



First report of anatoxin-a encoding gene in isolated cyanobacterial strains from Malaysia

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ABSTRACT

Aims: This study focus on the presence of cyanobacterial toxin in Malaysia and anatoxin-a-encoding gene was detected in this study and the status of cyanobacterial toxins in Malaysia can now be clarified.

Methodology and results: As part of status determination of cyanobacterial toxins in Malaysia, cyanobacterial strains have been isolated from different environments and identified using cyanobacterial 16S rRNA gene sequence. PCR assay was carried out to detect the presence of cyanobacterial toxin-encoding genes in these isolated strains by amplifying genes encoded for microcystin, anatoxin-a, cylindrospermopsin and saxitoxin. Using molecular identification of 16S rRNA gene sequences, a total of forty-two cyanobacterial strains were identified, which belongs to eighteen different genera of *Synechococcus*, *Cyanobium*, *Synechocystis*, *Chroococcidiopsis*, *Leptolyngbya*, *Nodosilinea*, *Limnothrix*, *Pseudanabaena*, *Cephalothrix*, *Aerosakkonema*, *Oscillatoria*, *Alkalinema*, *Pantanalinema*, *Planktolyngbya*, *Scytonema*, *Nostoc*, *Hapalosiphon* and *Symphyonemopsis*. The toxicity of these strains was tested using PCR amplification of toxin-encoding genes using specific primers.

Conclusion, significance and impact of study: Anatoxin-a (ATX) gene, which involved in the biosynthesis of anatoxin-A was detected in two isolated strains namely *Limnothrix* sp. B15 and *Leptolyngbya* sp. D1C10. This study focus on the the presence of cyanobacterial toxin in Malaysia can now be determined as potential threat because anatoxin-a-encoding gene was detected in this study and the status of cyanobacterial toxins in Malaysia can now be clarified.

Keywords: anatoxin-a, cyanobacterial 16S rRNA, cyanobacterial toxins, PCR amplification, toxin-encoding gene

INTRODUCTION

Cyanobacteria also known as blue-green algae are photosynthetic prokaryotic algae and can be found in most of water column. Cyanobacteria can multiply rapidly in water surface and form blooms during favorable conditions. Cyanobacterial blooms can be found in eutrophic water bodies of freshwater, estuarine as well as marine ecosystem. Species responsible forming cyanobacterial blooms in freshwater ecosystem mainly consisted of *Microcystis*, *Anabaena* or *Cylindrospermopsis*, while estuarine ecosystems mainly consisted of *Nodularia* and *Aphanizomenon*. Cyanobacterial blooms in marine ecosystem consisted of *Lyngbya*, *Synechococcus* and *Trichodesmium* (O'neil *et al.*, 2012).

Cyanobacterial blooms can degrade water quality causing foul odors and tastes, deoxygenation of bottom waters, toxicity, aquatics kills and disruption of food web (WHO, 1999). They also produce toxins known as

cyanotoxins that can severely affect animals as well as human health (Paerl *et al.*, 2001). A wide range of toxins produced by cyanobacteria can be classified into few distinct categories in terms of their structure and mode of action. The toxins can be divided into hepatotoxins; microcystins and nodularins, cytotoxins; cylindrospermopsins, neurotoxins; anatoxins and saxitoxin, and dermatotoxins; lyngbyatoxins (Dittmann *et al.*, 2012). Problems with these toxins are they persists in the water even after the algal blooms collapsed making the water unsuitable even for irrigation

Identification of cyanobacterial strains based on morphological characteristics and 16S rRNA gene sequences can be used to detect the presence of cyanobacteria in the samples. Although these approaches enable the identification of unknown strains, these are limited to only taxonomic characterization without the recognition of strains being toxin or non-toxin producers. This is because production of cyanotoxin is not species specific but gene-specific. Thus, detection of toxin-

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encoding gene using PCR amplification of specific sequences is likely the best options.

