CULTURE AND CHARACTERIZATION OF Microcystis spp. AND THEIR EFFECTS ON CLADOCERAN POPULATION GROWTH

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CULTURE AND CHARACTERIZATION OF *Microcystis* spp. AND THEIR EFFECTS ON CLADOCERAN POPULATION GROWTH

By

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Chairman : Fatimah Md. Yusoff, PhD
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Toxic cyanobacterial species such as *Microcystis* spp. can form harmful blooms that cause water quality deterioration and negatively impact aquatic life in addition to triggering health risks towards human. This study aimed to isolate *Microcystis* spp. that produce a toxin, microcystin, and assess their impacts on the growth and reproductive capacity of a cladoceran zooplankton which feeds on microalgae as its main diet. Two *Microcystis* spp. were isolated and identified with both conventional and molecular methods. Species and toxicity identification for both species were done by using polymerase chain reaction (PCR) with the use of 16S rRNA and *mcy*B gene sequence. Apart from molecular approach, nuclear magnetic resonance (NMR) was used to detect the presence of microcystin in both isolates. Samples were obtained during the exponential phase, freeze dried and kept in -80˚C freezer prior to toxin extraction. Lyophilized cells were extracted using 75% methanol and dried *in vacuo* at 40˚C. Each sample was transferred to 1.5 ml amber vial before analysis. 10% of both *Microcystis* culture (at exponential phase) was transferred into the culture medium with limited nutrient availability (25% reduction = N_{75} and P_{75}; 50% reduction = N_{50} and P_{50}; 75% reduction = N_{25} and P_{25} from initial concentration (15g L^{-1}). Growth was determined by cell density, optical density and dry weight measurements. *Moina micrura* was used in population growth study and chronic bioassays. For the population growth study, *M. micrura* was exposed to three different species of microalgae; *Microcystis aeruginosa*, *Microcystis viridis*, and *Chlorella vulgaris* as a control. For chronic bioassay, 20 neonates (< 24h) were individually reared in glass vials. All the glass vials were checked daily (at 12h intervals) to determine age at first reproduction (day), fecundity (no of eggs $female^{-1}$), total offsprings (no. of offsprings $female^{-1}$) and longevity (no. of days). The chronic bioassays were terminated when all the cladocerans died (13 days).

Based on 16S rRNA and *mcy*B genes sequences, two potential microcystin producer *Microcystis* spp. were successfully isolated, purified and identified as *Microcystis aeruginosa* (UPMC-A0038, GenBank ID number KX447651.1) and *Microcystis viridis* (UPMC-A0039, GenBank ID number KY009735.1). Both isolates varied substantially in terms of morphological features such as cell size, colonial formation and cell arrangement. In addition, ^{1}H NMR results showed the presence of Adda group had confirmed microcystin in both *Microcystis* species. Both *Microcystis* spp. growth decreased under low nutrient concentrations. Nitrogen and phosphorus play an equal roles in the growth of *Microcystis*. Compared to *M. aeruginosa,* the growth of *M. viridis* was severely affected under low phosphorus level. In addition, *M. viridis* responded differently toward nitrogen limitation and exhibited adaptive mechanism in low nutrient environment. Both *Microcystis* spp. were toxic to *M. micrura*. The mortality rates of *M. micrura* subjected to *M. aeruginosa* and *M. viridis* were significantly higher ($p<0.05$) than the control treatment. *Moina micrura* exposed to *M. aeruginosa* did not reach maturity as their mean body size only reached 627.80±31.4 µm compared to *M. micrura* fed with *C. vulgaris* (814.94 ±21.84 µm) and *M. viridis* (914.21±12.64 µm). The population growth rate of *M. micrura* fed with *C. vulgaris* was 0.28 day^{-1} while growth rates were negative when fed with *M. aeruginosa* (-0.23 day^{-1}) and *M. viridis* (-0.20 day^{-1}). Longer exposure of *M. micrura* to *M. aeruginosa* resulted in delayed production of *M. micrura*'s first offspring, which only occurred on day 6 compared to *M. micrura* fed with *C. vulgaris* which produced their first offspring on day 3. In conclusion, both *Microcystis* spp. were microcystin producer species and nutrients play an important role in promoting *Microcystis* growth. This study also indicated that toxicity of both *Microcystis* spp. negatively affected *M. micrura* growth, survival as well as their reproductive capacity.

