MEDWIN PUBLISHERS
Committed to Create Value for Researchers

Physical Science & Biophysics Journal
ISSN: 2641-9165

Simultaneous Detection of Multiple Carotenoid Pigments in Algae by Raman Spectroscopy and Classical Least Squares (CLS) Analysis

Sarkar P[1], Gupte Y[2], Kumbhar P[1], Talukdar J[2], Dasgupta S[2] and Bhakthavatsalam V[1]*

[1]Advanced Analytical Sciences, Research and Development Centre, Reliance Industries Limited, India

[2]Algae to Oil, Research and Development Centre, Reliance Industries Limited, India

Research Article

Volume 4 Issue 2

Received Date: July 27, 2020

Published Date: August 27, 2020

***Corresponding author:** Vishnupriya Bhakthavatsalam, Advanced Analytical Sciences R&D Center (TC30C-G1, CA01) Reliance Corporate Park Navi Mumbai, India - 400701, Tel: +91 8928523802; Email: Vishnupriya.B@ril.com

Abstract

Synthetic carotenoid pigments derived from petrochemicals are used for feed industry. However, due to health safety concerns these synthetic forms cannot be used for direct human consumption. Algae cells naturally produce multiple carotenoid pigments, including astaxanthin and lutein that have antioxidants, antitumor and antiviral properties. Hence, these natural carotenoids have high market value as nutraceutical supplements, and also as natural food coloring and cosmetics ingredient. Cost-efficient extraction and purification of these high value carotenoids from algae is challenging as the pigment profiles of algae cells is complex and dynamic. Cells contain a mixture of multiple pigments dissolved in lipids and the composition varies with growth stages, changes in environment, and nutrient availability. To be commercially beneficial, harvesting and extraction of algal cultures should be done at an optimal time point, when both biomass and the desired high-value carotenoid accumulation are at its peak. Pigment composition and quantity are conventionally determined by chromatography and UV/IR spectroscopy. These methods although can provide high accuracy level, are dependent on multi-step process of pigment extraction, needs large volume of algal biomass, expensive chemicals, laboratory equipments, and hours of skilled manpower. In this paper, we present a high-throughput method of *in situ* pigment profiling by Raman spectroscopy. Pigment spectra could be obtained under 5 min from live algae cells, without any extraction, drying or grinding. Identification and relative quantification of 3-6 carotenoid pigments simultaneously from the spectra was possible using CLS analysis method. The method was found to be sensitive, versatile and robust by statistical analysis. This approach can be used for pigment profiling and screening of live algal cell cultures with population variability for real-time assessment of their health and productivity. It additionally has the potential to be used for live pond monitoring using portable Raman spectrometers.

Keywords: Algae culture; Carotenoid pigments; CLS analysis; Pond monitoring; Raman spectroscopy; Rapid analysis

Introduction

Carotenoids are organic pigments that are synthesized from petrochemicals and used by animal and fish feed industry for flesh pigmenting. The synthetic carotenoids are not advisable to use for direct human consumption due to safety concerns [1]. Around 600 unique carotenoids are naturally produced by all plants and algae, some bacteria,

fungi, as well as few animals. Microalgal carotenoids such as astaxanthin, β-carotene, canthaxanthin, lutein, lycopene, and zeaxanthin have highly positive health benefits due to their high antioxidant, antitumor and antiviral properties [2-4]. These natural pigments have higher market value compared to the synthetic forms as they are found to be safe for direct human use in form of nutraceuticals, cosmetics and food colour enhancers [1,2,5]. The global market for carotenoids reached $1.5 billion in 2017, and estimated to reach $2.0 billion by 2022, at a compound annual growth rate of 5.7%, according to BCC Research Report, 2018. Such a steady growth in carotenoid market value has raised interest in commercial culturing of carotenoid-producing microalgae such as *Haematococcus pluvialis*, *Dunaliella salina, Isochrysis galbana, Coelastrella oocystiformi.* Cost analyses from pilot plants show that production cost of some carotenoids from algal biomass could be lower than that of their chemical synthesis, giving a significant profit margin [5]. Algae grow using carbon dioxide and produces oxygen and hence, algae culturing also helps in reducing our carbon footprint. Cost-effective production of these high value carotenoids from algal biomass however would require tight control on culturing, harvesting and extraction of the biomass.

Biosynthesis of carotenoids in microalgae initiates from dimethylallyl pyrophosphate (DMAPP) that forms phytoene and then lycopene. Lycopene is channelled either as α-carotene or β-carotene based on lycopene cyclase activity. Lutein is formed by the cytochrome P450 hydroxylase from the α-carotene pool, whereas β-carotene leads to cryptoxanthin formation via hydroxylase activity, and to echinenone via ketolase activity. It is known that the β-carotene ketolase and hydroxylase are two enzymes that determine the fate of β-carotene pools in an alternate or progressive manner. The enzyme activity hence controls the amount of various pigments such as zeaxanthin, canthaxanthin, astaxanthin, antheraxanthin, violaxanthin, neoxanthin, diadinoxanthin, fucoxanthin, etc. produced in each cell [6]. The number and quantity of carotenoid pigments produced in the algal cells vary at different growth stages. Nutrient composition of growth media as well as environmental conditions such as light and temperature affect the rate of biomass production and pigment profile [7,8]. Some algal strains such as *Coelastrella oocystiformis* can produce and store up to 9 carotenoids at any given time [9]. Hence, harvesting and extraction of culture should be done at the optimum time point when both biomass and accumulation of the targeted high-value carotenoid pigments are at its peak to be commercially beneficial.

