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Research Article

Simplified Bioanalytical Approaches for the Characterization of Microalgae and Cyanobacteria

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Abstract

Harmful microalgae and cyanobacterial bloom cause deteriorated water quality and raises severe health concerns. This study discussed the demonstrations of simplified bioanalytical tools to enhance the natural resources of microalgae and cyanobacteria, to be given its importance, and how this knowledge can be used for wealth creation. Three bioanalytical approaches; (1) gas chromatography-mass spectrometry (GC-MS), (2) Raman spectroscopy, and (3) universal plastid biomarker, were successfully incorporated in selected microalgae and cyanobacteria. GC-MS analysis via solvent extraction characterized the organic compounds such as hydrocarbons and silicones derivatives. Air-dried thin layer of microalgae and cyanobacteria analyzed in Raman spectroscopy corresponded to three major spectra peaks, which fingerprints saffron and carotene photosynthetic pigments. Lastly, the taxonomic classification was simplified via the plastid 23S ribosomal ribonucleic acid (rRNA) biomarker, whereby, the Neighbor-Joining tree showed a clear separation between the phylogenetic clade of brown and green microalgae (eukaryotic), and cyanobacteria (prokaryotic). In short, this simplified multi-approaches; using molecular biomarkers provided an accurate identification on the species of microalgae and cyanobacteria, chemical biomarkers GC-MS characterized the organic compounds for sustainable green technology in industrial application, and finally, Raman spectroscopy can be future developed as a miniaturized handheld device for field sample detection for its fingerprinting characterization.

Keywords: Microalgae; Cyanobacteria; Plastid 23S rRNA; GC-MS; Raman Spectroscopy

Introduction

Characterization studies of microalgae and cyanobacteria are important to confirm the origin of species lineage, and to understand its fundamental operations. The conventional characterization methods of microalgae and cyanobacteria require laborious sample preparation, technical expertise, and moderately expensive instruments. The conventional approaches such as microscopic analysis hinder characterization, as different cells may share similar morphological characteristics [1]. Biochemical assays are expensive, as the cell-specific antibody is required for expression [2]. Besides, high-performance liquid chromatography and liquid chromatography-mass spectrometry are reliable and robust for environmental monitoring [3]. However, the solvents and the reference standards used are expensive, and high maintenance cost, as residual clean-up is often needed when the column is clogged [3].

GC-MS is known as the 'gold standard' for identification of organic compounds, in known or unknown materials [4]. The samples are propelled by the Helium gas, and the GC column allows for the separation of organic compounds, based on its volatility [5]. The chromatogram peak will be detected when the compounds are eluted. With GC-MS, the naturally occurring organic compounds in a biomaterial can be detected quickly, which can be further bioengineered for industrial applications.

In addition, Raman spectroscopy is incorporated for the studies of the vibrational effect of microalgae and cyanobacteria, during the inelastic scattering (Raman scattering), from the monochromatic rays [6]. In Raman scattering, the electrons in the virtual state fall to a different vibrational state and emit a photon with a different energy than the incident photon [6]. This frequency shift is known as the Raman shift. Raman spectroscopy provides a direct assessment of the sample to detect fingerprinting peaks of novel compounds, ideal to be tested for fieldwork samples [7].

The conventional primers namely the 16S rRNA, 18S rRNA or the internal transcribed spacer region primers established in past studies, are a hassle in polymerase chain reaction (PCR), which sometimes indicates false-positives due to the wrong pairing of primers [1]. According to Sherwood and Presting; Presting [8,9], a universal biomarker, the plastid 23S rRNA (p23SrV) primer, targeting the conserved region of cyanobacteria and plastids of microalgae, simplified the classification.

Materials and Methods

Sample collection

The marine microalgae cultures obtained from the International Institute of Aquaculture and Aquatic Sciences, Universiti Putra Malaysia, Malaysia, comprised of *C. calcitrans*, *I. galbana*, *Chlorella* spp, and *Tetraselmis* spp. Meanwhile, freshwater cyanobacteria strain purchased from the Culture Collection of Algae and Protozoa (CCAP), Scotland, United Kingdom comprised of *M. aeruginosa* (CCAP 1450/16) and *Nostoc* spp. (CCAP 1453/25).

