IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS)
e-ISSN: 2278-3008, p-ISSN:2319-7676. Volume 10, Issue 1 Ver. II (Jan -Feb. 2015), PP 15-21
www.iosrjournals.org

Energy-Dispersive X-Ray Spectroscopy Procedure for Analysing Cellular Elemental Affinity of Pigmented Phototrophs

[1,2,]*Dr. Olawale Faith-Anthony Akinnuoye
[1]*Academic Research and Administration, MFM Group Sdn Bhd, Kuala Lumpur, Malaysia*
[2]*Formerly in School of Biosciences and Biotechnology, Faculty of Science, National University of Malaysia, 43600 Bangi, Selangor DE, Malaysia*
**dr.akinnuoye@gmail.com*

Abstract: *Four phototrophic bacterial species Blastochloris sulfoviridis, Rhodocista pekingensis, Rhodopseudomonas palustris and Rhodomicrobium vannielii, isolated from hot springs were analysed for cellular elemental affinity between them and to determine possible relationship between physiological features and the constituent elements. A novel methodology using Energy-dispersive X-ray spectroscopy (EDS/EDX) of pigments produced by the bacteria was adopted and applied. Results did show close affinity of cellular elements with little or no difference in weighted and atomic percentages of the constituent elements. There was also little or no inference in the effects of these elements on the pigments and other features of the bacteria such as colour and morphological differences could not be fully attributed to the elemental inclusions. It was concluded that systemic factors that were responsible for extraneous features such as pigmentation, pigment density etc, could be a combination of elemental inclusion variations, genetics and other factors in-between rather than one. Required energy sources and metabolic factors were assumed to play key roles in contents and types of cellular elements in relation to pigmentation.*

Keywords: *Cellular element, Elemental affinity, Energy-dispersive X-ray spectroscopy, Pigmented bacteria.*

I. Introduction

The presence of phototrophic bacteria is naturally observable in their habitat with the appearance of pigmented blooms which in many cases gives vivid colouration of the water in which they are found. Tit has been shown that purple non-sulphur bacteria photo-assimilate a wide range of organic compounds and the extent to which water is polluted with organic matters usually determines the presence phototrophic bacteria in water. Alphaproteobacterium *Rhodomicrobium vannielii* was first identified and reported by Duchow and Douglas [1]. Its morphology and proliferation patterns have been used in its characterisation and differentiation from other photosynthetic bacteria. These features are common to *Rhodopseudomonas palustris* which on the other hand has a versatile nature and characteristics which include CO_2 fixation, aerobic and anaerobic biodegradational abilities for many organic pollutants and toxic compounds as does *Rhodocista pekingensis* [2], biofuel production and complex metabolic capability. *Rhodopseudomonas palustris* could grow in the dark with oxygen and under 2kilolux of light intensity anaerobically [3]. *Rhodomicrobium vannielii* and eubacteria such as *Rhodopseudomonas palustris* have some resemblance in terms of their growth in culture media [4]. Though *Rhodomicrobium vannielii* and Hyphomicrobium are the sole representatives of their genera, there are distinct differences that separate them morphologically from each other.

Rhodomicrobium vannielii and *Rhodopseudomonas palustris* have morphological features that have been known to be peculiar to both organisms. These two bacteria bud and divide from polar sides of matured cells: a feature that differentiates them from other photosynthetic bacteria. However, the structure of *Rhodopseudomonas palustris* shows some similarities with those of *Blastochloris sulfoviridis* and *Rhodocista pekingensis* with only a few differences [3][4]. The bacteria are carotenoid-producing autotrophs consisting of photoautotrophs, chemolithotrophic autotroph or chemoautotroph and chemoorganotrophic autotroph [2][5]. The medical importance of bacteriocarotenoids from some of the bacteria has recently been tested [6][7] and biological evaluation of bacterial carotenoid extracts was reported few years afterward [8].

