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Cellular Protein/Peptide Expression Profiles (PEPs): an alternative approach for easy identification of cyanobacterial species

Fred Wang-Fat Lee[1,2], Kin-Chung Ho[2], Daniel Yun-Lam Mak[1], Junrong Liang[3], Changping Chen[3], Yahui Gao[3], Samuel Chun-Lap Lo*[1]

[1]The Proteomic Task Force, Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hong Kong; [2]School of Science and Technology, The Open University of Hong Kong, Hong Kong; [3]Key Laboratory of the Ministry of Education for Coastal and Wetland Ecosystems, and School of Life Sciences, Xiamen University, Xiamen 361005, China.

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ABSTRACT

Cyanobacterial harmful algal blooms (CyanoHABs) are recognized as an expanding and serious global problem that threatens human health. Timely and accurate identification of cyanobacteria is of vital importance for public health. Morphologic characteristics of cyanobacteria have been used for classical taxonomic studies and identification purposes. However, misidentification may occur either due to subjective judgment by the operators or inability to recognize natural variations of morphotypes. To circumvent problems of morphology-based identification methods, we reported previously a rapid and simple method for the identification of dinoflagellates using protein/peptide expression profiles (PEPs) of whole cell protein extracts generated by MALDI-TOF-MS (Lee FWF *et. al*., 2008). In the present study, we applied this method in the identification of harmful cyanobacteria. Our results showed that various species of the cyanobacteria can be easily distinguished from each other using their PEPs.

Keywords: Cyanobacteria; Harmful Algal Bloom; Identification; MALDI-TOF-MS; Protein Expression Profiles (PEPs).

Abbreviations

CHCA: α-cyano-4-hydroxycinnamic acid; **DHB**: 2,5-dihydroxybenzoic acid; **HABs**: Harmful Algal Blooms; **PEPs**: Protein Expression Profiles; **SA**: sinapinic acid.

1. Introduction

Cyanobacteria, also known as blue-green algae, play an important role as primary producers of the food web [1, 2]. Most of them are single-celled organisms that can be found in almost every conceivable environment, from lakes, ponds, rivers brackish and marine waters, bare rock to soil throughout the world. Cyanobacteria can also grow extensively, resulting in harmful algal blooms (HABs) that can cause serious negative impacts on human health and the aquatic ecosystems. Some of the cyanobacteria produce powerful toxins including neurotoxic, hepatotoxic, dermatotoxic, as well as other bioactive compounds. Blooms of these toxic cyanobacteria pose additional threat if the blooms occur in reservoirs and other drinking water sources [3-5]. These cyanotoxins are responsible for fresh and brackish water intoxication and the intoxication of animals has been widely reported around the world [6]. Scientists generally name the blooms caused by cyanobacteria as CyanoHABs. These blooms have dramatically increased in recent decades all over the world. The recent freshwater blue-green algal bloom that occurred in Taihu (Lake Tai), Jiangsu Province, China (starting end of May 2007) is an example showing how serious the effects can be. A population of more than two million people living around Lake Tai was affected by the bloom because Taihu was the city's sole water supply, leaving them without drink-

***Corresponding author:** Samuel Chun-Lap Lo. The Proteomic Task Force, Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hong Kong. Tel: (852) 3400 8669. Fax: (852) 2364 9932. Email-Address: samuel.chun-lap.lo@polyu.edu.hk

ing water for at least a week [7]. Truck-loads of bottled drinking water had to be sent from surrounding provinces for temporary relief. At the initial stages of the bloom, there was additional fear on whether the causative agent was toxic or not. Hence, fast and accurate cyanobacterial species identification is important for surveillance and possibly prevention and control of CyanoHABs.

Cyanobacteria are a morphologically diverse group of organisms ranging from unicellular, colonial to filamentous forms. The class of Cyanophyceae includes 150 genera and around 2000 species. They are now placed within the group Eubacteria in the phylogenetic taxonomy [8]. Traditionally, identification and classification of cyanobacteria has been based on their morphological features such as cell size, shape and arrangement, pigment coloration and the presence of characters such as gas vacuoles and a sheath [9]. However, these types of identification are time-consuming, laborious and a high-level of expertise is required for identifying and distinguishing morphological characters for identification purposes. More than 50% of the known strains identified using morphological-based strategies were estimated to be misidentified [9]. The lack of distinguishing morphological features in some strains/species, the changes and variations of some diagnostic characters during different growth or environmental conditions, as well as the subjective judgment by the operators can lead to errors, resulting in incorrect species identification. Limitations of the morphology-based species identification method implicated a pressing need for the development of more reliable and rapid methods for the identification of cyanobacteria. Nowadays, DNA technologies have been routinely applied in areas of species identification of microorganism including cyanobacteria. For example, one of the common approaches is the analysis of DNA sequence similarities of 16S rRNA gene [10] and 16S-23S rRNA internal transcribed spacer (ITS) [11]. However, these PCR-based methods are labor-intensive as well as difficult to meet demands of high-throughput and especially in cases of previously unknown cyanobacterial strains.

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) can be used to analyze the protein expression profile of a cell. This method has emerged as a new technology for species identification for various microorganisms and has been extensively reviewed [12, 13]. The MALDI-based identification method is praised as objective, fast, simple and reliable. It usually requires minimal amounts of biological material and is suitable for high-throughput routine analysis. Therefore, it has great potential for applications in clinical microbiology and environmental monitoring [13, 14]. The central idea of this method is to generate PEPs from intact unicellular cells, or cell lysates. Despite the fact that only ionized proteins/peptides that are in abundance in the cells of interest are detected in the MALDI-TOF-MS, the PEPs generated are usually genus-, species- and sometimes even strain-specific (Dworzanski and Snyder, 2005; Fenselau and Demirev, 2001). It is possible to identify the species within a few minutes [12, 13, 15]. Furthermore, Mellmann et al. (2008), showed that the MALDI-TOF-MS based identification method is more robust than the 16S rRNA gene sequencing method for species identification of non-fermenting bacteria. It is because the MALDI-TOF-MS method can provide differentiating information even when that 16S rRNA- based identification fails [15]. Although the MALDI-TOF-MS based method has been applied to a wide range of microorganism, for example bacteria [15-19]; parasites [20] and fungi [21-24], the successful application of protein-expression-profiles (PEPs) for species identification of blue-green algae has never been reported.