Propagation of microalgae and cyanobacteria

Conway media was prepared by adding macronutrient (1 ml), trace metals (0.5 ml), and vitamins (0.1 ml), into a 1L of seawater, at 30 ppt salinity, pre-sterilized [10]. The silicate solution (1 ml) and the nitrate solution (1 ml) were added for the propagation of diatoms. With a serological pipette, 20 ml of microalgae stock was transferred into a 30 ml of Conway media, in an Erlenmeyer culture flask, aseptically. Meanwhile, Blue-Green medium (BG11) was prepared by adding sodium nitrate (100 ml), macronutrients (10 ml of each stock), and trace metal (1 ml), into a 1L of deionized water, pre-sterilized [11]. A pH of 7.1 was attained with 1 M hydrochloric acid or sodium hydroxide. With a serological pipette, 5 ml of cyanobacteria strain was transferred into a 50 ml of BG11, in the culture flask, aseptically.

Microalgae and cyanobacteria cultures were propagated in a cooling shaking incubator (N-BIOTEK NB-205LF, Korea), at 24°C and 150 rpm rotational speed. The cultures were maintained under white fluorescent lamps with 12-hour photoperiods. A serial subculture was performed once a week and the cultures were harvested after numerous subcultures. The cell pellets were centrifuged (Eppendorf 5415R Centrifuge, Germany) and stored in -80°C, prior to deoxyribonucleic acid (DNA) extraction. The remaining cultures were propagated for the next harvest.

GC-MS

Solvent extraction was carried out via a non-polar organic solvent, hexane (HPLC grade), transferred into a separatory funnel [12]. The harvested cell suspension (5 ml) was transferred into the same separatory funnel. Then, the separatory funnel was shaken vigorously, vented and swirled, to allow the two layers to settle down. The bottom layer was discarded, and repeated with the remaining 5 ml of the cell suspension. The organic phase was filtered over a nylon membrane filter and stored in a vial.

The GC-MS analysis was performed using the Agilent 6890N Network GC system, equipped with Agilent J&W DB-5 capillary column (30.00 m length × 0.24 mm internal diameter × 1.00 µm film thickness). The sample (1 µl) was propelled into the column with the help of the helium carrier gas [5]. The temperature of the GC oven was pre-set at 40 °C for 5 min to a final temperature at 260°C for 5 min, with an increment rate of 10 °C/min. The flow rate was at 1 ml/min. The ionisation voltage of the Agilent 5973 Network Mass Selective Detector was at 70 eV. The mass spectrum of the eluted compounds was identified using the NIST98.L library match.

Raman spectroscopy

The glass slides were soaked in dishwashing soap and immersed in an ultrasonic bath (Thermo-6D Thermo-Line, Australia) for 15 min. Followed by rinsing in deionised water, acetone, and isopropyl alcohol, respectively for 5 min, and a final

rinse in deionised water [13]. The glass slides were blow-dried with nitrogen gas gun. Next, 5 ml of each of the cell suspension of microalgae and cyanobacteria were centrifuged at 13,000 rpm, followed by washing the pellet twice with deionised water. The pellet was evenly layered on the sterilised glass slides and dried overnight. The dried thin film was then used for spectral acquisition.

The Renishaw In Via Raman microscope system at 514 nm laser was operated. For calibration, the Raman spectrum of the silicon wafer was measured at peak 520-521 cm^{-1}. The surface of the sample was examined at 20X or 50X magnification. The region of interest was positioned to the centre of the crosshair of the laser and the Raman spectra of the samples were measured. Spectral analysis was performed via the Bio-Rad KnowItAll Informatics System software. The identification of the query component with its respective hit score and functional groups search were analyzed.

p23SrV markers

The phenol-chloroform DNA extraction protocol was followed [14]. Tris/Ethylenediaminetetraacetic acid (Tris/EDTA) buffer (561 µl) and 10% sodium dodecyl sulphate (SDS) (30 µl) were added to the microalgae and cyanobacteria cell pellet. Followed by, Proteinase K, RNase A and 2-Mercaptoethanol, each added at a volume of 3 µl, respectively, and incubated for 1 hr at 37°C. Next, 5M sodium chloride (100 µl) and cetyltrimethylammonium bromide/ sodium chloride (80 µl) were added, and incubated for 10 min at 65°C. Chloroform/isoamyl alcohol was added in equal volume and centrifuged at 13,000 rpm for 5 min. Phenol/chloroform/ isoamyl alcohol was added to the supernatant in equal volume and centrifuged. A 0.6 volume of isopropyl alcohol was added to the supernatant, stored in -20°C overnight, and centrifuged at 13,000 rpm for 20 min. The pellet was washed with 70% ethanol (700 µl), centrifuged, and air-dried. Lastly, Tris/EDTA buffer (100 µl) was added and kept in -20°C freezer. The genomic DNA was analysed in a Nanodrop spectrophotometer.

