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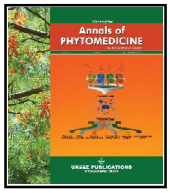

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Antioxidant properties of carotenoid pigments from *Rhodovulum viride* strain JA814

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Abstract

Pigment producing bacteria commonly occur in nature and have intense colour. Bacterial secondary metabolites-pigments are secreted during unfavorable conditions. The exponential growth of bacterial pigments augmented several times as they are more beneficial over synthetic pigments. Biologically originated carotenoid pigments are significant for their wide distribution, structural diversity, varied functions and their actions. It is very crucial to try because bacterial pigments contain certain restorative characteristics including cytotoxicity, antibacterial activity, anticancer activity and exceptional antioxidant activity. Hence, it is important to study a pigment producing photoheterotrophic purple non-sulfur bacterial species. The present study reports on the general characteristics of a photosynthetic bacterium, *Rhodovulum viride* strain JA814, extraction of carotenoid pigments and evaluation for antioxidant properties. Strain JA814 was identified through 16S rRNA gene sequencing analysis showing high sequence similarity value (100%) with closely related species, *Rhodovulum viride* JA756. Since all other species of the genus Rhodovulum are brown to red in colour, except *R. viride* JA756 and the strain JA814 is green in pigmentation. UV-visible spectrophotometric analysis indicated the presence of carotenoids. Carotenoid composition is determined through HPLC and revealed the presence of neurosporene contributing as a major carotenoid (19.44%) and minor components of dihydroxy neurosporene (13%), 1,2 dihydroneurosporene (14.02), hydroxyl dimethyl spheroidenone (9.4%) and dimethylspheroidene (10.45%). The crude extract of carotenoids having potential/radical scavenging activity was evaluated by *in vitro* assay methods such as total antioxidant capacity and DPPH assay. The crude pigment extracts in methanolic solutions showed maximum antioxidant activity at moderate/minimum concentrations. The sample extracts significantly inhibited the DPPH free radical by a proportion of 92 per cent at 800 µg/ml. The standard reference L-ascorbic acid showed inhibition of DPPH radical 85%. In the phosphomolybdenum assay of total antioxidant capacity, the efficiency of antioxidant potential is 0.25 mg/g ascorbic acid equivalents at a concentration of 100 µg/ml. The standard reference value of L-ascorbic acid is 1.07 mg/g equivalents. These findings suggest that the carotenoid pigments proved as promising bioactive compounds and can be exploited in therapeutics.

1. Introduction

Natural pigments have much economic importance as they are more beneficial and have significant properties such as anticancer and antioxidant (Numan *et al.*, 2018). Because of their simple extraction processes and simple cultivation procedures, bacterial pigments are generally preferred. In addition to certain of these chemicals, pro-vitamin A action, carotenoid pigments have been linked to a lower chance of acquiring chronic diseases like cancer, cardiovascular disease and impairments in eye function, making them even more crucial in a human diet (Bhat *et al.*, 2020; Agarwal *et al.*, 2020). The chemicals known as antioxidants scavenge free radicals and lessen oxidative stress by preventing the cellular damage giving beneficial effect on human health (Gezici *et al.*, 2020). Naturally occurring antioxidant compounds are more beneficial over synthetically derived molecules as the synthetic compounds are more toxic, carcinogenic and cause teratogenic effects (Das *et al.*, 2019; Vijayalakshmi *et al.*, 2022). Biologically, source of antioxidants are from plants which have been evaluated and potentially safe. The possible source of antioxidants used in the agricultural, industrial and medical fields is bioactive chemicals derived from microorganisms. Due to popular concern over synthetic food additives, the generation of pigment from microbial sources has received a lot of interest (Saha *et al.*, 2008). Carotenoids are, therefore, regarded as one of the most valuable groups of molecules for advantageous applications, such as in the chemical, pharmaceutical, animal feed and food sectors (Koes *et al.*, 1995; Shreeja *et al.*, 2021). Because microorganisms have diverse metabolic processes and can use a variety of substrates, including agro-industrial wastes and because it is possible to control operating conditions such as pH, temperature, dissolved oxygen and light intensity production of microbial carotenoids has emerged as a potential replacement for artificial pigments. Despite technological, economic and legal restrictions carotenoids are crucial for the functioning of living

