JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Dec. 2014. Vol. **8**(6), p. 4983-4988

# Evaluation of Antioxidant Activity and Characterization of Carotenoid Pigment from *Rhodotorula mucilaginosa* Isolated from Parambikulam, Tiger Reserve

**M. RA. Manimala* and R. Murugesan**

Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore - 641003, Tamil Nadu, India.

(Received: 10 October 2014; accepted: 15 November 2014)

**Pigmented yeasts like *Rhodotorula* sp., *Phaffia rhodozyma* and *Sporobolomyces* sp.producedcarotenoid represent a group of valuable molecules for the pharmaceutical, chemical, food and feed industries, not only because they can act as vitamin A precursors, but also for their coloring, antioxidant and possible tumor-inhibiting activity. The carotenoid pigment producing yeast isolated from soil sample of Parambikulam Tiger reserve, India was identified as *Rhodotorula mucilaginosa*. In the present study antioxidant assays *viz*., DPPH scavenging and hydroxyl radical scavenging assays were carried out with yeast carotenoid pigment. The maximum antioxidation characteristics of carotenoid by DPPH and hydroxyl radical scavenging antioxidantassays (77.18 and 74.74 %) were achieved by pigmentation of*R. mucilaginosa*at the concentration of 100 $\mu$g ml$^{-1}$. The pigment from*R. mucilaginosa* was separated by thin layer chromatography (TLC) yielding three major fractions, *viz.*, yellow, orange and red fractions. These fractions were further purified through high performance liquid chromatography (HPLC) before subjecting to FT-IR spectral analysis for their structural elucidation. The carotenoid pigment from *R. mucilaginosa* resulted in three peaks at retention time of 3.35, 4.55 and 5.92 min respectively.**

**Key words:** Carotenoid pigment, *Rhodotorula mucilaginosa,* Antioxidant activity, Characterization, Purification, Natural food colourants.

Carotenoids are one of the pronounced and most important groups of naturally occurring pigments. It has been estimated that nature produces about 100 million tons of these pigments per year.They are great interest in many scientific disciplines because of wide distribution and diverse functions. Owing to their ubiquitous occurrence, many functions and interesting properties. Carotenoids are subject to interdisciplinary research in biochemistry, biology, chemistry, medicine microbiology, physics and many other branches of science[3].

* To whom all correspondence should be addressed.
E-mail: maniarchana20@gmail.com

Carotenoids have the ability to act as antioxidants and thus protect cells against photo oxidation. The ability of carotenoids to quench singlet oxygen is well known and reactions with radical species have also been studied [4].Dietary carotenoids inhibit onset of many diseases in which free radicals are thought to play a role in initiation, such as atherosclerosis, cataracts, age-related macular degeneration, multiple sclerosis and most importantly cancer. Antioxidant properties of carotenoids in cosmetics preparations were reported to be effective in preventing various kinds of damage resulting from oxidation and exposure to UV light. Astaxanthin has also health benefits in cardiovascular disease prevention, immune system boosting, bioactivity against *Helicobacter pylori*, and cataract prevention due to its high antioxidant activity[7].

Now a days biological sources of carotenoid pigment received major focus because of the stringent rules and regulations applied to chemically synthesized/purified pigments. Compared with the extraction from vegetables or chemical synthesis the microbial production of carotenoid pigment have paramount interest, mainly because of overcoming the problems of seasonal and geographic variability in the production and marketing of the colourants from plant origin and because of the economic advantages of microbial processes using natural low-cost substrates as carbohydrate sources[6]. Biopigments have been produced from large number of bacterial, yeast and mold species. Among different microorganisms, *Rhodotorula* sp., *Achromobacter* sp., *Blakeslea* sp. and *Monascus* sp. are common pigment producing microbes [7]. Moreover, industrial interest is now gradually shifting away from the yellow carotenoids such as ²-carotene and lutein towards the considerably more valuable orange-red keto-carotenoids, such as torularhodin and torulene, for which at present no commercially exploitable plant or animal sources exist[9]. *Rhodotorula* is well known for its characteristic carotenoids "torulene, torularhodin and ²-carotene". Carotenoids are used in food industries as colourants, feed additive, vitamin A sources and are being preferred to synthetic pigments. The interest in the natural pigments has increased in recent years due to the consumer concern about the harmful effects of synthetic pigments on health and with the development of new food products based on natural ingredients[2]. In the present study we report the antioxidant potential and characterization of carotenoid pigment extracted from *R. mucilaginosa*.