Production of carotenoid pigments in algal culture are assessed by thin-layer chromatography (TLC), high pressure liquid chromatography (HPLC), ultra violet - visible (UV-Vis) spectroscopy, or Fourier Transform Infrared (FT-IR) spectroscopy [10-17], which require extraction of total pigments from the algal cultures. The extraction process requires large quantities of sample and time. Due to light-sensitive nature of pigments and variability in extraction efficiency, there could be inaccuracy in analysis of pigment extracts. Sophisticated chromatographic methods such as HPLC also require skilled analyst and frequent calibration of the equipment using expensive standards. Raman spectroscopy can be used as a tool to perform real-time carotenoid analysis of aqueous cell cultures without any extraction, drying or grinding steps. Using a bench-top micro-Raman spectrometer, carotenoid detection can be done from even a single cell [13,14,17,18]. Hence, diversity within the cell culture can also be monitored in real-time. An advanced feature of micro-Raman spectrometer, the mapping tool, has been used to detect astaxanthin localization within different parts of single algae cells [13]. Portable and on-line Raman spectrometers have been used for high-throughput carotenoid analysis of fungal, bacterial and algal cultures in field [19,20].

In Raman spectroscopy, an algae cell is illuminated by a laser beam. Incident photons with high energy interacts with the biomolecules present within the sample, and in that process, photons return to ground state and emits a scattered light with a different frequency (inelastic Raman scattering). Each biomolecule produces a "fingerprint spectra" based on its unique chemical structure that allows their identification [21]. In case of carotenoids, the fingerprint spectra of individual pigments are similar due to similarities in their chemical structure. Three major peaks can be observed in Raman spectra of all carotenoid pigments at around 1005, 1157, and 1525 cm^{-1} due to ($C–CH_3$ deformation), (C-C stretching) and (C=C stretching) respectively [14,22]. The exact value and relative intensity ratio of these peaks however, vary slightly for individual carotenoids due to the minor structural differences such as variation in chain length, side chains etc. Additional smaller characteristic peaks are also observed for various carotenoids due to the above differences [13,22].

A sample with multiple components produces a complex spectrum due to overlaps in fingerprint peaks. When the molecular structures of these components have many similarities, such as in case of carotenoid pigments within any cell, complexity of spectrum further increases. The minor differences between these spectra are difficult to differentiate manually. Advanced spectral analysis methods, such as Multivariate Analysis (MVA), is hence required to identify the various molecular components present in such complex samples. MVA are statistical techniques used to analyse data that consists of multiple measurements made simultaneously on several individuals, objects or data samples [23]. Various MVA approaches are available for

analysing spectroscopy data of mixtures. CLS method is an easy and fast to use MVA approach. CLS model assumes that spectrum of a mixture of multiple components is the sum of spectra of the pure components and requires reference spectra of all pure components [24]. CLS method has been used for separating total carotenoid signal from UV-spectra [25] and for distinguishing between two carotenoid by Raman spectroscopy in human retina [26]. Decomposition of complex Raman spectra into individual pure carotenoids using CLS method has not been reported yet.

In this paper, we report simultaneous detection of up to six carotenoid pigments and their relative quantification. We used *in situ* Raman spectroscopy of live algae cell cultures followed by CLS spectral analyses. This method can be used for real-time screening of live algae cultures in lab or outdoor. Use of this technique can ease routine monitoring of rapid changes in carotenoid pigment profile, which would enable rapid decision-making during algae cultivation.

Materials & Methods

Algae Culture

Algae cultures of *Haematococcus pluvalis* were grown in Bold Basal Media in glass bottles or flasks in lab, under 100 µmol m^{-2} s^{-1} light 12:12 light:dark period, with continuous 2% CO_2, unless any variation is mentioned below for specific experiments.

Raman Spectroscopy of Fresh whole Cell Cultures

Freshly collected cell cultures were taken on an aluminium covered glass slide with wells. Excess water was removed carefully to minimize cell movement. Individual cells were located, and Raman point spectra were collected through 50X objective lens of micro-Raman spectrometer (Horiba LabRAM HR Evolution). Spectra of at least 5 individual cells were collected for each sample. All spectra were collected with the 532 nm laser incident at 2.5 mW for 5 iterations of 5 s exposure.

CLS analysis of Raman Spectra

CLS fitting option available in Horiba LabSpec6 Spectroscopy Suite software [27,28] was used for decomposition of complex spectra into known carotenoid pigment spectra. CLS reference library was created with six pure carotenoid pigment standards: alloxanthin, antheraxanthin, astaxanthin, lutein, violaxanthin and zeaxanthin (Sigma-Aldrich and DHI pigments). CLS decomposition of each complex spectrum from algae samples was done after normalizing the six standard spectra using the "normalize loadings" option in the CLS module. Scores obtained for the 6 known components by CLS decomposition were normalized using "normalize scores" option in the same software. Presence/absence of these 6 pigments and their relative amount were obtained for each sample spectrum.

Validation by HPLC

Pigment detection method by Raman spectroscopy of whole cells was validated by conventional HPLC of cell extracts. For this, Raman spectra was collected for 3 visibly different cell cultures from different growth stages. Spectra analyses for each was done for pigment identification as described above. For HPLC analysis, 2 mL each of same cell cultures (= 2.44 mg dry biomass) were extracted in 1 mL of 100% acetone/ Dimethyl sulfoxide (DMSO) in a bead beater for 15 min with intermittent shaking after every 5 min. Extracts were centrifuged at 10,000 rpm for 2 min and the supernatant collected in amber coloured vials. The extraction steps were repeated until both pellet and supernatant obtained were colourless. Extracts were analysed immediately or stored at 20°C until further use. All extracts were analysed by HPLC (Agilent 1260 Infinity) using 10 mL of extract as injection volume, mobile phase A- Methanol: ACN: 0.25 M ammonium acetate (50:25:25) and B- Methanol: ACN (40:60), running for 70 min at 40°C, with flow of 0.5 mL/min on phenomenex column (c-18_2, 10amst, 150*4.6mm).

Application in Algae Strain Screening and Real-Time Monitoring of Algae Cultures

For strain screening, five fresh algae cultures were analysed by collecting Raman spectra of individual cells (n=10 cells for each sample), followed by the CLS method described above. The production level of six pigments: alloxanthin, antheraxanthin, astaxanthin, lutein, violaxanthin and zeaxanthin, in each sample were then compared to determine the relative differences in pigment productivity. Mean and standard error were calculated for relative amount of each pigment found in each sample.

