Chemistry and Materials Research
ISSN 2224- 3224 (Print) ISSN 2225- 0956 (Online)
Vol.7 No.1, 2015

Elution Protocols of Intracellular Bacteriocarotenoid Extracts

AKINNUOYE Olawale Faith-Anthony[1,2,*]
1.Academic Research and Administration, MFM Group Sdn Bhd, Kuala Lumpur, Malaysia
2.Former Address, School of Biosciences and Biotechnology, Faculty of Science National University of Malaysia, 43600 Bangi, Selangor DE, Malaysia
* E-mail of corresponding Author: faithanthony.medic@yahoo.com

Abstract
Bacteriocarotenoids of thermophilic species of *Rhodopseudomonas palustris* isolated from a well of hot spring in Malaysia was extracted and characterized. Multiple spectrophotometeric absorption maxima of the bacteriocarotenoid, thin-layer chromatography and confirmatory high performance liquid chromatography (HPLC) were used in the mapping, separation and identification of the extracts from bulk-bacteriocarotenoid extracts. Results of the TLC detections with glowing spots of reddish isolated compounds at distinct R_f under low radiation UV lamp, indicated the presence of bacteriocarotenoids. Moreover, results from HPLC analyses did identify two of the extracts as β-carotene with spectral detections at 450 nm, 474 nm wavelengths, and rhodopin with spectral detections located at 453nm, 482 nm, 521 nm wavelengths.
Keywords: *Rhodopseudomonas palustris*, Bacteriocarotenoid, Elution, Eluate, Thin-layer chromatography

1. Introduction

Rhodopseudomonas palustris is a Proteobacterium of the Alphaproteobacteria class and it is one of the many photosynthetic bacteria of importance. Bacterial pigments were discovered long before the discovery of bacteriochlorophyll (Bchl) in 1963. The activation of an elaborate system of intracytoplasmic membrane vesicles which are used by photosynthetic bacteria when necessary compounds are deficient, have been explored (Goodwin and Osman 1953; Lang and Oesterhelt, 1989a; Aksu and Tugba 2005), with few research works on the bioactivities of isolated carotenoids of bacterial origins (Akinnuoye *et al*, 2011). Recently preliminary test was done and reported (Akinnuoye *et al*, 2012).

As at a decade ago, there over 200 carotenoid pigments isolated from microbes and other marine animals. Most of these pigments were reported to have been acquired either through photoheteroptropism or dietary intake (Misawa *et al*, 1995). These include pigments from a number of bacteria such as *Rhodospirillum rubrum* (Goodwin and Osman 1953), *Gibberella fujikuroi* (Garbayo *et al*, 2003) and *Chlorella zofingiensi*s (Po-Fung and Feng, 2005). Moreover, other reported bacteria from which carotenoids have been extracted include *Haematococcus pluvialis* (Garcia-Malea *et al*, 2005), from *Rhodotorula mucilaginosa* (Aksu and Tugba 2005) among numerous others. Photosynthetic bacteria utilize photosynthesis in bioconversion of light energy into chemical-free energy. This could be done by oxygenic mechanism in which organic compounds are used in place of carbon dioxide with the release of oxygen in the process. Alternative process is by anoxygenic process involving Gram negative facultative anaerobes most of which are purple non-sulphur bacteria such as *Rhodopseudomonas palustris*. Photosynthetic bacteria are able to use trapped light in the photosystems in metabolic activities (Kohring *et al*, 2003).

2. Materials and Methods

2.1 Growth and maintenance of Rhodopseudomonas palustris

The culture of *Rhodopseudomonas palustris* was isolated on Malate Yeast-Extract agar as described by Akinnuoye *et al*, (2008). Maintenance of pure culture of the bacterium was done by selecting from distinct isolated colonies in MYE broth and incubated at 45(±3)^{o}C automated INFORS AG® incubator (Infors AG, Rittergasse 27 CH - 4103 Bottmingen, Switzerland) in which hydrogenated carbon dioxide (Oxoid® H_2-CO_2) was added. Standardization of culture was prepared from single colony of MYE agar-grown isolates as reported by Akinnuoye *et al*, (2012).