Our group has previously shown the successful application of using PEPs generated by MALDI-TOF-MS for the identification of different dinoflagellates species (one of the major HAB causative agents) [25]. To further extend its application, in this study, we evaluated the use of MALDI-TOF-MS based methodology for rapid identification of different cyanobacterial species/strains. We had further adopted and simplified the sample preparatory procedures for cyanobacterial investigations. Besides being more effective, this protocol is a refined version when compared to the one our group reported earlier [25]. The eventual establishment of a common protocol for all HAB causative agents will facilitate the development of automatic systems for environmental surveillance purposes. Lastly, identification of some cyanobacterial strains based on MALDI-TOF-MS detection of their low-mass toxins presented has been reported previously [26]. However, to our knowledge, this is the first report to demonstrate the potential use of protein expression profiles (PEPs) generated by MALDI-TOF-MS for rapid and objective identification of cyanobacteria.

2. Material and Methods

2.1 *Cyanobacterial species and strains*

Cyanobacteria species used in this study are listed in Table 1. Four isolates of *Synechococcus sp*. were isolated by Prof. Gao Ya-Hui of The Xiamen University. The other species, including *Lyngbya aestuarii* (CCMP473), two isolates of *Planktothrix agardhii* (CCMP600 and CCMP601), *Arthrospira platensis* (CCMP1295), *Oscillatoria sp.* (CCMP1731), *Cylindrospermopsis raciborskii* (CCMP1973) and *Anabaena sp.* (CCMP2066) were purchased from The National Center for Marine Algae and Microbiota (NCMA, previously Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP)). NCMA had extensively verified identities of the cyanobacterial cultures before they were sold while identities of other cultures were confirmed by DNA sequences of 16S-23S rRNA ITS.

2.2 *Culture conditions*

Primarily, f/2 or DY-V media were used for culturing the cyanobacteria. Stock cultures of all cyanobacteria were kept at exponential growth phase by transferring to new medium

Table 1. Cyanobacteria species and strains used in this study

Species/strains	Collection site
Anabaena sp. (CCMP2066)#	Red River, Trollwood Park, Fargo, North Dakota, USA
Arthrospira platensis (CCMP1295)#	Yunnan Province ,China
Cylindrospermopsis raciborskii (CCMP1973)#	Florida, USA
Lyngbya aestuarii (CCMP473)#	Woods Hole, Massachusetts, USA
Oscillatoria sp. (CCMP1731)#	Isla do Sol, Cape Verde Islands, Africa
Planktothrix agardhii (CCMP600)#	Lake Kolbotnvatn, Akershus, Norway
Planktothrix agardhii (CCMP601)#	Lake Kolbotnvatn, Akershus, Norway
Synechococcus sp. (FACHB-460)*	Wuhan, China
Synechococcus sp. (FACHB-562) *	Wuhan, China
Synechococcus sp. (FACHB-940) *	Wuhan, China
Synechococcus sp. (FACHB-7820) *	Wuhan, China

CCMP: The Provasoli-Guillard National Centre for Culture of Marine Phytoplankton (Current name NCMA: National Center for Marine Algae and Microbiota).
FACHB: Freshwater Algae Culture Collection of the Institute of Hydrobiology.
*The species were kindly provided by our Chinese partners in Xiamen University and were identified by the DNA sequences of 16S-23S internal transcribed spacer (ITS) region (see supplementary information, Figure S1).
According to the information provided, species has been identified by taxonomists.

every two week in a ratio of 1:10 (v/v). Vegetative cells from cultures in mid- or late-exponential phase of growth were inoculated into freshly prepared culture medium. Possible contamination of the culture was monitored by regular microscopic examination. The cultures were grown at 20 °C under a 12-h light:12-h dark cycle at a light intensity of 120 μE lx $m^{-1}s^{-1}$ provided by cool white fluorescent tubes in a Conviron growth chamber (Model EF7).

2.3 *16S-23S rRNA ITS sequencing*

DNA extraction was performed according to the procedures reported previously [25]. 16S-23S rRNA ITS region was amplified from the extracted DNA using the cyanobacterial 16S-specific primer CSIF: 5' GYCACGCCCGAAGTCRTTAC 3' and 23S-specific primer ULR: 5' CCTCTGTGTGCCWAGGTATC 3' [27]. PCR were performed under conditions: 95℃ 5min; 35 cycles of 94℃ 45s, 50℃ 45s and 72℃ 2min; 72℃ 10min. PCR products were cloned into pGEM-T easy vectors (Promega, USA) prior to DNA sequencing. DNA sequencing of all cloned plasmids were performed by commercial facilities using traditional dideoxy-methodolgy.

2.4 *Sample preparation to obtain PEPs using MALDI-TOF MS*

Cyanobacterial samples were prepared using methodology as described previously with minor modifications [25] and the workflow is shown in Figure 1. Briefly, exponential 100-200 ml cultures with 10^5 cells were collected by centrifugation (1500 x g for 15 min at room temperature). Cell pellets were re-suspended in 100 μL 0.1% trifluoroacetic acid (TFA) (Aldrich, USA). These cells were broken by quick sonication (a total of 2 min with short pulses of 10 s each) on ice. Cell debris was removed by centrifugation at 13,000 x g at room temperature for 5 min. Inorganic salts in the samples were cleaned up by absorbing the proteins onto C-18 zip tips (Millipore, USA) according to the manufacturer user manual. Proteins were eluted from the zip tip with 1-2 μL 0.1% TFA in 70% acetonitrile. Eluted proteins solutions were mixed with matrix solution in a ratio of 1:1 (v/v). Matrix solution contained saturated sinapinic acid (SA) in 1:1 (v/v) 0.1% TFA/acetonitrile. 1 μL of the resulting mixtures were then spotted onto a mass spectrometer target plate (MTP AnchorChip™ 600/384 T F) (Bruker, Germany).