For real-time growth and production monitoring, one algae culture was monitored over 21 days to determine the changes in its pigment profile with time. Raman spectra were collected for individual cells (n=10 cells for each time-point), followed by the CLS method described above. The production level of six pigments: alloxanthin, antheraxanthin, astaxanthin, lutein, violaxanthin and zeaxanthin, were then compared to determine the relative changes in pigment productivity with time. Mean and standard error were calculated for relative amount of each pigment found in each sample.

To determine the possibility of using Raman spectroscopy in field for high-throughput carotenoid production monitoring, portable Raman spectrometer (Bruker Bravo) was tested. Standard spectra of a benchmark sample with desired pigment content was obtained and saved as reference spectra in the handheld Raman spectrometer. Acceptable hit quality index (HQI) of 0.95 was set. 1 mL each of fresh cell cultures from algae samples with known pigment composition were taken in HPLC glass vials, Raman spectra collected by the handheld Raman spectrometer, and compared with the reference spectra. Each sample was tested at least 3 times.

Results & Discussion

Raman spectra for carotenoid pigment detection could be obtained from whole algae cells by simply putting a drop of live cell cultures on a regular microscopy slide covered with aluminium foil. Creating wells in the foil helped in minimizing the quantity of cell culture needed for analysis (Figure 1A). Total pigment signal with prominent carotenoid peaks around 1005, 1157, and 1525 cm^{-1} due to ($C–CH_3$ rocking), (C-C stretching) and (C=C stretching) respectively, was detected consistently in various live algae cells. Only sample preparation step required was removal of excess water from the slide carefully to minimize cell movement. Sample preparation steps such as cell centrifugation, drying or chemical extraction that are required for conventional pigment analysis methods such as, HPLC and UV/IR spectroscopy, were eliminated. Additionally, Raman spectra for pigments could be obtained consistently with dried algae cells and extracted pigments as well (data not shown) showing versatility of this method. Using Raman microscope (Horiba LabRAM HR Evolution), individual algae cells of varying shape, size and colour could be located for spectra collection, allowing to correlate the chemical and structural characteristics of the individual cells. For example, Figure 1B shows oval green cells of *H. pluvalis* culture in growth stage that were 25-35 µm in diameter with multiple pyrenoids. Figure 1C shows spherical red cells, around 25-30 µm with a smooth, featureless cell wall from the same culture after growth stops. Raman spectra of the two algae cultures could be collected for comparison (Figure 1D) under 10 min.

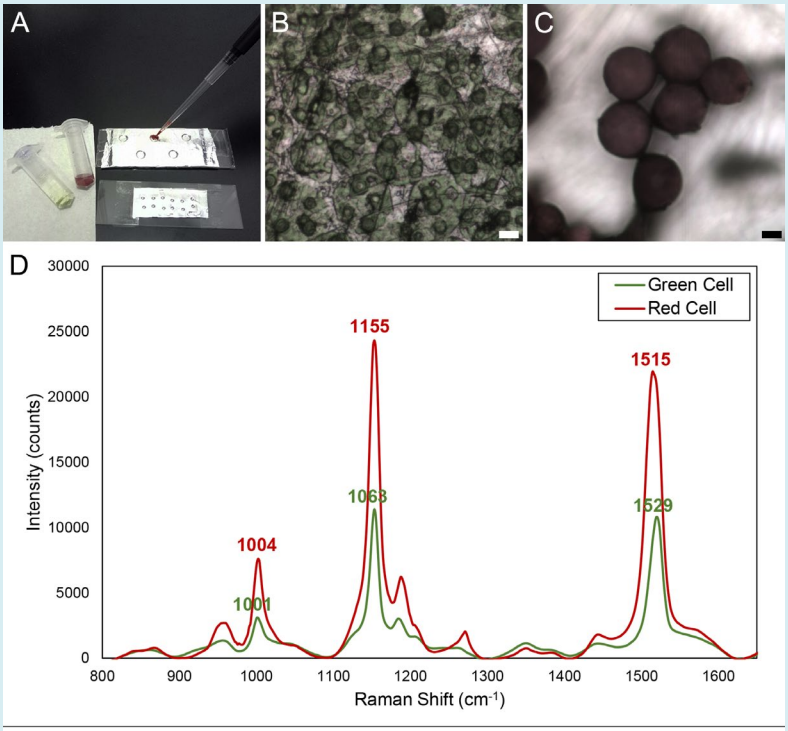

Figure 1: Raman spectroscopy of individual algae cells using Raman micro-spectrometer. (A) Freshly collected cell cultures were taken on an aluminium covered glass slide and excess water was removed carefully to minimize cell movement. No additional processing was required. (B-D) Cell localization of individual algae cells of varying shape, size and colour with simultaneous spectra collection for correlating chemical and structural characteristics. (B) Algae culture of oval green cells, 25-35 µm in diameter with multiple pyrenoids. (C) Algae culture of spherical red cells 25-30 µm in diameter with smooth featureless cell wall. Scale bars = 10 µm. (D) Raman spectra of green and red cells for comparative analysis showing major peaks of carotenoids.

The complex Raman spectra from whole cells that contain signal from multiple carotenoids could not be decomposed into the individual carotenoids manually due to close similarity in their chemical structure. CLS fitting (available in Horiba LabSpec6 Spectroscopy Suite Software) was used for decomposition of such complex spectra into the individual pure carotenoid components. For this, a CLS library was first created with spectra of pure carotenoid standards (pure components) collected at same settings as of algae samples (Figure 2A; Table 1). The pure carotenoid pigment standards used were: alloxanthin, antheraxanthin, astaxanthin, lutein, violaxanthin and zeaxanthin, which are known to be present in microalgae [6]. After normalizing the six standard spectra, they were fitted into each complex spectrum from algae samples by CLS algorithm. The differences in characteristic peaks (Table 1) that were difficult to separate manually could be separated by this fitting approach. Presence/absence of the six standard pigments and their relative quantity could hence be obtained from each complex spectrum (Figure 2B) as soon as the spectra is collected. This combination of Raman spectroscopy and CLS spectra analysis can therefore detect multiple structurally similar pigments simultaneously in real time without any sample preparation of the cell cultures. For example: CLS fitting of the spectrum in Figure 2B showed that majority of the carotenoid signal was contributed by zeaxanthin, alloxanthin and antheraxanthin that are usually found in algae cells at biomass growth stage. However, small amount of high value pigments such as, astaxanthin and lutein were also detected, which indicates that the cells were starting to go into stressed stage when these pigments are usually formed. This method can hence be used to study cells at various stages of growth and pigment production. This method could also be a handy tool to quickly identify enzymatic routes of carotenoid synthesis in algae cultures. Only relative quantification was possible by this rapid method, as fragile nature of live cells and cell-to-cell variation did not allow for absolute quantification.