However, Phototrophic purple bacteria have been found in extreme conditions [9] such as permanently frozen Antarctic lake and in hot springs with temperature of 55-60^{o}C[10]. Moreover, many phototrophic bacterial species have been isolated from habitat with normal growth conditions for other non-photosynthetic bacteria [2][3][4]. Strains of photosynthetic bacteria from habitat with extreme conditions such as permanently frozen lakes and water springs with high temperatures have also been reported [8]. Factors responsible for the adaptation have been attributed to evolutionary and genetical modifications. Other obscured factors such as cellular inclusions such as cell elements and other intracellular contents constitute may have constituted principal cellular integrity and major factors that should be explored further. Unfortunately this has not been reported. It was deemed

necessary to explore the similarities and variations in the cellular elemental contents of photosynthetic bacteria with different pigmentation patterns to assess if pigment colours were affected by what elements the bacteria contain rather than attributing most features to genetical factors which in itself contains some of the basic elements and compound that are being considered for assessment. A novel adoption and adaptation of Energy-dispersive X-ray spectroscopy (EDX/EDS) method was first adapted, successfully used and reported earlier [4] and followed-up with this work.

.

II. Materials and Methods

2.1 Bacterial propagation and growth

The medium used for isolation and growth of the organisms was malate yeast-extract (MYE) broth as described **[11]** and modified [3][4] incubation was at 47-50^{o}C. Pigmented growth in broth was sub-cultured by spread-plating 1µL of pigmented broth on MYE agar prepared as previously reported [3]. Pure cultures of the bacteria were transferred into semi-solid MYE agar, prepared with addition of 8g/L of distilled H_2O and managed as with MYE agar, then stored at 4^{o}C.

2.2 Scanning Electron Microscopy and Energy-dispersive X-ray spectroscopy (EDX/EDS)

Samples for scanning electron microscopy, SEM were prepared as described **[3]** with modifications **[4]**. Energy Dispersive X-ray spectroscopy (EDX/EDS) of elemental constituents was done as earlier described **[3][4]**. Modification of end-point was done by knocking off possible interfering extraneous elements such as aluminium and gold found in materials used in the preparation of samples. Details have been fully described [4]. Following elements were standards C, $CaCO_3$, O, SiO_2, Na, (Albite), Mg, MgO, Cl, KCl, K, (MAD-10 Feldspar), Ca, (Wollastonite), Br, KBr, Nb, Pt. No peak was omitted in spectrum processing. All results were in Weight percent and all elements were analysed in Normalised format.

III. Results and Discussion

3.1 Bacterial propagation and growth

Growth pigmentation was unique and peculiar to each bacterium with diffused pigmentation marked by evenly distributed pigmentation patterns in the broth medium. This diffused pigmentation was common to *Blastochloris sulfoviridis, Rhodocista pekingensis* and *Rhodopseudomonas palustris* except *Rhodomicrobium vannielii* in which debris of interwoven bacterial cells was observed in the bottom of growth MYE broth. The cells of *Rhodomicrobium vannielii* and *Rhodocista pekingensis* drifted towards light and were found amassed to the walls of the transparent culture bottles at light intensity of 1000 to1500 lux (Fig. 1).

a **b** **c** **d**

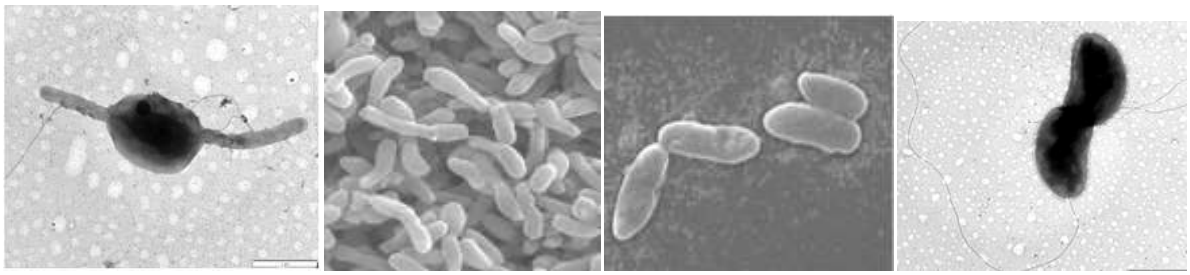
Figure 1: Physiological features of the bacterial species on MYE broth (a) *Blastochloris sulfoviridis,* (b) *Rhodocista pekingensis,* (c) *Rhodopseudomonas palustris* and (d) *Rhodomicrobium vannielii*

