CONTROL OF TOXIN-PRODUCING CYANOBACTERIAL POPULATION USING SELECTED AGRICULTURAL WASTES

by

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LIST OF ABBREVIATIONS

AI – Air Itam

BLAST – Basic local alignment search tool

EFB – Empty fruit bunch

EFB (P) – Empty fruit bunch powder

mcy – microcystin synthetase

NCBI – National Centre for Biotechnology Information

NRPS – Non-ribosomal peptide synthetase

OPT – Oil palm trunk

OPT (P) – Oil palm trunk powder

PCM – Phase-change materials

PCR – Polymerase chain reaction

PKS – Polyketide synthase

SCB – Sugarcane bagasse

TAE – Tris base, acetic acid and EDTA

TA – Tasik Aman

TB – Teluk Bahang

TH – Tasik Harapan

WBL – Wheat bran leachate

PENGAWALAN POPULASI SIANOBAKTERIA YANG MENGHASILKAN TOKSIN MENGGUNAKAN SISA TANAMAN TERPILIH

ABSTRAK

Sianobakteria, juga dikenali sebagai alga hijau-biru, adalah prokariot fotosintetik yang menghasilkan ledakan di dalam air apabila keadaan persekitaran sesuai. Sianobakteria telah menjadi masalah di seluruh dunia dan menimbulkan isu kesihatan, terutamanya spesies yang menghasilkan toksin. Penggunaan algisid kimia seperti kuprum sulfat untuk mengawal populasi adalah berkesan tetapi ia mempunyai spektrum ketoksikan yang luas terhadap organisma lain. Penggunaan agen biologi seperti jerami barli telah terbukti berkemampuan dan digunakan secara meluas di Eropah dan Amerika Utara bagi mengawal pertumbuhan sianobakteria tetapi negara seperti Malaysia mempunyai sumber barli jerami yang terhad. Sisa pertanian seperti hampas tebu dan biomas kelapa sawit boleh didapati dengan banyak dan bersesuaian menganalisis potensi mereka untuk mengawal pertumbuhan sianobakteria. Sehingga kini, belum ada kajian tentang spesies sianobakteria di Malaysia terutama spesies yang menghasilkan toksin atau penggunaan sisa pertanian untuk mengawalnya. Tujuan kajian ini adalah untuk menyelidik potensi sisa pertanian terpilih, batang kelapa sawit (OPT), tandan kosong sawit (EFB) dan hampas tebu (SCB) untuk mengawal mekar sianobakteria dengan menggunakan sianobakteria yang diasingkan dari Pulau Pinang. Pelbagai spesies sianobakteria yang diasingkan daripada empangan Air Itam, empangan Teluk Bahang, Tasik Harapan dan Tasik Aman di Pulau Pinang telah dikenal pasti dengan menggunakan kaedah morfologi dan molekul 16S rRNA. Pengesanan untuk gen mengekodkan *microcystin, anatoxin, cylindrospermopsin* dan *saxitoxin* dilaksanakan dengan menggunakan primer tertentu. Kesan perencatan sisa pertanian terpilih terhadap sianobakteria diuji dengan

menganalisis kandungan klorofil a. Secara keseluruhannya, 25 strain telah diasingkan daripada empat tempat di Pulau Pinang. *Microcystis aeruginosa* yang diasingkan daripada empangan Air Itam telah dikesan mengekod gen toksin microcystin. OPT dan EFB menunjukkan perencatan terpilih terhadap spesies yang berlainan. Sementara itu, kesan perencatan SCB pada pertumbuhan sianobakteria lebih berpontensi berbanding OPT dan EFB.

CONTROL OF TOXIN-PRODUCING CYANOBACTERIAL POPULATION USING SELECTED AGRICULTURAL WASTES

ABSTRACT

Cyanobacteria, also known as the blue-green algae, are photosynthetic prokaryotes which form blooms in water when the conditions are favourable. Cyanobacterial bloom has been a nuisance around the world and pose health issue especially those related to toxin producing cyanobacteria. The use of chemical algaecide such as copper sulphate to control its population was effective but it has a wide spectrum toxicity towards non-target organisms. The use of agricultural wastes such as barley straw has been proven capable and widely used in Europe and North America to control cyanobacterial bloom but country like Malaysia has limited access to bulk barley straw. Agricultural wastes like sugarcane bagasse and oil palm biomass are available in abundance and suitable to study of their potential towards control of cyanobacterial bloom. So far, no study has been done on cyanobacterial species in Malaysia especially toxin producing species or the use of these agricultural wastes to control cyanobacteria. This study aims to investigate the potential of selected agricultural wastes, oil palm trunk (OPT), empty fruit bunch (EFB) and sugarcane bagasse (SCB) to control cyanobacterial bloom by using locally isolated cyanobacteria. Variation of cyanobacterial species isolated from Air Itam dam, Teluk Bahang dam, Harapan Lake and Aman Lake in Penang, were identified using morphology and molecular 16S rRNA. Detection for microcystin-, anatoxin-, cylindrospermopsin-, and saxitoxin-producing genes were done using specific primers. Then, selected agricultural waste was tested with isolated cyanobacteria for inhibitory effect by measuring chlorophyll a content. In total, 25 strains were isolated from the four locations in Penang, Malaysia. *Microcystis sp*.-specific mcyE gene was