The primer pair used was "p23SrV_F1 (5'-GGA CAG AAA GAC CCT ATG AA-3') and p23SrV_R1 (5'-TCA GCC TGT TAT CCC TAG AG-3')" [8]. A 25 µl of PCR master mix comprised of 5.0 µl of 5X colorless GoTaq reaction buffer, 1.5 µl of 25 mM magnesium chloride, 0.5 µl of 10 mM dNTP mix, 0.25 µl of GoTaq DNA polymerase, 1.0 µl of 10 mM of forward and reverse primer, and 1.2 µl of the DNA template, was prepared. The pre-denaturation at 94°C for 2 min; followed by 35 cycles of denaturation (at 94°C for 20 sec), annealing (at 46-50°C for 30 sec), and extension (at 72°C for 30 sec); and a final extension at 72°C for 10 min, was performed in the Eppendorf Mastercycler EP gradient S thermal cycler (Eppendorf, Hamburg, Germany). ClustalW was used for sequence alignment and Neighbor-Joining tree was constructed based on the Kimura 2-parameter substitution model with 1000 bootstrap replication.

Results and Discussion

GC-MS

The GC-MS chromatogram represents the organic compounds detected in microalgae species (*C. calcitrans* and *Tetraselmis* spp.) (Figure 1) and cyanobacteria species (*M. aeruginosa* and *Nostoc* spp.) (Figure 2), with its respective retention time. As the samples were vaporized and propelled forward by the helium carrier gas (mobile phase), the GC capillary column (stationary phase) allows for the separation of the compounds, with the most volatile compound was eluted first. The 1-Hexanol, 2-ethyl- has the lowest boiling point (the most volatile), compared to 3,5-Octadiyne, 2,7-bis[t-butoxycarbonyl)amino]-1,8-diphenyl-, as the least volatile compound (Table 1). However, 3,5-Octadiyne, 2,7-bis[t-butoxycarbonyl)amino]-1,8-diphenyl- was the first to be eluted in *M. aeruginosa*, being the most polar compound, as reported in PubChem [15]. This is due to the highest topological polar surface area, which quantifies polarity [16]. Furthermore, the non-polar DB-5 GC capillary column has a higher affinity in retaining non-polar compounds, therefore, polar compounds hit the detector the fastest, or vice-versa with polar GC column.

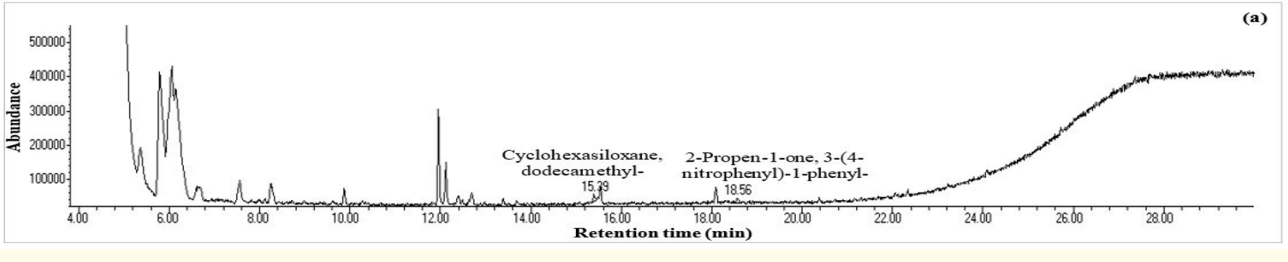

Figure 1: GC-MS chromatogram of organic compounds detected in: (a) C. calcitrans; (b) Tetraselmis spp., with its respective retention time.