Rhodomicrobium vannielii is a photoheterotrophic anaerobic bacterium which possesses the general physiological properties associated with the Athiorhodaceae except that it does not require the addition of growth factors required by the latter. Electron microscopy and processed bacterial cell observations of negatively stained cells did show peritrichous flagella in newly formed cells of *Rhodomicrobium vannielii* with the formation of narrow hyphae emerging from polar ends (Fig. 2a), with the development of cross-walls of hyphae developing dense-septa as hyphae grew older. The shapes were elongated ovoid or lemon-shaped with width between 0.9 to 1.4 µm and length was between 2.0 to 3.0 µm, all of which were similar or close to previously published data [12] including originally described morphology [13]. The vegetative growth as shown (Figs. 2a), developed into tube-like hyphae between next new cell formation emerging from the tips of the hyphae. This was most common features peculiar only to *Rhodomicrobium vannielii*.

The growth of *Rhodopseudomonas palustris* was prolific in MYE broth and produced light red to deep red pigments. *Rhodopseudomonas palustris* red pigment turned to brownish red with advances in incubation time on MYE agar. The colour of pigment was glossy red at the on-set of growth but the glossy appearance later became dull and crackly as cultures aged. *Rhodopseudomonas palustris* was rod and sometime irregularly shaped with some elongated cells three or more times longer than other average cells (Fig. 2b).

The colour of *Rhodocista pekingensis* was deep pink to red in MYE broth. Growth in both was respectively marked with pink red pigmentations. Each of the negatively stained cells possessed single flagellum attached to the polar ends of the cells. The shape was spiral or vibriod with length range of 0.6-0.8 µm (Fig.2d).

Blastochloris sulfoviridis grew with green to greenish yellow pigments and cells were ovoid to rod-shaped with sub-polar flagella (Figs. 1b and 2b). The size range was 0.6-2.3 µm in length and 0.5-0.7 µm wide. Reproduction was by budding and rosettes were formed due to clogging of the flagella of motile swarmer cells.



a **b** **c** **d**

Figure 2: Negatively-stained SEM micrographs of proliferation patterns of (a) *Rhodomicrobium vannielii* ×5500 (b) *Rhodopseudomonas palustris* ×12300 (c) *Blastochloris sulfoviridis* ×15670 (d) dividing cells of *Rhodocista pekingensis* ×12500, Bar 1µm.

3.2 Analytical points and response by Energy Dispersive X-ray Spectroscopic analyses

From the Analytical points of Energy-dispersive X-ray spectroscopy for elemental contents, the results for *Rhodomicrobium vannielii* showed that the average carbon content was between 68.33 % and 71.19 % with min-max C contents in overall analyses respectively given as 68.87 % and 73.53 %. The average oxygen content in bulk selection was 17.72 % while it was higher with 20.48 % in the single cell selection analysis. The peak content for oxygen was 20.48 % (Fig 3, Table 1).

