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Halophilic Bacteria-A Potent Source of Carotenoids with Antioxidant and Anticancer Potentials

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Halophiles are known to be potent sources of commercially exploitable substances. Recent interest in the halophile research has its focus on the medicinal properties of these microorganisms. In the current study, an attempt was made to isolate carotenoids from two strains of halophilic bacteria – *Halobacterium salinarium* and *Haloferox volcanii*. Media optimization was carried out and *H. salinarium* was found to produce the maximum yield of carotenoids among the strains. The isolated carotenoids were tested for their free radical scavenging activity by DPPH assay and the results validated the known antioxidant activity of carotenoids. A further analysis of the cytotoxic properties of the carotenoids against human liver cancer cell lines hinted at their anticancer properties. This work is a contribution to the recent research on alternative natural sources of medicine.

Key words: Halophilic bacteria, Anticancer potentials, Carotenoids.

Carotenoids represent one of the most widespread groups of naturally occurring pigments. These compounds are responsible for the red, yellow, and orange colour pigments. Over 40 different carotenoids can be found in the fruits and dark green vegetables out of which only some can be converted to vitamin A. Those that can be converted are called provitamin A carotenoids[1,2]. Tocopherols, tocotrienols, ascorbic acid and carotenoids react with free radicals, remarkably peroxyl radicals and singlet molecular oxygen and prevent oxidation. Carotenoids, such as beta-carotene, lycopene, and some other oxycarotenoids exert antioxidant functions in lipid phases by reducing the free radicals[3]. In addition to their antioxidant and immune-enhancing activity, carotenoids have shown the capability to stimulate cell to cell communication and demonstrate anticancer property[4,5].

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Although carotenoids are rich in leafy plants, it is also present in various bacterial sources. The European Union committee[6] regards *Phycomyces* and *Mucor circinelloides* as potential source for Beta-carotene and fermentation of *Blakeslea trispora* to yield Beta-carotenoid equivalent to chemically synthesized materials. Halophiles are heterogeneous group of microorganisms that requires NaCl for its survival. Their cellular machinery is adapted to high NaCl concentrations by having charged amino acids on their surfaces, allowing the cell to keep its water molecules around these components. They produce various pigments such as phytoene, beta-carotenoid, lycopene, derivatives of bacterioruberin, and salinixanthin. Most important of these halophiles are *Halobacterium salinarium* and *Haloferox volcanii* species.

Halobacteria are extremely halophilic microorganisms usually found in highly saline lakes such as the Great NaCl Lake, the Dead Sea, and Lake Magadi[7]. Arginine mediates substrate level

phosphorylation and allows the cells to grow anaerobically. During anaerobic conditions, bacteriorhodopsin and light provides pigments to the cells[8].

Haloferox volcanii survives in extreme NaCl concentrations. They are mostly found in NaCl ponds and lakes and exists in the form of dormant or living cells, biopolymers in rocks, NaCl crystals, or as evaporates in desert regions[9] and are distinct from other halophytes in few ways[10]. *H. volcanii* respire as their sole source of ATP, unlike several other halobateriacae, such as *Halobacterium salinarium*. They are incapable of photophosphorylation as they lack the necessary bacterioruberin. In both these microorganisms, the pigment secreted is used to repair their DNA when exposed to harmful ultra violet radiation[11].

This study was undertaken to extract carotenoids from halophilic microorganisms and to analyze its antioxidant and anticancer activities.

MATERIALS AND METHODS

Sample collection and identification

Salt brine samples were collected from Chennai and Tuticorin. The collected samples were inoculated in the halophilic broth with different concentration of NaCl. *Halobacterium salinarium* and *Haloferox volcanii* were isolated from the grown culture broth with 25% NaCl (Merck, India) and 12% NaCl (Merck, India) respectively. The isolated strains were inoculated into different media for carotenoid isolation and analysis.

