Indian Journal of Geo-Marine Science
Vol 45(8) August 2016,pp. 1029-1034

Isolation of carotenoids producing marine red yeasts

MK Dhaliwal[1] & N Chandra[2]
[1]Department of Microbiology, Birla College, Kalyan, M.S.-421304, India.
[2]Department of Botany-Biotechnology, Birla College, Kalyan, M.S.-421304, India
[E.Mail : dmkaur@gmail.com]

Received 10 February 2014; revised 07 July 2014

Marine red yeasts were enriched and isolated using sea-water samples from different locations as well as various marine animals. Two red yeast isolates selected were studied for their carotenoids production. Two selected isolates were identified as *Rhodosporidium paludigenum and Sporobolomyces novazealandicus* based on their cultural, morphological and biochemical characters. The isolates were studied for their carotenoid content using HPTLC and HPLC. The *Rhodosporidium paludigenum* isolate was observed to produce β-carotene only, whereas isolate *Sporobolomyces novazealandicus* produced higher amounts of total carotenoids, with an additional pigment.

[**Key words:** Carotenoids, torulene , lutein , environments]

Introduction

Carotenoids are a group of over 600 types of molecules which can be found in plants and certain micro-organisms and fulfill diverse functions, ranging from their original evolutionary role as photosynthetic or light-quenching pigments to antioxidants, precursors of vitamin A, or pigments involved in the visual attraction of flower pollinators. Several micro-organisms, including bacteria, algae, molds and yeasts are able to produce carotenoids naturally. Majority of carotenoids are hydrocarbons of 40 carbon atoms which contain two terminal rings joined by a chain of conjugated double bonds. The biosynthesis of different commercially important carotenoids (β-carotene, torulene, torularhodin and astaxanthin) by several yeast species belonging to the genera *Rhodotorula, Rhodosporidium*, *Sporobolomyces* and *Phaffia* has led to consider them as potential pigment sources. Yeasts are more convenient than algae or molds for large-scale production in fermentors, due to their unicellular nature and high growth rate [1]. The red yeasts develop pink, orange or reddish colonies due to their ability to produce carotenoids or carotenoid-like compounds[2]. Scientific interest in dietary carotenoids (α-carotene, lutein, zeaxanthin, lycopene, β-cryptoxanthin, fucoxanthin, astaxanthin), as well as β-carotene has increased in recent years because of their beneficial effects on human health, such as lowering the risk of cancer and enhancement of immune system function, which are attributed to their antioxidant potential[3]. Carotenoids are of commercial interest as vitamin precursors and effective antioxidants[4]. Industrial application of red yeasts for the production of carotenoids through biotechnological processes has been studied[5,6].

The enrichment and isolation of red yeasts from marine sources would enhance the understanding of carotenoids production in extreme environments. Also the extremophiles so isolated may provide a cheaper alternative carotenoids production method.

Materials and Methods

The marine water samples from Thane creek, Kalyan creek, Murud Zanzira, Gorai beach, Alibaug beach, Bombay High (Maharashtra), Daman beach (Daman), Silvassa (Gujarat) and Panjim (Goa) were collected in 1L plastic container and used for study[7](Table I). Water samples were filtered using Sietz filter assembly with Millipore (0.45 mm) membrane. The membrane was then placed on the surface of seawater-YEM agar (yeast extract- 3 g l^{-1}, malt extract- 3 g l^{-1}, peptone- 5 g l^{-1}, agar-3 g l^{-1}, aged sea- water-1l, pH-5.0) plates. All the plates were incubated at room temperature till the emergence of yeast colony. The marine animals like squid, crab, salmon, shark, eel and Bombay duck were collected from Bombay High and

washed with sterile seawater. Washings were then directly plated on Seawater YEM agar plates in triplicate, by surface spreading technique. Colonies were selected based on their pigmentation, studied microscopically for presence of large ovoid unicellular yeast cells and then transferred to YEM agar slants for further analysis. Marine isolates were referred to as M1 (Panaji, Goa) and M2 (Squid from Bombay high). Identification of the pigmented yeast strains was based upon characteristics of vegetative growth, vegetative reproduction (Dalmau test), biochemical tests such as fermentation of carbohydrates assimilation of carbon and compounds, growth at 37^0 C, formation of extracellular amyloid compounds, production of ammonia from urea[2].

