MORPHOLOGY, TOXICITY AND TOXIN PROPERTIES OF CULTURED TOXIC CYANOBACTERIA SPECIES ISOLATED FROM AQUACULTURE POND OF SARAWAK AND SABAH

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This thesis is submitted in fulfillment of the requirement for the degree for Master of Science (Aquatic Toxicology)

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DECLARATION

No portion of the work referred to this dissertation has been submitted in support of an application for another degree or qualification of this or any other university or institution of higher learning.

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ABSTRACT

In this study, potential-toxin cyanobacteria were isolated from selected aquaculture ponds in Indigenous Fisheries Production and Research Center Sarawak and Babagon Fisheries Center Sabah. A total of 12 cyanobacterial strains from Orders Chroococcales, Oscillatoriales and Nostocales were classified morphologically based on published references. Out of this isolated strains, only six strains were successfully maintained and mass cultured, namely; Microcystis aeruginosa (MIC1), Anabaena spp. (ANA1 and ANA4), Cylindrospermopsis sp. (CYL1), Nostoc sp. (NOS1) and Lyngbya sp. (LYN1). Distinct morphological features were observed during sequential developmental stages of MIC and NOS1. For nutrient assessment, strain Anabaena spp. ANA1 and ANA4 showed the highest cell density in medium ASN3 with K$_2$HPO$_4$. Growth rate, $\mu$ for ANA1 was maximal in medium ASN3 with NaNO$_3$ and K$_2$HPO$_4$ while ANA4 was maximal in medium ASN3 with NaNO$_3$. Strain Cylindrospermopsis sp. CYL1, achieved the highest cell density in medium ASN3 containing K$_2$HPO$_4$ and the maximal growth rate, $\mu$ recorded in medium ASN3 with NaNO$_3$. For the light assessment, all strains of ANA1, ANA4 and CYL1 achieved the highest cell density under light intensity of 10 $\mu$mol photons m$^{-2}$s$^{-1}$. Strain ANA1 recorded the maximal growth rate under light intensity of 20 $\mu$mol photons m$^{-2}$s$^{-1}$. Growth rate, $\mu$ for ANA1 and CYL1 were maximal under light intensity of 10 $\mu$mol photons m$^{-2}$s$^{-1}$. For toxicity assessment, the result shows that only MIC1 extracts was toxic to brine shrimp at 24 hours in a dose dependent manner in which the LC$_{50}$ recorded was below 50 $\mu$g/ml. While for mouse bioassay MIC1 was found to be the most toxic to mouse followed by ANA1 and NOS1. For TLC detection, only MIC1 and ANA1 showed the same Rf value (0.97) when compared to Rf value of standard Microcystin-LR (0.97). The chromatograph peak of standard Microcystin-LR appeared at retention time of 8.18 min, while for Microcystis sp. at 8.18 and
Anabaena sp. at 8.19 min, respectively. The results indicate that MC-LR toxin was found in Microcystis sp. MIC1 and Anabaena sp. ANA1.

Keywords: Cyanobacteria, cultured, brine shrimp, mouse assay, TLC, HPLC
MORFOLOGI, KETOKSIKAN DAN SIFAT TOKSIN BAGI SPEIES SIANOBAKTERIA YANG DIPENCILKAN DARI KOLAM AKUAKULTUR DI SARAWAK DAN SABAH