2.2 Determination of bacteriochlorophylls and inclusive carotenoids

Bacteriochlorophylls (Bchl) in live bacterial cells was determined following the method of Lorquine et al (1997) as modified by Akinnuoye *et al*, (2012) for purification of the pigments during which cells were pilleted and extracted with cold acetone-methanol at 7:2 (v/v). This was followed by spectral analysis at 300-900 nm. Cellular bacteriocarotenoid extraction was performed as described by Liaaen-Jensen and Jensen (1971) and modified by Nelis and De Leenheer (1989).

Top ether layer of bulk-bacteriocarotenoids was collected while the ether solvent was vacuum-distilled and added into the column of alumina N, with elution with diethyl ether. The extract was processed to dryness and quantified by pipetting 15 mL of the extracts into bottles, transferred into a desiccator, connected to a GAST®

electrical vacuum pump. The solvent was dried under pressurized vacuum for 72 hours in the dark until no liquid was observed. The extract was dried in vertical automated liquid nitrogen pressurized drier of 0.250 mBar with collector temperature of -53^{o}C for 7 days. The dried samples were stored at -20^{o}C until needed.

2.3 Thin-layer chromatography (TLC) methods

Bacterial extracts were analysed using thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). Glass sheets coated with TLC silica gel 60PF_{254}® (Merck KG_GA®, Germany) were used in the purification of the eluate as follows: Clean 20 x 20 cm size glass sheets were set on TLC coating track by lifting up the two track holders on each side of the aluminium-alloyed metal track. The glass were then arranged tip-to-tip on the track and held down with the glass holders while the holders knob was turned at 180^{o} to hold the glass sheet in position and lock the holders. The glass surface was then cleaned with acetone (analytical grade), swiped through with cotton wool or paper towel.

Gypsum-containing silica gel powder 60 PF_{254} was prepared as follows: Fifty grams of the gel powder was weighed into 100 mL-sized beaker and transferred into a conical flask through a funnel, followed by addition of 110 mL of cold distilled water to the gel powder and shaken vigorously for 30 seconds until homogenous slur was formed. Coating was done by pouring the homogenized silica gel into a spreader well with 0.5 mm thick dispenser, with the dispensing side facing the outside part of the track. The gel was spread quickly over the glass sheets. The holder's edge was tapped several times to clear off aberration on the gel surface. The coated plates were allowed to set for at least 3 hours and afterwards dried in the oven at 110^{o}C for at least an hour or until when needed.

Spotting of coated TLC plates for preparative elution was done closely until all spots from the same extract were joined together. Different plates were used for one type of extract at a time. Spotting of the silica gel coated glass sheets with the bacteriocarotenoid samples and development of spots were done using an eluent made from the combination of dichloromethane-ethyl acetate (95:5 v/v). The separated compounds were viewed and mapped in the dark or under mercury light. The mapped spots were individually scrapped into small Eppendorf® tube, reconstituted with hexane and kept in the dark. Spectral analyses of the compounds were made using spectrophotometer. The separated compounds were viewed under the long wave ultraviolet light at 365 micro minutes (λ). Both the compound and solvent fonts were marked and R_f was calculated as R_f = A/B; where A= distance travelled by compound and B = distance travelled by solvent.

2.4 The use of granulated iodine developer

One of the developing agents was the conventional granulated iodine in a developing tank. The iodine granules were put at the bottom of the tank and allowed to saturate the tank. Saturation was confirmed with brownish coloration of the glass tank. The spotted and separated TLC plate was placed in the tank and covered for about 20 to 30 seconds depending on the type of TLC plate used and the brand of silica gel used during plate coating. The plate was carefully removed and the spots of separated compounds were mapped, scrapped off the TLC plate and extracted with methanol for HPLC analysis.