**Microorganism and Culture Conditions**

The microorganism used in this study was isolated from soil, collected from Tiger reserve Parambikulam, Kerala, India. Stock cultures were maintained on yeast malt extract agar slants at 4ºC after being incubated at 25-30ºC for 4-5 days. The basal medium for liquid culture contained 30.0 g glucose, 2.5 g $(NH_4)_2SO_4$, 1.0 g $K_2HPO4$, 0.5 g $MgSO_4.7H_2O$ and 4.0 g yeast extract (per litre).

**Extraction of carotenoid pigment**

The yeast cultures were inoculated on to yeast malt extract broth and incubated at 28±1ºC for 5 days.A known amount (500mg) offreeze-dried red yeast was hydrolyzed with 1 ml of 1N hydrochloric acid in water bath at 70°C for one and half hour. After removal of excess acid by washing with water, the cells were soaked overnight in acetone: methanol (1:1) solution. The pigment was extracted with acetone until the entire colour was leached out from the cells. Acetone extracts were transferred to light petroleum (20ml) at (40 - 60°C) in a separating funnel and washed thrice with distilled water. The absorbance of the light petroleum phase was documented at 474 nm. The carotenoidyieldis reportedonthebasisofcellmass (¼g $g^{-1}$dried cellweight) [8].

**Determination of antioxidant activity of yeast carotenoid pigment**

Diphenyl-2-picrylhydrazyl (DPPH) scavenging and hydroxyl radical scavenging antioxidant powerassays were used to determine antioxidant activity of carotenoid produced by *R. mucilaginosa* that was carried out by following standard method [1, 10].

**Thin Layer Chromatography (TLC) and HPLC analysis for separation and purification of the pigment fractions**

**Thin layer chromatography**

Thin layer chromatographic separation of the different fractions from the carotenoid pigment of *R. mucilaginosa* was carried out using TLC plates using benzene and petroleum ether (85:15, v/v)as a mobile phase and determined their Rf values. The samples were identified by comparing the distance travelled by the standard to the distance travelled by the test sample (*R. mucilaginosa*) ²-carotene. The Rf values is a mathematical representation of the ration of the distance travelled by the solvent [15].

**HPLC-High Performance Liquid Chromatography**

The purity of the different fractions was checked by HPLC using a reverse phase-C18 column. For mobile phase (HPLC grade solvents were used) and samples were filtered through 0.25¼m membrane filter, C18 column consists of acetonitrile, isopropanol and ethyl acetate (40:40:20, v\v\v) with flow rate at 1 ml/min[18].

**Structure determination of carotenoid pigments**

The structure of three fractions were determined using FT-IR absorption spectra. FT-IR spectrometer (Impact 400D, Nicolet, Madison,WI) was used to measure the infrared spectra of extract

solution in the wave number of 400-4000 $cm^{-1}$ at room temperature. For each IR spectrometer samples 32 scans at 4 $cm^{-1}$ resolution was collected in the transmittance mode.

## RESULTS AND DISCUSSION

Carotenoids are mainly colored pigments present in plants and microorganisms. The epidemiological studies have revealed that an increased consumption of a diet rich in carotenoids is correlated with a lower risk of age-related diseases. Carotenoid contains conjugated double bonds and their antioxidant activity arises due to the ability of these pigments to delocalize unpaired electrons [16]. The double bond present in the pigment is responsible for the ability of carotenoids to physically quench singlet oxygen without degradation and for the chemical reactivity of carotenoids with free radicals. The efficacy of carotenoids for physical quenching is related to the number of conjugated double bonds present in the molecule, which determines their lowest triplet energy level. They can also scavenge peroxy radical thus preventing damage in lipophilic compartments [17]. The carotenoid ²-carotene can also act as a pro-oxidant causing an increase in lipid peroxidation [13].

The highest percentage of DPPH scavenged radicals was recorded in pigment produced from *Phaffia rhodozyma* for an addition of 0.05 per cent carotenoid extract (94.58 per cent), while the other extracts at different concentrations *viz*., 0.02 and 0.10 per cent were slightly weaker scavengers [13]. It is well known that antioxidants can seize the free radical chain of oxidation and form stable free radicals, which would not initiate further oxidation. In the present study, the scavenging activities of DPPH exerted by *R. mucilaginosa* pigment extract at the concentration of 100 µg $ml^{-1}$ exhibited 77.18 per cent inhibition whereas the standard BHA at the same concentration exhibited 86.44 per cent inhibition. It gives the impression that carotenoid pigment also has got the capacity to secrete antioxidants almost equal to BHA.

