Preliminary Evaluation of The Pigments Content from Rhodobacter Sphaeroides at Stages During Photosynthetic Growth

by Tatas Hardo Panintingjati Brotosudarmo

2nd Humboldt Kolleg in conjunction with International Conference on Natural Sciences,
HK-ICONS 2014

Preliminary Evaluation of the Pigments Content from *Rhodobacter sphaeroides* at Stages during Photosynthetic Growth

Chan Jia Hui[a], Monika Nur Utami Prihastyanti[b],
Tatas Hardo Panintingjati Brotosudarmo[b]*

*[a]Department of Cell and Molecular Biology, Faculty of Biotechnology and Science Biomolecule, Universiti Putra Malaysia,
43400 UPM Serdang, Selangor, Malaysia*
[b]Ma Chung Research Center for Photosynthetic Pigments, Villa Puncak Tidar N-01, Malang 65151, Indonesia

Abstract

Under photosynthetic growth, purple non–sulfur photosynthetic bacterium *Rhodobacter sphaeroides* can use a diverse array of substrates for the source of carbon donor. Substrates such as acetate and succinate are most commonly used to study energetic and metabolic networks, especially in the production and consummation of NADPH during the citric acid cycle (TCA cycle) and ethylmalonyl–CoA pathway, respectively. Although the utilization of both substrate, the bacterium will grow at different growth rate and this also influence the biosynthesis of photosynthetic pigments as important components for overall photosynthesis. For this study, *Rhodobacter sphaeroides* strain 2.4.1, GA and G1C have been grown in acetate and succinate. Here, preliminary results on the evaluation the pigment ratio at different stages of the growth is reported, especially on the growth in succinate substrate.

Keywords: Bacteriochlorophylls; carotenoids; pigment ratio; *Rhodobacter sphaeroides*.

Nomenclature			
LH	light–harvesting complexes	EM–CoA	ethylmalonyl–Coenzyme A
RC–LH	core complexes	Cars	carotenoids
Bchl	bacteriochlorophylls	NADPH	nicotinamide adenine dinucleotide phosphate

* Corresponding author. Tel.: +62 821 4149 0052; fax: +62 341 550 175.
E-mail address: tatas.brotosudarmo@machung.ac.id

1. Introduction

R. sphaeroides is a purple photosynthetic bacterium, which can grow under several condition, such as aerobic and anaerobic respiration, fermentation, and also anoxygenic photosynthesis[1]. Several group of carbon sources have been determined to be the most utilized carbon in the growth of *Rhodobacter sphaeroides*. D–glucose is one of the carbon sources used by *R. sphaeroides* to support the growth due to the ability to produce NADPH through assistance from glucose–6–phosphate dehydrogenase enzyme based on glycolysis pathway. Other example of carbon source is acetate which requires anabolic process to convert acetate into ethylmalonyl–CoA in Em–CoA pathway. This process also involves the consumption of NADPH . Thus, it takes time to increase optical density of the cells. The third group of carbon source is succinate which is able to enter TCA cycle and make the cells have a rapid growth as compared to cells grown in acetate[1]. On the other hand, *R. sphaeroides* is able to produce high amount of fatty acids, photopigments and other components of the photosynthetic apparatus[2].

Under anaerobic condition, cytoplasmic membrane of *R. sphaeroides* undergoes changes where invagination process occurred and developing intracytoplasmic membrane system, which is also known as chromatophore. The intracytoplasmic membrane is physically continuous with cytoplasmic membrane but both of them performing the different functions and structures. In these resulting specialized domains, containing the pigments and proteins that are essential for light reaction of photosynthesis. Basically, chromatophore is light–reflecting organelles that can be found in the membrane of photosynthetic microorganisms, which can absorb light energy by using light–absorbing antenna complexes. For some organisms, there are two types of antenna complexes in the chromatophore, which are known as light harvesting complex 1 (LH1) and 2 (LH2)[3]. In the last 25 years, the antenna system from this group of bacteria has been studied extensively and perhaps the best-analyzed antenna complex is LH2.