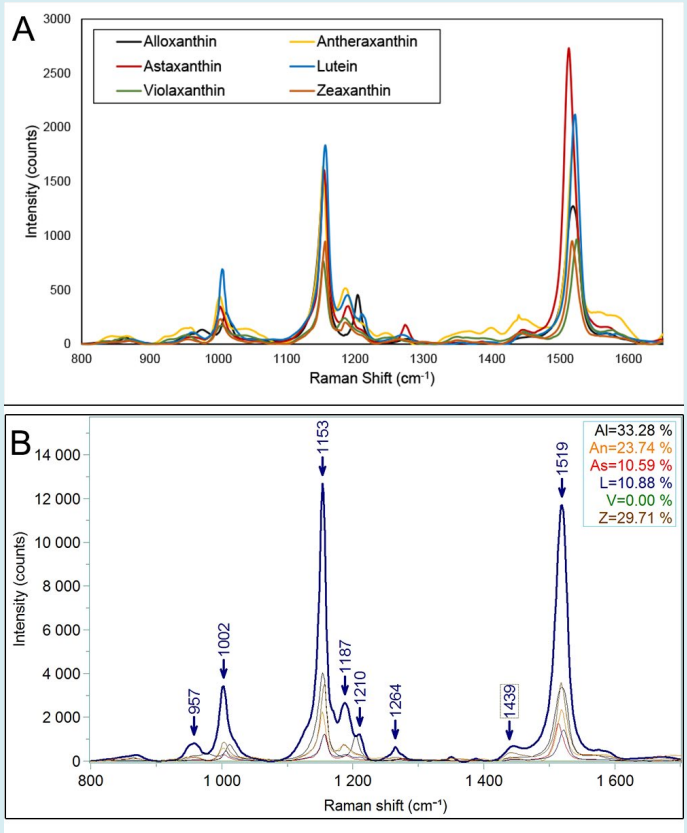

Figure 2: CLS analysis of Raman spectra of carotenoids. (A) Spectra library of pure components prepared with pure carotenoid standards. (B) CLS fitting of complex spectra of algae samples with the 6 carotenoid standards loaded in CLS library. (Inset) Relative amount of the 6 carotenoids obtained by CLS decomposition.

Alloxanthin	Antheraxanthin	Astaxanthin	Lutein	Violaxanthin	Zeaxanthin
977 w	959 w	952 w	962 w	954 w	956 w
1012 m	1002 m	1004 m	1006 m	1004 m	1004 m
1153 s*	1153 s-	1157 s-	1157 s-	1153 s-	1157 s-
	1186 m	1189 m	1189 m	1186 m	1186 m
1204 m			1210 m		
1276 w	1245 w	1274 w	1268 w	1252 w	1265 w
1449 w	1439 w	1444 w	1450 w	1447 w	1441 w
1518 s-	1519 s*	1513 s*	1522 s*	1524 s*	1518 s*

Table 1: Characteristic Raman peaks observed in xanthin pigment standards. s: strong (s* and s- denotes relative intensity ratio between the two strong peaks. s* is of higher intensity that respective s- for each standard); m: medium; w: weak.

For validation of this Raman spectroscopy-CLS fitting approach, algae cells were analysed by the Raman method and extracts of same algae cultures were analysed by HPLC for comparison. Figure 3 shows examples of this validation test with three visibly different algae cultures – green (sample 1), brown (sample 2), red (sample 3). Raman spectroscopy with CLS fitting could detect significant amount (arbitarily set to above 5% of total signal) of lutein and violaxanthin in sample 1 (Figure 3A); lutein, violaxanthin and astaxanthin in sample 2 (Figure 3B); astaxanthin, zeaxanthin, lutein and alloxanthin in sample 3 (Figure 3C). The same pigments could be detected by HPLC analysis of pigment extracts from the same cell cultures (Figure 3D-F). HPLC could detect few additional unknown peaks (Figure 3E) as cell extract is likely to have additional components that we did not consider for Raman-CLS analysis. Use of additional pure components for CLS or use of advanced spectral analysis methods that are independent of prior knowledge of pure components could be tested to detect all components in cell extracts. CLS of Raman spectra could distinguish between isomers (lutein and zeaxanthin) in Sample 3 (Figure 3C) due to their structural difference, and hence can be very effective in such samples.

