FUNGI *Lichtheimia* SP. ENHANCED PALM OIL TRUNK AS CYANOBACTERIAL BIO-CONTROL

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ABSTRACT

Efficient cyanobacterial bloom management is important because a bloom in a water body may cause problems such as unpleasant odour and taste, and most importantly, toxins production that are potentially fatal to human and animals. Previous researches have shown that various plants, including barley straw were able to inhibit the growth of cyanobacteria. It was also showed that the ability of barley straw to control cyanobacteria might likely involved complex microbial degradation. Therefore, experiments were set up to test the effect of fungi-degraded palm oil trunk on cyanobacterial growth. In the study, 1 g of palm oil trunk was pre-treated with fungus Lichtheimia sp for 30 days to allow degradation to occur. After the incubation, the fresh and degraded palm oil trunk was introduced to cyanobacterial culture for 30 days. Cyanobacterial growth was measured based on chlorophyll a concentration. This studies showed an increase ability of fungi-degraded palm oil trunks in inhibiting cyanobacterial growth. The results strengthened the theory of involvement of microbial degradation in controlling cyanobacterial growth.

Keywords: Biological control, cyanobacteria, fungal degradation, palm oil trunk.

INTRODUCTION

Although cyanobacteria are important as a potential source for renewable energy, biofertilizers, and for facilitation in degradation of complex organic compounds such as oil and herbicides [1], excessive growth of cyanobacteria may forms blooms in the water. The blooms may cause several problems such as unpleasant odours and taste, and, most importantly, toxin production. Presence of toxic bloom was recorded earliest by Francis [2]. The cyanobacterial toxins are generally categorized into 4 major groups based on its toxicology effects, namely hepatotoxic toxin (microcystin and nodularin), neurotoxin (anatoxin-a, anatoxin-a(s), and saxitoxin), cytotoxic toxin (cylindrospermopsin) and dermatotoxin (aplysiatoxins, lyngbyatoxin-A) [3]. Consumption or direct contact with these toxins has caused severe health consequences. For examples, microcystin leads to the death of dialysis patients in Brazil [4], hospitalisation of 148 children in the Palm Island, Australia due to cylindrospermopsin toxication [5], and several cases of animal death [6, 7].

Increasing concern on harmful and unpleasant cyanobacterial blooming in the freshwater environment leads to extensive researches on efficient cyanobacterial growth control. Currently, the most widely used chemical for water treatment, copper (II) sulphate ($CuSO_4$) has harmed a wide spectrum of species, risking a secondary pollution in the water environment [8]. While physical treatments such as sedimentation has lower secondary pollution risk, the treatments can injure other organisms and are usually energy consuming and expensive [8]. Hence, more scientists are in search of biological-derived treatments as an alternative.

So far, the most effective and researched cyanobacterial bio-control is barley straw. Other researchers also observed inhibition of cyanobacterial growth by different terrestrial plant and herbs, such as sugarcane bagasse, palm oil trunk [9] and oak trees [10], aquatic plant such as *Myriophyllum spicatum* [11] and *Hydrilla verticillata* [12]. Although many of the studies showed biological-derived compounds to be effective, several studies indicate that effectiveness depended on cyanobacterial species. For instance, palm oil trunk able to inhibit *Microcystis* sp. effectively, but unable to inhibit the growth of *Synechocystis minuscula* [9]. Similarly, various studies on barley straw also indicate dependent of cyanobacterial species [13]. Types of barley straw also showed different effectiveness in inhibiting cyanobacteria [14]. Researches have also hypothesized that the composition and complexity of microbial in degrading lignin in barley straw influenced its capability to control algae growth, such as a study conducted by Murray, Parsons [15] using barley straw pre-treated with fungus. However, currently, information on if in fact, similar to barley straw, fungus also able to assist palm oil trunk in inhibition of cyanobacteria is not known. Therefore, experimentations were set up to test the effect of fungi-degraded palm oil trunk in compare to fresh palm oil trunk on growth of four cyanobacterial species.

METHODOLOGY

Materials

Cyanobacterial strains were collected and identified from Dr. Japareng's laboratory, School of Industrial Technology, Universiti Sains Malaysia (USM), Penang, Malaysia. Four isolated cyanobacteria species were selected. *Synechococcus* sp., *Synecocystis* sp. and *Planktothrix* sp. isolated from Teluk Bahang (TB) dam, Penang, Malaysia, and *Pseudanabaena* sp. isolated from Ayer Itam (AI) reservoir, Penang Malaysia. All species were cultured and maintained in conical flask containing sterile 100 mL BG 11 liquid media.