R. sphaeroides contains two antenna complexes which absorb at different wavelengths. The LH1 complex is core antenna complex, which exists as an integral membrane pigment-protein complex. They form a ring that physically surrounding the reaction center, presenting RC–LH1 complex. From the intact bacterial cell of *R. sphaeroides*, it exhibits an intense band at ~875 nm. A single subunit of LH1 is consisting of a pair of α and β protein subunits. It also contains two bacteriochlorophyll (bchl) molecules which have an absorbance value at around 820 nm[4]. However, there a shifting of absorbance band to ~875 nm which representing the aggregation of subunits forming the intact LH1, these are known as B875 pigments.

LH2 integral membrane antenna complex in purple photosynthetic bacteria is similar but clearly distinct to LH1. Bacteriochlorophyll pigments in LH2 exhibit spectrally different compared to those from LH1. There are two well–separated absorption bands which centered at around 800 nm and 850 nm, referring to B800 and B850 pigments. For B800 pigments, the bacteriochlorophyll exist in monomeric form and forming a ring that parallel to the plane of the membrane that the complex embedded in, while for the B850 pigments, each subunit consists of two bacteriochlorophylls, in the form of arrangement of coupled dimer.

2. Materials and methods

2.1. Isolation of bacterial membrane

Three different strains of *Rhodobacter sphaeroides*, namely *R. sphaeroides* 2.4.1, Ga, and G1C were incubated at room temperature, 2 500 lux (1 lux = 1 lm $\cdot$ m^{-2}) under anaerobic condition in four rectangular bottles respectively which containing succinate medium[4] with sodium succinate ($C_4H_4Na_2O_4$) or sodium acetate (CH_3COONa) as carbon source. The bacteria cultures which have achieved initial log phase and late log phase were harvested respectively by centrifugation at 6 000 rpm (60 rpm = 1 hertz) for 10 min at 4 °C after 3 d to 4 d and 5 d to 7 d after inoculation. The harvested bacterial cells were resuspended well with 20 mM of Tris HCl (pH 8.0) buffer, and disrupted by using a sonicator for 10 min. Then the bacterial membrane which is in the pellet form of each strain of *R. sphaeroides* was collected by carrying out centrifugation at 11 500 rpm for 15 min at 4 °C.

2.2. Pigment extraction

For pigment extraction, 0.1 g of bacterial membranes was taken. Rapid extraction was carried out in this experiment. Solvent used to extract pigment is acetone-methanol which was prepared in 7 : 3 (v / v) proportions. Three mL of solvent was added to bacterial membrane sample and vortex for 2 min in a conical tube. Extraction process was carried out at room temperature under green light condition.

2.3. Determination of pigment ratio

The electronic absorbance spectra of the pigment component were recorded, at room temperature, by using a Spectrophotometer UV-Vis-NIR (UV1700–Shimadzu). Sample was prepared in 1 : 10 ratio (0.5 ml of sample diluted with 4.5 ml of solvent). The crude pigments extracted from both initial and late log phases of *R. sphaeroides* were subjected to HPLC analysis.