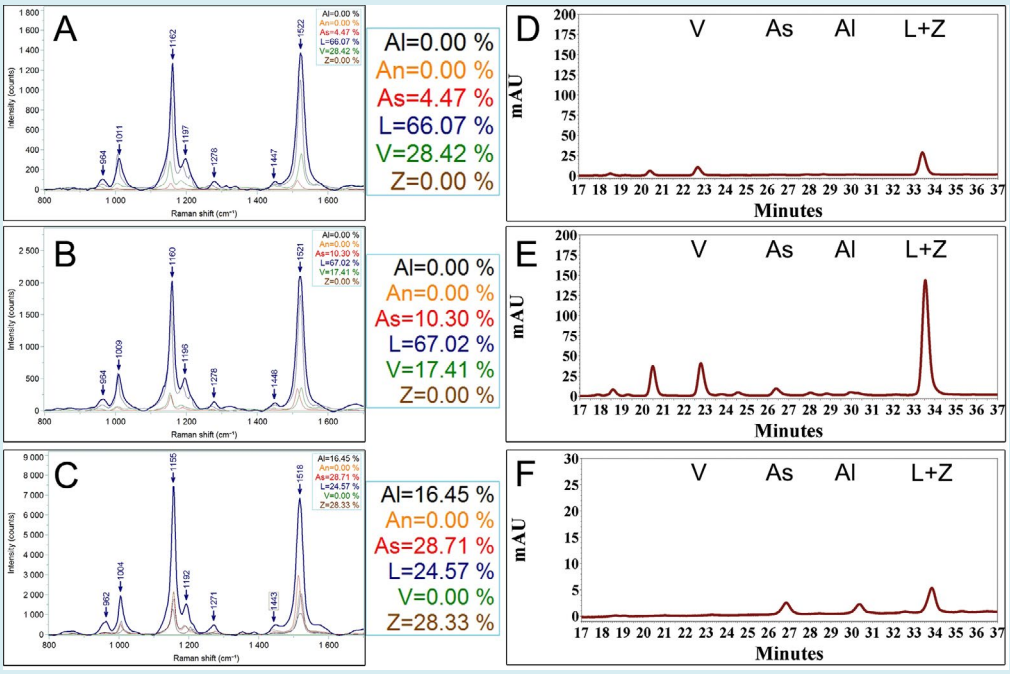

Figure 3: Validation of Raman-CLS method by HPLC analysis (A-C) Detection of pigments by Raman spectroscopy of three different cultures of algae samples, followed by pure pigments detected by CLS fitting. (D-F) Pigment profiles of extracts of same algae cultures obtained by HPLC. *Abbreviations:* Al = Alloxanthin, An = Antheraxanthin, As = Astaxanthin, L = Lutein, V = Violaxanthin, Z = Zeaxanthin.

Carotenoid pigment profiles showing relative levels of 3-6 carotenoids could be obtained for different cell cultures by Raman spectroscopy and CLS spectra analysis (Figure 4). Some samples produced as high as six carotenoids (sample 3), while some had only 3 carotenoids (samples 4-5). Presence of more components did not affect the robustness of CLS method. The standard error for lutein and violaxanthin in sample 3 was similar or even less than that in the other samples with fewer pigments. This indicates that the variabilities observed for pigments are not from experimental errors. The high variabilities observed in some pigments of sample 3, such as astaxanthin and zeaxanthin, are hence only from variability in cell populations. Additionally, the small error bars for pigments present in very small quantity such as, antheraxanthin in sample 2 and 3, indicates high sensibility of the CLS method. This robust and sensitive method can hence be used for rapid relative quantification also in addition to simultaneous detection of multiple pigments. This can help in quickly screening multiple cell cultures to select strains that have the highest amount of desirable high value pigments. For example, if an astaxanthin producing facility must select one strain for scale up from the five strains used for Figure 4, they would need to check multiple factors for their commercial viability. Apart from good growth and high astaxanthin production level, choice of good strain will also depend on variability within population and efficiency of pure astaxanthin extraction from the cell cultures. From Raman analysis of the five samples, it can be quickly concluded that samples 1 and 2 are not astaxanthin producers. Sample 3 although has the highest astaxanthin content, also has the widest population variability. It also has 5 other pigments in the same cells at the same time, which could make extraction process in downstream more challenging. On the other hand, sample 4 and 5 that although have slightly lower astaxanthin productivity, have much narrower population variability with only 3 types of pigments. The later factors possibly would result in higher astaxanthin purification efficiency for samples 4 and 5 compared to sample 3, making them a better choice for scale up. The above Raman analysis can be done under an hour, while conventional analysis by HPLC or UV/IR spectroscopy of the same samples would require 1-2 days. Raman analysis would allow taking decisions such as, which strain to continue growing faster with detailed reasoning available on-site.

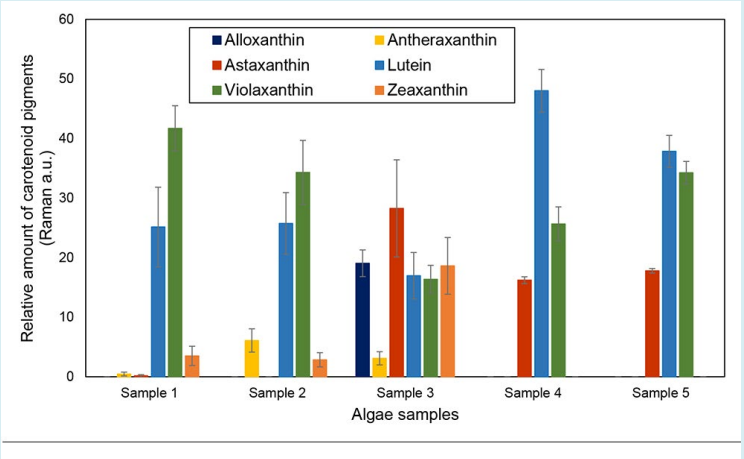

Figure 4: Production level of six carotenoids in live algae cultures as determined by Raman spectroscopy with CLS spectra analysis. Each bar represent mean (n=10) with standard error.

The Raman-CLS method could also be used to monitor changes in pigment profile of a specific algae culture during different stages of its growth over many days (Figure 5A). Relative quantity of each of the six pigments were determined by calculating the mean (n=10), and variance in population was determined by calculating standard error. During biomass growth stage of the culture (Control or 0-day), pigments such as violaxanthin and antheraxanthin were the main carotenoids, lutein was present in small amount in few cells, while the other three pigments were not detected at all. The pigment profile started showing noticeable changes between 1-6 days. Violaxanthin and antheraxanthin signals started decreasing, while lutein and astaxanthin signals started to increase, indicating a shift from growth stage to stressed stage. These trends continued with minor fluctuations and by 14th day, pigment profiles reached a steady state with little change in relative amount of pigments and variability. The culture showed the presence of high amount of lutein and increased amount of astaxanthin consistently. Violaxanthin also reached a steady lower amount than in early growing

stages. Antheraxanthin was not detected at this point. Alloxanthin and zeaxanthin were rarely detected in this cell culture. Variability in each pigment quantity again increased by Day 21, which indicates that some cells are reaching the end of their life cycle. Daily monitoring of cell cultures can allow taking real-time decisions to regulate growth parameters such as temperature, pH or nutrient supply.