MATERIALS AND METHODS

Sampling locations

The sampling locations in this study involved three states in Malaysia which were Penang, Perak and Sarawak due to the easy accessibility to the sampling locations. Sampling points were selected based on the place that potentially exposed to human activity either for recreational or drinking water purposes. The Air Itam Dam, Teluk Bahang Dam, Waterfall Reservoir, Mengkuang Dam, Banding Lake, Teluk Intan, Kuala Gula Sanctuary, Bukit Merah Lake and Miri, Sarawak.

Isolation and culturing of cyanobacterial strains

Cyanobacteria present in the sample materials were isolated by numerous streaking on BG 11 full strength agar plates until single species was obtained. Unialgal isolates were maintained in sterile liquid or slanted agar BG 11 media at ambient temperature.

Extraction and purification of genomic DNA

DNA from the sample was extracted using a commercial kit; Bacterial DNA Extraction Kits (Vivantis Technologies, Malaysia). The procedures were carried out according to the handbook provided by the manufacturer. Extracted DNA was stored at -20°C to prevent DNA degradation in the absence of buffering agent.

Molecular identification using cyanobacterial 16S rRNA gene

Isolated strains were identified using 16S rRNA gene sequence analysis using primer pairs CYA106F, CYA781R(a) and CYA781R(b). PCR protocols to amplify cyanobacterial 16S rRNA involved an initial denaturation for 2 min at 95°C ; followed by 30 cycles, each consisting of 60 sec at 94°C , 60 sec at 60°C , and 60 sec at 72°C ; and a final extension of 7 min at 72°C (Nübel *et al.*, 1997).

Molecular detection of toxin-encoding genes

The samples were tested for the presence of generic microcystin (*mcyE*) gene, polyketide synthase (PKS) and peptide synthetase (PS) genes, genes encoding anatoxin-a, cylindrospermopsin and saxitoxin. Generic microcystin (*mcyE*) gene amplified using forward primer; *mcyE*-F2 combined with reverse primer; *mcyE*-R4 (Rantala *et al.*, 2004; Rantala *et al.*, 2006). PCR protocols was performed as followed: An initial denaturation step of 2 min at 95°C followed by 35 cycles of 30 sec at 94°C , 30 s at 56°C and 60 sec at 72°C and a final extension of 10 min at 72°C (Rantala *et al.*, 2006). Polyketide synthase regions (PKS) gene was amplified using degenerate

oligonucleotide forward primer; DKF and reverse primer; DKR (Moffitt and Neilan, 2011). PCR protocols to amplify PKS gene involved an initial denaturation for 2 min at 95°C ; followed by 30 cycles, each consisting of 10 sec at 94°C , 20 sec at 50°C , and 60 s at 72°C ; and a final extension of 7 min at 72°C (Schembri *et al.*, 2001). For samples positive general PKS gene, PKS specific primer pairs were used to detect genes encodes for anatoxin-a and cylindrospermopsin. The PKS specific primer pair of *atxoa*f and *atxar* was used to amplify the gene encodes for the PKS fragment of the putative anatoxin-a (ATX) biosynthesis gene cluster. PCR protocols was performed as followed: The first step was an initial denaturation step of 2 min at 95°C followed by 30 cycles of 10 sec at 94°C , 20 sec at 55°C and 60 sec at 72°C and a final extension of 10 min at 72°C (Ballot *et al.*, 2010a). Another PKS specific-cylindrospermopsin primers, M4 and M5 were used to detect the presence of cylindrospermopsin gene. PCR protocols was performed as followed: The first step was an initial denaturation step of 2 min at 95°C followed by 30 cycles of 10 sec at 94°C , 20 sec at 55°C and 60 sec at 72°C and a final extension of 7 min at 72°C (Sarma, 2012). Peptide synthetase (PS) degenerate primers; MTF2 and MTR were used to amplify the general PS regions. PCR protocols was performed as followed: The first step was an initial denaturation step of 4 minutes at 94°C followed by 30 cycles of 10 s at 94°C , 20 sec at 50°C and 60 sec at 72°C and a final extension of 7 min at 72°C (Schembri *et al.*, 2001). Primers pair of *sxta*f and *sxta*r used to amplify saxitoxin (*sxtA*) gene cluster. PCR protocols was performed as followed: The first step was an initial denaturation step of 5 min at 94°C followed by 30 cycles of 10 sec at 94°C , 20 sec at 55°C and 60 sec at 72°C and a final extension of 10 min at 72°C (Ballot *et al.*, 2010b). All PCR amplifications were performed with a Mastercycler® ep PCR System (Eppendorf, Germany). PCR reaction were carried out in 25 μL reaction mixtures containing 12.5 μL of 2x Taq Master Mix (Vivantis Technologies, Malaysia), 0.25 μL of each forward and reverse primers and 2 μL of DNA sample combined with sterile distilled water to make up a volume of 25 μL of total reaction. 2x Taq Master Mix consisted of 0.05 u/ μL , 2x Vbuffer A, 0.4 mM dNTPs and 3.0 mM MgCl_2 . All primers used in this study were synthesized by Integrated DNA Technologies, US.