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beings. According to studies carotenoids work along with other biomolecules like proteins and lipids to increase their antioxidant activity (Ligia *et al*., 2017). Though, the bacterial carotenoids have wide range of applications, received less attention when compared to other microorganisms such as algae and cyanobacteria. Among the industrially important microorganisms like actinomycetes which produces phenolic compounds, lactobacillus and bifidobacterium though act as a probiotics also has antioxidant potentials (Chandra *et al*., 2019). The microbial pigments used in commercial products include astaxanthin, canthaxanthin, carotenoids, melanins, granadaene, indigoidine, flavins, quinones and more specifically prodigiosin, pyocyanin, rubrolene, scytonemin, violacein and phycocyanin, which have a variety of functions including anti-oxidant, anticarcinogenic, anti-inflamatory and antiobesity properties (Venil *et al*., 2020; Sekeroglu *et al.*, 2019). The main drawback of these organisms is their long cultivation period. Therefore, it has become a research interest to search for bacteria having short cultivation period. Photosynthetic purple non-sulfur bacteria are widely distributed in almost all kinds of habitats such as aquatic fresh water, polluted lakes, marine water, lagoons and in hyper saline environments. The photoassimilation of a variety of organic substances, including fatty acids, primary and secondary alcohols, organic acids, carbohydrates, proteins and aromatic compounds is one of their many recognized physiologically diverse roles. Phototrophic purple bacteria are one such group which is under explored and very less studies were reported having benefits to human health (Ramprasad *et al*., 2013). One such application which is not documented well so far in the genus of *Rhodovulum* in phototrophic purple non-sulfur bacteria is antioxidant property of carotenoids. In the present communication, an attempt has been made to extract the carotenoid pigments, and evaluation followed by free radical scavenging assay methods to exploit its potentials.

2. Materials and Methods

2.1 Organism and cultivation

Rhodovulum viride strain JA814 was grown in a photoheterotrophically (anoxygenic conditions provided with light 2400 lx and medium sourced with pyruvate (0.03%, w/v) as carbon source/ electron donor) in a fully filled screw capped bottles containing a modified bieble and pfennigs medium (Divyasree *et al*., 2016). Inoculated culture medium was incubated at 28 ± 2^{o}C till growth appears as turbidity.

2.2 Pigment identification

The lmax of methanolic pigment extract of strain JA814 was determined by UV × visible spectrophotometer (Spectronic Genesys 2) between 300 and 460 nm. The carotenoid profile of strain JA814 was confirmed by C_{18} high-performance liquid chromatography (HPLC; eluted with acetonitrile : methanol: ethyl acetate; 5 : 4 : 1 v/v; flow rate, 1 ml min^{-1}; absorption at 450 nm) using a photodiode array detector (Srinivas *et al*., 2014).

2.3 Strain identification

The genomic DNA for 16S rRNA amplification was extracted using a commercial DNA extraction kit (Nucleopore g DNA Fungal Bacterial Mini Kit). The 16S rRNA gene was amplified and sequenced in accordance with methods previously described by Subhash *et al.* (2013). By performing a BLAST search on the EzBioCloud database, it was possible to determine the strain's 16S rRNA gene sequence similarities (Yoon *et al*., 2017).

2.4 Antioxidant activity

2.4.1 Studies on antioxidants *in vitro*

2.4.1.1 Effects on free radical scavenging

The inhibition is the basis of *in vitro* techniques. When samples are added to a system that produces free radicals, the amount of free radical action that is inhibited is evaluated, and this inhibition is correlated with the antioxidant activity of the sample. Therefore, the *in vitro* antioxidant activity of the test sample JA814 and the reference (L-ascorbic acid) was evaluated using two standard techniques, namely; the phosmolybdenum assay and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical assay. Under laboratory circumstances, processes were standardized to ensure their effectiveness (Rajesh *et al*., 2011).

2.4.2 Evaluating of total antioxidant activity

2.4.2.1 Phosphomolybdenum assay

The phosphomolybdenum approach was based on the formation of a green phosphate/Mo (V) complex with a maximum absorption at 695 nm after Mo (VI) was reduced to Mo (V) by phosphomolyb date.