ABSTRAK

Dalam kajian ini, sianobakteria yang berpotensi menghasilkan toksin telah dipencilkan dari kolam akuakultur terpilih di Pusat Penyelidikan dan Pengeluaran Ikan Tempatan Darat (Bahagian Perikanan), Sarawak dan Pusat Perikanan Babagon, Sabah. Sebanyak 12 jenis sianobakteria dari Order Chroococcales, Oscillatoriales dan Nostocales telah diklasifikasikan mengikut morfologi berdasarkan kekunci dan rujukan. Hanya enam strain berjaya dikultur iaitu: Microcystis aeruginosa (MIC1), Anabaena spp. (ANA1 dan ANA4), Cylindrospermopsis sp. (CYL1), Nostoc sp. (NOS1) dan Lyngbya sp. (LYN1). Terdapat beberapa beberapa ciri perbezaan morfologi telah dikenalpasti sepanjang pertumbuhan MIC1 dan NOS1. Untuk penilaian nutrien, strain Anabaena spp. ANA1 dan ANA4 menunjukkan sel kepadatan tertinggi dalam medium ASN3 dengan K2HPO4. Kadar pertumbuhan, μ untuk ANA1 maksima dalam medium ASN3 mengandungi NaNO3 dan K2HPO4 manakala ANA4 maksima dalam medium ASN3 mengandungi NaNO3. Strain Cylindrospermopsis sp. CYL1, mencapai sel kepadatan paling tinggi dalam ASN3 mengandungi K2HPO4 dan kadar pertumbuhan yang maksimal, μ direkodkan dalam medium ASN3 mengandungi NaNO3. Untuk penilaian cahaya, semua strain ANA1, ANA4 dan CYL1 mencapai sel densiti paling tinggi di bawah keamatan cahaya 10 foton µmol m-2s-1. Strain ANA1 mencatatkan kadar pertumbuhan maksima di bawah keamatan cahaya 20 foton µmol m-2s-1. Kadar pertumbuhan, μ untuk ANA1 dan CYL1 maksima di bawah keamatan
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Kata kunci: Sianobakteria, kultur, anak udang, bioesi tikus, KLT, KCPT
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CHAPTER I

INTRODUCTION

1.1 General Introduction

Blue green algae, scientifically known as Cyanobacteria are prokaryotic algae that have existed for over three billion years (Sze, 1993). It belongs to Kingdom Monera, Class Cyanophyceae, which divided into four Orders namely Chroococcales, Nostocales, Oscillatoriales and Stigonematales (Euzéby, 2004; Skulberg 1993). Cyanobacteria, which formerly classified as algae, were later considered to be affiliated to bacteria, owing to their biology and absence of a differentiated nucleus (Reyssac and Pletikosic, 1990). They have a relatively simple prokaryotic structure and lack membrane-bound organelles, which are structurally and physiologically, like other gram-negative bacteria although they conduct photosynthesis like plants in aquatic systems (Rogers, 2008).

Cyanobacteria are widely distributed and known as common phytoplankton found in freshwaters (i.e. lakes, ponds, rivers and reservoirs) and brackishwaters (i.e. seas, estuaries and lakes) waters (Metcalf and Codd, 2004; Castro et al., 2004). The ability to widespread and colonize in different environment are due to their special characteristics including cell buoyancy, nitrogen fixing akinetes and unfavourable condition resistant heterocysts (Sze, 1993; Oberholstcre et al., 2004). In addition, the combined effects of environmental factors such as temperature fluctuations, water movement, grazing and overloading of nutrients, which also known as eutrophication contributes to their proliferation in freshwater environment (WHO, 1998).
Excessive growth of cyanobacteria, known as bloom, which is characterized by the sudden appearance of large number of cells, represents a serious threat to aquatic ecosystem (Kroggman et al., 1986). In aquaculture system, cyanobacteria can out-compete algae for nutrients, thrive with low dissolved oxygen, and photosynthesize more efficiently at low light levels and produce bloom that contributes to unfavourable condition (Rodgers, 2008).

Aquaculture ponds could be experience cyanobacteria bloom as they provide favorable condition due to excessive fertilizer (Jewel et al., 2003). The increase demand of protein source has shifted to intensive aquaculture activities, in which growth of cultured species is supported by the natural productivity towards intensively operated production system (Prommana et al., 2006). The intensive aquaculture activity often requires high stocking densities and supplemental feed to achieve high productivity per unit volume, commonly resulting in eutrophic to hypereutrophic conditions (Tucker, 1996).

Eutrophication and cyanobacterial bloomin freshwater ecosystem have become a worldwide problem which can become serious when bloom-forming species release potent water soluble toxins (WHO, 1998; Falconer, 1993, Metcalf and Codd, 2004, Vasconcelos, 2001). Till date, cyanobacteria known to produce many bioactive compounds that are structurally and biochemically diverse which are classified as odorous metabolites and bioactive metabolites (Smith et al., 2008). Furthermore, Rodgers (2008) revealed that algal toxins can cause problems in the freshwater aquaculture organism by three different conditions namely as off-flavor, indirect toxicity through changes in water quality and direct toxicity. Aquaculture species are susceptible to odorous and bioactive secondary metabolites through the ingestion of cyanobacteria, consumption of contaminated food items and absorption of dissolved compound from the water column (Smith et al., 2008). Odorous compounds such as
geosmin (GSM) and 2-methylisoborneol (2MIB) are metabolites produced by certain species of cyanobacteria (Phoslock, 2008).