Fresh palm oil trunk obtained from local supplier used in the study was kindly obtained from Professor Rokiah Hashim, from School of Industrial Technology, USM and dried at 37℃ for one week. The palm oil trunk was degraded by fungus by incubating 1 cm of fungus plug with the palm oil trunk for 30 days to allow degradation to occur.

Lichtheimia sp. fungus was isolated from fruit brunch by Sim Yi Jing from the School of Industrial Technology was selected for the study, and was identified using morphological and molecular approaches. The fungus was periodically

re-cultured by transferring small amount of stock fungus using inoculating loop into a new potato dextrose agar (PDA) media. The culture was then incubated at 37℃.

Maintenance of Cyanobacteria

All cyanobacteria species were maintained in 250 mL conical flask (Fisher) containing 100 mL of autoclaved liquid BG 11 culture medium. Each flask was sealed with cotton wool bungs whilst allowing ventilation. The cultures were placed under the continuous light condition on incubation shaker at 95 rpm at room temperature. For each subculture, 1-2 mL of the previous culture, depending on the density was added into a new medium.

Biological Assays

To determine the effect of fungi-degraded palm oil trunk to the cyanobacterial growth, 5 different tests were carried out. For set up that used fungus, a fungus plug of about 1 cm was cut from 4 days of incubated fungus, while 1 gL^{-1} concentration of palm oil trunk was added in each set up that requires the palm oil trunk.

Test 1: Cyanobacterial growth without treatment (Control group).

Test 2: To observe the direct effect of the fungus to the growth of cyanobacteria. 1 cm fungi plug was added into cyanobacterial culture

Test 3: To observe the effect of palm oil trunk to the growth of cyanobacteria without the presence of the fungus. 1 gL^{-1} of palm oil trunk was added into cyanobacterial culture.

Test 4: To investigate the effect of the palm oil trunk and the fungus without 30 days degradation incubation on cyanobacterial growth.

Test 5: To test the effect of the biological agents that was pre-treated with the fungus plug for 25 - 30 days on the cyanobacterial growth.

Growth of cyanobacteria in laboratory condition were observed for 30 days by transferring 1 – 2 mL of the stock culture, depending on the density into conical flask (Fisher) containing 100 mL of autoclaved liquid BG 11 medium and the biological agents to be tested. Each flask was sealed with cotton wool bungs whilst allowing aeration. The cultures were placed under continuous light condition at approximately 23 μmol/m2/s on an incubation shaker at 95 rpm at room temperature. Growth of each cyanobacterial species was recorded by harvesting the cells for extraction of chlorophyll *a* reading periodically between 24 - 72 hours throughout the 25 - 30 days

Cyanobacterial Growth Measurement

Cyanobacterial population density were measured based on concentration of chlorophyll *a*. In each cell harvest for the extractions, 1 mL of cell culture was centrifuged for 2 min at 10,000 rpm (Eppendorf). Then, 0.5 mL of supernatant was removed. The remaining sample was further centrifuged for two minutes at the same speed. Afterwards, the rest of the supernatant was completely removed. Chlorophyll *a* reading were taken by re-suspended harvested cells in 1 mL of 90% methanol containing 10 mg/L magnesium carbonate ($MgCO_3$) and incubated for one hour at room temperature in the dark. After the incubation, extracted chlorophyll *a* was centrifuged for five minutes at 10,000 rpm. The absorbance of the supernatant was measured at 665 nm by using UV-Visible Spectrophotometer (Shimadzu) using 90% methanol containing 10 mg/L of $MgCO_3$ as reference blank. The chlorophyll *a* content was calculated using the following formula:

$$\text{Chlorophyll } a \text{ content (mg/L)} = OD_{665} \times 12.9447$$

Where OD_{665} = Absorbance at 665 nm, and 12.9447 = Constant

RESULTS

Previous researches hypothesized the ability of barley straw to control cyanobacterial growth is due to lignin composition. However, the effectiveness and efficacy varies depending on cyanobacteria species and type of barley straw. It was also showed that the barley straw after pre-treated with fungi has enhanced ability as algae bio-control. Additionally, previous research also indicates potential of palm oil trunk as bio-control, but with different effectiveness and outcomes. Therefore, the objective of the experiment is to observe the effect of fungus in enhancing control of cyanobacteria by the palm oil trunk.