2.4.2.1.1 Extract preparation and activity

The test samples were prepared by successive solvent extraction with methanol from whole cell pellet. Approximately, 10 mg/ml of lyophilized powder of strain JA814 was suspended in methanol. Vortex it well to form a uniform mixture. Aliquot the supernatant in to 6.25 mg, 12.5 mg, 25 mg, 50 mg and 100 mg dilutions per ml for antioxidant assay (Elkhamlichi *et al*., 2017). Along with the standard of same concentration, the control and test samples were incubated with reagent solution according to the protocol given by Preito *et al* (1999). Different volumes of samples were added and volume was made upto 100 µl and added reagent solution in ml (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The sample tubes were sealed, and they were heated to 95°C in a hot water bath for 90 min. Each sample's absorbance at 695 nm was measured in comparison to a blank after the samples had been cooled to room temperature. The reference was L-Ascorbic acid. ascorbic acid equivalents are used to express the overall antioxidant activity (ASAE).

2.4.2.1.2 Standardization of a reference compound L-ascorbic acid

Ascorbic acid standard curve was generated by plotting graph with series of concentrations (20, 40, 60, 80, 100, 110, 120, 130, 140, and 150 µg) *vs* absorbance values using UV×visible spectrophotometer (Figure 3). 1mg/ml of stock concentration is used for experiment.

2.4.2.1.3 Calibration curve

Calibration of standard L-ascorbic acid was done under laboratory conditions. Statistical interpretations were carried out by R square or linear regression analysis. The statistical indicator of how closely the data resemble the fitted regression line is called R-square. R-squared values are always between 0 and 1. The better the model

matches the data, in general, the greater the R-squared. Calibration curve was constructed by plotting absorbance *vs* concentration which gives y = m x + c (0.022X + 0.20) was found with an r^2 equal to 0.99 reflects its reproducibility and significance.

2.4.3 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity

Scavenging of free radical activity of the extract was carried out using the stable radical 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and the modified method given by Braca *et al.* (2002). 0.9 ml of 0.003% of DPPH prepared in methanol was mixed with the extracts of different concentrations. These solution mixtures were kept in dark for 30 min and absorbance was measured at 517 nm against methanol (1ml) with DPPH solution as blank. 10 mg/ml of lyophilized powder of sample was extracted in methanol by vortexing well and further extract was diluted in a series of 200, 400, 800, and 1000 mg/ml. Along with the test samples, standard ascorbic acid and control solutions were incubated in dark for 30 min as described. Experiment was performed in triplicates.

When the 2, 2-Diphenyl 1-picryl hydrazyl (DPPH) free radical reacts with hydrogen donors, it is reduced to a corresponding hydrazine. The DPPH radical is purple in colour and turns yellow when it reacts with hydrogen donors. It is a discoloration assay in which the antioxidant is added to a DPPH solution in ethanol or methanol and the decrease in absorbance is measured. The reduction in solution absorbance caused by the substances proton donation was measured at 517 nm. As a positive control, L-ascorbic acid was used. The following formula was used to calculate the percentage of radical scavenging activity.

DPPH radical scavenging activity [%] = $[(A_c - A_s)/A_c]$ x 100

where, Ac and A_s are absorbance of control and sample respectively.

3. Results

3.1 Strain identity and carotenoid determination

Strain JA814 was identified to be belonged to *Rhodovulum viride* JA756 with 100.0 % pair-wise sequence identity based on BLAST search of the 16S rRNA gene sequence. Acetone-methanolic extracts of strain JA814 gave green colored pigment which has absorption maxima at 327, 357, 393, 441, and 473 nm, indicated the presence of carotenoids (Figure 1). Further confirmation of carotenoids is determined by HPLC revealed the composition with neurosporene (19.44%) as the major carotenoid and minor components of dihydroxy neurosporene (13%), 1,2 dihydroneurosporene (14.02%), hydroxyl dimethyl spheroidenone (9.4%), and dimethylspheroidene (10.45%) (Figure 2).

3.2 Antioxidant studies

3.2.1 Phosphomolybdinum assay

In this quantitative method of assessing total antioxidant capacity, all of the extracts demonstrated varying levels of activity, as shown in Figure 4. The antioxidant activity of crude extracts increased with concentration. However, at higher concentrations (100 µg/ml), methanolic extracts of strain JA814 have less total antioxidant capacity, equivalent to 0.25 mg/g ascorbic acid equivalents per 10 mg of dry cell weight.