Pigment ratio was determined using HPLC equipped with photodiode array detector. Analytical separations were carried out by using a Waters Symmetry C_8 COLUMN (150 mm × 4.6 mm, 3.5 μm particle size, 100 Å pore size) as the stationary phase. While for the mobile phases, eluent A was a mixture of methanol:acetonitrile: queous pyridine solution (0.25 M pyridine) (50 : 25 : 25 v / v / v) while eluent B was mixture of methanol:acetonitrile:acetone (20 : 60 : 20 v / v / v). Organic solvent that was employed to prepare mobile phases were HPLC–grade. The aqueous pyridine solution (0.25 M) was prepared by 10 mL of acetic acid and 20 mL of pyridine were added to 900 mL of milli–Q (Millipore) water in a 1 L flask and mixed by using a magnetic stirrer. Acetic acid was then added dropwise until the pH was 5.0. The mixture was diluted to 100 mL with distilled water to achieve final pyridine concentration of 0.25 M. All procedures were carried out in a fume hood. The pyridine solution was filtered after mixing with methanol and acetonitrile (eluent A). Different gradient profiles were adjusted for minimizing differences of equipment dwell volume. The flow rate was fixed at $1\ mL \cdot min^{-1}$ [5].
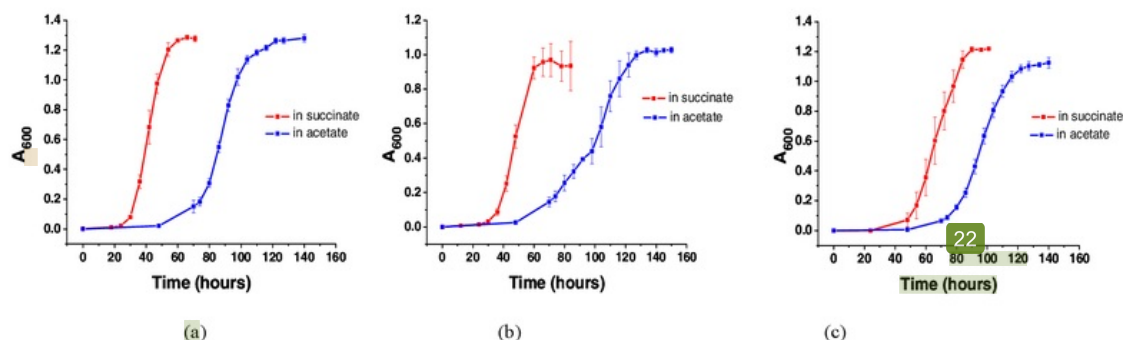
3. Results and discussion

3. 1. Growth analysis

Fig.1. Growth curves of *R. sphaeroides* strain (a) 2.4.1., (b) GA, (c) G1C with succinate and acetate as carbon sources

In order to extract pigment from bacteria of different growth phases, growth analysis was carried out to generate comparable growth curves for both carbon sources. Acetate and succinate are carbon sources that capable of supporting photosynthetic growth of *R. sphaeroides* along with some differences on metabolic activity. Growth of all three strains of *R. sphaeroides* when using acetate as carbon source, were significantly impaired (Figure 1) compared to those which were cultured with succinate as carbon source. A long lag phase was exhibited by every strain before log phase started. It is because growth of *R. sphaeroides* on acetate occurred via the ethylmalonyl-CoA

(EM-CoA) pathway. In this metabolic pathway, longer time is needed for acetate to be converted into succinyl-CoA and condensed with glyoxylate, which is the EM-CoA pathway product[6,7], resulting in formation of malate before entering tricarboxylic cycle (TCA cycle). It should be noted, however, there is shorter lag phase before growth commenced. Succinate is able to directly enter the TCA cycle without passing through EM-CoA cycle.

3.2. Absorption spectra

By referring to growth curves from Figure 1 as comparable data, crude pigment was extracted from bacterial culture with succinate as carbon source at both initial log and late log phases by using solvent acetone:methanol (7 : 3 v / v). Figure 2 shows the electronic absorption spectra of extracted crude pigments from three strains of *R. sphaeroides* respectively. Their Q_y absorptions appear at same wavelength 770 nm, which represents the bchl while their carotenoids (cars) absorb at different wavelength ranging from 400 nm to 500 nm.