To achieve the objective, several treatment groups were set up, they were: no treatment (control) group, fungi only group, palm oil trunk only group, and fungi and palm oil trunk group. One to two microliter of pure culture of cyanobacterial strain was added in the treatment groups. Fungus used in the experiment was 1 cm fungi plug of *Lichtheimia* sp. grown for 4 days.

Control of Cyanobacterial Growth by Palm Oil Trunk in the Presence of Fungi

Figure 1 and 2 show growth pattern of *Synechococcus* sp. (TB) and *Planktothrix* sp. in all four treatments mentioned above. The growth patterns in each treatment were observed for 30 days. Growth was measured based on chlorophyll *a* concentration.

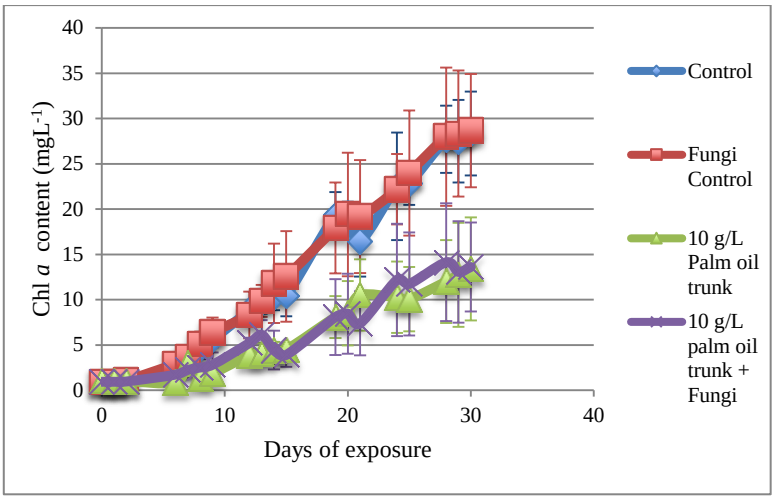

Figure 1. *Synechococcus* sp. (TB) growth in presence of palm oil trunk and fungi in compare to the growth of the cyanobacteria without treatment (Control group). The growth was observed for 30 days at a controlled laboratory conditions. The y-error bars represent standard deviation from the mean.

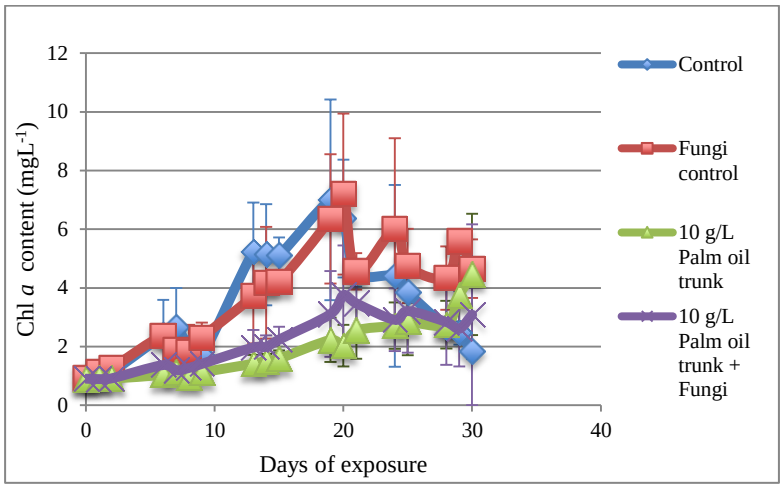

Figure 2. Shows *Planktothrix* sp. growth for 30 days in different treatments and without any treatment. Each treatment was conducted in three replicates, under continuous shaking at 95 rpm at room temperature. The y-error bars represent standard deviation from the mean.