Table 1: Pigmented yeast isolates enriched from marine water and animal samples collected from different locations.

Sr.No.	Location of sample collection		Total number of yeast colonies	Identity of red yeast isolate
Marine water				
1.	Panaji, Goa		128	M1
2.	Thane creek, Maharashtra		19	-
3.	Kalyan creek, Maharashtra		56	-
4.	Murud Zanzira, Maharashtra		158	-
5.	Gorai beach, Maharashtra		9	-
6.	Alibaug beach, Maharashtra		132	-
7.	Bombay High, Maharashtra		26	-
8.	Daman beach, Daman		14	-
9.	Silvassa, Gujarat		54	-
Marine animal samples from Bombay High				
10.	Squid	*Sepia* sp.	12	M2
11.	Crab	*Sylla serrata*	39	-
12.	Salmon	*Polynemous tetradactilus*	16	-
13.	Shark	*Scoliodon* sp.	6	-
14.	Eel	*Anguilliformes* sp.	24	-
15.	Bombay duck	*Herpadone neherius*	45	-

The carotenoids from the marine isolates were extracted using Sedmark *et* al[8] method and estimated using An *et al.*[9] method. Absorbance of the pigment extract was measured at 478 nm using spectrophotometer (Elico, SL 159 UV-Vis Spectrophotometer). β-carotene was detected by HPTLC (Perrier *et al.*[1]). Chromatographic runs were performed on 20x10 cm Silica gel G plates with mobile phase toluene: acetone (4:1). For High-Performance Liquid Chromatography

(HPLC), samples and the β-carotene standard (Sigma-Aldrich) were analyzed by diode-array UV detector (Waters 990). Carotenoids were separated by HPLC analysis on a reversed-phase Zorbax ODS C18 column (250×4.6 mm; 4.65 µm particle size), Agilent, USA and sphere diameter 5 µm. Temperature of the column was maintained at 20°C. The mobile phase was an isocratic solvent system consisting of acetone - water (67.4: 32.6, v/v) that was delivered by a LaChrom pump (L-7100) at a flow rate of 0.5 ml min^{-1} and monitored at 430 nm, using D_2 lamp. HPLC device (LC Autosampler, LC Pump and UV/Vis detector (L-7420) was from Merck Hitachi LaChrom.

Results and Discussion

Of the 15 different marine water and animal samples, only two samples showed presence of red pigmented yeast colonies (Table I). Marine pigmented yeast isolate M1 produced orange colored, spherical, mucoid with smooth and glistening surface, entire margin and convex elevation colnies. The ovoid yeast cells showed presence of asymmetric ballistospores as studied by Dalmau test. The mode of vegetative reproduction was monopolar budding, with stalk formation between the mother and daughter cells. the marine isolate was thus identified as *Rhodosporidium paludigenum* on the basis of morphological and biochemical tests. Isolate

M2 from marine squid, produced orange colored butyrous colonies, asymmetrical ballistospores, assimilated nitrate, nitrite and urea. On the basis of its biochemical and morphological characters, the isolate was identified as *Sporobolomyces novazealandicus* (Table II).

Table II: The biochemical profile of the pigmented yeast isolates enriched from marine sources