2.5 *MALDI-TOF MS analysis*

Proteins expression profiles (PEPs) of all samples being studied were obtained with a MALDI-TOF spectrometer (Autoflex III Smartbeam, Bruker, Germany) in linear mode at an accelerating voltage of 20 kV by using a 300 ns delay time and over a mass range of 2000–16000 Da. For each sample, spectra from 500 laser shots at several different positions were combined to generate a mass spectrum. The mass spectra were calibrated externally using Protein Calibration Standard I (Bruker, Germany) and were used to provide a miminium mass accuracy of at least 1 part in 3000. The calibrant mixture contains insulin (5734.51 Da),

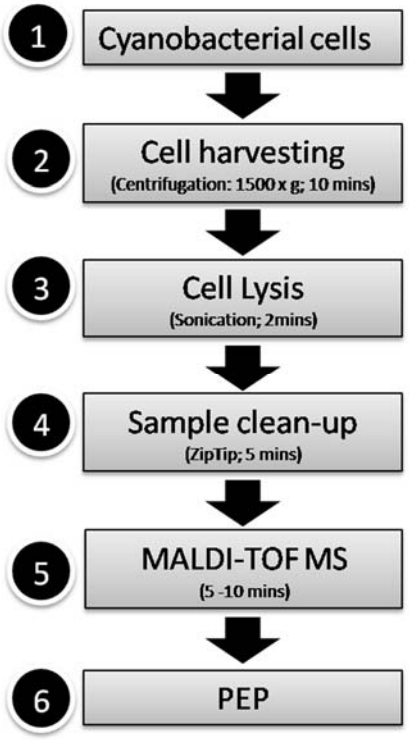

Figure 1. Summarized workflow of the HAB species sample preparation for MALDI-TOF-MS analysis. Time spent on MALDI sample preaprations include sample spotting and crystallization as well, indicating the short turn around time of results.

ubiquitin (8565.76 Da), cytochrome c (12360.97 Da) and myoglobin (16952.3 Da). Internal calibration was performed by adding purified insulin (5734.51 Da) and ubiquitin (8565.76 Da) directly into the sample mixture. Depending on the range of peak mass ions of interest, the combined strategy of calibration ensured the mass accuracy of the spectrum to be within 0.5-2 Da. Fresh calibration was performed for different samples and for different individual experiments. Prior to data analysis, each spectrum was baseline corrected and smoothed according to the TopHat and Savitzky-Golay smoothing algorithm. Normalized spectra were transferred to the software "Flex Analysis" ver3.0 (Bruker, Germany) for automated peak extraction and analysis as described in the user manual. PEPs shown in the manuscript were the representative data obtained from a triplicate analysis of 3 different batches of the samples.

3. Results and Discussion

3.1 *Sample preparation for MALDI-TOF-MS analysis*

The methodology described for sample preparation is instrumental for MALDI-TOF-MS analysis because it determines whether a high-quality spectrum of the PEP can be obtained. There are several factors that are most critical in affecting the quality of spectra obtained. These include types of matrix, extraction solvent and sample preparation procedures such as sample clean-up. We had adopted the workflow of the methodology we described previously [25] for application in cyanobacterial samples (Figure 1). In order to have appropriate conditions leading to good-quality mass spectra, we have tested these three factors in this study.

The choice of matrix is important in MALDI-TOF-MS detection because it can promote ionization of the analytes. The three most commonly used matrices are: α-cyano-4-hydroxycinnamic acid (CHCA), 2,5-dihydroxybenzoic acid (DHB) and 3,5-dimethoxy-4-hydroxycinnamic acid/ sinapinic acid (SA). These matrices were screened for compatibility with the analysis of cyanobacterial samples. Among the three matrices tested, only samples with SA show a detectable and high-quality mass spectrum (data not shown). With this matrix, it was possible to observe the ion peaks within the mass window between 2000-20000 Da. Previous reports highlighted that most of the ion peaks observed were also within this mass range [13, 25]. However, for the species detected in the present study, no significant peaks were observed in between 16000–20000 Da. Therefore, results with mass window of 2000-16000 Da was displayed in this study. Signals of other two matrices (i.e CHCA and DHB) were in low resolution with high background noise in the corresponding mass range. The findings were comparable to our previous study [25]. Hence these matrices are inappropriate for MALDI-TOF-MS analysis of cyanobacterial samples.

The conditions of protein extraction can also affect the appearance and quality of the mass spectra. One of the explanations is the solubility of proteins in a given solvent varies according to their degrees of *pI* and hydrophobicity [28]. Acid is used in the sample preparation to provide charges on the protein. Some studies showed that trifluoroacetic acid (TFA) gave a better signal intensity than other common extraction solvents such as formic acid [13, 23, 25, 29]. This solvent was shown to allow the detection of a large number of mass peaks in the mass range of interest. Therefore, this solvent was used in the present study for protein extraction. We have evaluated different concentrations of TFA used for optimal protein extraction, including 0.1%, 1% and 5 %. Among the concentrations of TFA studied, 0.1% TFA gave the best result in term of signal intensity (data not shown). The result was also comparable to our previous work on dinoflagellates [25].

Interferences such as salts and pigments that are present in the sample can also affect spectrum quality. Of these, salt is one of the major concerns as it would suppress or modify the desorption / ionization proprieties of proteins so that some ions are detected preferentially to others [30]. Therefore, in order to obtain a high quality MS spectrum, it is important to obtain a clean and desalted sample prior to mass spectrometry. For sample absorption with C-18 ziptips was found to be a simple and effective method for sample clean-up in dinoflagellate samples [25]. Subsequently, we evaluated the same method for desalting the cyanobacterial samples

and found that much more peaks were obtained with the desalted sample than that of the sample without desalting (data not shown). One of our aims in this study was to determine the simplest sample preparation procedure that is applicable for various types of HAB causative agents, including dinoflagellates and cyanobacteria (blue-green algae). Based on the results in the present and previous studies, we have summarized the sample preparation procedure and workflow (Figure 1). We believe the procedures described are generally applicable in MALDI-TOF-MS-based species identification in the studies of HABs.