Amount of bchl and cars are found to be higher in late log phase. Figure 2 shows an evident that the quantity of bchl and cars produced increases as cell density is increased from initial log phase to late log phase[8]. However, it should be noted that the increasing of bchl is not significant because of shading effect occurred during the bacterial growth. The shading effect is the result due to the increasing cell densities, thus reduce the effectiveness of the light intensity. It is predicted that the ability of bacteria to develop chromatophore is reduced when the effectiveness of light intensity is also reduced and repressed both of the amount of intracytoplasmic membrane synthesized and pigment produced[1].
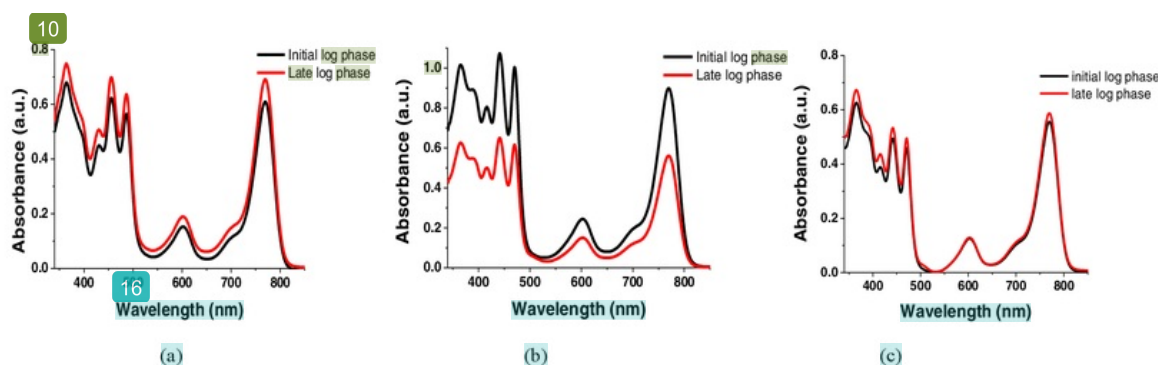

Fig. 2. Absorption spectra of pigments *R. sphaeroides* (a) strain 2.4.1, (b) strain GA, and (c) strain G1C

Furthermore, amount of bchl and cars in late log phase of *R. sphaeroides* GA are decreased compared to initial log phase instead of increased like the other strains. General absorption patterns of both spectra are highly similar. It is proposed that the decrease of bchl and cars is the result of degradation. During the fractionation process on sucrose column by Holt[9], products of degradation were observed. Moreover, deliberate photo-oxidation was carried out by them to study the consequences on bchl. After complete fractionation and spectral analysis, they had revealed that the bchl oxidized to produce an unstable fraction, which has similar absorption pattern with bchl but in a relative low amount[10]. Here, it is predicted that photo-oxidation reaction occurred during the incubation of bacterial cultures and resulting the decrease in amount of bchl. Over exposure to light during incubation of bacterial culture will speed up the degradation process of pigments. It is predicted that the pigment from *R. sphaeroides* GA is less stable compared to the other two strains.