PCR products were loaded on 1% agarose gel that was prepared by adding 0.25 g of agarose (Vivantis Technologies, Malaysia) to 25 mL of 1x TBE buffer. 2.0 μL of gel stain (TransGen Biotech Co., Ltd, Beijing) added into the hot agar. 2 μL of 6x loading dye (Vivantis Technologies, Malaysia) added into the PCR products. Gels were running at 70 V for 40 min and viewed using a gel imaging system (Gel Doc™ XR+ camera, Image Lab™ software, BioRad) and the gel image was captured.

Purification of PCR product and sequencing

To purify DNA from the PCR product, a commercial kit; Ambiclean Kits – PCR & Gel (Vivantis Technologies, Malaysia) was used. The procedures were carried out

according to handbook provided by the manufacturer. The purified was sent to Center for Chemical Biology, USM (CCB) for sequencing using the same primers used for PCR amplification. Sequence similarities between 16S rRNA gene sequences were compared with the available data from National Centre for Biotechnology Information (NCBI) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) using nucleotide basic local alignment search tool (BLAST).

RESULTS

Identification of isolated cyanobacterial strains using molecular analysis

Total of forty-two strains were isolated from different locations in Malaysia. The isolated stains were unialgal but nonaxenic Molecular identification was done due to limitation of morphological identification. In this study, a combination of primer pair, CYA106F, CYA781R(a) and

CYA781R(b) were used to amplify a 654-699 bp of DNA fragment of isolated species. The PCR products were run by agarose gel electrophoresis and viewed under UV light to ensure that the gene had been amplified and produced a right size for cyanobacterial 16S rRNA gene. The PCR products were purified and sequenced. The similarities of the isolated species with the species available in NCBI GenBank was compared for the molecular identification.

Detection of toxin-encoding genes in isolated strains

All forty-two isolated strains had been identified using cyanobacterial 16S rRNA gene. Due to the presence of toxin-encoding gene in some environmental samples and some the strains have history of producing some cyanotoxins these strains were tested for the presence of the toxin-encoding genes. Results for detection of toxin-encoding genes in isolated strains are shown in Table 1.

Table 1: Summary results for detection of toxin-encoding genes in isolated strains.