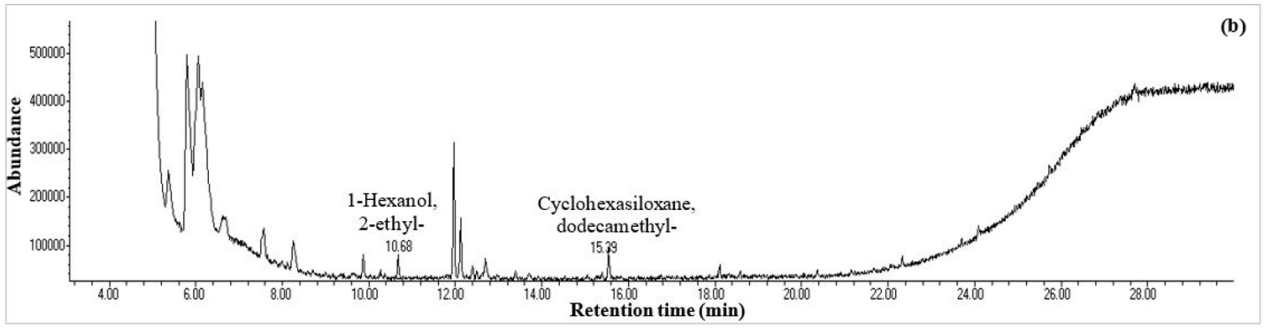

Figure 1: GC-MS chromatogram of organic compounds detected in: (a) C. calcitrans; (b) Tetraselmis spp., with its respective retention time.