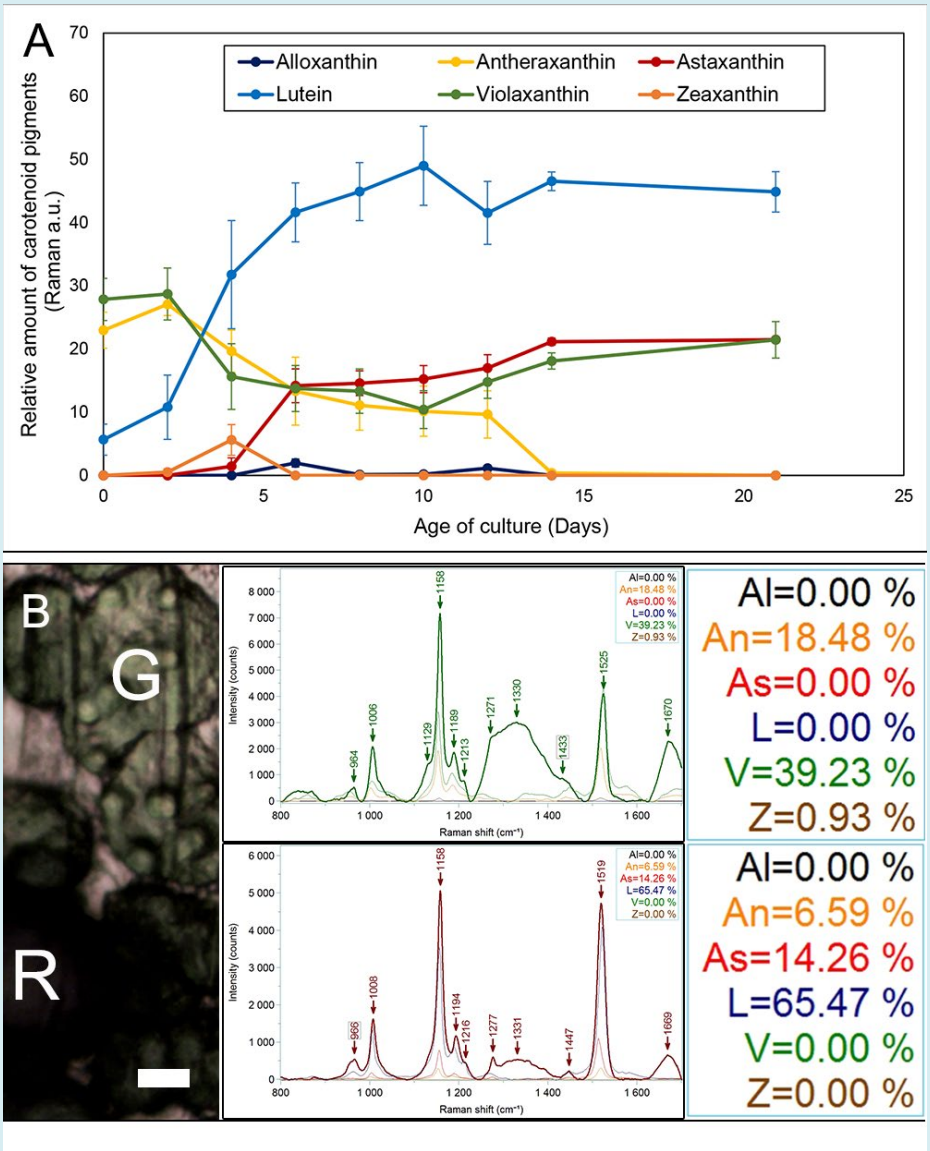

Figure 5: Growth and pigment profile monitoring of live algae cell culture. (5A) Each line in graph represents the changes in relative quantity of the six carotenoid pigments detected in an algae culture on various days over 3 weeks. Each data point represents mean (n=10) with calculated standards error bars. (5B) Simultaneous live cell imaging and Raman spectroscopy showing pigment profile of individual cells within a heterogenous cell culture. Scale bar = 10 µm. *Abbreviations:* G = Green cells, R = Red cells.

For cultures with individual cells at varying stage of growth and development, such as the culture at Day 3 in Figure 5A, the high variability in population can be further analysed at individual cell level by Raman microscopy. In this method, simultaneous optical imaging and Raman spectroscopy of visibly different cells show the pigment profile in cells at various growth stages present within same culture (Figure 5B). Even though the entire culture might appear green, close look at individual cells showed presence of few red cells. Raman with CLS analysis confirmed that those

red cells have elevated amount of lutein and astaxanthin; and decrease in antheraxanthin and violaxanthin compared to the green cells. This helps in early detection of growth phase completion of a population. Precise decisions can be made about when the culture has reached its maximum biomass increase stage and is ready for adding stress inducers for maximizing production of stress pigments such as astaxanthin. In a situation as above, one could save 1-2 days of culture time by adding stress inducers after Day 3, instead of waiting until Day 6 when the entire culture starts turning red after growth completion of all cells in the population. This could mean saving valuable time and resources without compromising on biomass or pigment productivity, and hence could increase cost efficiency.

Handheld Raman spectrometer (Figure 6A) could be further used to determine similar pigment profiling and relative comparison of live algae cultures. Each sample could be accepted or rejected under 2 min by comparing its Raman spectra with that of benchmark sample. Acceptable closeness of fit between sample and benchmark spectra can be set as hit quality index (HQI) in the instrument. Figure 6B-E shows two examples of such testing. Spectra of first sample matched well with that of benchmark sample (Figure 6B) and was instantly accepted for further processing (Figure 6C). Spectra of second sample however did not match the benchmark sample (Figure 6D) and was immediately rejected (Figure 6E), saving time and resources needed for further extensive analysis. This method can be further extended for relative quantification with systematic analysis of diverse field samples whose composition is pre-determined by benchtop Raman spectrometer and HPLC. This portable method can be used for real-time culture monitoring at outdoor pond or photobioreactors.