Off-flavour is the most economically significant problems encountered in freshwater aquaculture which are mainly caused by the absorption of odorous compounds from the water (Smith et al., 2008; Phoslock, 2008). A number of commercially important species have been affected by this problem including Nile tilapia, Oreochromis niloticus (Yamparoon and Noomhorn, 2000), shrimp (Whitfield et al., 1988), Atlantic salmon, Salmo salar (Farmer et al., 1995) and rainbow trout, Salmo gairdneri (From and Horlyck, 1984).

Moreover, aquaculture species also are vulnerable to bioactive metabolites that can cause mortality, initiate or promote tumors, or deteriorate the health of cultivated species or their prey species by affecting feeding, growth, or immune defense (Smith et al., 2008). Indeed, certain species of cyanobacteria were reported to give hazardous effect on fishes (Reysacc and Pletikosic, 1990) and massive mortality particularly among carp due to cyanobacterial intoxication was recorded (Prescott, 1948). The cause of mortality was postulated due to toxic cellular materials from cyanobacteria blooms which been released into the water during the cell lysis process (Jewel et al., 2003).

Yet, the aquatic organisms (e.g. fish, molluscs, crustacean) may accumulate cyanobacterial metabolites (Smith et al., 2008) via ingestion of cyanobacteria cells in contaminate food (Funari and Testai, 2008). Some aquatic organism such as molluscs (Prepas et al., 1997), crustaceans (Prommana et al., 2006) and fishes (Jewel, 2003; Liqiang et al., 2005) were identified as a vector and able to transfer the cyanotoxins (e.g. microcystins) along the food chain that may risk the human.

In Thailand, toxic cyanobacterial blooms found in many water bodies of the regions, including drinking water sources and fisheries activity area. Prommana et al. (2006) reported
that cyanobacteria species (i.e. *Microcystis* sp) and its toxins in prawn cultivation ponds possibly pose a hazard to aquatic organisms and to humans through food webs. It is proven as the laboratory experiments indicated that prawn hepatopancreas, heart and brain are primary organs for hepatotoxic bioaccumulation (Prommana et al., 2006).

Likewise, molluscs also reported as potential vectors for cyanotoxins (Funari and Testai, 2008). High concentration of toxin was found in the visceral mass of some individual’s clams due to increased exposure to selective bioconcentration of *Microcystis* (Prepas et al., 1997). In addition, Oberholster et al. (2006) reported histopathological investigations of toxic cyanobacterial blooms in Sheldon Lake showed that rainbow trout death due to cyanobacterial blooms, which indicated by damage of gills and fins. Gill damage, probably caused by the high pH induced by cyanobacterial photosynthesis activity prior to the bloom collapse, together with the higher level of ammonia arising from the decomposition of the cyanobacteria (Sivonen and Jones, 1999).

In tropical countries, very few studies have been done on cyanobacteria in comparison to temperate countries (Gires et al., 2002), even though it poses a high impact to human and aquatic environment. Specifically in Sarawak, only three published reports on cyanobacteria studies were conducted in selected freshwater ecosystem that mainly in Kuching District (Ramlah, 2005; Mardhiah, 2006; Nazriq, 2007). For diversity study done by Ramlah (2005), nine cyanobacterial genera namely *Anabaena, Anacystis, Calothrix, Chamaesiphonales, Gloeotrichia, Lyngbya, Microcystis, Oscillatoria, and Spirulina* were identified in which some are classified as toxigenic cyanobacteria based on Skulberg et al. (1993). Mardhiah (2006) and Nazriq (2007) have successfully isolated and established a pure culture of potentially toxin producing cyanobacteria namely *Microcystis* sp. which was collected from aquaculture pond of Indigenous Fisheries Research and Production Centre (IFRPC), Tarat, Serian, Sarawak.
Consistently, several cyanobacteria monitoring were conducted on respective pond as part to monitor the bloom occurrence and to identify the reason of fish mortality (pers comm, 2010) in the IFRPC aquaculture pond. Hence, this study were conducted to identify potential toxic cyanobacteria and it’s toxin properties that might be a turning point to help the centre for better aquaculture ponds management. In brief, IFRPC are consisted of two different types of freshwater aquaculture ponds which are; earth pond and earth pond that layered with black HDPE (High Density Polyethylene). Fishes such as Tor species and catfish are cultured in the ponds size which are varied from 60 x 60m² to 120 x 60m² using natural water system from the nearest water source. Besides of that, Babagon Fisheries Centre (BFC), which is located in Penampang Sabah also been selected as study sites, since it is facing a common problem as in Tarat. BFC had practised basic water system using freshwater source from the highland at the nearest area and the cultured fishes are common carp and tilapia in various size of earth pond.