Noted that the growth of *Synechococcus* sp. (TB) and *Planktothrix* sp. in the presence of fungi shows similar growth pattern with the cyanobacterial culture without treatment. The results show that the fungus used in the media unable to utilize the cyanobacteria or BG 11 culture media as nutrient source, and thus do not directly inhibit cyanobacteria growth. While the addition of palm oil trunk reduces the growth of both cyanobacterial strains, the treatment was not able to completely inhibit the cyanobacterial growth. In addition, as the incubation time increases, the growth of cyanobacteria increases. Besides that, the cyanobacterial growth in the palm oil trunk with fungi plug shows no significant differences with the group of palm oil trunk-only, possibly indicates requirement for prior treatment of fungus to the palm oil trunk.

Control of Cyanobacterial Growth by Palm Oil Trunk Pre-Treated with Fungi

As palm oil trunk bioassay in the presence and absence of fungi prior to pre-treatment show no significant differences, the ability of the fungus to enhance anti-cyanobacterial properties of the palm oil trunk was investigated by pre-treating the palm oil trunk with the fungus for 30 days before the bioassay experiment was conducted. In this study, 4 cyanobacterial species were selected: *Synechococcus* sp. (TB), *Planktothrix* sp., *Synechocystis* sp. and *Pseudanabaena* sp. (Figure 3, 4, 5 and 6)

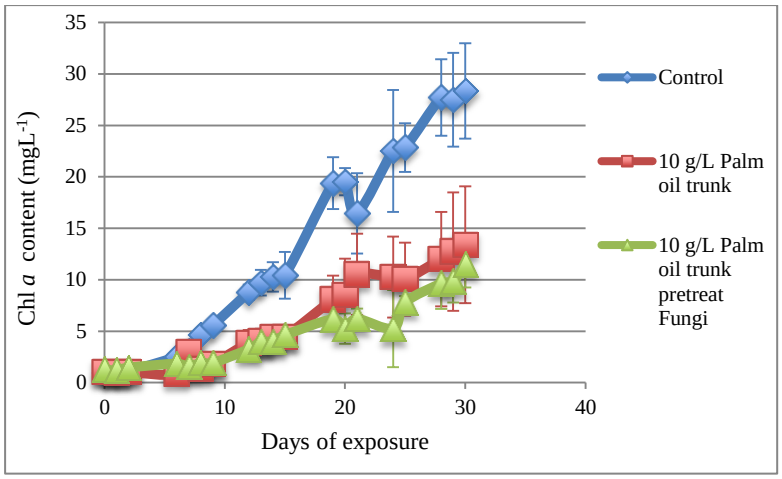

Figure 3. Indicates *Synechococcus* sp. (TB) growth for 30 days in the presence of pre-treated palm oil trunk with fungi in comparison to the control group (no treatment) and in the presence of palm oil trunk. The y-error bars represent standard deviation from the mean.

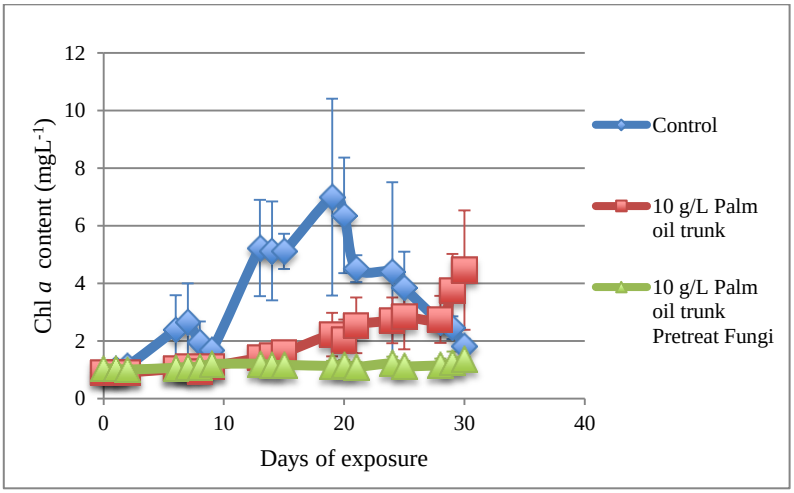

Figure 4. Indicates *Planktothrix* sp. growth for 30 days in the presence of pre-treated palm oil trunk with fungi in comparison to control group (no treatment) and in the presence of palm oil trunk group. The y-error bars represent standard deviation from the mean.