Sample	**M1**	**M2**	***Rhodosporidium paludigenum***	***Sporobolomyces novazealandicus***
Carbohydrate fermentation				
Glucose	-	-	-	-
Maltose	-	-	-	-
Xylose	-	-	-	-
Sucrose	-	-	-	-
Carbohydrate assimilation				
Lactose	-	-	-	-
Xylose	+	+	+	+
Maltose	+	-	+	-
Fructose	+	+	+	+
Dextrose	+	+	+	+
Galactose	+	-	+	±
Raffinose	-	-	-	-
Trehalose	+	-	-	-
Melibiose	-	-	-	-
Sucrose	+	-	+	-
L- Arabinose	-	-	-	-
Mannose	+	+	+	+
Inulin	-	-	-	-
Sodium gluconate	-	-	-	-
Glycerol	+	+	+	+
Salicin	-	-	-	-
Glucosamine	+	+	+	+
Dulcitol	-	-	-	-
Inositol	-	-	-	-
Sorbitol	-	-	-	-
Mannitol	+	-	+	-
Adonitol	+	+	+	+
α-methyl-D-glucoside	+	-	+	-
Ribose	-	-	-	-
Rhamnose	-	-	-	-
Cellobiose	+	-	+	-
Melezitose	+	-	+	-
A-methyl-D- mannoside	-	+	-	+
Xylitol	-	-	-	-
ONPG	-	-	-	-
Esculin hydrolysis	-	-	-	-
D-Arabinose	+	+	+	+
Citrate utilization	+	+	+	+
Malonate utilization	+	+	+	+
Sorbose	+	+	+	+
Blank	-	-	-	-
Nitrogen utilization				
Nitrate	+	+	+	+
Nitrite	+	+	+	+
Starch production	-	-	-	-
Urease activity	+	+	+	+
Growth at 37^0C	+	+	+	+
Growth with 10% NaCl	+	+	+	+

Key: + = growth; - = no growth

Nagahama *et al.*[11] have isolated two novel species *Rhodotorula benthica* and *Rhodotorula calyptogenae* respectively from tubeworm and giant white clam, collected from the deep-sea floor of the Pacific Ocean. Gadanho and Sampaio[12] identified four pigmented yeasts associated with deep-sea hydrothermal systems of the Mid-Atlantic Rift. Approach of isolating pigmented yeasts from different ecological niches for possible commercial application in carotenoid production aids in discovery of hyper pigmented wild type strains.

The total carotenoids content of the two selected marine isolates showed that isolate M2 produced high total carotenoids as compared with the isolate M1 (Table III). Hence the use of marine isolate for commercial carotenoids production can be explored. Marine isolate M2 produced total carotenoids content of 7.43 ± 0.93 mg g^{-1}, which is significantly high.

Table III: The Cell dry weight (CDW), Total carotenoid content (TCC) and Total carotenoid production (TCP) of various pigmented yeast isolates

Samples	CDW (g l^{-1})	TCC (mg g^{-1})	TCP (mg l^{-1})
M1	2.0 ± 0.01	0.24 ± 0.73	0.47 ± 7.3
M2	1.6 ± 0.01	7.43 ± 0.93	11.89 ± 9.3

Values are mean of three sets of determinants

The orange color of the two isolates is due to presence of C_{40}- carotenoids (Goodwin[13], 1992). Garcia *et al*[14] studied the occurrence of culturable yeasts in glacial meltwater and found the pigmented yeasts *Rhodotorula, Rhodosporidium* and *Sporobolomyces* sp. Yurkov *et al*[15] studied strains of basidiomycetous yeasts isolated from different sources in order to determine the content of carotenoid pigments and ubiquinone Q_{10}. The high total carotenoids content was revealed in the representatives of the following species: *Rhodosporidium diobovatum, Rhodosporidium sphaerocarpum, Rhodotorula glutinis, Rhodotorula minuta* and *Sporobolomyces roseus.* These compounds were identified using UV-Visible spectrophotometer spectral data, HPTLC relative factor and HPLC retention time. Carotenoids absorb maximally at three wavelengths (533nm, 468nm and 341nm), resulting in three peak spectrum, which is characteristic of carotenoids. The pigment extracts of *Rhodosporidium paludigenum* and *Sporobolomyces novazealandicus* showed a typical spectrum and the absorption of petroleum ether pigment extracts was studied at 478nm. The carotenoids produced by *Rhodosporidium paludigenum* and *Sporobolomyces novazealandicus* were analyzed by HPTLC and HPLC to separate the carotenoids (Fig.1). Both the isolates showed presence of β-carotene in their pigment extracts, though isolate M2 had another pigment too (Table IV, V). Godinho and Bhosale[16] studied marine bacterial isolate *Microbacterium arborescence* strain using HPLC to identify the production of lycopene.