1.2 Cyanobacteria characteristics
Cyanobacteria are photosynthetic prokaryotic algae (Oberholster et al., 2006) which belong to Division Cyanophyta and contain chlorophyll a as their major pigment (Funari and Testai, 2008). They contained accessory pigments such as phycobiliprotein, which comprises of phycoerythrin, phycocyanin, allophycocyanin and phycoerythrocyanin (Sze, 1993; Briand et al., 2003). Cyanobacteria reproduce asexually by binary fission, spore production, or fragmentation, forming singular cells, colonies and filaments (Mur et al., 1999). They are divided into non-filamentous and filamentous type, with the filamentous species further subdivided by the presence or absence of specialized cells called heterocyst and akinetes (Sze, 1993).
Vegetative cells are the normal photosynthetic cells that are formed under favorable growing conditions. During stress condition, including anoxic state, heterocysts will act as converter and fix nitrogen from the air into ammonia (NH₃), nitrites (NO₂⁻) or nitrates (NO₃⁻) form, which can be absorbed by plants (Ahern, 2002). In heterocysts, the respiratory pathways and part of the photosynthesis system function to provide energy in the form of ATP and reduced compounds to accomplish nitrogen fixation. Meanwhile, akinetes are the climate-resistant spores that may form under the inappropriate environmental conditions. Thick-walled akinetes will form at the end of a period of growth and survive in a dormant state until conditions are again favourable to grow (Sze, 1993).

Blue-green algae are advantageous over other algae because of their ability to control buoyancy to access areas of increased nutrients and light (Phoslock, 2008). Many species of cyanobacteria possess numerous gas vacuoles (Oberholster et al., 2006). These are cytoplasmic inclusions that enable buoyancy regulation and are gas-filled, cylindrical structures (Mur et al., 1999). Gas vesicles are important device to ensure positive buoyancy so that they float on the surface and act to regulate the position of cells in the water column (Sze, 1993). Moreover, Mur et al. (1999) stated that cyanobacteria use different environmental stimuli such as photic gravitational, chemical, thermal to optimize their position, and thus to find a suitable niche for survival and growth. Therefore, they can tolerate adverse conditions such as the complete drying of a pond or the cold winter temperatures (Vincent et al., 1993).

1.3 Habitat of cyanobacteria

The ability of cyanobacteria to survive under extreme of environmental stress such as desiccation, temperature fluctuation, high light intensities and oligotrophic low nutrient condition make them an ubiquitous organism that can be found virtually in all ecosystem
habitats on earth. Habitats occupied range from freshwater lakes and rivers, through to the oceans, including hot springs, and deserts, ranging from the hottest to the cold dry valleys of Antarctica (Hitzfeld et al., 2000; Acreman, 1994; Vincent et al., 1993).

Cyanobacteria are found nearly everywhere, occurring in typical aquatic and terrestrial habitats as well as in such extreme sites as hot springs with temperatures as high as 71°C and crevices of desert rocks (Büdel, 1999) and bare soil (Mur et al., 1999). For example, *Synechoccus* that can tolerate temperatures up to 74°C while other species of cyanobacteria such as *Phormidium* and *Lyngbya* are found in Antarctic lakes (Sze, 1993). Cyanobacteria also tolerate high salt concentrations, as they may occur in tidepools and lakes when evaporation concentrates salts. The filamentous cyanobacterium *Spirulina* commonly occurs in lakes with a high soda content and high pH (Sze, 1993). Cyanobacteria have a number of special properties which determine their relative importance in phytoplankton communities. They tend to favor neutral or basic conditions and are less common at low pH (Sze, 1993). The optimum temperature for toxin production in cyanobacteria is between 20°C and 25°C (Watanabe and Oishi, 1985). These optimum temperatures are higher than for green algae and diatom which cause most cyanobacteria in temperate region bloom during summer (Funari and Testai, 2008).