detected in *Microcystis aeruginosa* isolated from Air Itam dam and was confirmed with microcystin strip test. OPT and EFB showed selective inhibition towards different species. Meanwhile, the inhibitory effect of SCB on cyanobacterial growth was more promising than OPT and EFB.

CHAPTER ONE: INTRODUCTION

1.1 Background

Cyanobacteria, also known as the blue green algae are photosynthetic prokaryotes which present in most water bodies. Sometimes they grow to large populations known as blooms which are mostly harmful due to the fact that certain species are capable of producing toxins. Cyanobacterial blooms can cause severe water quality deterioration including scum formation, toxin production, hypoxia, foul odours and tastes (Paerl et al., 2001). Of all these, the production of active toxic compounds known as cyanotoxins is the primary concern because it can pose lethal and sub-lethal effects in both humans and animal (Wood et al., 2012a). Caruaru Incident indicated that these toxins can be fatal to human through haemodialysis (Jochimsen et al., 1998). Toxic cyanobacteria poisonings have been reported in animals such as birds, cattle, and sheep (Carmichael, 2001) and have caused over 350 cases of suspected or confirmed poisonings or deaths in the U.S. between the 1920s and 2012 (Backer et al., 2013).

The direct way to control cyanobacterial bloom would be the application of algaecides or simply any chemicals but it may cause harmful effects to the natural environment itself and may even risk the accumulation of those compounds in sediments (Mason, 2002). Biological approaches on the other hand will carry less ecological risk to the environment. Many studies were done showing range of aquatic and terrestrial plants that exhibit inhibitory effect towards cyanobacteria (Shao et al., 2013). Agricultural by-products or waste were also shown to exhibit inhibitory effects towards cyanobacteria which included wood (Pillinger et al., 1995), leaf litter (Ridge et al., 1995), straw and hull of rice (Park et al., 2009), fruit peels especially

citrus peels (Liang et al., 2010) and wheat bran leachate (Shao et al., 2010). Among them, barley straw was the most popular waste studied (Ridge et al., 1999).

Microbial decomposition of barley straw had been shown to inhibit the growth of cyanobacteria (Barrett et al., 1996; Everall and Lees, 1996). Newman and Barrett (1993) suggested the algistatic effect may be due to the incomplete decomposition of lignin while Everall and Lees (1997) have identified several phenolic compounds produced during barley straw decomposition that may be toxic to the cyanobacteria.

Agricultural wastes such as oil palm biomass are rich in lignin (Meier and Faix, 1999; Demirbaş, 2000), which may enable them to be as effective as barley straw to control cyanobacterial growth. In general, oil palm tree has an economic life span of about 25 years, in which it will need to be replanted in order to maintain its oil productivity (Abdul Khalil et al., 2010). Replanting will generate enormous amount of solid wastes including oil palm trunks, fronds and empty fruit bunch which can be utilized. Malaysia as a world leading palm oil producer generates approximately 3 million tonnes of oil palm trunk per year (Abdul Khalil et al., 2012). Aside from oil palm waste, sugarcane (*Saccharum officinarum*) of the yellow cane variety, is a very popular sugarcane cultivar grown for juice production in Malaysia (Salunkhe and Desai, 1988) and with that, sugarcane bagasse are among the abundant biomass waste available in our country that are rich in lignin (Hong et al., 2011).