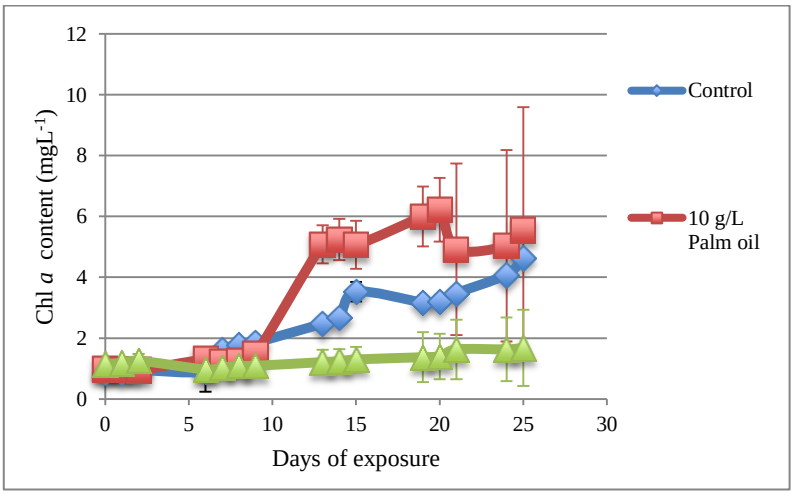

Figure 5. Shows *Synechocystis* sp. growth for 25 days in the presence of pre-treated palm oil trunk with fungi compared to the control condition (no treatment) and in the presence of palm oil trunk. The y-error bars represent standard deviation from the mean.

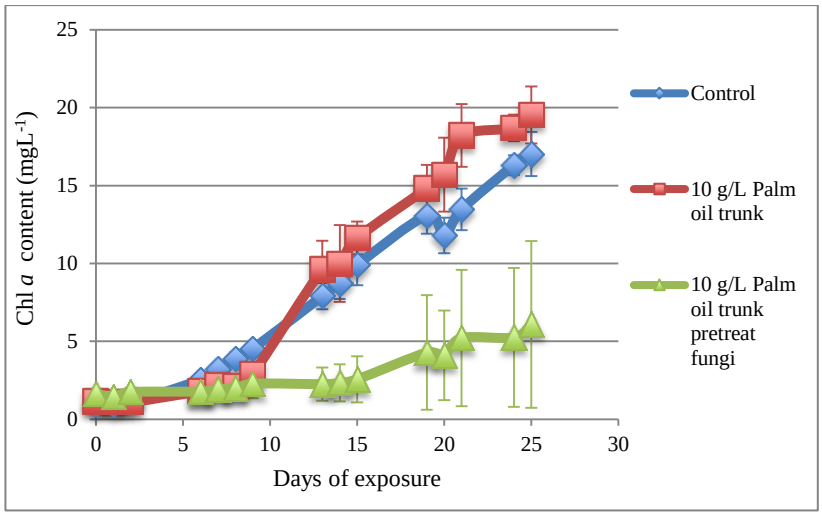

Figure 6 Shows *Pseudanabaena* sp. growth for 25 days in the presence of the pre-treated palm oil trunk with fungi in comparison to the control group (no treatment) and in the presence of palm oil trunk group. The y-error bars represent standard deviation from the mean.

Synechococcus sp. (TB) and *Planktothrix* sp. (Figure 3 and 4 respectively) show no significant differences in the growth pattern between palm oil trunk-treated group and palm oil trunk pre-treat with fungus group. Even so, in both treatments, cyanobacterial growth decreases significantly compared to control group. However, it was also observed that the growth of *Planktothrix* sp. in palm oil trunk-only treatment increases steadily after day 19, which is in the opposite to the control group, where the growth decreases, while the growth of the strain in pre-treated group remained low. And after day 28, it was observed that chlorophyll *a* count in the palm oil trunk-only group is higher than both of the control group and the fungus pre-treated group (Figure 4).

As for *Synechocystis* sp. (Figure 5), all of the three treatment groups show different response, where the presence of palm oil trunk enhances the growth of the strain, whereas palm oil trunk pre-treat with fungus group successfully control the growth of the cyanobacteria. Meanwhile, in *Pseudanabaena* sp. (Figure 6), no significant different was observed between control group and palm oil trunk-only treatment group. However, similar to *Synechocystis* sp., the pre-treated palm oil trunk group shows enhancement in inhibiting the growth of the strain.