3.3. Determination of pigment ratio

To determine pigment ratio present in initial log and late log phase, crude pigments were extracted from individual cells harvested at different growth phases as described. Pigments isolated from the individual strains were analyzed by HPLC (Figure 3).
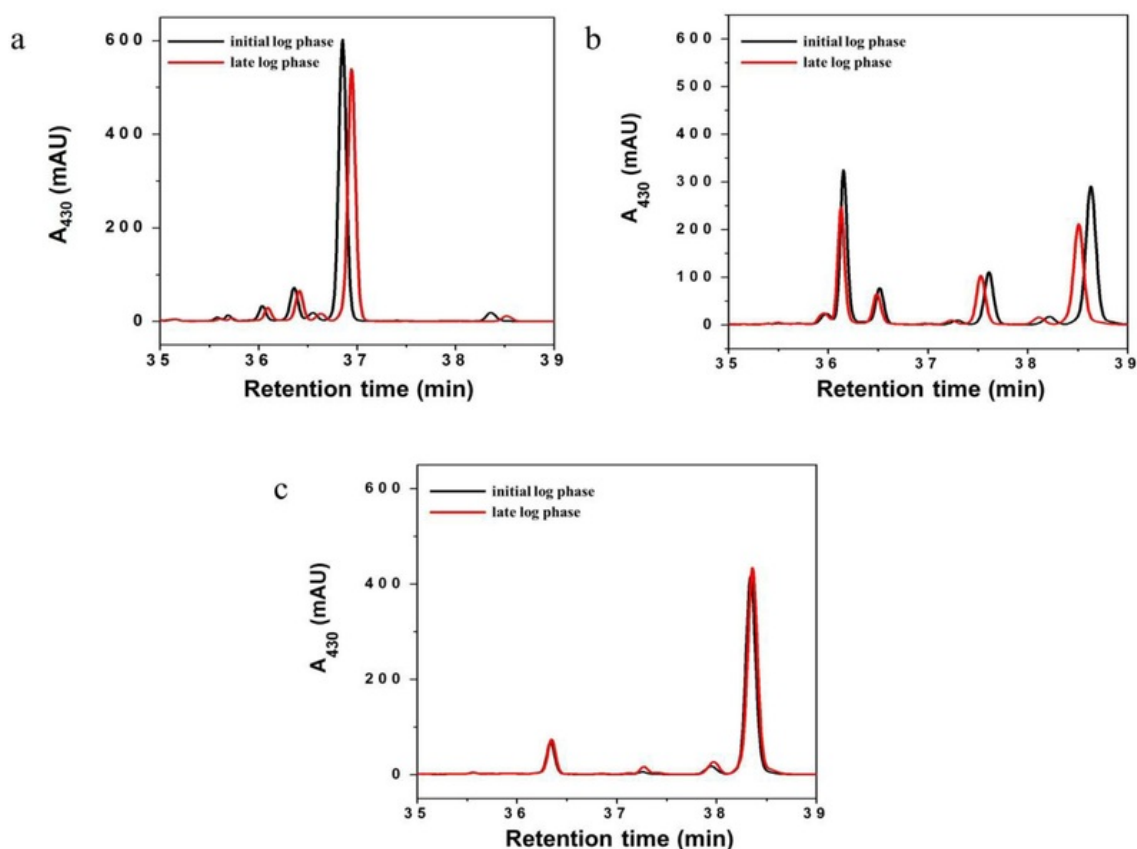
Fig. 3. Chromatogram of carotenoid extracted from 0.1 g of bacterial membrane of (a) *R. sphaeroides* 2.4.1, (b) *R. sphaeroides* GA and (c) *R.sphaeroides* G1C using acetone : methanol (7:3 v/v). Black line represents initial log phase, red line represents late log phase

Table 1. Ratio of carotenoids, detection was at 430 nm.

R. sphaeroides strains	Peak area		Ratio of initial : late log phase
	Initial log phase	Late log phase	
2.4.1	3 423 707	3 142 245	1.09 : 1
GA	4 697 636	3 589 859	1.39 : 1
G1C	3 025 434	3 265 852	1 : 1.08

The general pattern of peaks between initial log phase and late log phase are same, which indicates that the main composition of carotenoid has no changes. *R. sphaeroides* GA has three dominant peaks of cars (36.15 min, 37.6 min, 38.62 min). Peak appeared at 36.36 min, 36.51 min and 36.33 min represent bchl. In the other hand, *R. sphaeroides* 2.4.1 and G1C have one dominant peaks which appeared at 36.58 min and 38.34 min in initial log phase, although slight shift was obtained. However, absorption spectra of each peaks were used for identification of pigment peaks. Small peaks were not included due to unclear absorption spectra and extremely low intensity. According to the HPLC analysis data, amount of cars slightly decreased in all strains of *R. sphaeroides* during late log phase, except in *R. sphaeroides* G1C (Table 1). In addition, among all three strains of *R. sphaeroides*, the