3.2 *MALDI analysis of cyanobacterial species/strains*

To investigate the discriminatory ability of different cyanobacteria species by MALDI-TOF-MS, a total of eleven cyanobacterial strains, representing seven species were analyzed (Table 1). They are all environmentally important species and most of them, including *Anabaena* sp, *Cylindrospermopsis* sp, *Lyngbya* sp, *Oscillatoria* sp, *Planktothrix* sp and *Synechococcus* sp, are responsible for CyanoHABs. All seven reference species tested in the study showed unique PEPs in a mass range between 2000 and 16000 Da (Figure 2). For each PEP, the number of mass ion peaks observed is around 10-15. MALDI-TOF-MS-based identification of other microorganisms showed similar numbers of discrete mass ions observed in the same mass range [13]. Beside the unique PEPs observed from different species, a number of unique and consistently occurring species-specific ions can also be annotated from the spectra. Typically, 5 to 10 mass ions are reported to be sufficient to discriminate bacteria at the species level [14]. Although the species-specific ions found in the present study, based on the mass ion peaks observed in the mass range of 2000–16000 Da, cannot be regarded as biomarkers for the corresponding species until more PEPs of all existing cyanobacteria are analyzed in the future, we clearly demonstrated that either the PEPs or the individual specific ions can be used unambiguously for the identification of different cyanobacteria. Furthermore, whether a sample contains toxin can be determined if biomarker ions specific for toxin production can be found and used. Otherwise, other techniques, such as HPLC, are needed for analysis of toxin production. Nonetheless, based on known characteristics of toxic species/strains, the PEP technology can be used as an initial screening tool for high-throughput.

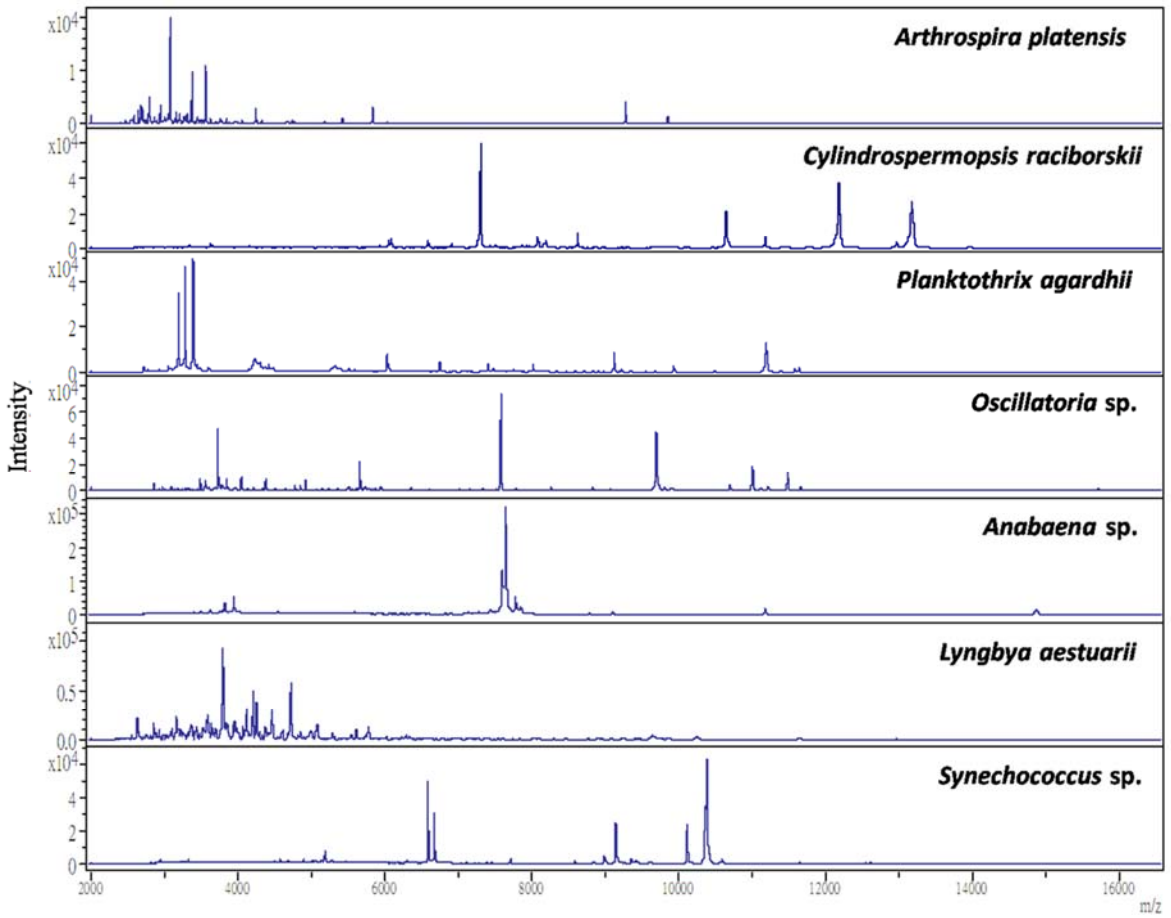

Figure 2. MALDI-TOF-MS protein expression profiles of cyanobacterial species in different genus (*Planktothrix agardhii* (CCMP600) and *Synechococcus sp.* (FACHB-940) were used in the experiment).