DISCUSSION

In general, ideal anti-cyanobacterial compounds are characterized by strong inhibition to cyanobacteria, non-toxic to other organisms, readily degraded in the environment, inexpensive and safe to the environment [8]. Effectiveness of the compounds which is influenced by the hydrophilicity and hydrophobicity is also one of the important characteristic [16]. In addition, ideally, anti-cyanobacterial compounds should be able to inhibit most cyanobacterial species. If the inhibition is species specific, the compounds may enhance the growth of other cyanobacterial species, which is undesirable if the enhanced cyanobacterial species are toxin-producing species [13].

As observed in this study, the presence or absence of fungi plug in control group and palm oil trunk group has no effect on cyanobacterial growth. However, different growth pattern in *Planktothix* sp., *Synechocystis* sp. and *Pseudanabaena* sp. were observed between fresh palm oil trunk group and palm oil trunk pre-treated with fungi group. This indicates that the fungus able to increase the release of anti-cyanobacterial properties of palm oil trunk after the 30 days incubation prior to the bioassays. Although it is best to screen all cyanobacterial strains isolated from Malaysian water environment, it would take much more resources and time. Therefore, from many isolated cyanobacterial strains, four strains are selected. *Synechococcus* sp. and *Synechocystis* sp. were chosen in the experiment as they were recorded as bloom-forming cyanobacteria, meanwhile cyanobacterial species *Planktothrix* sp., and *Pseudanabaena* sp., have been recorded previously as toxin-producing species and therefore, potentially encode toxin-encoding gene cluster.

Based on molecular and morphological results, the fungus used in the study was identified as *Lichtheimia* sp.. A study showed that *Lichtheimia ramose* synthesized β-Glucosidases, carboxymethylcellulase (CMCase), xylanase, and β-xylosidase enzymes that are able to catalyse different enzyme-substrate reactions [17]. Therefore, it is highly likely that the products from the enzymatic actions enhance the control of cyanobacterial growth. However, it is also important to note that the palm oil trunk in the study was not autoclaved prior to 30 days incubation with *Lichtheimia* sp. as it may lead to lose of volatile anti-cyanobacterial compounds. Autoclaving could also lead to destruction of plant-derived active compounds, as depicted for example in *Armesia annua*-derived compound, artemisinin as malaria treatment [18] which was later studied as anti-algae compound [19]. Therefore, when incubation occurred at moisturized conditions, the growth of other microbial may be enhanced as well, and subsequently may also involved in the degradation of the palm oil trunk that leads to cyanobacterial control enhancement. Hence, further research using autoclaved palm oil trunk and pure culture of *Lichtheimia* sp. can be conducted.

Overall, based on the experimental results, incubation of palm oil trunk with fungi for 30 days enhance its ability as cyanobacterial bio-control. The outcomes are similar to previous studies, which showed that incubation of fungi with barley straw enhance inhibition of algae *Scenedesmus* sp. [15] and studies by Lalung [13], which showed communities of microbial in rotten barley straw increases inhibition of cyanobacterial growth. The outcomes show in the study is similar to many

previous researches that showed release of anti-cyanobacterial compounds from plants. However, studies on the isolation of specific compounds and understanding of its mechanisms are limited due to high workload and complexity in separation and characterization of plants compounds [14]. Even so, in 2014, a research group has successfully isolated and proved that two compounds under the group of flavonolignin, namely salcolin a and salcolin b from barley straw act as algistatic and algicidal toward cyanobacteria respectively [14]. As such, there is possibly that the compounds play similar role in the palm oil trunk.

CONCLUSION

Currently, most researches focuses on anti-cyanobacterial compounds derived from waste and plant biomass and so far the most effective and researched cyanobacterial bio-control is barley straw. Several studies have indicated microbial degradation enhances anti-algae ability of barley straw whilst other study indicates variable degree of palm oil trunk effectiveness in controlling cyanobacterial growth. In this study, fungi pre-treated palm oil trunk shows higher effectiveness as bio-control in compare to fresh palm oil trunk, indicating involvement of microbial in enhancing the release of anti-cyanobacterial compounds from the palm oil trunk. For future direction, research can be conducted at environmental scale, as laboratory scale is too simple to reflect actual circumstances. Evaluation of toxicity of the compounds to other organisms should be also conducted and further evaluations on the active compounds of anti-cyanobacteria by using fractionated techniques such as high performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR) spectroscopy or gas chromatography mass spectrometry (GC-MS) for further understanding on the molecules and mechanisms.

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