Cyanobacterial strains	Generic microcystin (<i>mcyE</i>) gene	Generic PKS gene	PKS specific-cylindrospermopsin (CYN) gene	PKS specific-anatoxin (ATX) gene	Generic PS gene	saxitoxin (<i>sxtA</i>) gene
<i>Leptolyngbya subtilissima</i> EcFYyyy700	-	-	n.a	n.a	-	-
<i>Leptolyngbya</i> sp. Tir_cyanD	-	-	n.a	n.a	-	-
<i>Leptolyngbya</i> sp. CENA520	-	-	n.a	n.a	-	-
<i>Nodosilinea nodulosa</i> UTEX 2910	-	-	n.a	n.a	-	-
<i>Nodosilinea</i> cf. <i>nodulosa</i> LEGE 10377	-	-	n.a	n.a	-	-
<i>Nodosilinea</i> sp. IS-EAG2	-	-	n.a	n.a	-	-
<i>Limnothrix redekei</i> BTA657	-	-	n.a	n.a	-	-
<i>Limnothrix planktonica</i> CHAB763	-	+	-	-	-	-
<i>Limnothrix</i> sp. B15	-	+	-	+	-	-
<i>Limnothrix redekei</i> BTA65	-	-	n.a	n.a	-	-
<i>Limnothrix</i> sp. PUPCCC 116.2	-	-	n.a	n.a	-	-
<i>Pseudanabaena thermalis</i> PUPCCC 106.4	-	-	n.a	n.a	-	-
<i>Pseudanabaena</i> sp. 40C_1_20	-	-	n.a	n.a	-	-
<i>Pseudanabaena mucicola</i> PMC269.06	-	-	n.a	n.a	-	-
<i>Pseudanabaena limnetica</i> PUPCCC 106.2	-	-	n.a	n.a	-	-
<i>Cephalothrix lacustris</i> CCIBt 3261	-	-	n.a	n.a	-	-
<i>Cephalothrix komarekiana</i> SAG 75.79	-	-	n.a	n.a	-	-
<i>Aerosakkonema uniforme</i> strain Lao26	-	-	n.a	n.a	-	-
<i>Oscillatoria</i> sp. OF9	-	-	n.a	n.a	-	-
<i>Oscillatoriales</i> cyanobacterium OF9	-	-	n.a	n.a	-	-
<i>Oscillatoria acuminata</i> PCC 6304	-	-	n.a	n.a	-	-
<i>Alkalinema pantanalense</i> CENA531	-	-	n.a	n.a	+	-
<i>Pantanalinema rosaneae</i> CENA539	-	-	n.a	n.a	-	-
<i>Planktolyngbya circumcreta</i> CHAB5683	-	-	n.a	n.a	+	-
<i>Scytonema</i> sp. U-3-3	-	-	n.a	n.a	-	-
<i>Hapalosiphon welwitschii</i> M5	-	-	n.a	n.a	-	-
<i>Westiellopsis</i> sp. NQAIF324	-	-	n.a	n.a	-	-
<i>Mastigocladopsis</i> sp. CCG2	-	-	n.a	n.a	-	-
<i>Nostoc</i> sp. TO1S01	-	-	n.a	n.a	+	-

'+' indicates the positive result while '-' indicates the negative result and 'n.a' indicates no reaction was carried out.

Detection of generic polyketide synthase (PKS) gene in isolated strains

All forty-two isolated strains were tested for the presence of generic polyketide synthase (PKS) gene using the same degenerate primer pair of DKF and DKR. Amplification of this gene generated a DNA fragment of 650 bp. Among forty-two isolated strains tested, only three strains were positive to the generic PKS gene namely *Leptolyngbya* sp. D1C10, *L. planktonica* CHAB763 and *Limnothrix* sp. B15 (Table 1). Amplification of generic PKS gene in these isolated strains was shown in Figure 1.

PCR product positive to generic PKS gene for both strains *Leptolyngbya* sp. D1C10 and *Limnothrix* sp. B15 were purified and sent for sequencing. Sequences obtained from the sequencing analysis were compared to the available database in NCBI using BLAST. Sequences with query length of 653 bp and 582 bp shows highest percentage similarity of 74% and 79% that identical to *Pseudanabaena* cf. *curta* LEGE 07160 clone 1 polyketide synthase gene and *Oscillatoria* sp. PCC 6506 anatoxin-a and homoanatoxin-a biosynthetic gene cluster (sequence data not shown).