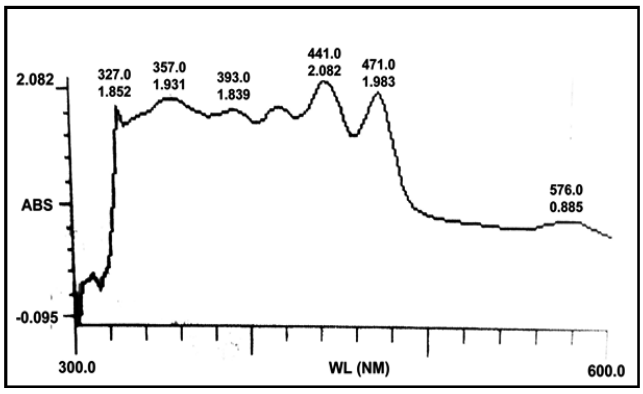

Figure 1: Acetone absorption spectrum of strain JA814 showing carotenoids.

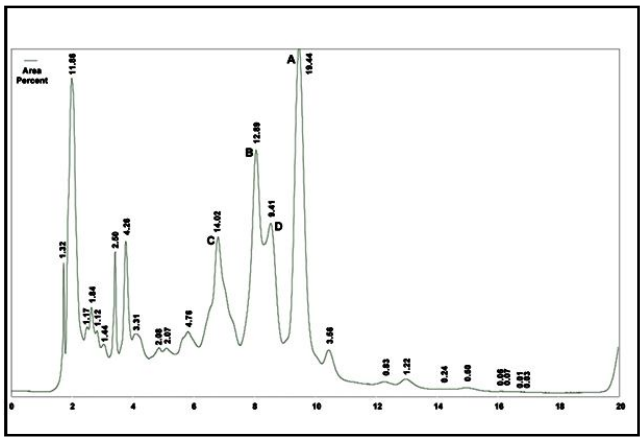

Figure 2: HPLC chromatogram depicting carotenoid composition.

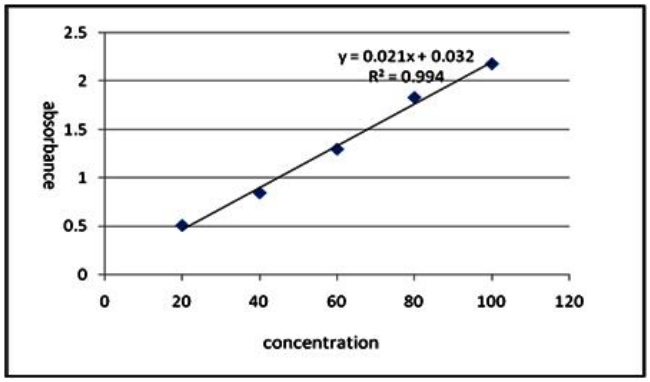

Figure 3: Calibration curve of L-ascorbic acid references.

3.2.2 DPPH radical scavenging activity

The DPPH free radical scavenging activity is depicted graphically in Figure 5. At 517 nm, scavenging activity increased with increasing concentrations of sample extracts, but the activities tend to decrease beyond 800 µg. At 800 µg/ml concentration, the maximum scavenging activity is observed with 92 per cent inhibition.

The 1, 1-diphenyl-2-picrylhydrazyl (DPPH) antioxidant assay method depends on the capacity of a putative antioxidant to scavenge the stable radical of this compound. This stable free radical has an odd electron and is responsible for a significant absorption spectrum in the 515 - 520 nm range. The antioxidant scavenger molecule can donate an electron to DPPH in the presence of antioxidant chemicals, which results in a more stable DPPH molecule. The reduced form of DPPH is a light yellow colour, which denotes an antioxidant compound's enhanced capacity to scavenge free radicals. With the crude extracts of the sample, the assessment of free radical inhibition considerably demonstrated the scavenging potential (Figure 5).