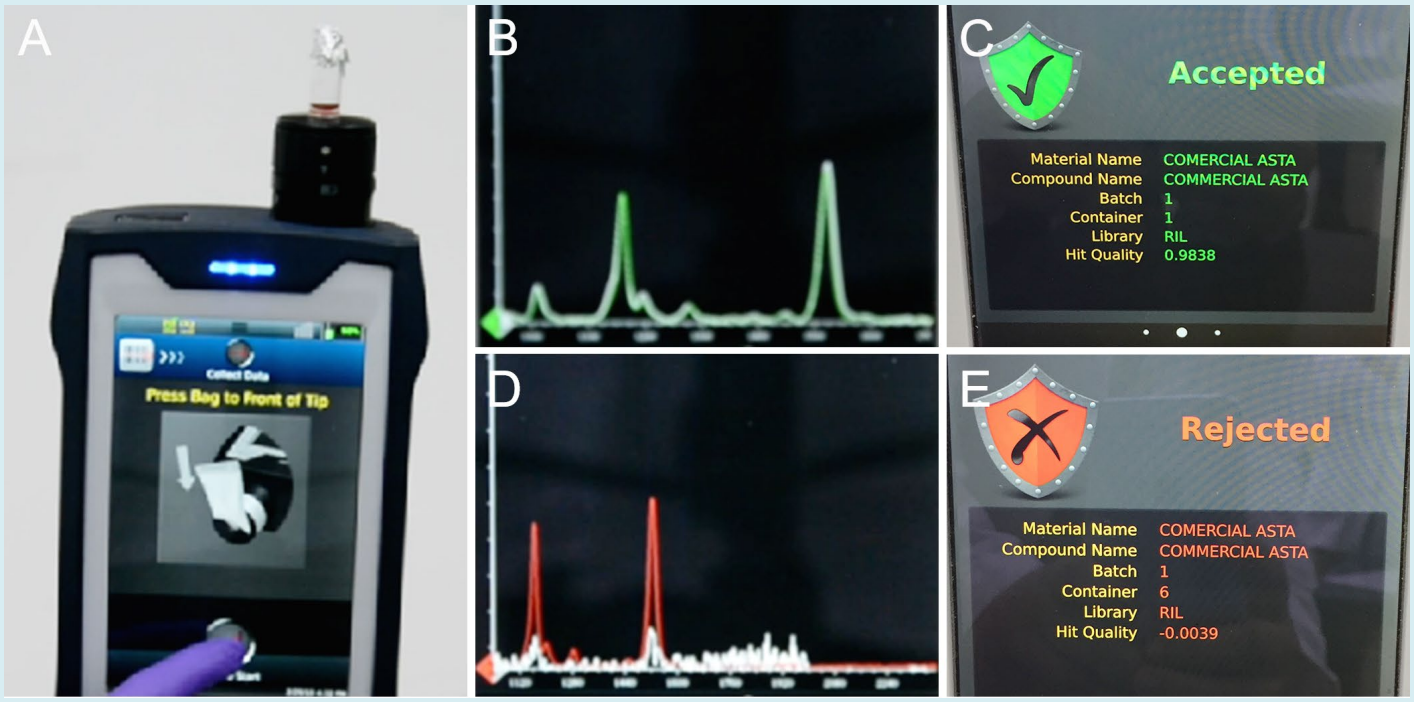

Figure 6: (A) Carotenoid production monitoring by handheld Raman spectrometer. (B, D) Sample spectra (white) is matched with benchmark spectra (green/red) and (C, E) accepted or rejected based on pre-set hit quality index.

Conclusion

Raman spectroscopy with CLS fitting could detect up to six structurally similar carotenoid pigments: alloxanthin, antheraxanthin, astaxanthin, lutein, violaxanthin and zeaxanthin from algae, and their relative quantity could also be obtained at various growth stages. Real-time analysis could be done with live cell cultures without any extraction, drying or grinding, and this method is versatile to analyse dried algae cells and extracted pigments as well. Presence of more pigments did not increase experimental variability that shows robustness of this analytical approach. Qualitative carotenoid detection by Raman and CLS was validated by HPLC. CLS method of spectra decomposition however has the disadvantage of detecting only the components whose pure spectra are available, and advanced multivariate spectra analysis methods could be tested for overcoming this drawback. This method when used with a Raman microscope

allowed to correlate chemical and structural characteristics of individual algae cells within a diverse population. This method if transferred to a portable Raman could be used for real-time culture monitoring at outdoor pond or photobioreactors and can help in taking rapid decisions on-site. This would save valuable time and resources without compromising pigment productivity, and hence could increase commercial viability.

Conflict of Interest

The authors do not have any conflict of interest.

Acknowledgements

The authors are thankful to Dr. Chandra Saravanan, Dr. Manish Shukla, Dr. Meenakshi Chelliah and Dr. Vinod Nagle for supporting this work. Thanks to Ms. Divya Borkar & HPLC team of AAS-RIL for help with data collection, Dr. Rudra Choudhury for inputs on spectroscopy, and Bruker for providing demo portable Raman spectrometer.

Funding

This work was funded by Reliance Industries Limited.

References

1. Capelli B, Bagchi D, Cysewski GR (2013) Synthetic astaxanthin is significantly inferior to algal-based astaxanthin as an antioxidant and may not be suitable as a human nutraceutical supplement. Nutrafoods 12: 145-152.

2. Vílchez C, Forján E, Cuaresma M, Bédmar F, Garbayo I, et al. (2011) Marine carotenoids: Biological functions and commercial applications. Mar Drugs 9(3): 319-333.