In order to test whether the method can differentiate different cyanobacteria at levels below the species level, strains of *Synechococcus sp* and *Planktothrix agardhii* were used as examples for MALDI-TOF-MS analysis under identical conditions (Figure 3). These strains are difficult to distinguish from each other using the traditional morphological -based identification method and even molecular gene sequencing. For instance, identification of *Synechococcus* species is unclear and there are numerous limitations with the current taxonomy of *Synechococcus* [31]. Recently, 16S-23S rRNA ITS sequence has been shown to be variable in different strains of cyanobacteria and thus can be used as a reliable identification tool [32]. 16S-23S rRNA ITS region of the four strains of *Synechococcus spp.* were sequenced (see supporting information, Figure S1). Identities of the strains were confirmed by GenBank BLAST searches. Except at most with two nucleotides, the ITS sequences of the four strains were nearly identical to each other. This result suggested that the ITS sequence alone may not sufficient to differentiate the polymorphism of the four *Synechococcus* strains. On the other hand, the PEP of the four strains obtained from the MALDI analysis display high levels of overall similarity (Figure 3A). They shared most of the common peaks (e.g "A" peaks). However, some distinguishing strains-specific ions ("B" peaks) could be easily pinpointed from the spectra (Figure 3B). These "B" peaks can only be observed from the spectra of specific *Synechococcus* strains, and their corresponding spectrum is highly similar to each other. For example, peak with ~6888 Da can only be found in FACHB-7820). Based on this finding, it was suggested that these two strains which may belong to the same group, which is different from the other two strains (FACHB-562 and FACHB-7820). Similar result was observed in the MALDI-TOF-MS analysis of two *Planktothrix agardhii* strains (Figure 3C). PEPs of the two strains were highly similar to each other, but some strain-specific biomarkers were found for each individual strain ("C" peaks). Therefore, our results demonstrated that the mass spectral data (including the PEPs and the bi-

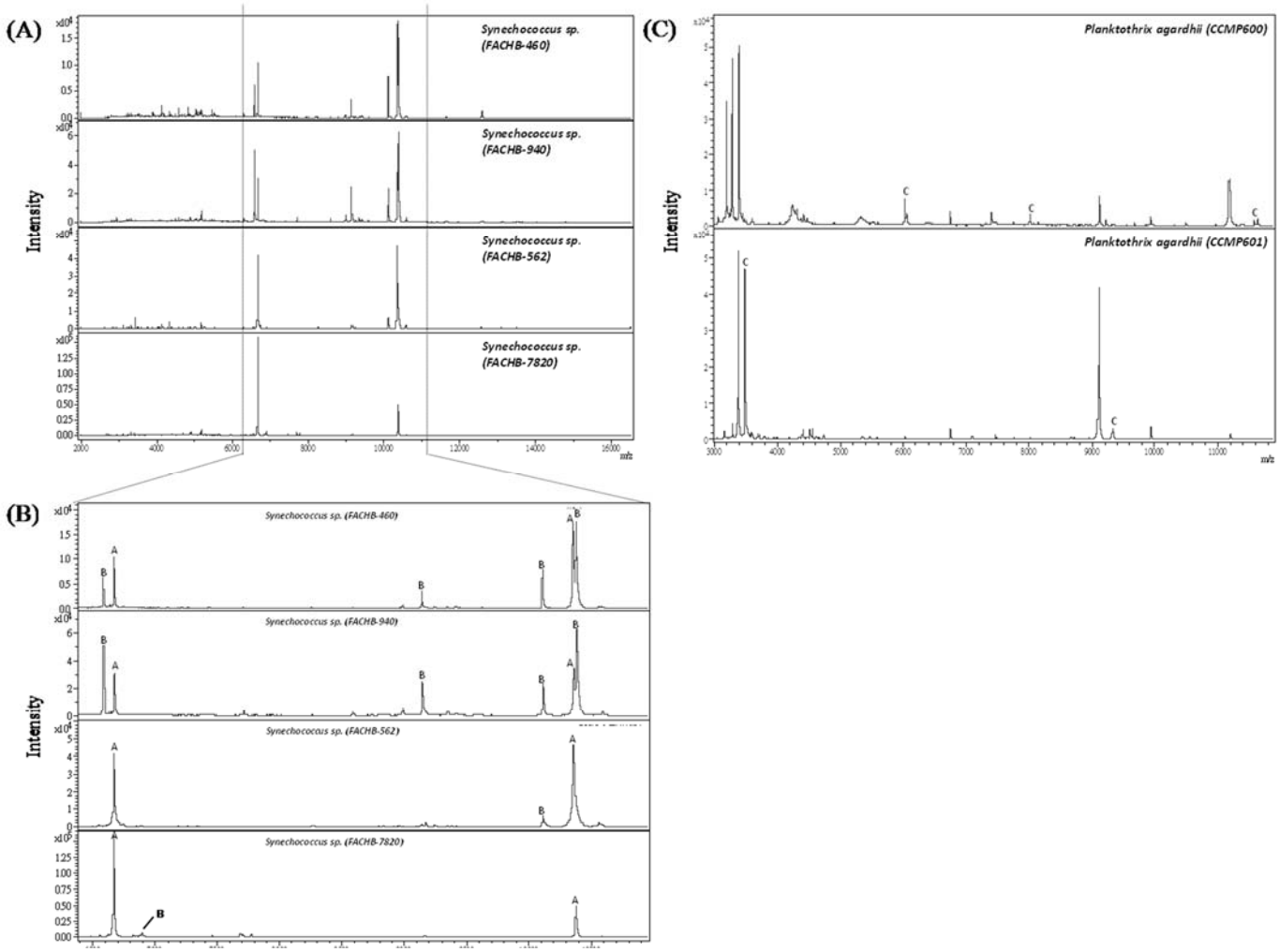

Figure 3. MALDI-TOF-MS protein expression profiles of cyanobacterial strains including (A) 4 strains of *Synechococcus sp*. and (C) 2 strains of *Planktothrix agardhii*. (B) The enlarged region of the *Synechococcus* strains spectra ranged from 6500-11000 m/z. Peaks labeled with "A", "B" and "C" representing the common peaks of all the 4 *Synechococcus* strains, the respective signature peaks of the corresponding *Synechococcus* strains and the respective signature peaks of the corresponding *Planktothrix* strains respectively.

omarkers) of the strains are not only sufficient to distinguish cyanobacteria of different genera and species but also potentially to differentiate different strains.