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PENGKULTURAN DAN PENCIRIAN Microcystis spp. DAN KESANNYA TERHADAP PERTUMBUHAN POPULASI CLADOCERAN

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Spesies sainobakteria yang bertoksik seperti *Microcystis* spp. boleh membentuk ledakan berbahaya yang menyebabkan kemerosotan kadar kualiti air dan memberi impak negatif terhadap hidupan akuatik serta menambahkan risiko kesihatan terhadap manusia. Kajian ini bertujuan untuk mengasingkan *Microcystis* spp. yang menghasilkan toksin, mikrosistin, dan menilai impaknya terhadap pertumbuhan dan keupayaan pembiakan zooplankton kladocera yang memakan mikroalga sebagai diet utama. Dua spesies *Microcystis* diasingkan dan dikenalpasti menggunakan dua kaedah iaitu konvensional dan molecular. Identifikasi spesies dan toksin untuk kedua-dua spesies dibuat menggunakan tindak balas rantai polimerase (PCR) dengan jujukan gen 16S rRNA dan *mcy*B. Selain daripada pendekatan molekular, resonans magnetik nuklear (NMR) juga digunakan untuk mengenal pasti kehadiran mikrosistin dalam kedua-dua spesies. Sampel biojisim diambil pada fasa eksponential, dibeku kering dan disimpan dalam peti sejuk pada suhu -80˚C sehingga toksin diekstrak. Sel diekstrak menggunakan 75% methanol dan dikeringkan pada 40˚C. Setiap sampel dimasukkan ke dalam botol legap 1.5 ml sebelum dianalisis. 10% daripada kedua-dua kultur asal (fasa eksponential) dipindahkan ke media dengan nutrisi yang terhad (25% pengurangan = N_{75} dan P_{75}; 50% pengurangan = N_{50} dan P_{50}; 75% pengurangan = N_{25} dan P_{25} dari kandungan asal (15g L^{-1}). Pertumbuhan ditentukan berdasarkan kandungan sel, kandungan optik dan biojisim. *Moina micrura* telah digunakan dalam kajian pertumbuhan populasi dan keupayaan pembiakan. Untuk kajian pertumbuhan populasi, *M. micrura* telah didedahkan kepada tiga spesies mikroalga; *Microcystis aeruginosa*, *Microcystis viridis*, dan *Chlorella vulgaris* sebagai rawatan kawalan. Untuk kajian jangka panjang, 20 neonat (< 24h) di besarkan di dalam bekas kaca. Semua bekas kaca diperiksa setiap hari (setiap 12 jam) untuk menentukan umur kelahiran pertama (hari), kesuburan (bilangan telur), jumlah anak (bilangan anak) dan jangka hayat (bilangan hari). Kajian jangka panjang ditamatkan setelah semua kladocera mati (13 hari).

Berdasarkan jujukan gen 16S rRNA dan *mcy*B, dua *Microcystis* spp. yang berpotensi untuk toksik telah berjaya diasingkan, dan dikenalpasti sebagai *Microcystis aeruginosa* (UPMC-A0038, GenBank ID nombor KX447651.1) dan *Microcystis viridis* (UPMC-A0039, GenBank ID nombor KY009735.1). Kedua-duanya berbeza dari segi morfologi seperti size sel, pembentukan koloni dan susunan sel. Sebagai tambahan, hasil keputusan ^{1}H NMR menunjukkan kehadiran kumpulan Adda yang mengesahkan kandungan mikrosistin di dalam spesies *Microcystis*. Pertumbuhan Kedua-dua *Microcystis* spp. menurun di bawah kadar nutrisi yang rendah. Peranan nitrogen dan fosforus adalah sama penting dalam pertumbuhan *Microcystis*. Berbanding *M. aeruginosa,* kekurangan fosforus amat memberi kesan terhadap pertumbuhan *M. viridis*. Tambahan pula, *M. viridis* memberi tindak balas yang berbeza terhadap kekurangan nitrogen dan menunjukkan mekanisme adaptasi terhadap persekitaran yang rendah nutrisi. Kedua-dua *Microcystis* spp. ini adalah bertoksik terhadap *M. micrura.* Kadar kematian *M. micrura* yang diberi *M. aeruginosa* dan *M. viridis* adalah tinggi dan siknifikan ($p<0.05$) berbanding rawatan kawalan. *Moina micrura* yang diberi *M. aeruginosa* tidak mencapai tahap kematangan dan saiz badan mereka hanya mencapai 627.80±31.4 µm berbanding *M. micrura* yang diberi *C. vulgaris* (814.94 ±21.84 µm) dan *M. viridis* (914.21±12.64 µm). Kadar pertumbuhan populasi *M. micrura* yang diberi *C. vulgaris* adalah 0.28 day^{-1} dan kadar pertumbuhan adalah negatif apabila diberi *M. aeruginosa* (-0.23 day^{-1}) dan *M. viridis* (-0.20 day^{-1}). *Moina micrura* yang terdedah dengan *M. aeruginosa* menghasilkan anak lebih lewat, iaitu pada hari ke-6 berbanding *M. micrura* yang diberi *C. vulgaris* yang menghasilkan anak pada hari ke-3. Kesimpulannya, kedua-dua *Microcystis* spp. adalah menghasilkan mikrosistin dan nutrisi memainkan peranan yang penting dalam menggalakkan pertumbuhan *Microcystis*. Kajian ini juga menunjukkan toksik *Microcystis* spp. memberi impak negatif terhadap pertumbuhan, kelangsungan hidup dan keupayaan pembiakan *M. micrura*.

