figure 3.2 | Chromatograms of DEAE measured at 238 nm …….. | 50 |
| Figure 3.3 | [Dha$^7$]MC-LR eluted from Strata-X cartridges with aqueous methanol from 30 to 80% ……. | 51 |
| Figure 3.4 | Reversed phase HPLC and MS/MS spectrum of [Dha$^7$]MC-LR and MC-LR with parent ion spectrum for MS-MS channels set up ……. | 52 |
| Figure 3.5 | Reversed phase HPLC and MS/MS spectrum of [Dha$^7$]MC-LR with daughter ion spectrum for MS-MS channels set up ……. | 53 |
| Figure 3.6 | MS/MS spectrum of M-1 to M-5 fractions after DEAE and Strata-X purification…….. | 55 |
| Figure 4.1 | MC-LR degradation pathway by *Sphingomonas* strain ACM-3962 …… | 78 |
| Figure 4.2 | Degradation of [Dha$^7$]MC-LR and MC-LR by the isolates NV-1, NV-2 and NV-3 …… | 97 |
| Figure 4.3 | Transmission electron micrograph of the bacterium isolate NV-3 ……. | 98 |
| Figure 4.4 | Scanning electron micrograph of the bacterium isolate NV-3 …… | 98 |
| Figure 4.5 | Transmission electron micrograph of the bacterium isolate NV-1…….. | 101 |
| Figure 4.6 | Growth curves of the bacterial isolate NV-3 at varying temperatures …….. | 102 |
| Figure 4.7 | Demonstrates bacterial growth curve from absorbance measurements ($OD_{600}$) and viable count …… | 103 |
| Figure 4.8 | Biodegradation of [Dha$^7$]MC-LR and MC-LR with bacterial isolate NV-3 at temperatures of 10, 15, 20, 25, 30, 35$^o$C ……..... | 104 |
| Figure 4.9 | Varying rates of [Dha$^7$]MC-LR and MC-LR biodegradation with varying bacterial concentrations …….. | 105 |
| Figure 4.10 | [Dha$^7$]MC-LR and MC-LR biodegradation at varying MC concentrations …… | 106 |

| | | |
|---|---|---|
| Figure 4.11 | MS/MS spectrum of [Dha$^7$]MC-LR and MC-LR in ESI$^+$ with parent ion spectrum for MS-MS channels set up ……. | 107 |
| Figure 4.12 | MS/MS spectrum of [Dha$^7$]MC-LR in ESI$^+$ with daughter ion spectrum for MS-MS channels set up ……. | 108 |
| Figure 4.13 | MS/MS spectrum in ESI$^+$ with parent scan and daughter ion spectrum of the degradation product A and the degradation product B …… | 109 |
| Figure 4.14 | Detection of *mlr*A gene by first PCR and nested PCR in the isolates NV-1 and NV-3 ..... | 112 |
| Figure 4.15 | Detection of MC-degrading gene cluster, *mlr*A, B, C and D of the isolates NV-1 and NV-3 ……..... | 113 |
| Figure 4.16 | Multiple sequence alignment of MlrA protein from bacterium isolate NV-3 and its homologue proteins from BLASTP analysis …….. | 121 |
| Figure 4.17 | Multiple sequence alignment of MlrB protein from bacterium isolate NV-3 and its homologue proteins from BLASTP analysis ……. | 123 |
| Figure 4.18 | Multiple sequence alignment of MlrC protein from bacterium isolate NV-3 and its homologue proteins from BLASTP analysis ……. | 124 |
| Figure 4.19 | Multiple sequence alignment of MlrD protein from bacterium isolate NV-3 and its homologue proteins from BLASTP analysis ……. | 127 |
| Figure 5.1 | Growth curve of *M. aeruginosa* strain SWCYNO4 using optical density of 750 nm [$OD_{750}$] …… | 165 |
| Figure 5.2 | Growth inhibition effect of *Sphingomonas* isolate NV-3, as reflected in changes in the content of chlorophyll-*a*, on *M. aeruginosa* strain SWCYNO4 cultured from PYEM …… | 166 |
| Figure 5.3 | Growth inhibition effect of *Sphingomonas* isolate NV-3, as reflected in changes in the content of chlorophyll-*a*, on *M. aeruginosa* strain SWCYNO4 cultured from 1/10 NB …….. | 167 |
| Figure 5.4 | Effect of different volumes of ‘bacterial culture’ of *Sphingomonas* isolate NV-3 on growth of *M. aeruginosa* strain SWCYNO4, as reflected in changes in the content of chlorophyll-*a* …….. | 168 |
| Figure 5.5 | Effect of different volumes of ‘bacterial supernatant’ of *Sphingomonas* isolate NV-3 on growth of *M. aeruginosa* strain SWCYNO4, as reflected in the content of chlorophyll-*a* …… | 168 |

| | | |
|---|---|---|
| Figure 5.6 | Effect of different 'bacterial cells' concentrations of *Sphingomonas* isolate NV-3 on growth of *M. aeruginosa* strain SWCYNO4, as reflected in the content of chlorophyll-*a* …… | 169 |
| Figure 6.1 | Fundamental process of a drinking water treatment plant……. | 183 |
| Figure 6.2 | Ceramic honeycomb support (CHS), and IAL-CHS bioreactor …… | 194 |
| Figure 6.3 | Schematic diagram of IAL-CHS bioreactor …… | 195 |
| Figure 6.4 | IAL-CHS bioreactor, glass-cylinder bioreactor shell, ceramic honeycomb support (CHS), and glass-cylinder bioreactor shell for batch mode …… | 200 |
| Figure 6.5 | IAL-CHS bioreactor set up for batch mode experiment . | 201 |
| Figure 6.6 | The IAL-CHS bioreactor in a continuous flow-through mode experiment …….. | 203 |
| Figure 6.7 | Destained biofilm (measured at $OD_{600}$) of *Sphingomonas* isolate NV-3 biofilm formation assay on microtiter plate at 24, 48, 72 and 96 h. ……. | 204 |
| Figure 6.8 | Destained biofilm (measured at $OD_{600}$) of *Sphingomonas* isolate NV-3 in coupon biofilm formation assays on polystyrene, polyvinylchloride plastic, glass, stainless steel and ceramic coupons (1cm x 2cm) at 24, 48 and 72h …….. | 205 |
| Figure 6.9 | Initial losses of [Dha$^7$]MC-LR and MC-LR by adsorption onto the ceramic core resulting in a final concentration of 17.8 µg/ml in the synthetic wastewater of the bioreactor …… | 206 |
| Figure 6.10 | Scanning electron micrograph of the bacterium isolate NV-3 biofilm on the ceramic coupon, in the control (un-inoculated), and 24 h, 48 h and 72 h of inoculated culture on the ceramic …… | 207 |
| Figure 6.11 | HPLC chromatograms from biodegradation of [Dha$^7$]MC-LR and MC-LR by the *Sphingomonas* isolate NV-3 at time zero, 12 h, 24 h and 30 h in an IAL-CHS bioreactor as the batch experiment ……. | 208 |
| Figure 6.12 | Percentage of MC remaining in the bioreactor using batch experiment mode at an initial concentration of [Dha$^7$]MC-LR and MC-LR of 32.5 µg/ml at 30$^o$C …….. | 209 |
| Figure 6.13 | Percentage of MC remaining in the IAL-CHS bioreactor over a period of 36 h in continuous flow-through mode at 30$^o$C ……. | 210 |