3.3 *Reproducibility and PEPs under different conditions*

The PEPs presented in Figure 2 and 3 were representatives of three different batches of the same samples. With the sample preparation and MALDI-TOF-MS analysis performed under the same conditions, the acquired PEPs are identical between different individual batches of samples. On the other hand, growth parameters, such as different growth media and growth phase of the organism, were reported to be important for the reproducible generation of the mass spectra and should therefore be carefully controlled [33, 34]. With the aim of achieving reproducible and reliable identification of the species/strains, we compared the PEPs of two different species (*Oscillatoria sp*. and *Synechococcus sp*.) grown under different culture media (Figure 4). Under all culture media used with a defined condition, the spectra from both pair of samples showed a high degree of uniformity. It seems that the PEPs obtained were not affected by different cultivation media used. Growth age is another important factor for the MALDI-TOF-MS based identification of cyanobacteria. Interestingly, intensity of one of the "signature" peaks (~6661 Da, indicated by arrow in Figure 4) of the *Synechococcus* sp was found to be greatly diminished if very old cultures of cells (>25 days) were used for the study (Data not shown). Relationship between the culture cells in dead growth phase and the peak intensity remains to be elucidated in further studies. However, similar phenomenon was observed in previous study in the application of PEPs method to the field samples [35]. In order to determine the PEPs of the cells in different growth phases, MALDI spectra of two cyanobacterial species of different growth time were recorded (Figure 5). However, the patterns and number of peaks obtained from different growth ages varies from each other. Some peak mass ions were observed all the time in the exponential and stationary growth phases, and others appeared transiently in only one of these growth phases. For example, the number and patterns of peaks observed from 2000-7000 Da in the spectra of *Oscillatoria* species in different growth ages were different (Figure 5A). The protein expression changes may be explained by the alterations of metabolic activities related to different culture ages of the cells. However, some peak mass ions were constantly expressed and observed in

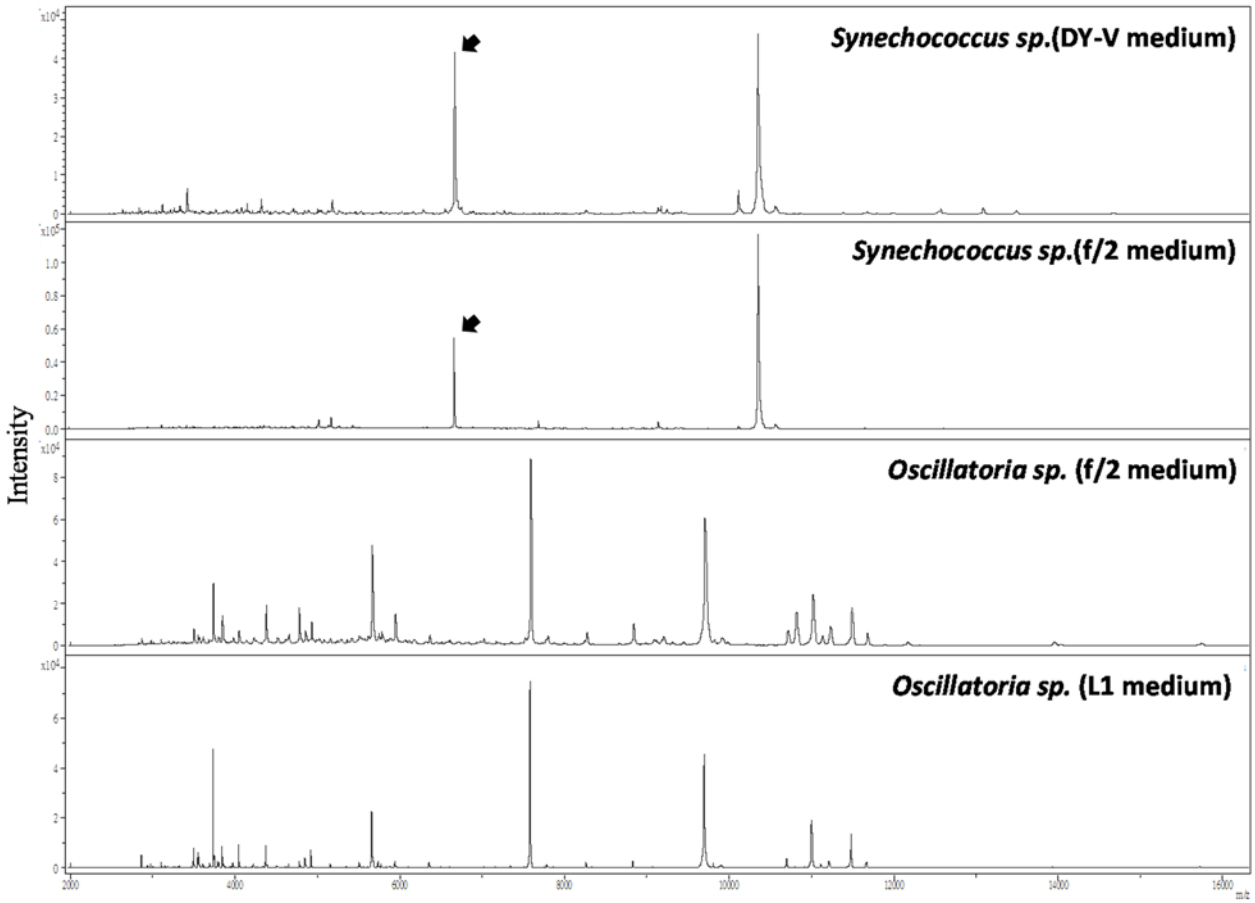

Figure 4. MALDI-TOF-MS protein expression profiles of cyanobacterial species grown in different cultivate media. (7-days cultures of *Oscillatoria sp* (CCMP1731) and *Synechococcus sp.* (FACHB-562) were used in the experiment). Arrow indicates the peak would greatly deduce in its intensity if old cultures of *Synechococcus* cells (i.e >25 days) were used for the experiments.