comparison of content of cars among three strains of bacteria also revealed that strain GA it has the highest amount of cars.
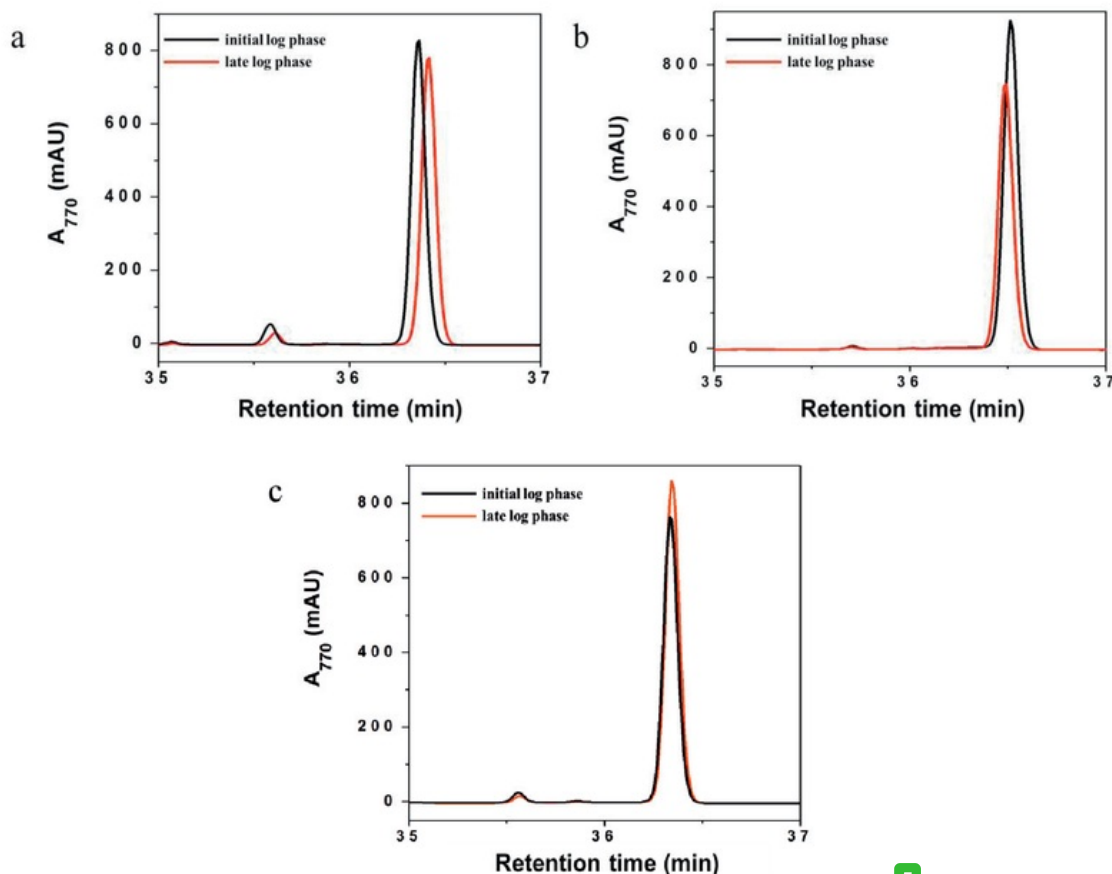

Fig. 4. Chromatogram of bacteriochlorohylls extracted from 0.1 g of bacterial membrane of (a) *R. sphaeroides* 2.4.1, (b) *R. sphaeroides* GA and (c) *R.sphaeroides* G1C using acetone : methanol (7:3 v/v). Black line represents initial log phase, red line represents late log phase

Bchl peaks of R. sphaeroides 2.4.1, R. sphaeroides GA and R. sphaeroides G1C appeard at 36.36 min, 36.51 min and 36.33 min, respectively. The ratio of bchl of all strains also have the same general patterns as of carotenoids for both growth phases. There is slight increase of bchl in *R sphaeroides* G1C along with an increase in cell density in late log phase. *R. sphaeroides* G1C has the highest amount of bchl at late log phase compared to other strains, while at initial log phase *R. sphaeroides* GA has the highest amount. However, there is a decrease of bchl content in *R. sphaeroides* GA and 2.4.1 and which is most likely due to photo-oxidation[8], however, further study is required to determine the exact degradation ratio of pigment.