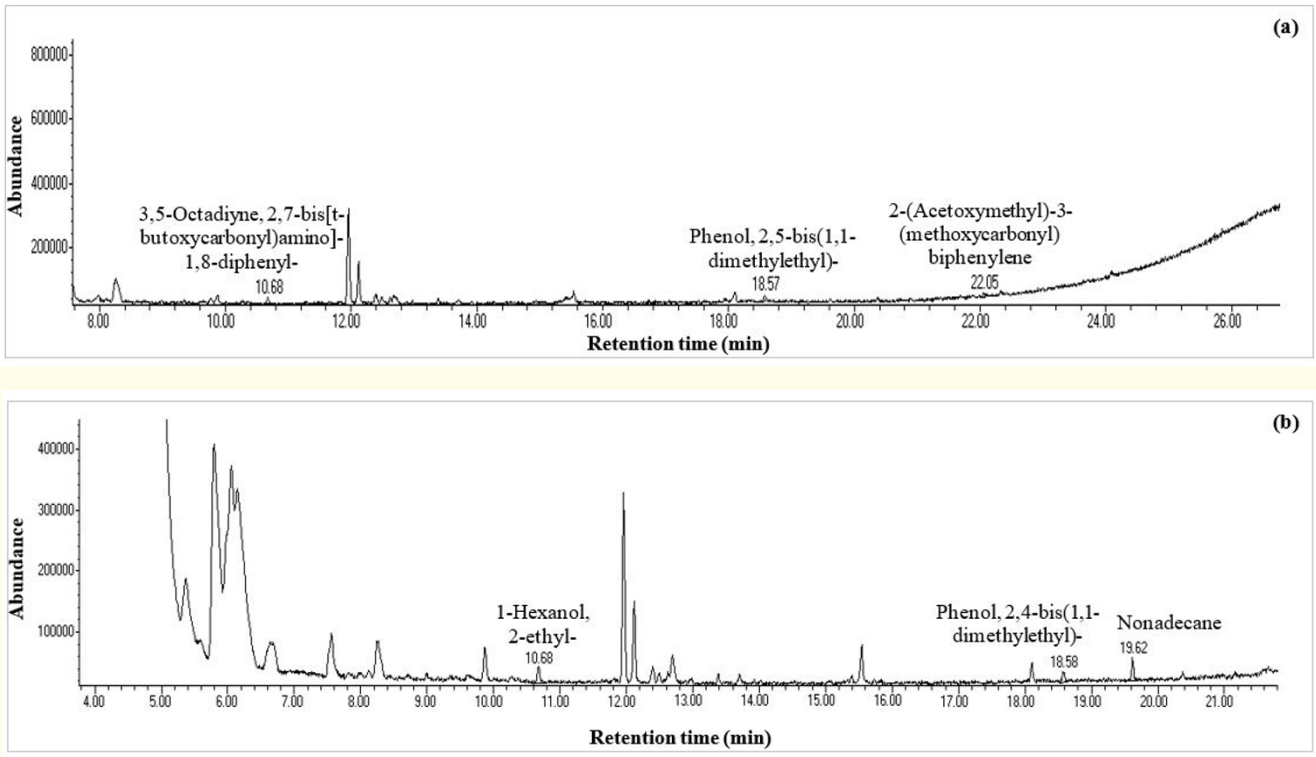

Figure 2: GC-MS chromatogram of organic compounds detected in: (a) M. aeruginosa; (b) Nostoc spp., with its respective retention time.

Besides, the concentration of the detected organic compounds which is proportional to the peak area was also quantified (Table 1). Based on the GC-MS chromatogram in Figure 1 and Figure 2, there were two to three peaks of organic compounds that were eluted in each microalgae and cyanobacteria species. The number of organic compounds eluted in the present studies was lower, compared to the previous studies reported by Devi and Mehta; Řezanka [17,18]. Transesterification and fatty acid methyl

extraction technique incorporated in the previous studies resulted in elution of various organic compounds namely the derivatives of fatty acids, hydrocarbons, and alcohol. Nevertheless, these solvent extraction methods are time-consuming with lengthy sample preparation and the prepared chemicals are expensive. In the present studies, hexane was used as the organic solvent to elute the organic compounds from microalgae and cyanobacteria [12]. Hence, minimizing the sample preparation time, yet eluting similar organic compounds with the previous studies.

Microalgae/ Cyanobacteria	Types of Organic Compound	Molecular Mass	Boiling Point (°C)	Retention Time (min)	Total Amount (%)
C. calcitrans	Cyclohexasiloxane, dodecamethyl-	429	245.0	15.39	47.30
	2-Propen-1-one, 3-(4-nitrophenyl)-1-phenyl-	253	399.2	18.56	52.70
Tetraselmis spp.	1-Hexanol, 2-ethyl-	112	185.0	10.68	42.43
	Cyclohexasiloxane, dodecamethyl-	429	245.0	15.39	57.57
M. aeruginosa	3,5-Octadiyne, 2,7-bis[t-butoxycarbonyl)amino]-1,8-diphenyl-	432	663.7	10.68	34.32
	Phenol, 2,5-bis(1,1-dimethylethyl)-	206	283.4	18.57	37.29
	2-(Acetoxymethyl)-3-(methoxycarbonyl) biphenylene	282	430.3	22.05	28.39
Nostoc spp.	1-Hexanol, 2-ethyl-	112	185.0	10.68	37.56
	Phenol, 2,4-bis(1,1-dimethylethyl)-	206	263.5	18.58	23.41
	Nonadecane	268	330.0	19.62	39.03

Table 1: The different types of organic compound eluted, and their respective molecular mass, boiling point, retention time and the total amount.

The organic compounds detected from the microalgae and cyanobacteria (Figure 1 and Figure 2) provided many beneficial applications. Cyclohexasiloxane, dodecamethyl- is an active ingredient in moisturizers, cosmetics, silicone breast implants, deodorants, sanitation, and varnishes [19]. 1-hexanol, 2-ethyl- can be used as a flavoring agent, lubricants, paint coatings and a solvent for the manufacture of PVC, fabric dye and resins [20]. Similarly, phenol, 2,4-bis(1,1-dimethylethyl)- is a good fuel antioxidant and a stabilizer for plastic production [21]. Lastly, petroleum, paraffin wax and detergent are made from nonadecane [22]. Despite their benefits to the consumers and industry, these compounds are detrimental to human health and aquatic life. Skin and lung irritation, nausea, ulcers, eye damage [23], bioaccumulation of toxins in tissues of aquatic organisms or even fatal death are observed, if exceeded the lethal dose (LD_{50}) regulation of the European Chemicals Agency.