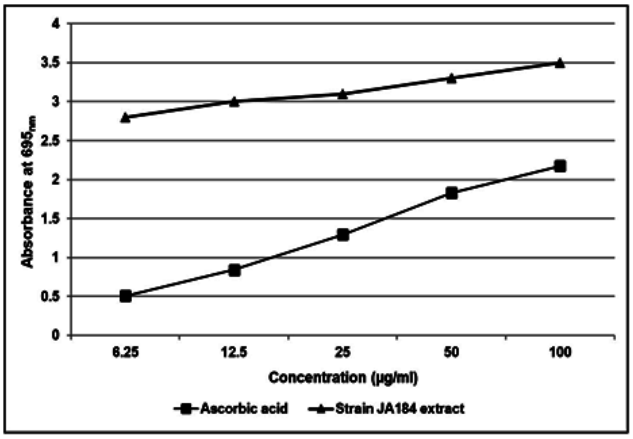

Figure 4: Total antioxidant activity (TAC) of methonolic extracts of strain JA814.

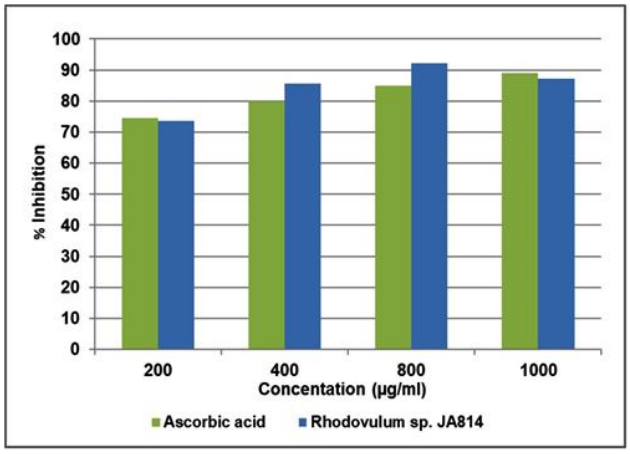

Figure 5: Antioxidant activities of methanolic extracts of strain JA814 measured by DPPH method.

4. Discussion

Natural pigments are derived from a variety of living organisms. Because of their stability and availability for cultivation, biopigments produced by microorganisms are preferred over those produced by plants. Bacteria, among microbes, have enormous potential to produce diverse bioproducts, one of which is pigments. Natural pigments use in food, dye stuff, cosmetics and pharmaceutical manufacturing processes has increased in recent years due to their non-toxicity. Violacein, produced by one of the gram-negative species, *Chromobacterium violaceum*, has antiprotozoan properties, anticancer, antiviral, antibacterial and antioxidant properties are all present (Hizbullahi *et al.*, 2017). Because of their positive effects, such as eco-friendliness, natural character, safety of use and medicinal properties, research on bacterial pigments should be expanded for application in industrial production.

The antioxidant potential of the green pigmented phototrophic purple non-sulfur bacterium strain JA814 was investigated in the present study. The crude extracts demonstrated antioxidant potency in single-proton/electron transfer reactions (DPPH and TAC assays). However, in comparison to standard ascorbic acid, these activities were manifested at a moderate intensity to maximise efficiency. The total antioxidant capacity, as measured by the phophomolyb denum assay, was found to be less active when compared to the reference compound L-ascorbic acid, which has the highest antioxidant potential. This is because crude extract may contain other intervening substances that limit its concentration to be readable. The crude extract's FRSA was calculated using the equation of the logarithmic best line of the graph drawn as DPPH per cent inhibition *vs* carotenoid extract concentration (µg/ml).

Clearance effects in the free radical scavenging activity of crude extracts as determined by DPPH tend to increase with increasing sample concentrations but decreased beyond 800 µg/ml. At 800 µg/ml concentration, the maximum scavenging activity is observed, with 92 per cent inhibition. This method variation significantly increased antioxidant activity. It is worth noting that extracts of strain JA814 can indeed be a good source of bioactive compounds relevant in the development of novel therapeutics, as well as a good source of natural antioxidants.

5. Conclusion

To the best of our knowledge, this is the first report on *Rhodovulum viride* strain JA814 antioxidant properties. The strain is notable for its vibrant green pigmentation. This study's antioxidant evaluation, which used two assay methods (total antioxidant capacity and DPPH), revealed that methanolic extracts have interesting antioxidant activity that correlates well with standard ascorbic acid. Furthermore, these findings offer a promising starting point for future therapeutics. For pharmacological studies, the crude extract of sample JA814 must be purified.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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