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- the research conducted and the writing of this thesis was under our supervision
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LIST OF ABBREVIATONS

LPS	Lipopolysaccharides
ELISA	Enzyme linked immunosorbent assay
HPLC	High performance liquid chromatography
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
mL	Millilitre
HABs	Harmful algae blooms
PSP	Paralytic shellfish poisoning
DO	Dissolve oxygen
DNA	Deoxyribonucleic acid
µM	Micromolar
µmol	Micromol photon
M	Meter
L	Litre
PS1	Photosystem I
PS II	Photosystem Ⅱ
CO_2	Carbon dioxide
HCO_3	Bicarbonate ions
MCs	Microcystins
CYN	Cylindrospermopsin
PPIA	Protein phosphate inhibition assay
UV	Ultraviolet
PDA	Photodiode array
TFA	Trifluoroacetic acid
MALDI-TOF	Matrix-assisted laser desorption ionisation time-of-flight
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RT-PCR	Real time polymerase chain reaction
µm	Micrometre
NRPS	Non-ribosomal peptide synthetase
PKS	

MC-LR	Polyketide synthetase
mcy	Microcystin- leucine arginine
%	microcystin synthetase
rpm	Percentage
RNA	Revolutions per minute
bp	Ribonucleic acid
nm	Base pair
mg	Nanometre
v/v	Milligram
TSP	Volume per volume
ppm	Trimethylsilylpropanoic acid
HSQC	Parts per million
HMBC	Heteronuclear single quantum coherence
Kb	Heteronuclear multiple bond correlation
ANOVA	Kilobase pair
	One way analysis of variance

CHAPTER 1

GENERAL INTRODUCTION

1.1. Introduction

Cyanobacteria generally are unique microorganisms that exhibit both the characteristic of a plant and bacteria. All cyanobacteria can undergo photosynthesis like plants and some can fix nitrogen like bacteria. They naturally can be found in a variety of ecological niches including freshwater, marine and terrestrial ecosystems in different climatic zones. In addition, having special characteristic such as gas vesicles that help in adaptation process make them strive in stressful condition (Reynolds *et al*., 1987). In aquatic environment, cyanobacteria act as a primary producer for zooplankton grazers (Ger *et al*., 2016). But when the growth of cyanobacteria intensify, they can form blooms. Two main factors that contribute to cyanobacterial blooms are climatic changes and increased in anthropogenic activities. Anthropogenic activities promote higher nutrient loading in water bodies thus lead to eutrophication (Paerl and Otten, 2013). In addition, climate changes particularly global warming stimulates cyanobacterial growth because cyanobacteria can strive at relatively high temperature compared to other eukaryotic algae (Paerl and Huisman, 2009). These cyanobacteria blooms have sparked the attention of many researchers because of the side effect they bring not only to the ecosystem and aquatic animal health (Landsberg, 2002) but also human health (Carmichael *et al*., 2001). Massive cyanobacterial biomass causes hypoxia, resulting in fish kill and water quality deterioration. In addition, cyanobacteria produce secondary metabolites such as oligopeptides and lipopolysaccharides (LPS) which have adverse effect on aquatic organisms as well as human beings (Best *et al*., 2002; Welker *et al*., 2004). Exposure of microcystin contamination through drinking water and during recreational activities resulted in poisoning and even death of human (Rastogi *et al*., 2014). Various methods include biological, chemical and analytical methods have been implemented in cyanobacterial toxin detection. Previously, mouse and *Daphnia* are widely used for initial detection of unknown toxin sample. But over the years, enzyme linked immunosorbent assay (ELISA) had replaced the laborious method of bioassay toxicity testing. They offered a very simple, rapid outcome and only need small amount of sample in detecting and quantifying toxins but not very sensitive toward different microcystin variant. In addition, analytical method such as high performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR) and mass spectrometry (MS) are powerful tools in toxin detection (Rastogi *et al*., 2014).

1.2 Problems Statement

Harmful algae blooms has been extensively studied especially in developing countries in term of distribution and toxicity. In Malaysia, studies on harmful algae blooms focused more in marine environment compared to freshwater ecosystems. Even if it is, it is about the species distribution and for ecological purposes only. In freshwater ecosystem, cyanobacteria are likely to blooms under certain circumstances such as physical and chemical factors. Nutrient and temperature are among the factors that accelerating cyanobacterial growth. Increase in anthropogenic activities and the hot tropical climate in Malaysia could increase the occurrences of cyanobacterial blooms. Less understanding of the implication of these cyanobacterial blooms probably the reason behind the lack of research regarding these issues. Freshwater ecosystem especially lakes are used for recreational activities may pose dangerous health risk to the public users. So, it is important to address this issues and more research needed in order to identify toxic species and understanding it occurrences as well as the impact of the cyanobacteria blooms.

1.3 Objectives

1. To isolate, culture and characterize the *Microcystis* spp. from Putrajaya lake.

2. To determine the effects of different nitrogen and phosphorus concentrations on the growth of *Microcystis aeruginosa* and *Microcystis viridis.*

3. To assess the impact of *Microcystis* spp. on the growth and reproductive capacity on a cladoceran zooplankton, *Moina micrura.*

1.4 Hypotheses

Null hyphothesis (H_o): *Microcystis* spp. does not affect the population growth of a cladoceran.

Alternative hyphothesis (H_a): *Microcystis* spp. affect the population growth of a clacoderan.

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