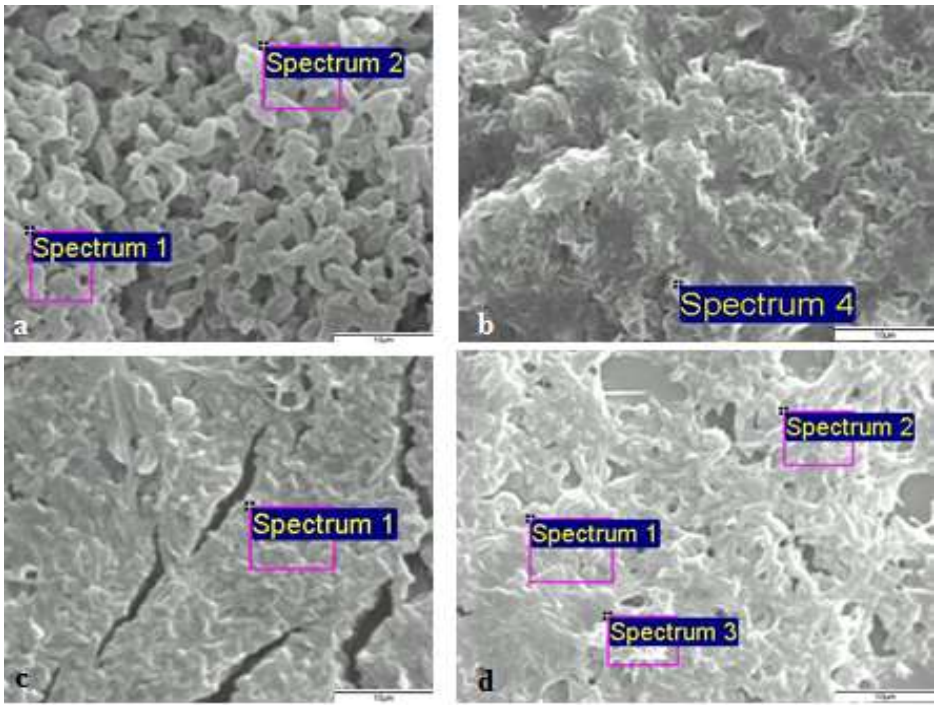


Figure 3: Analytical points of Energy-dispersive X-ray spectroscopy for elemental contents of pigmented bacteria (a) *Rhodomicrobium vannielii* (b) *Rhodopseudomonas palustris* (c) *Blastochloris sulfoviridis* (d) *Rhodocista pekingensis* ×5500, Bar 1µm

Generally, four spectral points were randomly selected and points 1, 2, and 3 represented pin-points of multiple bacterial cells while point 4 was single cell. The analyses were directed at determining the carbon, oxygen and other elemental contents of the bacterial cells from randomly selected parts of the samples. In the weighted percentages of elements and atomic inscriptions, there were variations in the contents from the individual element to the atomic percentage and these also differed with individual bacterial species (Fig. 4).