1.2 Problem statement:

Cyanobacterial blooms in freshwater bodies pose a worldwide problem, worsening by the production and release of a range of cyanotoxins (Codd et al., 1989). Climatic change scenarios predict rising temperatures in the future which favour harmful cyanobacterial blooms (Paerl and Huisman, 2009). Solution using chemical treatments pose long term detrimental effects on ecosystems (García-Villada et al., 2004). Until date, approaches using oil palm waste and sugarcane bagasse in controlling cyanobacterial bloom have not been studied and with such abundance of these wastes in Malaysia, their utilization for potential cyanobacterial control should be explored. This may be the solution to cyanobacterial bloom in the future. In addition, there is limited study on cyanobacteria in environment in Malaysia and until now there is still lack of information regarding toxin producing cyanobacteria in this country. Hence, this study has been conducted to fill the gap of data on toxin producing cyanobacteria in local water bodies in Penang, one of the state in Malaysia.

1.3 Research Scope:

The study focused on local water bodies in Penang Island which are Harapan Lake, Aman Lake, Air Itam dam and Teluk Bahang dam. Local isolated cyanobacteria from these selected water bodies in Penang Island have been screened for toxin producing potential and utilized as the test organisms to study the potential of selected agricultural wastes in controlling cyanobacterial growth. The agricultural wastes studied were oil palm trunk, empty fruit bunch and sugarcane bagasse.

1.4 Research Objectives:

The purpose of this study is to investigate the potential of selected agricultural wastes, namely the oil palm trunk (OPT), empty fruit bunch (EFB) and sugarcane bagasse

(SCB) to control cyanobacterial growth by using locally isolated species from water bodies in Penang Island, Malaysia.

Based on the above, this study has been conducted with the following objectives:

1. To isolate and identify cyanobacterial species from selected locations in Penang Island
2. To detect toxin-encoding genes in the isolated species
3. To investigate the ability of oil palm trunk, empty fruit bunch and sugar cane bagasse to control growth of the isolated cyanobacteria

CHAPTER TWO: LITERATURE REVIEW

2.1 Biology of cyanobacteria

Cyanobacteria are among the earliest organisms in the world with a record of date back to 3500 million years ago (Whitton and Potts, 2012). They were once classified as algae according to the Botanical Code and later in the 8th edition of Bergey's Manual of Determinative Bacteriology then cyanobacteria were first assigned to a separate division of the prokaryotes (Buchanan and Gibbons, 1974). Although they are now classified as bacteria, the term 'blue-green algae' is still popular among researchers. Being able to perform oxygenic photosynthesis, they are credited with their roles in oxygenation of biosphere and biogeochemical cycles (Blank and Sanchez-Baracaldo, 2010). Besides synthesizing chlorophyll a for photosynthesis, most cyanobacteria produce phycobilin pigment like phycocyanin, which is responsible for the bluish colour and thus the popular name, blue-green algae; in some cases the red pigment, phycoerythrin, is formed as well (Whitton and Potts, 2012).

Cyanobacteria are morphologically diverse group that range from simple unicellular to complex filamentous forms. Unicellular groups consist of two orders, the Chroococcales and the Pleurocapsales; whereas the filamentous groups which possess a variety of highly differentiated cells consist of three orders, the Oscillatoriales, Nostocales and Stigonematales (Castenholz and Waterbury, 1989). Cyanobacteria exhibit various physiological characteristics which allow them to react and adapt to changes in growth conditions. Among these is the evolving of multiple specialized cell types from vegetative cells, including nitrogen-fixing heterocysts, resting-stage akinetes, and the cells of motile hormogonia filaments. Heterocysts which supply nitrogen to the vegetative cells provide a division of labour between

oxygenic photosynthesis and anaerobic nitrogen fixation as nitrogenase, an enzyme responsible for nitrogen fixation is inactivated by oxygen (Kumar et al., 2010). Under extreme environmental conditions, heterocystous cyanobacteria generate akinetes which can remain dormant and viable for many years until suitable conditions are available, after which they germinate to produce new filaments (Adams and Duggan, 1999). Many filamentous cyanobacteria can form hormogonia which are responsible for the cell motility and dispersal (Whitton and Potts, 2000). Another special physiological feature is the gas-filled vesicles found within vacuoles inside the cells of cyanobacteria which gives cyanobacteria the ability to control their buoyancy or migrate vertically in the water column in response to light and nutrients (Brookes and Ganf, 2001). Both hormogonia and gas vesicles help cyanobacteria to optimize their position for survival and growth.

2.2 Cyanobacterial blooms

The natural occurrence of cyanobacteria in aquatic environments is beneficial due to their ability to fix nitrogen. However, they can be a nuisance or hazard when they appear in high population density, or known as bloom which decolorized the water and deteriorated water quality (Falconer, 1999). A number of freshwater phytoplankton are capable of forming blooms; however, the cyanobacteria are known to form the most notorious blooms. The most common bloom-forming genera include *Microcystis*, *Anabaena*, *Aphanizomenon*, *Oscillatoria*, *Cylindrospermopsis* and *Nodularia* (Codd et al., 2005b).