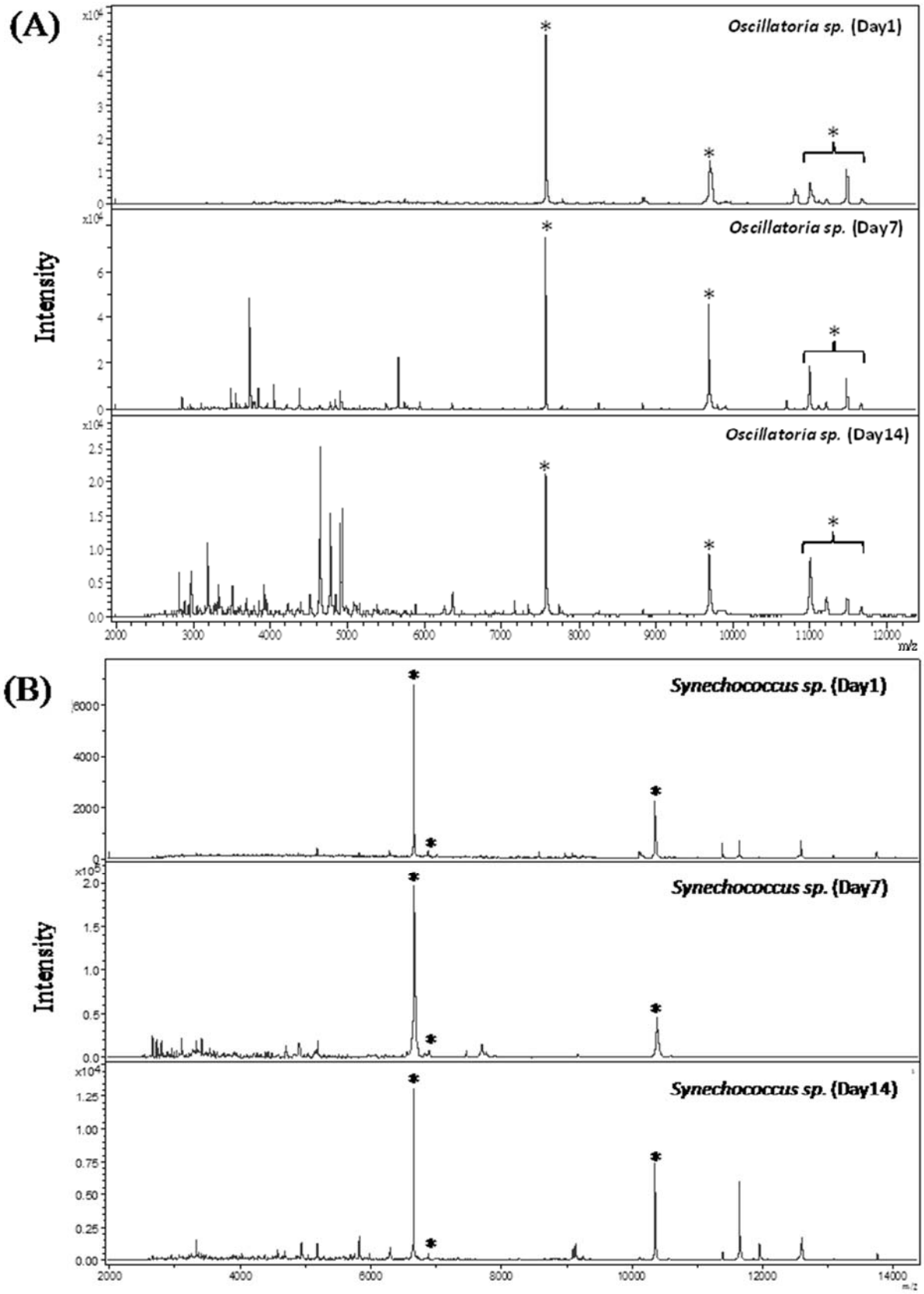

Figure 5. MALDI-TOF-MS protein expression profiles of cyanobacterial species (A) *Oscillatoria sp* (CCMP1731) and (B) *Synechococcus sp* (FACHB-7820),in different growth ages. Peaks labeled with asterisks are constantly expressed protein mass observed in the spectra irrespective of the growth ages.

the spectra irrespective of the growth ages of the cells (Figure 5, marked with asterisks). These sets of peak mass ions can be used as the "representative peaks" for the tentative identification of a specific species/strain. Whether these peaks can be used as the biomarkers should be subjected to more comprehensive studies of PEPs of other species in future studies. Further, Tao and co-authors (2004) has developed an interesting and efficient numerical method for the differentiation of microorganism irrespective of their growth stages [36]. This is a statistics-based algorithm to assign weight factors to individual mass ions observed in the mass spectra of different growth stages and the combined weight factors were used for species identification with satisfactory accuracy. Therefore, these distinguishing species-specific ions together with the established statistical algorithm will form an unambiguous reliable basis for cyanobacteria identification. Furthermore, with the aid of a commercially available software, such as "BioTyper" (from Bruker), automated species identification based on statistical analysis can be achieved.

Previously, we had demonstrated that it is possible to identify individual species in a mixed population of dinoflagellates based on species-specific signature ions in the spectrum [25]. In most of the cases of algal blooms, usually one to two algal species would predominate, usually with one succeed by another. Therefore, in this study, we evaluated whether it is also possible to differentiate different species/strains of cyanobacteria from a mixed culture comprising two dominatant species in 1:1 ratio (Figure 6). Two different species/strains, *Oscillatoria sp* and *Cylindrospermopsis raciborskii* (species of different genus) (Figure 6A); *Synechococcus sp* FACHB-460 and FACHB-7820 (strains of same species) (Figure 6B) were grown in a mixed culture (in ratio: ~1:1). The mixed cultures were analyzed by MALDI-TOF-MS with the same condition described previously. Our results showed that the species-specific (Figure 6A) or the strain-specific (Figure 6B) signature peak mass ions can be easily identified within the mass spectra of the mixed cultures even by simple visual inspection. The results are compatible to our previous data [25]. In Figure 6A, a set of signature peaks corresponding to *Oscillatoria sp* ("O" peaks) and *Cylindrospermopsis raciborskii* ("C" peaks) can be easily observed from the spectrum of the mixed culture. Although the signature peaks arise from the experiments cannot be proved to be species specific at this point in time, we would like to demonstrate the idea that strains from a mixed population comprising two domintant species can be identified based on the strain-specific peak mass ions. For instance, the two strains of *Synechococcus sp*, FACHB-460 and FACHB-7820, which are difficult to be distinguished from each other based on their morphology and even their 16S-23S rRNA ITS sequences, can be distinguished from each other based on their PEPs.