Raman spectroscopy

From the Bio-Rad KnowItAll Informatics System software, the Raman spectra of *C. calcitrans*, *Chlorella* spp., *Tetraselmis* spp. and *Nostoc* spp. correlates with saffron as the top hit followed by carotene (Figure 3). The Raman signal of saffron (Figure 3a) corresponded at wavenumber 1010 cm^{-1}, 1155 cm^{-1} and 1520 cm^{-1}, with the strongest peak at wavenumber 1520 cm^{-1}, in all the studied species. Similarly, carotene (Figure 3b) produced almost the same signals with saffron. The derivatives of carotenoid such as crocin, crocetin and safranal are found in saffron. As saffron is used in food colorings, as well as in traditional and modern medicines, crocetin is possible to be extracted from microalgae and cyanobacteria, which was reported previously in *Chlorella vulgaris* [24].

Furthermore, the fingerprinting functional groups and the vibrational mode of the studied species were mainly via stretching and bending (deformation) (Table 2). In this study, the vibration

Figure 3: Raman spectra of: (a) saffron pigment; (b) carotene pigment.

of the functional groups is mostly infrared (IR) active and Raman inactive, as they are vibrated in an asymmetric stretching or bending mode, which alters the dipole moment of the covalent bond [6].

Functional Group	Bond	Raman Shift (cm^{-1})	Intensity	Vibrational Mode	Studied species
Alcohol C(C)(C)C-OH	C-C-O	1210-1100	Medium-strong	Asymmetric stretching	All studied species
Hydrazide (PhCONHNHCOPh)	C-N-H	1535-1525	Weak	Deformation (Amide II)	All studied species
Phosphorus	P-O-C	1050-970	Medium-weak	Asymmetric stretching	All studied species
Alkane (Cyclopropyl)	C-H	1200-1180	Variable	Ring vibration (ring breathing)	*Chlorella* spp.
Ether (Epoxy)	CH_2	3060-3030	Medium-weak	Asymmetric stretching	*C. calcitrans*
Halosilane	SiF_3	980-945	Medium-weak	Asymmetric stretching	*Tetraselmis* spp.
Aromatics (Tetra-substituted benzene)	Ring	1465-1430	Medium	Asymmetric stretching	*Nostoc* spp.

Table 2: The functional group and the vibrational mode of the studied species, with its respective Raman shift.

Based on table 2, in tertiary alcohol, the higher electronegativity of the C-O bond than the C-C bond during the stretching created a net change in the dipole moment [6]. The simultaneous C-N bond stretch and the C-N-H bond bending of hydrazide (cyclic trans-amide) resulted in a weak Raman band and a strong IR band of amide II (stretch-bend vibration). In organophosphorus, the stretching of the O-C bond is greater than the stretching of the P-O bond, resulting in an altered net dipole moment [6]. On the contrary, the ring breathing vibration of cyclopropyl is highly polarized [6], thus, the peak is Raman active and IR inactive.

Lastly, the C-C bonds of the benzene ring in *Nostoc* spp. favored the semicircle stretch at the wavenumber 1465-1430 cm^{-1} (Table 2). The C-C bonds in one semicircle region are being stretched, while the other is being contracted [6], thus, the spectrum is IR active and Raman inactive. However, the fluorescence noise inhibited the characterization of the functional groups at higher frequencies (3000-1600 cm^{-1}) in *Nostoc* spp. The increase of the laser's exposure time or higher magnification of the objective lens might reduce the noise signal [25,26]. Despite this, the fingerprinting region (frequencies below 1600 cm^{-1}) are still manageable for analysis.

p23SrV marker

The DNA extraction of microalgae and cyanobacteria using the phenol-chloroform method, quantified the ratio of A260/A280 and A260/A230, at 1.89 to 2.09 and 1.99 to 2.23, respectively (Table 3). SDS added in this extraction was able to digest the thick peptidoglycan layer of cyanobacteria, which was also reported by Morin., *et al*. [27]. However, using the commercial extraction kits, the EasyPure Bacteria Genomic DNA and the GF-1 Plant DNA, the lysis buffer failed to digest the cell wall of microalgae and cyanobacteria, resulting in a low purity. Phenol-chloroform produced high yield with good purity nucleic acids. This method is cost-effective as it caters both eukaryotic and prokaryotic extraction. A thick intact band at amplicon size of 400 bp was seen in a 2% agarose gel electrophoresis (Table 3). The gel images were represented in the supplementary section.