2.5 High-performance liquid chromatography (HPLC) methods

The HPLC machine (Ultimate 3000® HPLC with Ice PL-ELS2100® auto sampler) was a product of Polymer laboratory. The machine was attached to a UPS system (Nova 1100AVR), then connected to a computer system and a printer (HP® computers). The HPLC machine was preconditioned to a nominal temperature of 35^{o}C with lower limit of 4^{o}C and upper limit of 45^{o}C. The ready temperature delta was 2^{o}C. Lower limit pressure was 200 per square inch (psi) and 3000 psi upper limit. The minimum and maximum flow ramps were 6 mL/min^{2}. Draw speed was 10 µL/second. The samples were dispensed into 1 mL volume in dark HPLC sampler bottles and arranged on the loader platform of the machine. The loader tray was covered and the samples were automatically loaded and analyzed. Analysis was done with acetonitrite-methanol-methanol in either 40:50:10 (v/v) or 70:15:15 (v/v) ratio.

The flow rate was at 1 mL/min and detector signal monitoring was 470 nm. Peak identification was done by comparing the retention times of unknown peaks with those of the reference carotenoids (standards). With the reference, quantitation was based on the measurement of peak-height ratio of compound of interest versus internal standard. A calibration curve was constructed by plotting peak height ratios versus known amounts of the compound determined. However, without reference, semi quantitative determination using peak-area ratios of compound of interest versus standard was used and corrected for differences in the molar absorption coefficient of the compound of interest and the analog respectively.

3. RESULTS AND DISCUSSION

The initial growth incubation temperature was 37^{o}C. This was done to avoid thermal stress in the first 5-6 hours of incubation. Growth of the culture from freezing temperature of ≤4^{o}C to maintenance temperature of 45^{o}C

spanned a 24 hour period in which light intensity was silica gel 60PF_{254}®, Merck KG_GA®, Germany) increased to 2000 lux. Pigmented growth was observed in the entire broth medium with orange to light red pigment being observed in the first 72 hours. The density of the pigments increased with length of days. Similarly the anaerobic growth on agar medium was characterised by the initiating red growth, the rate of colouration was a bit faster than in broth medium (Figure 1).

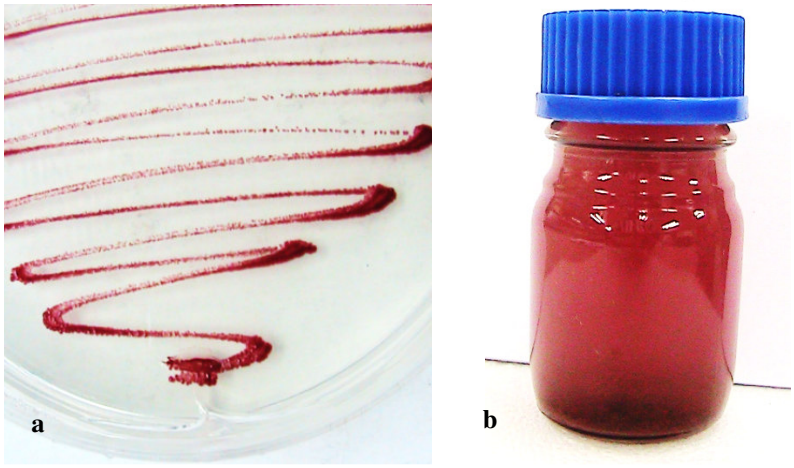

Figure 1: Characteristic anaerobic growth of *Rhodopseudomonas palustris* (a) in MYE agar (b) on MYE broth.

The extract separated into three to four layers with the top layer containing the bacteriocarotenoid compounds. Below the bacteriocarotenoid layer was the water phase followed by cell debris in the last two phases below the water phase. Graph showing typical bacteriocarotenoid peaks and valleys recorded in spectral analysis of carotenoid extracts from *Rhodopseudomonas palustris*. Thin-Layer chromatography (TLC) and High performance liquid chromatography (HPLC) were used in further identification processes. (Figure 2)