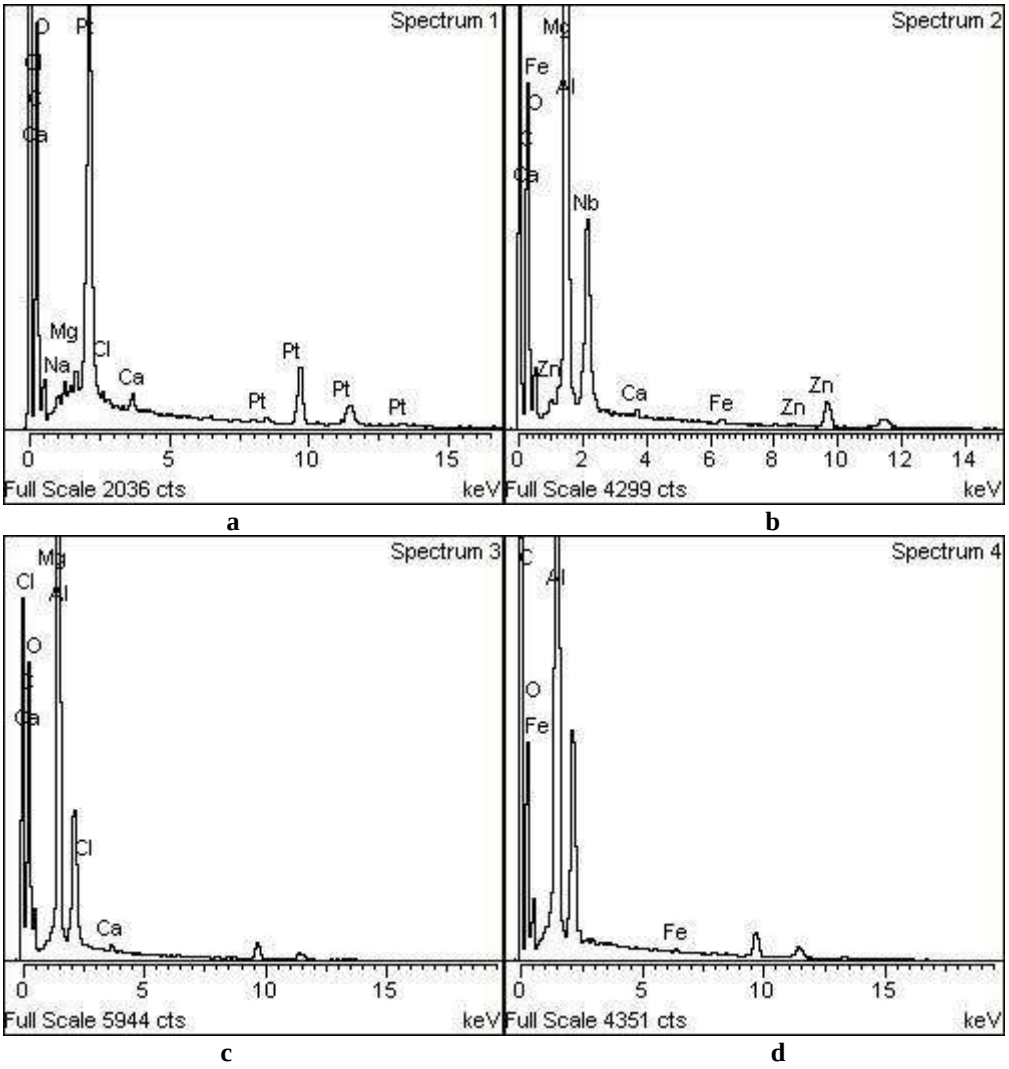


Figure 4: Pooled results of EDS/EDX spectra of cellular element analyses of (a) *Rhodomicrobium vannielii* (b)*Blastochloris sulfoviridis* (c) *Blastochloris sulfoviridis* and (d) *Rhodocista pekingensis*.

From analyses results shown in the spectrographs in Figure 4, major constituent elements in the studied pigmented bacteria were shown by the graphic spikes of each spectrum. Spectra were consistent with dominant elements showing closely related results with variation in the secondary and other elements recorded in smaller quantities represented by lower percentages.

However, the differences between results could be seen as constituent elements minor variations and a test of purity and homogenous patterns of the organisms. The difference between the multiple bacterial selection and single selection points was considerably marginal.

Energy-dispersive X-ray spectroscopy results for *Rhodomicrobium vannielii* in Table 1 (and Figs. 3a, 4a) indicated the dominance of carbon with mean value of 56.9 %, having 60.17 % peak and the lowest C content with 53.63 % in all the 4 spectra. Followed by oxygen with almost 9 % mean content having lowest O content of 7.02 % while highest was 8.37 %. Pt and Niobium (Nb) were 0.9 % while Mg was lowest in overall mean contents. As with others, all elements were in normalised format in data processing option and all results were weighted percent of the total elemental constituent with little difference in the carbon contents of bulk bacterial cells and a single cell (Table 1).

Table 1: Energy-dispersive X-ray spectroscopic analysis of elemental contents of *Rhodomicrobium vannielii*

Spectrum	C	O	Na	Mg	Al	Ca	Fe	Zn	Nb	Pt	Total
Spectrum 1	53.63	6.83			39.02		0.51				100
Spectrum 2	60.17	8.37		0.19	29.31	0.24	0.32	0.49	0.91		100
Spectrum 3	59.13	7.02	0.21	0.21	31.98	0.16	0.39			0.91	100
Spectrum 4	55.63	11.16			32.82	0.39					100
Mean %	56.9	8.99	0.21	0.20	34.17	0.28	0.42	0.49	0.91	0.91	

There was no difference in the mode of selection of bacterial cells for spectral analyses of *Rhodopseudomonas palustris* but the position of each spectrum differed. However, as in previous cases this did not adversely affect the results in any considerable way. Spectra 1, 2 and 3 were spectra of bulk bacteria and the fourth spectrum was a spectrum of a single bacterial cell (Fig. 3b).

This was a way of evaluating the cells individual characteristics based on the elemental contents of the cells. Therefore, the analysis of *Rhodopseudomonas palustris* showed that the peak contents for carbon was 73.53 % with mean content of 70.7 %. Calcium content in the reverse with an average content in the bulk selection standing were at 1.83 % and 0.83 % with mean value of 1.33% in the single selection analysis with the cut-off for calcium.

Magnesium bulk contents average was 1.07 % while the single selection analysis gave 0.84 % with a peak of 1.30 % and a cut-off of 0.84 %. Two major constituents were carbon and oxygen with the lowest level of C to O at 68.0 % to 14 % while the highest contents for the two were 73.5% and 20.4 % respectively, along with other detected elements at 0.4 % and 4.1 %, (Figs. 3b, 4b, Table 2).