Species	Nucleic Acid Concentration (ng/µl)	A260/ A280	A260/ A230	Amplicon Size (bp)
C. calcitrans	105.7	1.99	1.99	400
I. galbana	120.2	2.06	2.11	
Chlorella spp.	99.3	2.09	2.03	
Tetraselmis spp.	133.7	2.05	2.23	
M. aeruginosa	121.1	1.89	2.20	
Nostoc spp.	129.5	2.02	2.16	

Table 3: The concentration and purity of the extracted nucleic acids from the selected microalgae and cyanobacteria, with its respective amplicon size.

Based on the Neighbor-Joining tree in figure 4, there were two major clades: the microalgae and the cyanobacteria. The microalgae clade further diverged into subclades of red microalgae, brown microalgae, and green microalgae. The lineage of microalgae and cyanobacteria were separated. All the studied species were grouped in their respective clade. *Tetraselmis* spp. is closely related to *Tetraselmis chuii*, *Chlorella* spp. is closely related to *Parachlorella* spp., and *Nostoc* spp. is closely related to *Nostoc commune*. Briefly, the taxonomic classification of both microalgae (eukaryotes) and cyanobacteria (prokaryotes) was simplified with the use of a single primer. Presting [9] reported that the plastid sequences of algae, diatom, euglena, land plants, as well as cyanobacteria, are primed exactly at the conserved region of the p23SrV. Thus, any organisms that have the plastid organelles or its pigments are possible to be amplified by this universal biomarker.

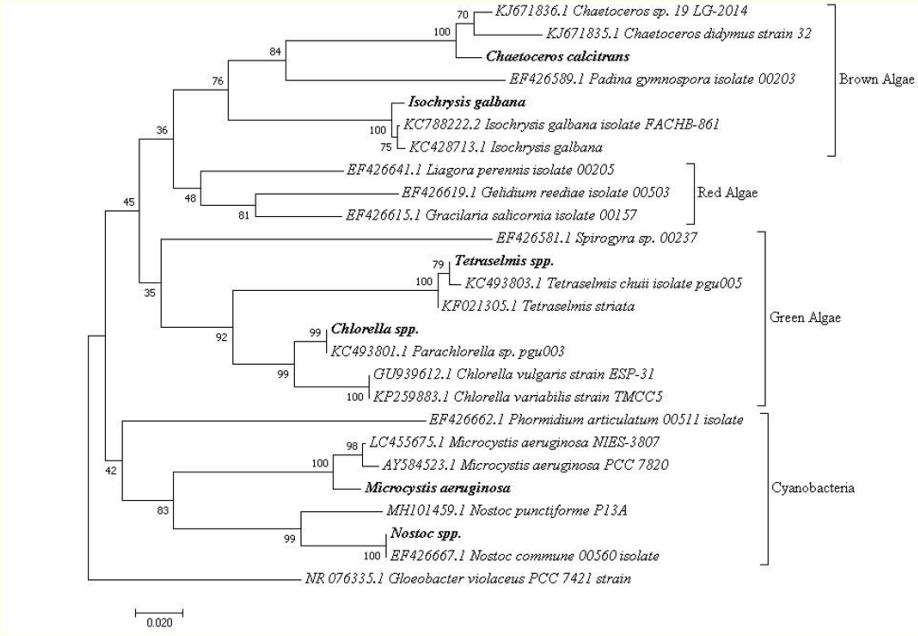

Figure 4: The Neighbor-Joining tree of the p23SrV sequence data of microalgae and cyanobacteria. The studied species were shown in bold.

Conclusions

The bioanalytical approaches investigated in this study simplified the characterization. The organic compounds detected in GC-MS can be used for industrial application, thus, a sustainable method to convert waste to wealth. Besides, the pigment analysis and the functional group vibrations obtained from the Raman scattering is fingerprinting to the studied species. This spectroscopy can be miniaturized into a handheld device for fieldwork in future, as it is non-destructive and measured directly, without the need for any sample preparation. Lastly, with the p23SrV biomarker, other multiple primers are not needed for the taxonomic characterization of microalgae and cyanobacteria. Perhaps, in future studies, this marker can be deployed in microalgae blooms of water sample for environmental monitoring.

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Conflicts of Interest

The authors declared there is no conflict of interest.

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