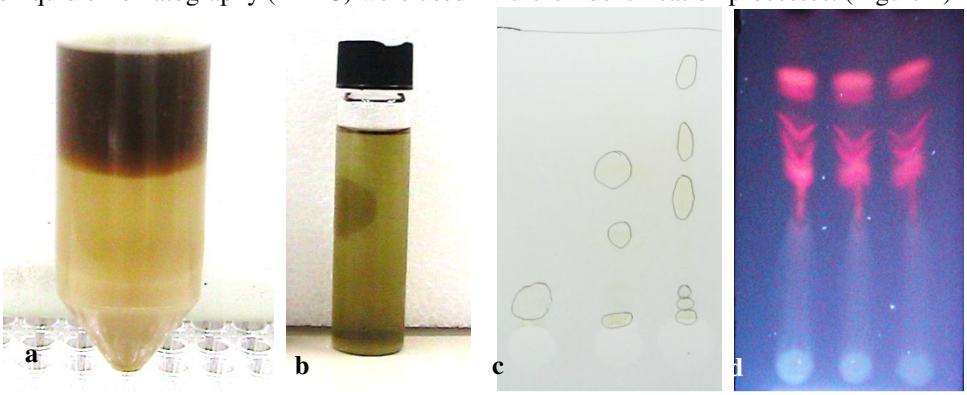

Figure 2: Images of (a) Bacteriocarotenoid extract (b) Purified stock of bacteriocarotenoids extract (c) Fonts of TLC-separated bacteriocarotenoids developed with crystal iodine (d) View of high emission UV spectrograph of bulk-bacteriocarotenoid extracts from *Rhodopseudomonas palustris*.

Bacterochlophyll (Bchl) type was determined by spectral analysis of living bacterial cells. Spectral absorbance maxima (λ-max) were indicative of the type of Bchl in containing bacterium. Predominant bacteriochlorophyll was Bchl *a* as shown by the λ-max of the cells. There were more bulk-bacteriocarotenoid yields (an embodiment of carotenoids) within the extract from diethyl ether. The ether extracts of bulk and isolate bacteriocarotenoids signalled a wide range of peak response with spectral analyses of each of the refined extracts.

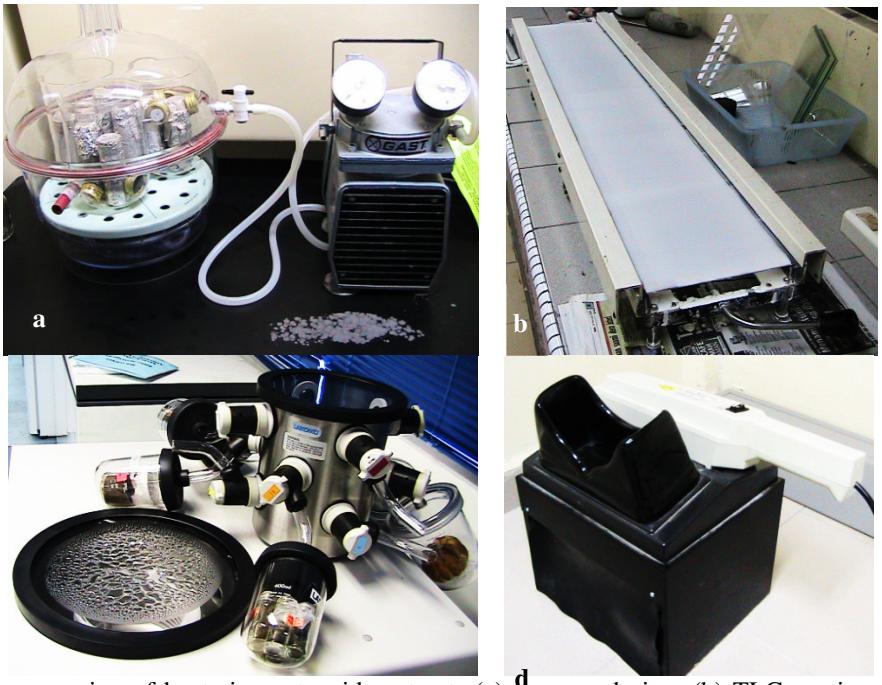

Figure 3: Final processing of bacteriocarotenoids extracts (a) vacuum drying, (b) TLC coating track, (c) ultra-lyophilization, (d) UV detection chamber.

Both normal and second generation HPLCs (TorfQ-HPLC) were used. *Rhodopseudomonas palustris* possessed Bchl *a* with detected peaks at wavelengths from 374-376, 590, 800-806 and 831-890. Bacteriocarotenoids were detected at wavelengths from 374-376, 590, 800-806 and 831-890 nm with multiple point-specific peaks of different bacteriocarotenoids some of which were detected with weak spikes. Other multiple peak detections were in the regions of 350 and 374 while some were between 442 and 453, 461 and 478, 495 and 503 nm wavelengths in the spectral range of 400 to 900 nm for bacteriocarotenoids (Table 2, Figure 4).