Conclusion

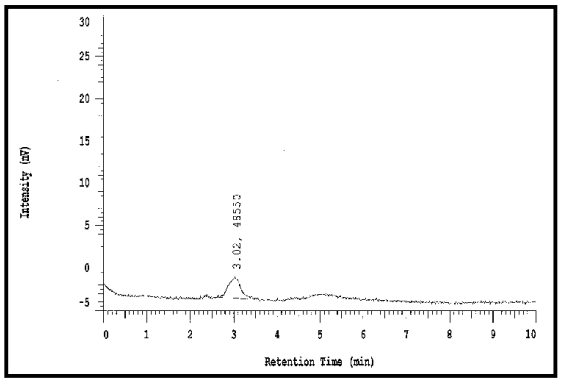
The isolation of pigmented yeasts from marine samples indicates the ecological niches of red yeasts. Further studies may demonstrate the use of these organisms as potential commercial β-carotene producers. Isolate M2 (*Sporobolomyces novazealandicus*) may be further studied to optimize its carotenoid production. Isolate showed promise as a good source of marine carotenoids producer and can be exploited by marine Aquaculture feed industry.

Table IV: HPTLC analysis of carotenoids extracted from pigmented marine yeast isolates and standard β-carotene

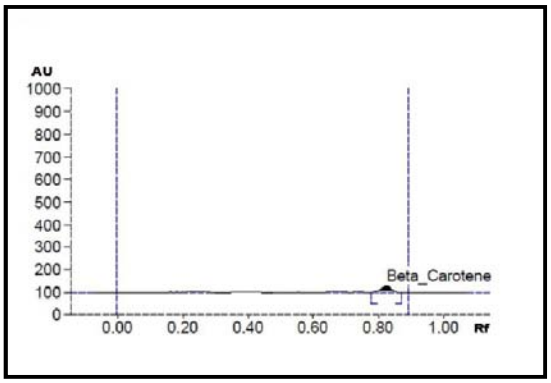
Sample	Rf	AUC	Area %	Approximate β-carotene concentration (µg ml-1)
Std.	0.83	5774.0	100	500
M1	0.83	747.0	100	31.27
M2	0.40	1397.3	56.36	
	0.83	1081.9	43.64	18.12

Table V: HPLC analysis of carotenoids extracted from pigmented marine yeast isolates and standard β-carotene

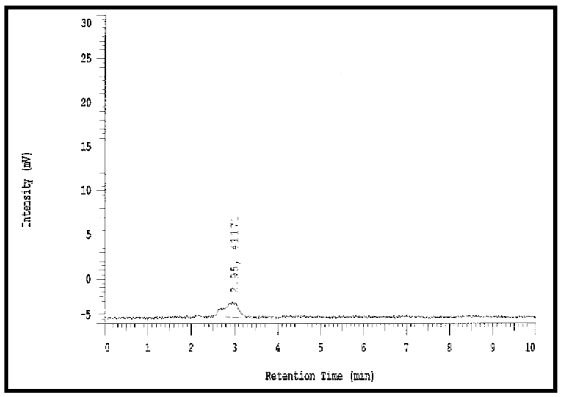
Sample	R_t	AUC	Approximate β-carotene concentration (µg ml^{-1})
Standard (100 µg ml^{-1})	3.01	4706	100.00
M1	3.02	48550	25.79
M2	2.95	41171	21.87



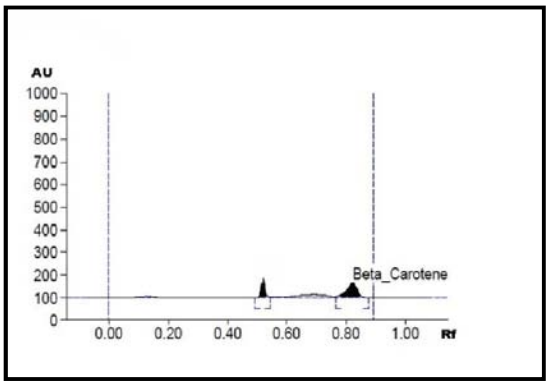
a.HPLC profile of carotenoid pigment extract of yeast isolate M1



b.HPTLC profile of carotenoid pigment extract of the yeast isolate M1



c. HPLC profile of carotenoid pigment extract of yeast isolate M2



d. HPTLC profile of carotenoid pigment extract of the yeast isolate M2

Fig. 1: Chromatograms of the pigment extract of the marine isolates.