Table 2: Results of elemental analysis of Rhodopseudomonas palustris showed highest percentage of carbon contents than other tested bacteria

Spectrum	C	O	Na	Mg	Cl	Ca	Nb	Pt	Total
Spectrum 1	72.17	19.78	0.93	1.11	0.84	1.54		3.63	100
Spectrum 2	73.53	19.02	1.01	0.97	0.54	1.05		3.88	100
Spectrum 3	67.87	14.36	0.86	1.3		1.83	7.16	6.62	100
Spectrum 4	68.33	20.48	0.81	0.84	0.38	0.83	4.1	4.23	100
Mean %	70.7	17.42	0.91	1.07	0.61	1.33	5.63	5.13	

Carbon was observed in *Blastochloris sulfoviridis* with mean detection of 57.4 % while other elements detected in the bacterial cells were considerably small after oxygen. The predominant constituent was as with other analysed bacteria, carbon. The mean percentage of carbon was maintained followed by oxygen at 7.7 %. Detectable level of Niobium was recorded only in spectrum 2 of the bulk-selected spectrum of analyses at 0.9 % however, not in other three spectra. Iron and calcium were present in the first three and the last three spectra at 0.42 % and 0.24 % respectively.

Table 3: Results of combined EDS/EDX elemental analysis of *Blastochloris sulfoviridis*

Spectrum	C	O	Na	Mg	Al	Ca	Fe	Zn	Nb	Pt	Total
Spectrum 1	53.63	6.83			39.02		0.51				100
Spectrum 2	60.17	8.37		0.19	29.31	0.24	0.32	0.49	0.91		100
Spectrum 3	59.13	7.02	0.21	0.2	31.98	0.16	0.39			0.91	100
Spectrum 4	55.63	11.16			32.82	0.39					100
Mean %	57.38	7.7	0.21	0.19	32.4	0.24	0.42	0.49	0.91	0.91	

The EDS/EDX analysis of *Rhodocista pekingensis* and the selection of bacterial cells for each spectrum were same (Fig. 3d) with differences found in the positions where the bacterial cells were selected for the four spectral points. Results did show the highest percentage of inclusive carbon making up 44.4 % followed by cellular O which constitute 3.42 % and Fe which was present at low level with 0.35% mean percentage.

Table 4: There were lesser numbers of overall cellular elemental contents in *Rhodocista pekingensis* with weighted percentage of detected elements

Spectrum	C	O	Na	Al	Fe	Total
Spectrum 1	44.52	3.24	0.13	51.66	0.44	100
Spectrum 2	40.32	3.6		55.66	0.42	100
Spectrum 3	55.96	2.9	0.14	40.72	0.28	100
Spectrum 4	44.22	5.66		49.86	0.25	100
Mean %	44.4	3.42	0.14	50.76	0.35	

An unusual spike in Aluminium was believed to be influenced by the aluminium materials used in sample processing. The pattern of detection was same as earlier discovered in *Rhodomicrobium vannielii* with sodium detected only in spectra 1 and 3.

Elemental affinity by comparison as shown in the graph (Fig. 5) did show the dominance of carbon as expected with *Rhodopseudomonas palustris* and *Rhodomicrobium vannielii* recording the highest contents. Naturally bacteria at large, possess carbon. This implies that bacterial species irrespective of characteristics and other features including pigment production are made up of carbon. This was corroborated as shown in the graph.