Hydroxyl radical produced may cause sugar fragmentation, base loss and leakage of DNA

**Table 1.** TLC separated fractions of carotenoid pigment from*R. mucilaginosa*

| Pigment fraction | Colour of the pigment fraction | Rf value (cm) | Absorption maximum(» max) |
|---|---|---|---|
| 1 | Yellow | 0.87 | 446 |
| 2 | Orange | 0.79 | 479 |
| 3 | Pink | 0.44 | 515 |

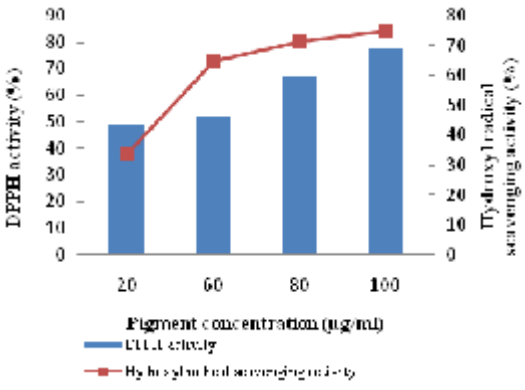


**Fig 1.** Determination of DPPH and hydroxyl radical scavenging antioxidant activity of pigment from *R. mucilaginosa*

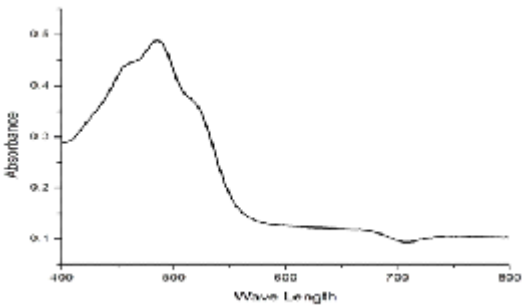


**Fig 2.** Spectrometric scanning of pigmentation in *R. mucilaginosa*

strand [19]. Hydroxyl radicals are the major ROS causing lipid peroxidation and enormous biological damage [12]. It is apparent from the present study that pigment extract of *R. mucilaginosa* not only scavenges off the free radical but also inhibits the generation of free radicals. The red pigment produced from *Penicillium purpurogenum* showed strong $Fe^{2+}$chelating activity even at the minimalconcentration of 20 mg $ml^{-1}$ and showed 51.37 per cent chelating rate[14]. In the present study, pigment extracted from *R. mucilaginosa*exhibited a maximum of 74.74 per cent hydroxyl radical scavenging activity at the concentration of 100µg $ml^{-1}$ whereas the standard BHA at the same concentration exhibited 90.11 per cent inhibition (Fig. 1). The results suggested that pigment scavenges off these free radicals and hence inhibit cellular damage.

**TLC, HPLC analysis and FT-IR spectra of pigment extracted from *R. mucilaginosa***

In the present study, TLC separation of the crude pigment with benzene: petroleum ether (85:15, v/v) has revealed presence of three major bands such as yellow, orange and pink whose Rf values and »$_{max}$ were presented in Table 1 and Fig 2. The Rf value of the yellow fraction is similar as that of standard ²-carotene spot and also there isa resemblance in their absorption spectra as well. Close agreement was obtained between absorption maxima of these fractions and Rf values which resembles with that of orange, yellow and red fractions as torulene, ²-carotene and torularhodin was published earlier[5,15, 16].

Based on absorption spectra three major fractions from *R. mucilaginosa* were identified as: fraction 1 as ²-carotene, fraction 2 as torulene and fraction 3 as torularhodin respectively[9].Moreover, in a recent report on thechromatographic analysis of the crude extract from *R.glutinis* showed that similar three carotenoid pigments,*viz*., ²-carotene, torulene and torularhodin were present inthe extract[15].