Blooms can have various appearance, forming colonies, mats, and scums with colour that can range from blue-green to black (Cheung et al., 2013). Most cyanobacterial blooms are hazardous due to the fact that the blooms include species of the toxigenic genera *Microcystis*, *Anabaena*, or *Plankthotrix* which can have lethal and sub-lethal

effects in both humans and animals (Wiegand and Pflugmacher, 2005; Wood et al., 2012a). In addition to that, the presence of cyanobacterial blooms may disrupt the size structure of zooplankton community and the stability of aquatic ecosystems in freshwater systems (Ghadouani et al., 2006).

Cyanobacterial blooms generally occur as a result of anthropogenic activities which lead to nutrient enrichment from sources such as agricultural fertilizer run-off and domestic or industrial effluents. The bloom was attributed to favourable environmental conditions such as high temperatures, high pH, elevated nutrient concentrations particularly total phosphorus and absence of predators (Bowling and Baker, 1996; Bouvy et al., 1999). Cyanobacterial mass occurrences are a frequent phenomenon worldwide. On average, 59% contain toxins, with hepatotoxic blooms being more common than neurotoxic blooms in freshwaters (Sivonen and Jones, 1999).

2.3 Cyanobacterial toxins

Toxigenic cyanobacteria have the ability to produce a variety of toxic secondary metabolites known as cyanotoxins (Wiegand and Pflugmacher, 2005), which may cause fatal poisonings of agricultural livestock, wild animals, birds and fish on a world-wide basis (Codd et al., 1989). There had been reports on human sickness due to cyanobacterial toxins as early as 1931 in the USA, Australia, Brazil, China, and England (Chorus and Bartram, 1999). One of the examples of human deaths associated with cyanotoxins was the Caruaru Incident occurred in Brazil in 1996 where 56 out of 131 patients died after receiving haemodialysis treatment in which the water was contaminated with microcystins (Jochimsen et al., 1998).

Major cyanotoxins include microcystins, cylindrospermopsins, nodularin, anatoxins and saxitoxins (Neilan et al., 2008) with several of them are among the most potent toxins known (Hudnell, 2010). These toxins can be further classified into hepatotoxins (liver damage), neurotoxins (nerve damage), cytotoxins (cell damage), dermatotoxins and irritant toxins which are responsible for allergic reactions based on their toxicological target (Wiegand and Pflugmacher, 2005).

Cyanobacterial toxins can find its way into water supplies either by the breakdown of a natural cyanobacterial bloom in a reservoir or river, or the addition of copper sulphate to control cyanobacterial bloom which bring about the lysis of cyanobacteria and the subsequent release of toxic compounds (Falconer, 1999; Hawkins et al., 1985). Humans are exposed to cyanotoxins through various routes: drinking of contaminated water, dermal contact with toxins during recreational activities such as swimming and canoeing, consumption of contaminated aquatic organisms, food supplements and haemodialysis (Drobac et al., 2013). Although significant research had been done on microcystins, cyanobacteria still required further research attention as they produce a wide range of currently unknown toxins (Blaha et al., 2009).

2.3.1 Polyketide synthase (PKS) and Non-ribosomal peptide synthetase (NRPS)

Polyketide synthase (PKS) and non-ribosomal peptide synthetase (NRPS) are large multimodular enzymes complexes responsible for the production of polyketide and peptide secondary metabolites in microorganisms, such as bacteria and fungi (Gallo et al., 2013). Secondary metabolites rarely have a role in primary metabolism such as growth, development, or reproduction but have evolved to somehow benefit the producing organisms to survive interspecies competition, provide defensive mechanisms against stress, and facilitate reproductive processes (Neilan et al., 1999;

Mandal and Rath, 2015). Cyanobacteria produce numerous and structurally diverse secondary metabolites, in particular nonribosomal peptide and polyketide structures. (Dittmann et al., 2001). Microcystins and nodularins are cyanotoxins that synthesize via a mixed NRPS/PKS pathways (Pearson et al., 2010).

2.3.2 Microcystins

Microcystins are cyclic peptide hepatotoxins (Codd and Carmichael, 1982) and the most prevalent toxin found in cyanobacterial blooms (Chorus and Bartram, 1999). So far, more than 100 different structural variants of microcystins have been discovered (Vichi et al., 2015). Microcystin is the most common toxin and several genera of cyanobacteria including *Microcystis, Anabaena, Oscillatoria, Planktothrix, Chroococcus and Nostoc* are known to produce microcystin (Pearson et al., 2010).