**1.4 Bloom of cyanobacteria**

Cyanobacteria are a common form of algae which are often referred to as "pond scum" (Acreman, 1994) and form large colonies which indicate by greenish appearance of scum, foam or mats on the surface of freshwater lakes and ponds (Epperson, 2002). Generally the blooms are most obvious when the cyanobacterium is one containing gas vacuoles that allow it to concentrate at the surface of the water to maximize light absorption for photosynthesis
Cyanobacterial blooms are often been relate with the eutrophication and enrichment of waters with nutrients (Metcalf and Codd, 2004) especially nitrogen and phosphorus (Sze, 1993).

Experimental data have indicated that the affinity of many cyanobacteria for nitrogen or phosphorus is higher than for many other photosynthetic organisms (Funari and Testai, 2008). Nitrogen is an essential element for plant growth, required for the synthesis of amino acids, proteins, nucleotides, nucleic acids, coenzymes, chlorophyll and other photosynthetic pigments (Bartram et al., 1999; Falconer, 2005 and Sze, 1993). Most cyanobacteria are nitrogen fixers, converting atmospheric nitrogen to ammonia via the enzyme nitrogenase, which aid to proliferation in low-nitrogen condition (Phoslock, 2008). Besides nitrogen, Ahern (2002) included phosphate as the main part in regulating metabolism and ultimately the growth of cyanobacteria. Bartram et al. (1999) stated that concentration of phosphorus less than 0.1mg/l is sufficient enough to induce a cyanobacterial bloom.

The phenomena of cyanobacterial bloom thus shifted many lakes from having phytoplankton communities dominated by diatoms to having phytoplankton communities dominates by cyanobacteria (Campbell, 2002). Bloom formation often results hazard effect to the environment. When cyanobacterial blooms occur, light penetration are limited by scum formation on the water surface, reducing the growth of other benthic producer organism such as epiphyton, benthic algae and rooted vascular plants (Havens, 2007). Besides that, decomposition of dead algal bloom may lead to the depletion of dissolved oxygen in the water, resulting secondary problems such as fish mortality (Bartram et al., 1999). Moreover, during intense blooms, photosynthetic activity depletes free CO$_2$ from lake water and cause the elevation of pH which may harmful to certain species of fish (Havens, 2007).
Although not all cyanobacteria blooms are toxic, some blooms can produce significant quantities of natural toxins (Kaebernick and Neilan, 2000). When highly active natural biotoxins produced, these blue-green algae blooms are considered as “Harmful Algal Bloom (HAB)” (Sivonen and Jones, 1999; Gires et al., 2002). The effects of algal blooms vary widely. Some algae are toxic only at very high densities, while others can be toxic at very low densities (Rodgers, 2008). These toxins can affect aquatic life, humans as well as terrestrial animals. If the toxins are persistent, they may form a health risk via transfer and accumulation in the food web (Lehtiniemi et al., 2002). The levels of toxin accumulation sufficient to pose a risk will depend on levels of human consumption and the severity of toxic blooms in the area where fish or shellfish are caught or collected (Kozlowsky et al., 2003).

1.5 Cyanobacteria and public health concern

Human may be exposed to cyanotoxins via several routes such as ingestion, inhalation, intravenous (dialysis) and skin contact (Vasconcelos, 2001). One of most well known occurrence episode is “Palm Island Mystery Disease” in Australia. In 1979, about 140 indigenous people, mostly children were hospitalized after drinking water where a dense cyanobacterial bloom on a water supply occurred (Metcalf and Codd, 2004; WHO 1998). In response to the growing concern about nonlethal acute and chronic effects of microcystins, the World Health Organization has previously set a new provisional guideline value for microcystin-LR of 1.0μg/L drinking water (Hitzfeld et al., 2000).

When surface waters infested by cyanobacteria are used for hemodialysis, they can represent a remarkable risk for patients; indeed, the paternal route of exposure considerably increases the internal dose of toxins, directly entering the blood stream (Funari and Testai, 2008). The most remarkable episode of human health resulted from hemodialysis from