3. Hegazy GE, Abu-Serie MM, Abo-Elela GM, Ghozlan H, Sabry SA, et al. (2020) In vitro dual (anticancer and antiviral) activity of the carotenoids produced by haloalkaliphilic archaeon *Natrialba* sp. M6. Sci Rep 10.

4. Santoyo S, Jaime L, Plaza M, Herrero M, Rodriguez-Meizoso, et al. (2012) Antiviral compounds obtained from microalgae commonly used as carotenoid sources. J App Phycol 24: 731-741.

5. Li J, Zhu D, Niu J, Shen S, Wang G (2011) An economic assessment of astaxanthin production by large scale cultivation of *Haematococcus pluvialis.* Biotechnol Adv 29(6): 568-574.

6. Ye MV, Bhatia SK (2012) Pathway engineering strategies for production of beneficial carotenoids in microbial hosts. Biotechnol Lett 34(8): 1405-1414.

7. Panis G, Carreon JR. (2016) Commercial astaxanthin production derived by green alga *Haematococcus pluvialis*: A microalgae process model and a techno-economic assessment all through production line. Algal Research 18: 175-190.

8. Ota S, Morita A, Ohnuki S, Hirata A, Sekida S, et al. (2018) Carotenoid dynamics and lipid droplet containing astaxanthin in response to light in the green alga *Haematococcus pluvialis*. Sci Rep 8.

9. Iyer G, Nagle V, Gupte YV, Desai S, Iyer M, et al. (2015) Characterization of high carotenoid producing *Coelastrella oocystiformis* and its anti-cancer potential. Int J Curr Microbiol App Sci 4(10): 527-536.

10. Rao R, Sarada AR, Baskaran V, Ravishankar GA (2009) Identification of carotenoids from green alga *Haematococcus pluvialis* by HPLC and LC-MS (APCI) and their antioxidant properties. J Microbiol Biotechnol 19(11): 1333-1341.

11. Lu M, Zhang Y, Zhao C, Zhou P, Yu L (2010) Analysis and identification of astaxanthin and its carotenoid precursors from *Xanthophyllomyces dendrorhous* by high-performance liquid chromatography. Z Naturforsch 65(7-8): 489-494.

12. Cerón-García MC, González-López CV, Camacho-Rodríguez J, López-Rosales L, García-Camacho F, et al. (2018) Maximizing carotenoid extraction from microalgae used as food additives and determined by liquid chromatography (HPLC). Food Chemistry 257: 316-324.

13. Collins AM, Jones HDT, Han D, Hu Q, Beechem TE, et al. (2011) Carotenoid distribution in living cells of *Haematococcus pluvialis* (Chlorophyceae). PLoS ONE 6(9): e24302.

14. Osterrothová K, Culka A, Němečková K, Kaftan D, Nedbalová L, et al. (2019) Analyzing carotenoids of snow algae by Raman microspectroscopy and high-performance liquid chromatography. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 212: 262-271.

15. Kushwaha K, Saxena J, Tripathi BK, Agarwal MK (2014) Detection of carotenoids in psychrotrophic bacteria by spectroscopic approach. J BioSci Biotech 3(3): 253-260.

16. Minyuk GS, Solovchenko AE (2018) Express analysis of microalgal secondary carotenoids by TLC and UV-Vis Spectroscopy. In: Barreiro C, Barredo JL (Eds.), Microbial

carotenoids. Methods in Molecular Biology, Humana Press, New York, USA 1852.

17. Liu JH, Huang Q (2016) Screening of astaxanthin-hyperproducing *Haematococcus pluvialis* using Fourier Transform Infrared (FT-IR) and Raman microspectroscopy. Appl Spectrosc 70(10): 1639-1648.

18. Kaczor A, Turnaub K, Baranska M (2011) *In situ* Raman imaging of astaxanthin in a single microalgal cell. Analyst 136(6): 1109-1112.

19. Cannizzaro C, Rhiel M, Marison I, Von Stockar U (2003) On-line monitoring of *Phaffia rhodozyma* fed-batch process with *in situ* dispersive Raman Spectroscopy. Biotechnol Bioeng 83: 668-680.

20. Jan J, Adam C, Lily M, Aharon O (2019) Comparison of miniaturized Raman Spectrometers for discrimination of carotenoids of halophilic microorganisms. Front Microbiol 10: 1155.

21. Mayo DW, Miller FA, Hannah RW (2003) Course notes on the interpretation of infra-red and Raman spectra. John Wiley & Sons, New Jersey, USA, pp: 567.

22. Jehlička J, Edwards HGM, Osterrothová K, Novotná J Nedbalová L, et al. (2014) Potential and limits of Raman spectroscopy for carotenoid detection in microorganisms: Implications for astrobiology. Phil Trans R Soc A 372(2030).

23. Anderson TW (1984) An Introduction to multivariate statistical analysis. Wiley, New York, USA, pp: 6-56.

24. Diaz TG, Guiberteau A, Burguillos JMO, Salinas F (1997) Comparison of chemometric methods: derivative ratio spectra and multivariate methods (CLS, PCR and PLS) for the resolution of ternary mixtures of the pesticides carbofuran carbaryl and phenamifos after their extraction into chloroform. Analyst 122(6): 513-517.

25. Abrahamsson V, Jumaah F, Turner C (2018) Continuous multicomponent quantification during supercritical fluid extraction applied to microalgae using in-line UV/Vis absorption spectroscopy and on-line evaporative light scattering detection. J Supercrit Fluids 131: 157-165.

26. Li B, George EW, Rognon GT, Gorusupudi A, Ranganathan A, et al. (2020) Imaging lutein and zeaxanthin in the human retina with confocal resonance Raman microscopy. PNAS 117(22): 12352-12358.

27. Lancelot E (2013) Perspectives on Raman Spectroscopy of graphene. Horiba Scientific application note: Nanotechnology Raman Spectroscopy.

28. Adar F, Leary P, Kubic T (2014) Raman Microscopy for Detecting Counterfeit Drugs-A Study of the Tablets Versus the Packaging. Spectroscopy 29(6).