Microcystins are synthesized non-ribsomally via a mixed polyketide synthase and non-ribosomal peptide synthetase called microcystin synthetase (mcy). The mcy gene cluster spanning 55 kb arranged in the order of *mcyJIHGFEDABC*, transcribed bidirectionally as two putative operons, *mcyABC* and *mcyDEFGHIJ* are responsible for microcystin production (Figure 2.1). The gene cluster encodes ten open reading frames (ORFs), including six multifunctional enzymes comprised of NRPS and PKS domains (Tillett et al., 2000).

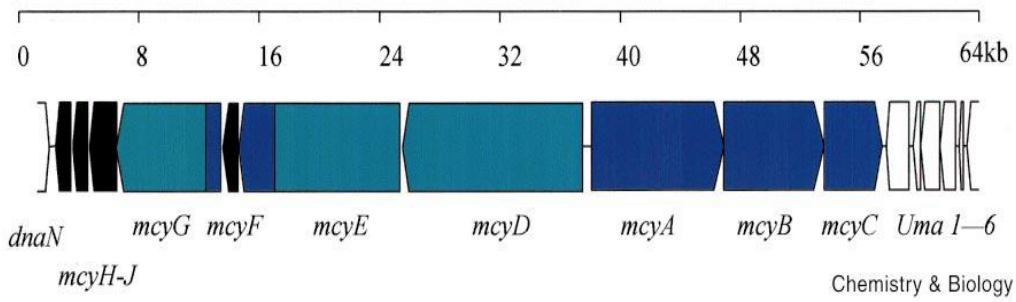

Figure 2.1. Structural organization of the microcystin synthetase gene cluster. Open reading frames (ORFs) are shown in relative sizes with the arrow denoting direction of transcription. ORFs in dark blue indicated the regions homologous to nonribosomal peptide synthetases and light blue indicated sequences homologous to polyketide synthases. Other microcystin synthesis genes are indicated in black. Non-microcystin synthesis genes are shown in white. Taken from Tillett et al. (2000).

Chronic exposure to low microcystins concentration has been linked to human hepatocellular carcinoma in China (Kuiper-Goodman et al., 1999) and it has been shown to promote tumours in animal experiments as well (Nishiwaki-Matsushima et al., 1992; Falconer and Humpage, 1996). Following various health risk issues, World Health Organization (WHO) had provided a provisional guideline value of 1 and 20µg/L microcystin for drinking and recreational water respectively (WHO, 1998; WHO, 2003).

2.4 Prevalence of cyanotoxin detection

Cyanobacteria studies in Malaysia are limited especially those regarding cyanotoxin and toxin producing cyanobacteria distribution in natural aquatic environment. In contrast to that, cyanotoxins have been found in Malaysia's neighbouring countries. For example, hepatotoxic microcystins were found to occur in some Thailand waterblooms (Mahakhant et al., 1998) and cyanotoxin cylindrospermopsin and deoxy-cylindrospermopsin were found from strain of *Cylindrospermopsis raciborskii* in pond in Bangkok (Li et al., 2001). Microcystin producing cyanobacteria was also identified at Kranji Reservoir in Singapore (Te and Gin, 2011). Countries around the world such as the Czech Republic, France, Japan, Korea, New Zealand, Norway, Poland, Brazil and Spain had adopted WHO guideline value of microcystin with some other countries such as Australia and Canada set up their own values based on the local conditions (Codd et al., 2005a). Brazil has a more comprehensive standard which cover not only microcystins but also saxitoxins and cylindrospermopsin. The mandatory standard for microcystin is 1 µg/L, and the recommended values for saxitoxins and cylindrospermopsin are 3 µg/L and 15 µg/L respectively (Codd et al., 2005a).

2.5 Cyanobacterial control

2.5.1 Technical and physical controls

2.5.1.1 Dilution and flushing

Generally, it is done by diluting nutrients for cyanobacterial growth while increasing water exchange rate leading to a faster loss of algae from the lake and previous study had shown to work successfully in several lakes (Welch, 1981). Examples include significant reduction of cyanobacteria in Moses Lake in Washington (Welch, 1981) and the shifting of dominant *Microcystis aeruginosa* to *Cyclotella sp.* in Lake Tega, Japan (Amano et al., 2012). However, due to huge amounts of water needed, this method is rarely applicable.