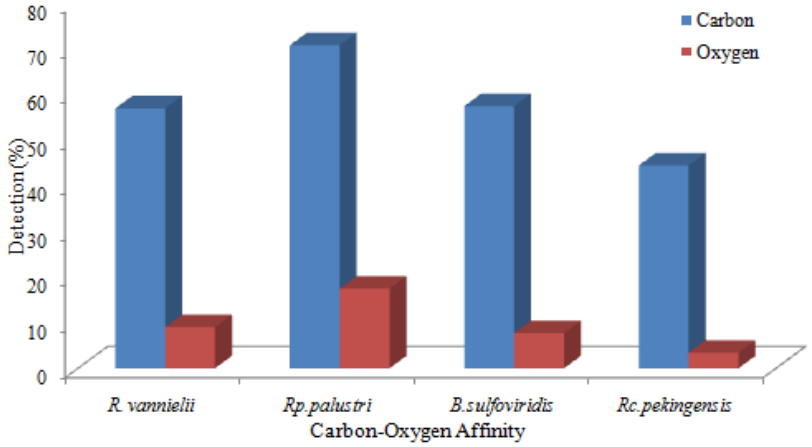


Figure 5: Afinity of Carbon and Oxygen by comparison with Carbon dominance for all the pigmented bacteria followed by cellular oxygen and carbon found much more in *Rhodopsedomonas* spp and *Rhodomicrobium* spp.

The sampling positions for spectra followed the same pattern as with others selected photosynthetic bacteria with selection of the first spectrum, run from the middle of the sample. The second and third were taken from the upper and lower right sides of the sample while the fourth single cell was selected from the extreme low left side of the sample. It should be noted however, that relationship of carbon, oxygen and other constituent elemental inclussions to physiological charateristics of each bcterium was not clear at this point with EDS/EDX analyses. What was known was that some cellular elemental materials were present in some and absent in others while some of the cellular elements were common to all the species of the pigmented bacteria.

In addition to cellular carbon and oxygen, sodium was common to all the bacteria. Other cellular elements such as magnesium, calcium, Nb and Pt were randomly found in three out of the four analysed pigmented bacteria, Zinc and iron were found in two while Cl was found in one of the four bacteria as summarised in Figure 6.

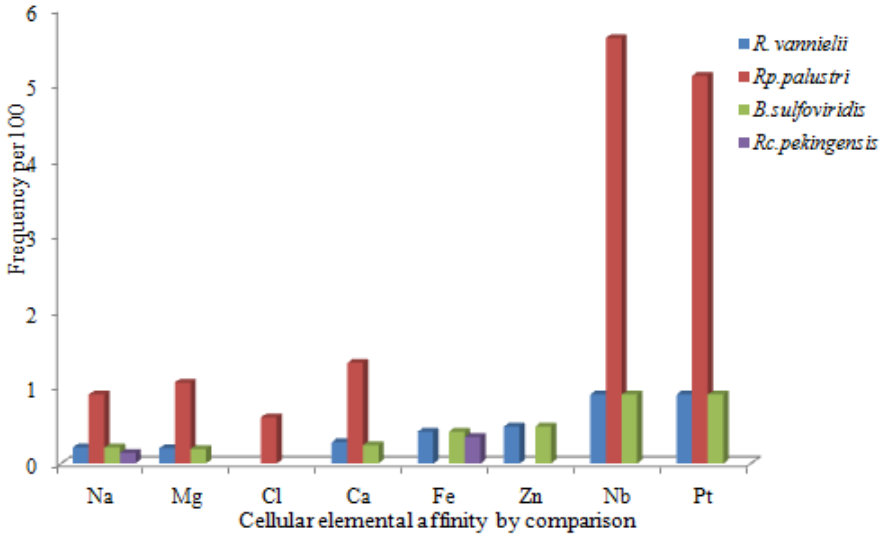


Figure 6: The affinity of cellular minor elements between the four studied pigmented bacteria showing some unique range of contents between different species of pigmeted bacteria.

IV. Conclusions

The analyses were directed at determining the carbon, oxygen and other cellular elements of the bacterial cells from randomly selected parts of the samples. Cellular carbon was observed largely both in quantity and in frequency. The physiological features of pigments produced by these bacteria differed in colours and in the densities of the colours but were not too clear what roles they play in the pigmentation process and in the determination of the pigment densities. Aluminium record was high because of the use of aluminium foil in the process and was therefore disregarded.

The total amount of carbon in the bacteria was elevated in some of the spectra than in other probably due to concentration of cells with detectable carbon on the concerned spectral point rather than the constancy of carbon in the bacterium of interest. This was substantiated by the fact that spectra with the least carbon contents were those of single bacterial cell while niobium was found in spectra two of the spectra. Pt, Ca, Mg and Na were recorded in all the spectra showing diversity of elemental inclusions though in marginally low numbers. However, the presence of some of the elemental inclusions in one bacterium and the absence of same in other bacteria could also be key factors in the morphological and chemical characterizations of each of the bacteria. This might include thermotolerance, phototrophic, metabolic nature and other features that are regularly and naturally displayed by the bacterial species. The detection of Nb and Pt was significant but it was unclear if these two actually was part of the functional inclusions of the bacteria from which they were detected. There is therefore the need for further exploration of what has been done through this work.