High performance liquid chromatographic analysis of the pigment fractions obtained from the thin layer chromatography depicted the separation of fractions. ²- carotene was used as

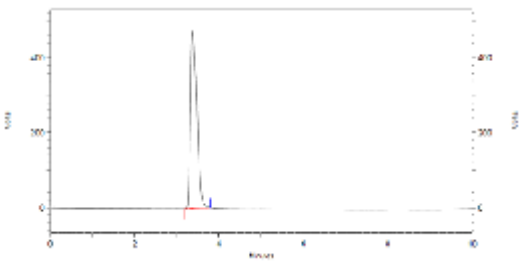

**Fig 3.** HPLC analysis of standard ²-carotene compound

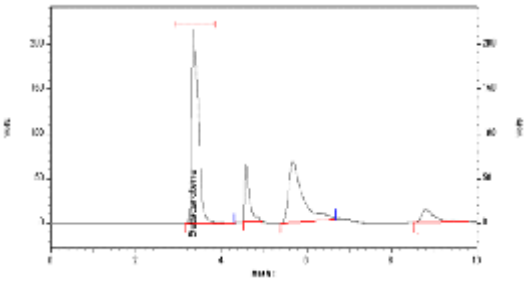

**Fig 4.** HPLC analysis of carotenoid pigment extract from *R. mucilaginosa*

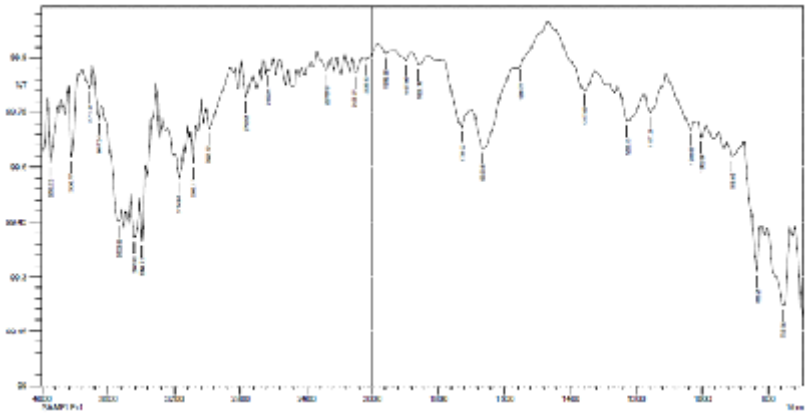

**Fig 5.** FT-IR spectrum of carotenoid pigment extractfrom *R. mucilaginosa*

standard. The result of this study showed that *R. mucilaginosa* accumulates carotenoids which were confirmed by TLC and HPLC analysis using ²-carotene as standard. The standard used which showed peak at retention time of 3.35 minutes and crude pigment was observed with three peaksat the retention time of 3.35, 4.55 and 5.92 minutes respectively(Fig.3 and 4). Thus the yellow fraction from pigmentwas similar to the standard ²- carotene at retention time of 3.35 minutes and thus we conclude that pigment from yeast confirms the presence of ²-carotene.

The identification of the major carotenoid fractions isolated from *R. mucilaginosa* was based on absorption, FT-IR spectra (Fig.5)showed the band at 2993 $cm^{-1}$ are due to asymmetrical stretching vibration of aliphatic $CH_3$ groups respectively and band at 2762 $cm^{-1}$ are due to symmetrical stretching vibration of same groups. Low intensity band at 1728 $cm^{-1}$ may be due to >C = O group indicate the presence of ester groups. The band at 1713 and 1738 $cm^{-1}$ is due to >C = O group probably an ester[9].The band at 1666 $cm^{-1}$ may be due to C = C due to alkenes which indicate the presence of an olefinic functional groups. The band at 3657 $cm^{-1}$ may be due to hydroxyl amino group. The band at 3086 $cm^{-1}$ is due to C-H due to methyl compound which indicate the presence of an olefinic functional group.

## CONCLUSION

At present, large quantities of pigments are actually satisfied through synthetic way. However, in food and cosmetics industries, the application of chemically synthetic carotenoid pigment is restricted because of their toxicity. Natural colourants are considered to be safer than synthetic ones, and their applications in foods, cosmetics and pharmaceuticals are growing rapidly. In this study, under *invitro*condition pigment efficiently scavenged free radicals DPPH and hydroxyl radical scavenging activity exhibited 77.18 and 74.74 per centinhibition at the concentration of 100 µg $ml^{-1}$. This study also identified that the presence of commercially important carotenoid compounds *viz*., ²-carotene, torulene and torularhodin were present in the extract. The commercial production of the carotenoid pigments using yeast has gained more importance owing to its highly efficient and easy manipulation. It is thought that the natural colour market will grow on a global scale at a greater rate than synthetic colours owing to a continued consumer pressure to 'go natural'.