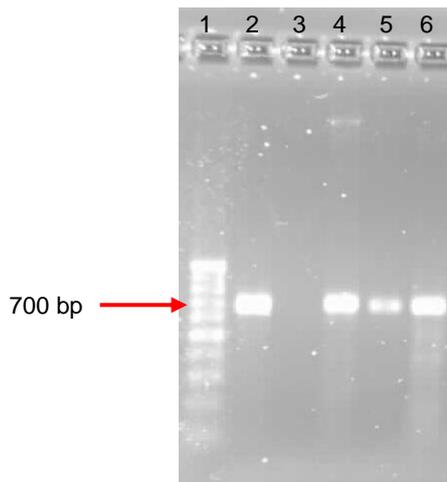


Figure 1: Agarose gel electrophoresis image of 650 bp of PCR product amplified using PKS-degenerate primer pair; DKF and DKR on DNA extracts from isolated cyanobacterial strains. 1 = Ladder (VC 100bp); 2 = Positive control; 3 = Negative control; 4 = *Leptolyngbya* sp. D1C10; 5 = *Limnothrix* sp. B15 and 6 = *Limnothrix planktonica* CHAB763.

Detection of PKS specific-anatoxin (ATX) and cylindrospermopsin (CYN) genes in isolated strains

Following the presence of generic PKS gene, *Leptolyngbya* sp. D1C10, *L. planktonica* CHAB763 and *Limnothrix* sp. B15 were tested to detect the presence of PKS specific-anatoxin (ATX) and cylindrospermopsin (CYN) genes. Amplification of both PKS specific gene generated DNA fragment of 434 bp and 650-725 bp for PKS specific to ATX and CYN genes, respectively. PKS

specific-anatoxin (ATX) gene was detected in two strains out of three tested strains namely *Leptolyngbya* sp. D1C10 and *Limnothrix* sp. B15 (Figure 2). For PKS specific-cylindrospermopsin (CYN) gene, none of these strains was detected for the presence of this gene as DNA was not visible on the gel electrophoresis images (data not shown).

PCR product positive to PKS specific-anatoxin (ATX) gene for both strains *Leptolyngbya* sp. D1C10 and *Limnothrix* sp. B15 were purified and sequenced. Sequences obtained from the sequencing analysis were compared to the available database in NCBI using BLAST. Sequences with query length of 453 bp and 437 bp showed highest percentage similarity of 100% and 94% that identical to *Anabaena* sp. 37 anatoxin-a synthetase gene cluster and *Oscillatoria* sp. PCC 6506 anatoxin-a and homoanatoxin-a biosynthetic gene cluster (sequence data not shown).

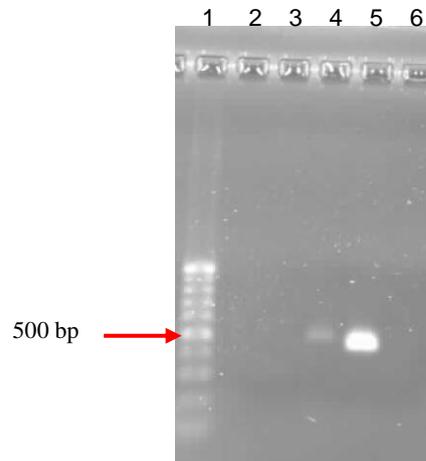


Figure 2: Agarose gel electrophoresis image of 434 bp of PCR product amplified using PKS-specific to anatoxin (ATX) gene primer pair; atxoaF and atxar on DNA extracts from isolated cyanobacterial strains. DNA was visible for *Leptolyngbya* sp. D1C10 (Lane 4) and *Limnothrix* sp. B15 (Lane 5). 1 = Ladder (VC 100bp); 2 = Empty; 3 = Negative control; 4 = *Leptolyngbya* sp. D1C10; 5 = *Limnothrix* sp. B15; 6 = *Limnothrix planktonica* CHAB763.