References

[1]. E. Duchow and H.C. Douglas. Rhodomicrobium vannielii, a new photoheterotrophic bacterium. *J. Bacteriol*. 58, **1949**, 409-416.

[2]. Akinnuoye Olawale Faith-Anthony, Nazlina Ibrahim, Akinnuoye Modupe Agnes, Ainon Hamzah. Inductive Toxic-Mopping (IT-m) and Carotenogenic Bioconversion Properties of Thermotolerant *Rhodocista pekingensis* Sp. Nov. *IOSR Journal of Environmental Science, Toxicology and Food Technology*. 9(1)1, **2015**, 1-4.

[3]. O.F.A. Akinnuoye, O. Othman, I. Nazlina and H. Ainon,Ultramicrotomy and structural analyses of two thermophilic non-sulphur photosynthetic bacteria. *Malaysian Journal of Microscopy* 6, **2010**, 23-29.

[4]. O.F.A. Akinnuoye, I. Nazlina and H. Ainon. Application of Electron Microscopy and Energy Dispersive X-Ray Spectroscopy in the characterization of *Rhodomicrobium vannielii*. *Journal of Advanced Microscopy Research* 6(1), **2011**, 1-7. Free edition could be downloaded at www.researchgate.net/.../0deec53b65df77fb69000000.pdf

[5]. F.W. Larimer. Complete genome sequence of the metabolically versatile photosynthetic bacterium *Rhodopseudomonas palustris*. *Nature Biotechnology* 22, **2003**, 55-61.

[6]. O.F.A. Akinnuoye, I. Nazlina and H. Ainon. Clinical potentials of bacteriocarotenoids: Rhodopin and β-carotene from phototrophic *Rhodopseudomonas palustris. IOSR Journal of Dental and Medical Sciences (IOSR-JDMS)*. 13(12)7, **2015**, 52-58.

[7]. O.F.A. Akinnuoye, O. Othman, I. Nazlina and H. Ainon, Preliminary Evaluation of the Antioxidative and Anticarcinoma Activities of Bacteriocarotenoids from Bacterium *Rhodopseudomonas palustris* (UKM 2-5A). *UKM/FST Post Graduate Colloquium* 2012(12), **2012**, 182-185.

[8]. K. Sasaki, M. Watanabe, Y. Suda, A. Ishizuka and N. Noparatnaraporn. Applications of photosynthetic bacterial for medical fields. *Journal of Bioscience and Bioengineering* 100 (5), **2005**, 481-488.

[9]. M.T. Madigan. Anoxygenic phototrophic bacteria from extreme environments. *Photosynth. Research* 76, **2003**, 157–171.

[10]. B.K. Pierson and R.W. Castenholz. A phototrophic, gliding filamentous bacterium of hot springs, Chloroflexus aurantiacus, gen. and sp. Nov. *Archives of Microbiology* 100, **1974**, 5-24.

[11]. H. Ainon, C.J. Tan and S. Vikineswary. Biological characterization of *Rhodomicrobium vannielli* isolated from a Hot Spring at Gadek, Malacca, Malaysia. *Malaysian Journal of Microbiology* 2(1), **2006**, 15-21.

[12]. E.S. Boatman and H.C. Douglas. Fine structure of photosynthetic bacterium *Rhodomicrobium vannielii*. *The journal of Biophysical and Biochemical Cytology* 11, **1961**, 469-483.

[13]. Murray and H.C. Douglas. The reproductive mechanism of *Rhodomicrobium vannielii* and the accompanying nuclear changes. *Journal of Bacteriology (ASM)* 59, **1950**, 157-167.

[14]. K. Sasaki, M. Watanabe, Y. Suda, A. Ishizuka and N. Noparatnaraporn. Applications of photosynthetic bacterial for medical fields. Journal of Bioscience and Bioengineering 100 (5), **2005**, 481-488.