2.5.1.2 Artificial mixing

Continual mixing of the water column destroys stratified conditions, disrupting cyanobacterial buoyancy for the advantage of faster growing green algae and other non-buoyant algae (Reynolds et al., 1984). Long term artificial mixing at lake Nieuwe (The Netherlands) showed a decrease in total algal biomass with the mass of *Microcystis* reduced up to 20 times and the cyanobacterial dominance was shifted to a mixed community of green algae, flagellates and diatoms (Visser et al., 1996). This method, however, is only applicable when the algal population is light limited. Otherwise, nutrient-limited phytoplankton can be promoted due to higher nutrient availability (Visser et al., 1996).

2.5.2 Chemical control

2.5.2.1 Copper sulphate

Chemical approaches can eliminate algae blooms rapidly and effectively. Chemicals such as copper sulphate ($CuSO_4.5H_2O$) are widely used as algaecide as its toxic effects to algae and cyanobacteria include the inhibition of photosynthesis, the

phosphorus uptake and the nitrogen fixation (Havens, 1994). However, in the process of removing harmful algae bloom, other non-harmful phytoplankton or aquatic organisms may also be eliminated or adversely affected due to the non-selective toxicity of copper sulphate. Introduction of concentrated copper sulphate into water bodies impairs food web functions (Havens, 1994) and often leads to the collapse of aquatic ecosystems. Another major concern of this chemical is it induces lysis of cyanobacteria and toxic compounds are released into the water as in the case of Palm Island in 1979 (Hawkins et al., 1985).

2.5.2.2 Other inorganic chemicals

Other inorganic biocides highly toxic to cyanobacteria that were used include potassium permanganate ($KMnO_4$, dose 1 - 3 mg/L) (Lam et al., 1995) and sodium hypochlorite (NaOCl, dose 0.5 - 1.5 mg/L) (Lam et al., 1995). Addition of aluminium sulphate can decrease phosphorus content and improves lake water quality of blooms but only in short term and with limited effectiveness (Hullebusch et al., 2002). Like copper sulphate, the application of these inorganic chemicals into natural aquatic environment is not conceivable due to their nonselective toxicity to many aquatic organisms.

Hydrogen peroxide (H_2O_2) has selective effects on cyanobacterial species and photosynthesis. H_2O_2 has been suggested as a promising compound for treatment of excessive cyanobacterial growth in lakes and reservoirs with effective dose of H_2O_2 vary from 0.3 to 5 mg/L, depending on particular cyanobacterial species, strains, conditions and light intensity. H_2O_2 does not lead to the accumulation of toxic residues in the environment and the compound is relatively cheap. However, it decomposes fast and is likely to damage other non-target organisms (Drábková et al., 2007).

2.5.3 Biological control

2.5.3.1 Grazing activities

Phytoplankton is the food source for planktivorous filter-feeding fish such as the silver carp (*Hypophthalmichthys molitrix*) and bighead carp (*Aristichthys nobilis*). Direct grazing of these fish eliminated cyanobacterial blooms as in Lake Donghu, China (Xie and Liu, 2001). However, it was experimentally proven that after passing through the gut of silver carp, metabolic activity of *Microcystis* from the excreta was able to recover fully (Gavel et al., 2004). Moreover, defecation of the fish contribute to nutrient enrichment in the water and cause ichthyoeutrophication (Datta and Jana, 1998) which can counteract the effect of grazing.

Zooplankton such as copepods and *Daphnia* have the potential to control cyanobacterial populations by grazing on them. Even so, toxic cells can deter the zooplankton from ingesting them (Panosso et al., 2003; Gobler et al., 2007) and *Microcystis* strains had been shown to increase their cell toxin production upon exposure to zooplankton (Jang et al., 2003)

2.5.3.2 Cyanophage and bacteria

Cyanophages are viruses specific to cyanobacteria which have the potential to control cyanobacterial populations. Manage et al. (1999) had reported the positive correlation between cyanophages density and *Microcystis aeruginosa* populations. However, through time cyanobacteria will develop resistance to cyanophages (Tucker and Pollard, 2005), thus minimizing the control effect. Other than cyanophages, host-specific lytic bacteria (family Cytophagaceae) also plays a role in selectively eliminating the bloom-forming cyanobacteria (Rashidan and Bird, 2001). One of the concern about the use of cyanophages and bacteria is the possibility of toxin released into the water during the lysis of the cells.