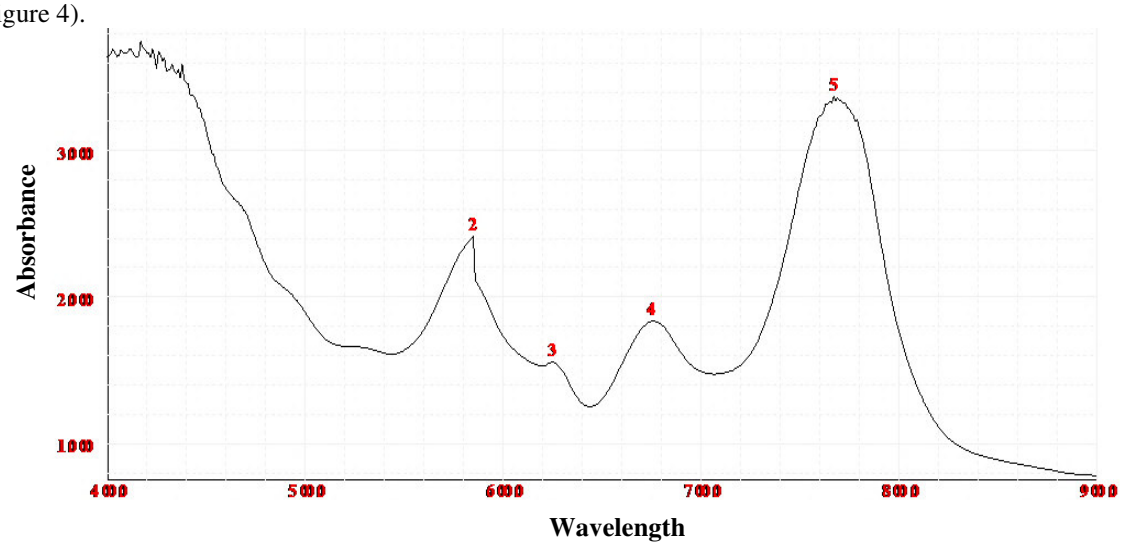

Figure 4: Spectral points of detection for bacteriocarotenoids of the photosynthetic bacterium *Rhodopseudomonas palustris*.

The results of peak analysis of *Rhodopseudomonas palustris* extracts there were at least five peaks of bacteriocarotenoids. There were multiple peak bacteriocarotenoids detections located at 350 and 374 nm, 442 and 453 nm, 461 and 478 nm, 495 and 503 nm wavelengths in the wavelength range of 350 to 900 nm. This was an indication of a wide range of bacteriocarotenoids (Figures 2, 4). There were up to nine separated compounds observed with TLC under high wavelengths of ultraviolet (UV) light (Figure 3). The differences between the UV observation and the iodine developed spots were in the safety of doing the mapping in a more accurate way as all spots glowed consistently and spots were clearly observed under the UV chamber, while these two factors were not the case with iodine chamber. However, safety of the iodine chamber developer was an advantage (Figure 2).

The colors of test-bacteriocarotenoids and their component carotenoids in extracts from the bacterium were

classified by automatically matching of the pre-set parameters of the standards with the detected bacteriocarotenoids. In the HPLC analysis results, there were detections of bacteriocarotenoids at 3.5, 4.2 and 24.3 minutes with 296, 290 and 274 max/z (Tables 1, 2).

Table 1: TORFQ HPLC detection of bacteriocarotenoids from *Rhodopseudomonas palustris*. Three detections were recorded at 3.5, 4.2 and 24.3 minutes.

S/N	RT [min]	Area	Int. Type	Intens.	S/N	Chromatogram	Max.m/z
1	3.5	44756	Chromatogram	5419	18.7	BPC 48.991-3000.681 +All	296.0663
2	4.2	623212	Chromatogram	14941	54.2	BPC 48.991-3000.681 +All	290.8473
3	24.3	213336	Manual	10569	26.0	BPC 48.991-3000.681 +All	274.2729

Spectra of raw extracts containing the bulk-bacteriocarotenoids with wide range of peaks were compared with what was obtained with the HPLC. The λ-max (nm) and suggested compound names by molecular weights were given where standards of the detected compounds were present in the analysis machine.