Table 2. Ratio of bacteriochlorophyll, detection was at 770 nm.

R. sphaeroides strains	Peak area		Ratio of initial : late log phase
	Initial log phase	Late log phase	
2.4.1	4 467 533	4 252 118	1.05 : 1
GA	5 180 486	4 053 425	1.28 : 1
G1C	4 122 859	4 611 884	1 : 1.19

It is predicted that degradation and differentiation have occurred. Although the general patterns are same, according to the presence of some small unknown peaks in the chromatogram (data not shown), it is also predicted that there are some other molecule other than cars and bchl that also absorb at 430 nm and 770 nm respectively. The amount of those molecules increased from the initial log phase to late log phase and resulting a higher absorbance value at 430 nm and 770 nm (Figure 2). These molecules might be the products of degradation or differentiation. It is believed that the cars and bchl molecules undergo degradation or differentiation throughout the incubation process.

4. Conclusion

In this preliminary work, different carbon source in the growth medium affects the growth and the production of photosynthetic pigments of three strains of *R. sphaeroides*. The amount of carotenoids and bacteriochlorophylls from 2.4.1 and GA in the initial log phase was found to be higher than in the late log phase. Although we postulated the products of degradation, further intensive examination should be taken for the future work.

Acknowledgements

References

1. Patricia JK, Samuel K. Molecular genetics of photosynthetic membrane biosynthesis in *Rhodobacter sphaeroides*. *Microbial Rev* 1988;52(1):50–69 .
2. Richard JC, Andrew G, Jürgen K. The architecture and function of the light–harvesting apparatus of purple bacteria :from single molecules to in vivo membranes. *Quarterly Reviews of Biophysics* 2006;39:227–324.
3. Paul AL, Pamela SP. Structure–function relationships in core light–harvesting complexes (LH1) as determined by characterization of the structural subunit and by reconstitution experiments. In: Robert EB, Michael TM, Carl EB, editors. *Advances in photosynthesis, anoxygenicphotosynthetic bacteria*. Dordrecht: Kluwer Academic Publishers; 1995. p. 437–471.
4. Bose SK. *Bacterial photosynthesis*. Ohio: Antioch Press; 1963.
5. Manuel Z, Fransisco R, José LG. Separation of chlorophylls and carotenoids from marine phytoplankton: a new HPLC method using a reversed phase C₈ column and pyridinecontaining mobile phases. *Mar Ecol Prog Ser* 2000;195:29–45.
6. Katrhin S, Rèmi P, Patrick K, et al. The ethylmalonyl–CoA pathway is used in place of the glyoxylate cycle by *Methylobacterium extorquens* AM1 during growth on acetate. *J Biol Chem* 2012;287(1):757–766.
7. Saheed I, Daniel RN, Timothy J. Global insights into energetic and metabolic networks in *Rhodobacter sphaeroides*. *BMC Systems Biology* 2013;**7**:89–104.
8. Alexei AY, Jesus ME, Samuel K. Differential carotenoid composition of the B875 and B800–850 photosynthetic antenna complexes in *Rhodobacter sphaeroides* 2.4.1: involvement of spheroidene and spheroidenone in adaptation to changes in light intensity and oxygen availability. *J Bacterio* 1996;178(20):5877–5883.
9. Holt AS, Jacobs EE. Spectroscopy of plant pigments. II. methyl bacteriochlorophyllsides and bacteriochlorophyll. *Am J Botany* 1954;41:718–722.
10. WS Kim. Complete fractionation of bacteriochlorophyll and its degradation products. *Biochimica Biophys Acta* 1966;112:392–402.