2.5.3.3 Crop wastes: Barley straw

The fact that rotting barley straw can control cyanobacterial populations has been documented by many researchers. The use of barley straw to control cyanobacterial bloom is well established with proven efficacy from laboratory study to field application (Newman and Barrett, 1993; Barrett et al., 1999). Recent study has suggested the inhibitory mechanism of flavonolignans, Salcolin A and Salcolin B in barley straw. Salcolin A was considered algistatic as it increased cyanobacterial intracellular ROS (reactive oxygen species) levels and inhibited esterase activity while Salcolin B were considered algicidal as it caused cytoplasm leakage in cyanobacteria (Xiao et al., 2014).

Studies indicated that the microbial decomposition of barley straw placed on water reservoirs releases a compound or compounds that inhibit the growth of algae and cyanobacteria (Barrett et al., 1996; Everall and Lees, 1996). The finding was confirmed by Iredale et al. (2012) where after few weeks of decomposition, whole barley straw releases either the growth inhibitory fraction, or its precursor, due to microbial activity.

Everall and Lees (1997) have identified several phenolic compounds such as p-coumaric acid and ferulic acid produced during barley straw decomposition which may be toxic towards cyanobacteria. Comparatively, Newman and Barrett (1993); (Barrett et al., 1999) also showed the release of phenolic compounds from the decomposition of straw cell walls, as well as other aromatic compounds from the incomplete decomposition of lignin may play roles in the algistatic effect.

Despite of the effectiveness reported, barley straw appears to demonstrate selective inhibition towards different species. Ferrier et al. (2005) found that *Microcystis aeruginosa* was susceptible to barley straw but *Anabaena flos-aquae* showed

otherwise. This species-specific inhibition may alter the phytoplankton composition. Hence, tolerance and resistance of target species must be taken into account prior to any application.

2.5.3.4 Crop wastes: Rice straw

Rice straw extract had been proven to inhibit the growth of *Microcystis aeruginosa* (Park et al., 2006a; Su et al., 2014). Rice hull, another residue from the cultivation of rice, also showed selective inhibition towards *Microcystis aeruginosa* with a small effect on green algae and *Daphnia* (Park et al., 2009). Park et al. (2006a) concluded the inhibitory properties of rice straw was due to the synergistic effects of various phenolic compounds from the rice straw and Park et al. (2009) showed that among 9 extractant from rice hulls, β-sitosterol-β-d-glucoside and dicyclohexanyl orizane showed significant inhibition (>60%) of *M. aeruginosa*.

2.5.3.5 Wheat bran leachate (WBL)

Shao et al. (2010) showed that wheat bran leachate (WBL) has an inhibitory effect on *Microcystis aeruginosa.* The study showed that oxygen evolution of *M. aeruginosa* was significantly reduced and intracellular ATP contents became lower with exposure to WBL. In addition, maximum electron transport rate was affected, impairing proper photosynthesis activities and cell lysis was observed.

2.5.3.6 Others

Many plants have been documented to be potential for cyanobacterial controls such as aquatic plants like *Myriophyllum spicatum* (Gross et al., 1996), *Najas marina spp. Intermedia* (Gross et al., 2003), *Phragmites communis* (Li and Hu, 2005), *Stratiotes aloides* (Mulderij et al., 2006) and terrestrial plants like those among families of Apiaceae (Meepagala et al., 2005), Rutaceae (Meepagala et al., 2010), Asteraceae (Ni et al., 2011) and Ephedraceae (Yan et al., 2012). Besides the whole plant, studies

on plant individual parts inhibitory potential were also done such as *Moringa oleifera* seeds (Lurling and Beekman, 2010), plants of the poppy family, Papaveraceae (Jančula et al., 2007), stem or leaves of nine oak species (Park et al., 2006b) and fresh mandarine skin and banana peel (Chen et al., 2004) have also been shown to be effective in controlling cyanobacterial populations.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Chemicals and raw materials

3.1.1 Cyanobacterial Growth Media (BG 11)

Two types of medium were used to culture cyanobacteria in this study, BG 11+N, with sodium nitrate and BG 11-N, without sodium nitrate (Stanier et al., 1971). Stock solutions were prepared using chemicals listed in Table 3.1 and 3.2. Liquid media were prepared by adding the appropriate volume of stock to 1L Duran laboratory bottle and topped up to 1L using distilled water and sterilized by autoclaving at 15 psi (121 ℃) for 15 minutes. Solid media were prepared by adding 1.5% molten agar (Merck) to the liquid media and autoclaved. The agar solution was then allowed to cool down to a temperature of around 60 ℃ before being poured into Petri dishes.

3.1.2 TAE Buffer

A 50x strength stock of TAE buffer was prepared according to Green et al. (2010). The components are shown in Table 3.3. To make a 1X strength solution, 20 mL of the concentrate was added to 980 mL water.