Detection of generic peptide synthetase (PS) gene in isolated strains

All isolated strains were also tested for the presence of generic peptide synthetase (PS) gene. Amplification of generic PS gene generate a DNA fragment of 1150 bp. Among forty-two isolated strains, four of them was positive for the detection of generic PS gene (Figure 3). These strains namely *Leptolyngbya* sp. D1C10, *Alkalinema pantanalense* CENA531, *Planktolyngbya circumcreta* CHAB5683 and *Nostoc* sp. TO1S01. While other strains tested negative for the detection of generic PS gene (Table 1). This positive result indicated the presence of the generic PS gene in the isolated strains.

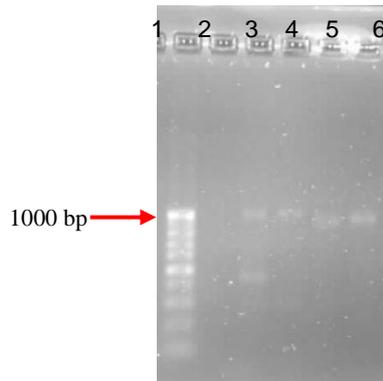


Figure 3: Agarose gel electrophoresis image of 1150 bp of PCR product amplified using PS degenerate primer pair; MTF2 and MTR primer; on DNA extracts from isolated cyanobacterial strains. 1 = Ladder (VC 100bp); 2 = Negative control; 3 = *Leptolyngbya* sp. D1C10; 4 = *Alkalinema pantanalense* CENA531; 5 = *Planktolyngbya circumcreta* CHAB5683; 6 = *Nostoc* sp. TO1S01.

Detection of microcystin (*mcyE*) gene and saxitoxin (*sxtA*) gene in isolated strains

All isolated strains were tested for the presence of generic microcystin (*mcyE*) gene and saxitoxin (*sxtA*) gene cluster. Amplification of *mcyE* gene using primer pair of *mcyE*-F2 and *mcyE*-R4 generate a DNA fragment of 809-812 bp, while amplification of saxitoxin (*sxtA*) gene cluster generated a DNA fragment of 650 bp using primer pairs of *sxtA*-F and *sxtA*-R. None of them tested positive for microcystin (*mcyE*) and saxitoxin (*sxtA*) gene (Table 1). Thus, the negative results indicate that these genes do not present in the isolated strains.

DISCUSSION

Toxin and non-toxin producing cyanobacterial strains can be distinguished using specific primers to amplify the presence of toxin-encoding genes. Some strains were reported to be non-toxic but has never been tested. Thus, this study was carried out to test the different species and confirming the negative results. Many cyanobacterial strains have not been reported to produce toxin, but no studies have been carried out to prove that the strains do not possess the toxin producing gene. The status of toxin producing in some cyanobacterial can now be clarified through this study.

16S rRNA gene can be used to detect the presence cyanobacteria in a sample as well as to identify unknown cyanobacterial. However, the method does not distinguish between toxic and non-toxic bacteria (Neilan *et al.*, 1997). Toxin production in cyanobacterial is gene-specific rather than species-specific (do Carmo Bittencourt-Oliveira, 2003), thus identifying the species does not indicates the status of the toxin in the strains.

From the results out of forty-two isolated strains, anatoxin-a (ATX) gene was detected in two strains

isolated from Malaysia. This gene was detected in two strains namely *Limnothrix* sp. B15 and *Leptolyngbya* sp. D1C10, while other strains shows the absence of toxin-encoding gene. This is the first times, anatoxin-a gene was detected in Malaysia. A PCR analysis was specifically developed for detection of a polyketide synthase (PKS) gene fragment of putative anatoxin biosynthesis gene clusters in both *Limnothrix* sp. B15 and *Leptolyngbya* sp. D1C10 strains.