References

1. Frengova, G.I. and Beshkova, D.M., Carotenoids from *Rhodotorula and Phaffia:* yeasts of biotechnological importance, *J. of Industrial Microbiol. and Biotech.,* 36(2009) 163-180.
2. Barnett, J.A., Payne, R.W. and Yarrow, D., *Yeasts: Characteristics and Identification* (Cambridge University Press: Cambridge, U.K.) 2000, pp-335-378..
3. Jaswir I., Noviendri, D., Hasrini, R.F. and Octavianti, F., Carotenoids: Sources, medicinal properties and their application in food and nutraceutical industry, *J. of Medicinal Plants Research*, 5, 33 (2011) 7119-7131.
4. Nelis, H.J. and De Leenheer A.P., Microbial sources of carotenoid pigments used in foods and feeds. *Journal of Appl. Bacteriol.,* 70(1991) 181-191.
5. Buzzini, P., An optimization study of carotenoid production by *Rhodotorula glutinis* DBVPG 3853 from substrates containing concentrated rectified grape must as the sole carbohydrate source. *J. of Industrial Microbiol. & Biotechnol*., 24(2000) 41-45.
6. Bhosale, P. and Gadre, R.V. Production of β-carotene by a *Rhodotorula glutinis* mutant in sea water medium. *Bioresource Technol.,* 76(2001) 53-55.

7. Libkind, D., Brizzio, S., Ruffini, A., Gadanho, M., Van Broock, M. and Sampaio, J.P., Molecular characterization of carotenogenic yeasts from aquatic environments in Patagonia Argentina. *Anton van Leeuwenhoek*, 84(2003) 313-322.
8. Sedmark J.J., Weerasinghe D.K. and Jolly S.O., Extraction and quantitation of astaxanthin from *Phaffia rhodozyma. Biotech. and Technol.* 4 (1990) 107–112.
9. An, G.H., Schuman, D.B. and Johnson, E.A. Isolation of *Phaffia rhodozyma* mutants with increased astaxanthin content. *Appl. and Environ. Microbiol.,* 55,1 (1989) 116-124.
10. Perrier, V., Dubreucq, E. and Galzy, P., Fatty acid and carotenoid composition of *Rhodotorula* strains. *Archives of Microbiology,* 164(1995) 173-179.
11. Nagahama, T., Hamamoto, M., Nakase T. and Horikoshi, K., *Rhodotorula benthica* sp. nov. and *Rhodotorula calyptogenae* sp. nov., novel yeast species from animals collected from the deep-sea floor and *Rhodotorula lysiniphila* sp. nov., which is related phylogenetically. *Int. J. of Systematic and Evolutionary Microbiol.*, 53(2003) 897-903.
12. Gadanho, M. and Sampaio, J.P., Occurrence and diversity of yeasts in the Mid-Atlantic Ridge hydrothermal fields near the Azores Archipelago. Springer Science and Business Media, Inc., 50(2005)408–417.
13. Goodwin, T.W. *Distribution of carotenoids*. *Methods in Enzymology*, **(***Academic* Press, UK)1992, pp-213.
14. Garcıa, V., Brizzio, S., Libkind, D., Buzzini, P. and Broock, M., Biodiversityof cold-adapted yeasts from glacial melt Water Rivers in Patagonia, Argentina. *FEMS Microbiol. and Ecology,* 59(2007)31-341.
15. Yurkov, A.M., Vustin, M.M., Tyaglov, B.V., Maksimova, I.A. and Sineokiy, S.P., Pigmented basidiomycetous yeasts are a promising source of carotenoids and ubiquinone $Q_{10.}$ *Microbiol.*, 77,1 (2008) 1–6.
16. Godinho, A. and Bhosale, S. Carotenes produced by alkaliphilic orange- pigmented *Microbacterium arborescens*- AGSB isolated from coastal sand dunes, *Indian J. of Marine Sci.* 37,3(2008) 307-312.