Table 3.1 Components of BG-11 stock solutions and volume required to make standard media

Chemical	**g/L (final concentration)**	**g in 250 mL H_2O for stock solution**	**mL of stock per litre medium**
$NaNO_3$*	1.5	75	5
$K_2HPO_4.3H_2O$	0.04	10	1
$MgSO_4.7H_2O$	0.075	18.75	1
$CaCl_2.2H_2O$	0.036	9	1
Citric Acid	0.006	1.5	1
Ferric Ammonium Citrate	0.006	1.5	1
EDTA	0.001	0.25	1
Na_2CO_3	0.02	5	1
A6 Microelements			1

* $NaNO_3$ was added to make BG 11+N, but not for BG 11–N media

Table 3.2 Composition of A6 microelements stock solution

A6 Microelements	**g/L for stock**
H_3BO_3	2.86
$MnCl_2.4H_2O$	1.81
$ZnSO_4.7H_2O$	0.222
$Na_2MoO_4.2H_2O$	0.391
$CuSO_4.5H_2O$	0.079
$Co(NO_3)_2.6H_2O$	0.049

Table 3.3 Composition of TAE buffer (pH 8)

Component	g/L medium	mL per litre medium
Tris base	242.0	n.a.
Glacial acetic acid	n.a.	57.1
di-sodium EDTA	18.61	n.a.

3.1.3 Cyanobacterial strain

Cyanobacterial strains used in this study were isolated from water bodies in Penang, Malaysia namely Harapan Lake, Aman Lake, Air Itam Dam and Teluk Bahang Dam. All cultures were non-axenic unicyanobacterial.

3.1.4 Oil palm trunk and empty fruit bunches

Oil palm trunk and empty fruit bunches obtained from local supplier were dried for several weeks before being used for experiments. Oil palm trunk was chopped into chunks of 2 cm and ground using a grinder into powder (Figure 3.1). Empty fruit bunch was sliced into smaller form and ground into powder (Figure 3.2). Both powders were passed through 1mm pore size sieve before being used.

3.1.5 Sugarcane bagasse

Sugarcane bagasse obtained from local supplier was dried and cut into shorter length of 5 cm prior to application (Figure 3.3).

Figure 3.1 Oil palm trunk chunk (left) and powder (right) used for experiment

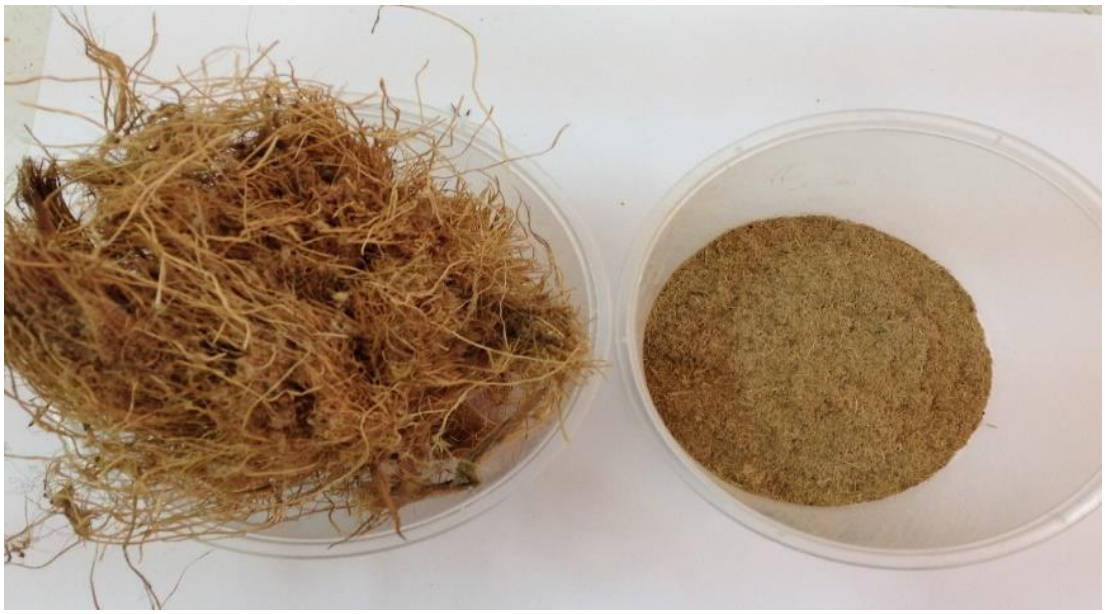

Figure 3.2 Empty fruit bunch (left) and powder (right) used for experiment