From the results obtained in normal HPLC and TORFQ HPLC, the general peak wavelengths were detected in all the extracts which indicated the presence of multiple bacteriocarotenoids with the identities of two confirmed. Spectra of raw extracts (bulk-bacteriocarotenoids) with wide range of peaks were also compared with the HPLC results. The λ-max (nm) and overall comparison did confirm the presence of the two isolate-bacteriocarotenoids as β-carotene with wavelengths of 450 nm and 474 nm and rhodopin with 453, 482 and 521 nm wavelengths (Table 2).

Table 2: The multiple absorption maxima of bacteriocarotenoid and the identified isolate carotenoids detected using spectrophotometer and HPLC

Bacteriocarotenoids	General and selective λ-max (nm)	Mol.Wt
Bulk-bacteriocarotenoid	410,428,442-453, 458, 461-478, 483, 495-503, 580	N/A
Rhodopin	453, 482, 521	554.89
β-carotene	450, 474	536.87

When reconstituted, and further studied, only two were identifiable through HPLC. Identification was based on matching the retention times of test-materials with those of the infused standards in TorfQ-HPLC and comparison of molecular weights and the standard peaks displayed by test-samples. Spectra of raw extracts containing the bulk bacteriocarotenoids wide range of peaks. When spectra for individual extracts were run, two major peak groups were observed representing the two of the three previously identified carotenoids of *Rhodopseudomonas palustris* (Nelis and DeLeenheer, 1989; Yurkov and Beatty, 1998; Table 2). The first group of peaks were 453, 482 and 521 with suggested common mol.wt of 554.89 while the second group of peaks were 450 and 474 with suggested common mol.wt of approximately 536.87. The component-bacteriocarotenoids were automatically matched with standards out of which two were identified by the peaks using spectrophotometry normal and by detailed descriptions using TORFQ-HPLC based on their suggested molecular weights. Rhodopin was confirmed for the group 1 peaks.

There were unclassed monocyclic carotenoid groups immediately following the first group. The second was the group 2 peaks and classed as β-carotene classed with bicyclic carotenoids. There were other unidentified component carotenoids and proteins because there were no matching standards. This findings and results were similar to previously reported findings (Yurkov and Beatty 1998). Anoxygenic photosynthetic bacteria have been known to possess vast number of carotenoids which were originally attributed to plant sources. However biological activities of the individual bacteriocarotenoids are yet to be determined. The combination of the first generation TLC method and the more recent HPLCs showed definite relevance now as in any previous generations in their discoveries of these methods (Nelis and DE Leenheer, 1989, Yurkov and Beatty, 1998).

Conclusions

The extraction and identification processes were combined in this work for efficiency. The combined protocols have been used successfully in multiple identification processes in which one method complimented the other. However, each of the methods adopted could independently be used with same quality results. Moreover results could vary based on types of solvents and TLC materials, eluents and other factors. Comparison of extracts analyses results with standardised and previously reported results confirmed reliability of methods adopted here. It is here suggested that activities of the extracts be tested to ascertain their properties and biological importance.

5 Acknowledgements

The Author heartily expresses appreciation to the Mountain of Fire Group, Malaysia and the National University

of Malaysia for supporting this work.

References

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

Akinnuoye, O.F.A., Nazlina I. and Ainon H. (2011). Application of electron microscopy and energy dispersive x-ray spectroscopy in the characterization of *Rhodomicrobium vannielii. Journal of Advanced Microscopy Research* **6** (1): 46-57.

Akinnuoye, O.F.A., Othman O, Nazlina, I. and Ainon, H. (2008). Comparison of Ultrastructure of four Thermophilic photosynthetic bacteria isolated from Malaysian hot springs. *10th Symposium of the Malaysian Society of Applied Biology*. Malaysian Society of Applied Biology, Malaysia.