Generic PKS gene was also detected in *Limnothrix planktonica* CHAB763, but tested negative for detection of PKS specific to anatoxin-a and cylindrospermopsin genes. From the sequence of the amplified PCR product of generic PKS gene, showed similarity of 85% to *Phormidium ectocarpi* SAG 60.90 clone 2 type I polyketide synthase gene. This result confirmed the presence of PKS gene in the samples, but none specific toxin was given, indicated that the gene might be new toxin-encoding gene which were not detected in this study. PS gene was also detected in four cyanobacterial strains namely *Leptolyngbya* sp. D1C10, *Alkalinema pantanalense* CENA531, *Planktolyngbya circumcreta* CHAB5683 and *Nostoc* sp. TO1S01. Both generic PKS and PS genes were detected in *Leptolyngbya* sp. D1C10. This result implies that both PKS and PS cluster genes were responsible for the production of anatoxin-a (ATX) gene.

Several genera of *Anabaena*, *Anabaenopsis*, *Aphanocapsa*, *Arthrospira*, *Hapalosiphon*, *Microcystis*, *Nostoc*, *Oscillatoria*, *Planktothrix*, *Snowella*, and *Woronichinia* has been described to produce hepatotoxic microcystin (Stewart *et al.*, 2006). Although *Hapalosiphon*, *Nostoc*, and *Oscillatoria* were identified in this study, these strains were non-toxic due to the negative result obtained *mcyE* gene was responsible for the biosynthesis of microcystin. *Anabaena*, *Aphanizomenon*, *Arthrospira*, *Cylindrospermum*, *Microcystis*, *Oscillatoria*, *Phormidium*, *Planktothrix* and *Raphidiopsis* were known to produce anatoxin-a (Stewart *et al.*, 2006). Most of these anatoxin-a producers was absence in the study, except two strains were identified as *Oscillatoria* spp. by 16S rRNA gene sequence analysis, but detection of anatoxin-a gene showed negative result. Thus, this strain can be determined as non-toxic. Cylindrospermopsin production has been identified in several genera of *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Raphidiopsis*, and *Umezakia* (Stewart *et al.*, 2006). But none of these toxic genera were identified and from the detection study no cylindrospermopsin gene was detected as well, thus it can conclude that no cylindrospermopsin gene was present in these isolated strains. Saxitoxin production has been reported in genera of *Anabaena*, *Aphanizomenon*, *Lyngbya*, *Planktothrix* and *Cylindrospermopsis* (Stewart *et al.*, 2006). Thus, according to negative result for the detection of this gene can proved as none of these genera were isolated and identified in this study. These results can be used to confirm to absence of these saxitoxin gene in other cyanobacterial genera.

The data presented for the detection of toxin-encoding gene can be proved that that production of toxin in cyanobacteria was gene-specific, not species-specific.

Although some species or genera have histories of toxin production, but detection of toxin-encoding gene in these strains resulted negative outcomes due to the absence of these genes in the species.

In order to differentiate both toxic and non-toxic cyanobacteria, PCR amplification of specific gene sequence could be the best options. However, there are still some limitation within this approach. This conventional PCR amplification are not able to quantify the numbers of gene present in the samples as well as the toxicity of the toxin-encoding gene. Presence of a toxin-encoding gene does not necessarily mean the existence of the toxin. Some strains might contain a certain toxin-encoding gene, but the toxins are not express due to some of the factors influencing the gene expression.

Toxicity of these gene can be tested using analytical or chemical analysis such as HPLC, GC-MS, immunology method, ELISA and high photodiode arrays detection in confirming and quantifying toxic content in environment (Głowacka *et al.*, 2011). However, most of these detections can only be applied to detect the toxin in the environment. Toxins are easily degraded in the environment and the half-life of these toxins is very short ranging from four to eight hours only, caused the analytic detection is rather difficult.

CONCLUSION

Toxin producing and non-toxin producing cyanobacterial strains can be distinguished using the molecular analysis by amplifying the toxin-encoding genes sequence. PKS specific to anatoxin-a gene was detected in two isolated strains by PCR amplification using specific primers. Potential ability of these strains to produce toxins also been determined using molecular analysis.

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