4. Concluding Remarks

Besides applicable to dinoflagellates [25], results of our present study extended our horizon that PEPs obtained from MALDI-TOF-MS are also applicable for identification of cyanobacteria species. To our knowledge, this is the first report to show the successful application of PEPs obtained by MALDI-TOF-MS for the identification of cyanobacteria. We believe that the methodology currently being developed can also be widely applied in other HAB causative agents, such as diatoms. Operation of this methodology is easy and would allow identification of various HAB species within hours. In the near future, further expansion of this MALDI-TOF-MS based methodology in generating more reference PEPs of various types of HAB causative agents and standardize the specific conditions employed will be very useful for rapid HAB identification. Given the advance development of the MALDI-TOF-MS for application in the field of proteomics and with the aid of bioinformatics examination such as the MALDI BioTyper software (Bruker, Germany), we believe this MALDI-TOF-MS approach can potentially become part of a standard platform for HAB species identification.

5. Supplementary material

Supplementary data and information is available at: http://www.jiomics.com/index.php/jio/rt/suppFiles/138/

Figure S1 - The 16S-23S internal transcribed spacer (ITS) sequences of 4 strains of Synechococcus sp. Nucleotides highlighted with dotted lines represent the differences among the 4 strains.

Table S1- Peak mass ions for the corresponding MS spectra from cyanobacterial species and strains used in this study.

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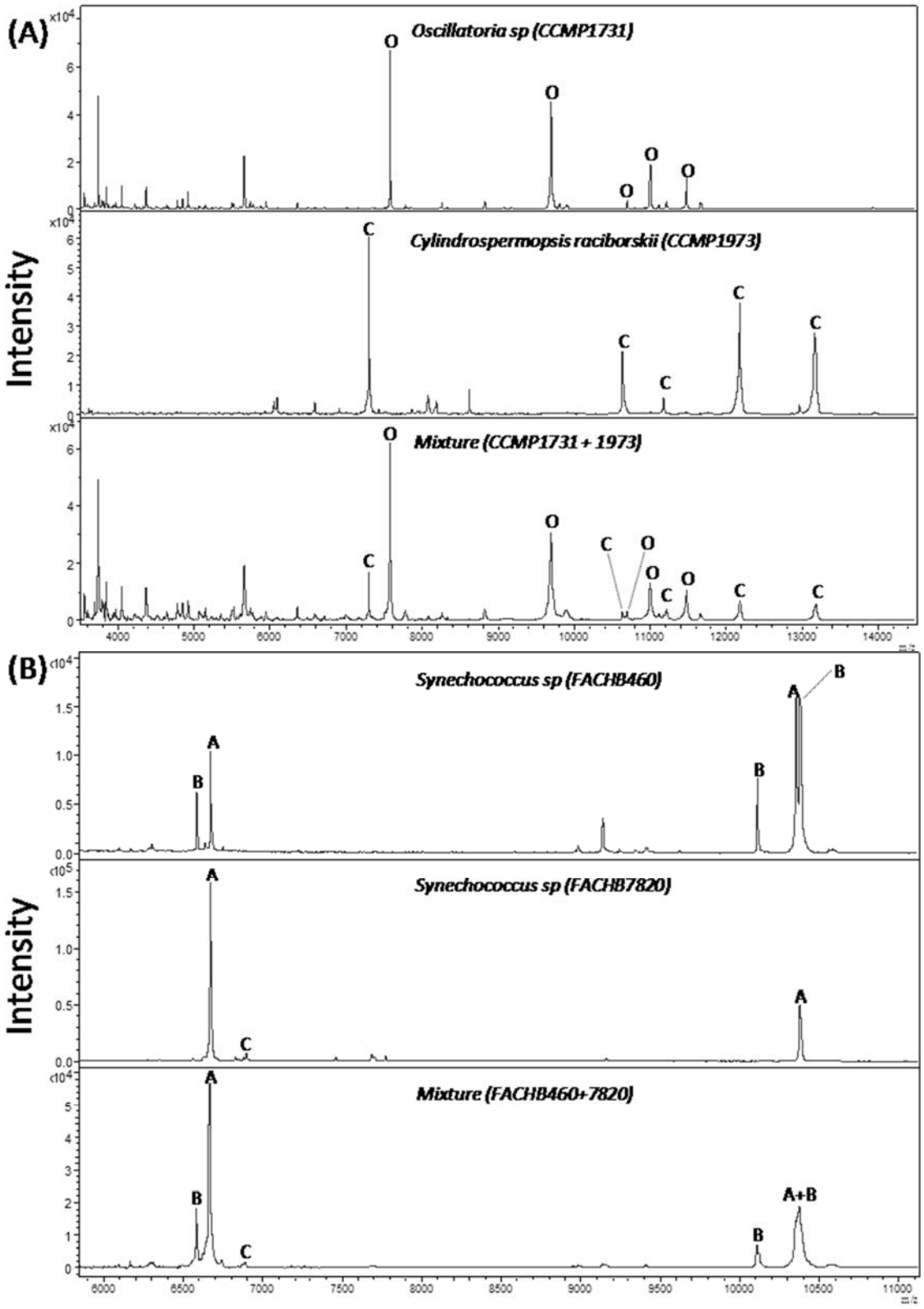

Figure 6. MALDI-TOF-MS protein expression profiles of mixed populations of cyanobacterial species. (A) PEP profiles of *Oscillatoria sp*, *Cylindrospermopsis raciborskii* and mixture of both species. Peaks labeled with "O" and "C" representing the signature peaks corresponding to *Oscillatoria* sp and *Cylindrospermopsis raciborskii* respectively. (B) PEP profiles of *Synechococcus* strains FACHB-460, FACHB-7820 and mixture of both strains. Peaks labeled with "A", "B" and "C" representing the common peaks shared by both strains, the signature peaks corresponding to FACHB-460 and the signature peak corresponding to FACHB-7820 respectively.

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