Aksu, Z. and Tugba, E.A. (2005). Carotenoids production by the yeast *Rhodotorula mucilaginosa*: Use of agricultural wastes as a carbon source, *Process Biochemistry* **40** (9): 2985-2991.

Garbayo, I., Vilchez, C. and Nava-Saucedo, J.E. 2003. Nitrogen, carbon and light-mediated regulation studies of carotenoid biosynthesis in immobilised mycelia of *Gibberella fujikuroi*. *Enzyme and Microbial Technology* **33**: 629-634.

Garcia-Malea, M.C., Brindley, C., Del Rio-Sanchez, E., Acien, F.G., Fernandez, J.M. and Molina, E. (2005). Modelling of growth and accumulation of carotenoids in *Haematococcus pluvialis* as a function of irradiance and nutrients supply. *Biochemical Engineering Journal*. **25**:187–194.

Goodwin, T.W. and Osman, H.G. (1953). Studies on carotenogenesis: General cultural conditions controlling carotenoid (Spiriloxanthine) synthesis in the photosynthetic bacterium *Rhodospirillum rubrum*. *Biochemical Journal* **53**: 541-546.

Kohring, G.W., Wiehr, P, Jeworski, M. and Giffhorn, F. (2003). Stereoselective oxidation of aliphatic diols and reduction of hydroxy-ketones with galactitol dehydrogenase from *Rhodopseudomonas sphaeroides* D. *Communication in Agricultural and Applied Biological Sciences* **68**: 209-211.

Lang, F.S. and Oesterhelt, D. (1989a). Gene transfer system for *Rhodopseudomonas viridis*. *Journal of Bacteriology*, **171**: 2827-2834.

Liaaen-Jensen, S., and Jensen, A. (1971). Quantitative determination of carotenoids in photosynthetic tissues. Methods Enzymol. **23**: 586–602.

Lorquin, J., Molouba F. and BL Dreyfu (1997). Identification of the Carotenoid Pigment Canthaxanthin from Photosynthetic Bradyrhizobium Strains. *Appl. Environ. Microbiol*. **63** (3). 1151-4. American Society for Microbiology.

Misawa, N., Satomi, Y., Kondo, K., Yokoyama, A., Kajiwara, S., Saito, T., Ohtani, T. and Miki, W. (1995). Structure and functional analysis of a marine bacterial carotenoid biosynthesis gene cluster and astaxanthin biosynthetic pathway proposed at the gene level. *Journal of Bacteriology* **177**: 6575–6584.

Nelis, H. J. and De Leenheer, A.P. (1989). Profiling and quantitation of bacterial carotenoids by LC and PAD. *Applied and environ. Microbiol* **55** (12) pp 3065-3071.

Po-Fung, I. and Feng, C. (2005). Production of astaxanthin by the microalgae *Chlorella zofingiensis* in the dark. *Process Biochemistry*. **40**: 733-738.

Yurkov, V.V. and Thomas Beatty, J.T. (1998). Aerobic anoxygenic phototrophic bacteria. *Microbiology and Molecular Biology Reviews* **62** (3): 695-724.

The IISTE is a pioneer in the Open-Access hosting service and academic event management. The aim of the firm is Accelerating Global Knowledge Sharing.

More information about the firm can be found on the homepage:
http://www.iiste.org

CALL FOR JOURNAL PAPERS

There are more than 30 peer-reviewed academic journals hosted under the hosting platform.

Prospective authors of journals can find the submission instruction on the following page: http://www.iiste.org/journals/ All the journals articles are available online to the readers all over the world without financial, legal, or technical barriers other than those inseparable from gaining access to the internet itself. Paper version of the journals is also available upon request of readers and authors.

MORE RESOURCES

Book publication information: http://www.iiste.org/book/

Academic conference: http://www.iiste.org/conference/upcoming-conferences-call-for-paper/

IISTE Knowledge Sharing Partners

EBSCO, Index Copernicus, Ulrich's Periodicals Directory, JournalTOCS, PKP Open Archives Harvester, Bielefeld Academic Search Engine, Elektronische Zeitschriftenbibliothek EZB, Open J-Gate, OCLC WorldCat, Universe Digtial Library , NewJour, Google Scholar