Preliminary Evaluation of The Pigments Content from Rhodobacter Sphaeroides at Stages During Photosynthetic Growth

6 Submitted to Universitas Diponegoro
Student Paper
1%

7 Submitted to Indiana University
Student Paper
1%

8 Kim, W.S.. "Complete fractionation of bacteriochlorophyll and its degradation products", BBA - Biophysics Including Photosynthesis, 19660314
Publication
1%

9 Submitted to University of Ulster
Student Paper
1%

10 Submitted to Pace University
Student Paper
1%

11 Tatas Hardo Panintingjati Brotosudarmo, Leenawaty Limantara, Heriyanto, Monika Nur Utami Prihastyanti. "Adaptation of the Photosynthetic Unit of Purple Bacteria to Changes of Light Illumination Intensities", Procedia Chemistry, 2015
Publication
1%

12 Monika N.U. Prihastyanti, Indriatmoko, Tatas H.P. Brotosudarmo. "Photostability Assay on Light-harvesting Complex as a Material of Biophotovoltaic", Energy Procedia, 2014
Publication
1%

13 Chai-Ling Ho, Phuoc Dang Nguyen, Chai-Ling Ho, Phuoc Dang Nguyen et al. "
1%

Sequence analysis and characterization of vacuolar-type H -ATPase proteolipid transcript from Vahl ", DNA Sequence, 2009
Publication

14 toc.proceedings.com
Internet Source
1%

15 Yanti, Yulmira. "Peroxidase Enzyme Activity of Rhizobacteria-introduced Shallots Bulbs to Induce Resistance of Shallot towards Bacterial Leaf Blight (Xanthomonas Axonopodis pv Allii)", Procedia Chemistry, 2015.
Publication
<1%

16 dspace.cc.tut.fi
Internet Source
<1%

17 Carles Curutchet, Benedetta Mennucci. "Quantum Chemical Studies of Light Harvesting", Chemical Reviews, 2017
Publication
<1%

18 www.jimmunol.org
Internet Source
<1%

19 www.frontiersin.org
Internet Source
<1%

20 Magdaong, Nikki M., Amy M. LaFountain, Jordan Alan Greco et al. "High Efficiency Light-Harvesting by Carotenoids in the LH2 Complex from Photosynthetic Bacteria: Unique Adaptation to Growth Under Low-
<1%

Light Conditions", The Journal of Physical Chemistry B
Publication

21 tel.archives-ouvertes.fr
Internet Source
<1%

22 ethesis.nitrkl.ac.in
Internet Source
<1%

23 www.biogeosciences.net
Internet Source
<1%

24 aem.asm.org
Internet Source
<1%

25 physwww.mcmaster.ca
Internet Source
<1%

26 Submitted to University of Glasgow
Student Paper
<1%

27 preview-bmcsystbiol.biomedcentral.com
Internet Source
<1%

28 krishikosh.egranth.ac.in
Internet Source
<1%

29 lib.dr.iastate.edu
Internet Source
<1%

30 The Phototrophic Prokaryotes, 1999.
Publication
<1%

31 Aleksandr Ashikhmin, Zoya Makhneva, Maksim Bolshakov, Andrey Moskalenko. "Incorporation of spheroidene and
<1%

spheroidenone into light-harvesting complexes from purple sulfur bacteria", Journal of Photochemistry and Photobiology B: Biology, 2017
Publication

32   Advances in Photosynthesis and Respiration, 1995.   <1%
Publication

33   jb.asm.org   <1%
Internet Source

34   Submitted to Jacobs University, Bremen   <1%
Student Paper

35   Yoshinori Kakitani, Junji Akahane, Hidekazu Ishii, Hiroshi Sogabe, Hiroyoshi Nagae, Yasushi Koyama. " Conjugation-Length Dependence of the T Lifetimes of Carotenoids Free in Solution and Incorporated into the LH2, LH1, RC, and RC-LH1 Complexes: Possible Mechanisms of Triplet-Energy Dissipation ", Biochemistry, 2007   <1%
Publication

36   Park, Hyun Je, Eun Jung Choy, and Chang-Keun Kang. "Spatial and Temporal Variations of Microphytobenthos on the Common Reed Phragmites australis bed in a Marine Protected Area of Yeoja Bay, Korea", Wetlands, 2013.   <1%
Publication

Exclude quotes	On	Exclude matches	Off
Exclude bibliography	On		