## ACKNOWLEDGEMENTS

The authors greatly acknowledge the Indian council of Agricultural Research (ICAR) for the financial support granted under the scheme "Application of microorganisms in Agriculture and allied sciences (AMAAS)" to carry out this study.

## REFERENCES

1. Blois, M.S. Antioxidant determinations by the use of a stable free radical, *Nature*, 1958; **29**: 1199 -1200.
2. Buhler, R.M.M., Dutra,A.C., Vendruscola,F., Moritz,D.E., Ninow,J.L. Monascus Pigment Production in Bioreactor Using A Co-Product of Biodiesel as Substrate, *Ciência e Tecnologia de Alimentos*.,2013*;* **33**: 9-13.
3. Chen,B.H., Peng,H.Y.,Chen, H.E. Changes of carotenoids, color, and vitamin A contents during processing of carrot juice, *J. Agric. Food Chem.*,1995; **437**: 1912-1918.
4. Edge, R., Mcgarvey, D. J., Truscott, T. G. The carotenoids as antioxidants: A review. *J. Photochem. Photobiol.*, 1997; **41**: 189-200.
5. Frengova,G., Simova,E., Pavlova,K., Beshkova, D ., Grigorora, D. Formation of carotenoids by *Rhodotorula glutinis* in whey ultrafiltrate,*Biotechnol Bioeng.*,1994; **44**: 888-894.
6. Frengova,G., Beshkova, M.Carotenoids from *Rhodotorula* and *Phaffia*: yeasts of biotechnological importance, *J.Ind. Microbiol. Biotechnol.*, 2009; **36**: 163-180.
7. Kirti. K., Amita, S., Priti, S., Mukesh Kumar, A., Jyoti, S. Colorful world of microbes: carotenoids and their applications,*Adv Biol.*, 2014; 1-13.
8. Latha, B. V., Jeevaratnam, K., Murali, H. S., Manja, K.S.Influence of growth factors on carotenoid pigmentation of *Rhodotorula glutinis* DFR-PDY from natural source, *Ind. J. Biotechnol.*,2005; **4**: 353-357.
9. Latha, B.V., Jeevaratnam, K.Purification and Characterization of the Pigments from *Rhodotorula glutinis*DFR-PDY Isolated from Natural Source, *Global J. Biotech and Biochem.*,2010; **5**(3):166-174**.**

10. Lloyd, R. V., Hanna, P. M., Mason, R. P. The origin of the hydroxyl radical oxygen in the Fenton reaction,*Free Radical Biol. Med.,* 1997; **22**(5): 885-888.
11. Michalowska, A .G., Stachowiak ,B. The antioxidant potential of carotenoid extract from *Phaffia rhodozyma, Acta Scientiarum Polonorum Technologia Aliment.*, 2010; **9**(2):171-188.
12. Morales, F. J.,Jimenez-Perez, S.Peroxyl radical scavenging activity of melanoidins in aqueous systems,*Eur. Food Res. Technol.*,2004; **218**(6):515-520.
13. Mortensen, A., Skibsted, L. H., Truscott, T. G. The interaction of dietary carotenoids with radical species, *Arch Biochem Biophs.*, 2001; 385:13.
14. Padmapriya ,C., Murugesan ,R., Gunasekaran ,S.In Vitro Antioxidant and Antibacterial Studies of Fungal Pigment Extracts from Parambikulam Tiger Reserve,*Trends in Bioscience,*2014; **7**(13)*:*1522-1527.
15. Park, P., Kim, E., Chu, J. Chemical disruption of yeast cells for the isolation of carotenoid pigments: *Sep Purif Technol.*, 2007; **53**: 148-152.
16. Perrier, V., Dubreucq,E., Galzy, P. Fattyacid and Carotenoid composition of *Rhodotorula* strains, *Archivesof Microbiology.*,1995*;***164**:173-179.
17. Stahl, W., Sies, H. Carotenoids and flavonoids contribute to nutritional protection against skin damage from sunlight, *Mol. Biotechnol.*, 2007; **37**: 26-30.
18. Tinoi,J., Rakariyatham, N., Deming, R. L. Simplex optimization of carotenoid production by *Rhodotorula glutinis* using hydrolyzed mung bean waste flour as substrate, *Process Biochem.*,2005; **40**: 2551-2557.
19. Xu, Q., Tao, W.,Ao, Z.Antioxidant activity of vinegar melanoidins,*Food Chem.*, 2007; **102**: 841-849.