Media Optimization

Halophilic media was prepared with five different component variations for *Haloferox volcanii* (12% NaCl) (labeled as Ia, Ib, Ic, Id and Ie) and six different media having component variations for *Halobacterium salinarium* (25% NaCl) (labelled as IIa, IIb, IIc, IId, IIe and IIf) as shown in table 1 to 4. The inoculated strains were incubated at 37°C for 7 days. The pigment (colour) intensity was measured at 494nm. The cell density was measured at 600nm. Based upon the O.D readings Fig.s were plotted for different media against absorbance at 494nm and 600nm.

Carotenoid Extraction

Carotenoids were isolated from the different media using standard protocol[12]. After 10 days of incubation of the cultures at 37°C, cells were harvested by centrifuging at 8000g for 10mins. Distilled water was added for cell lysate. Equal volume of methanol was added and stirred gently. This was followed by addition of equal volume of hexane (Merck, India) as the fat soluble carotenoid gets transferred to this phase. This set up was allowed to settle down and the hexane layer was collected separately and allowed to dry. The residue collected from hexane was considered as crude carotenoid. The dry weight of carotenoid was noted from different combinations of media. The results were plotted as amount of carotenoid present in mg/l.

Thin Layer ChromatoFig.y

Carotenoid samples from media Id and IIe were subjected to TLC along with a standard Beta carotenoid (Sigma Aldrich, India). Hexane (Merck, India) and ethyl acetate (Merck, India) were the solvents used with a ratio of 9:1. After separation was completed, individual compounds appeared as spots. Each spot has a retention factor (Rf) which is equal to the distance migrated over the total distance covered by the solvent. The Rf value for each of the sample was calculated using the following formula

$$Rf = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

DPPH assay

Antioxidant scavenging activity was studied using 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Sigma Aldrich, India) free radical[13]. 1mg/ml of the sample was added to methanol and then 100 µl of 0.1 % methanolic DPPH was added. The control was prepared by adding 100µl of 0.1% methanolic DPPH to 2.9ml of methanol (Merck, India). After 30 min of incubation at room temperature, the reduction in the number of free radical was measured by reading the absorbance at 517 nm. 0.16% of Butylated Hydroxy Toluene (BHT) (Sigma Aldrich, India) was used as reference standard. The scavenging activity of the samples corresponded to the intensity of quenching DPPH. All determinations were performed in triplicate. The percentage inhibition of the DPPH radical by the samples was calculated according to the formula[14].

$$\text{Percentage of inhibition} = \frac{(\text{Absorbance of control} - \text{Absorbance of test sample})}{\text{Absorbance of control}} \times 100$$

MTT assay

Human liver carcinoma cell lines HepG2

were purchased from the National Centre for Cell Sciences (NCCS Pune, India).The cells were grown in a 96-well plate in Minimum Essential Medium (HiMedia, India) supplemented with 10% fetal bovine serum (Gibco Laboratories, India) and antibiotics (streptomycin, penicillin-G, kanamycin, amphotericin B) (HiMedia, India). About 1ml cell suspension (10^5 cells/ml) was seeded in each well and incubated at 37° C for 48 hours with 5% Co_2 for the formation of confluent monolayer. The monolayer of cells in the plate was exposed to various concentrations of the carotenoid extract (100µg, 50µg and 25µg) and incubated for 24hrs. The cytotoxicity was measured using MTT assay[15] with MTT (5 mg/ml) (Sigma Aldrich, India). After incubation at 37ºC in a CO_2 incubator for 4h, the medium was discarded and 200µl of DMSO was added to dissolve the formazon crystals. Then the absorbance was read in a microplate reader at 570nm.

Cytotoxicity was calculated by the following formula:

Viability % = (Test OD/Control OD) X 100

Cell toxicity % = 100 – viability%

RESULTS

Carotenoids have received considerable attention due to their industrial applications and, more importantly, their potential beneficial effects on human health. Halophiles comprise a heterogenous group of microorganisms that need NaCl for optimal growth. The pigments produced by these organisms comprise ²-carotenoid, phytoene, lycopene, derivatives of bacterioruberin and salinixanthin[16].

In the current study an attempt was made to extract carotenoids from the halophilic bacterial strains isolated from NaCl brines, and to study their antioxidant and anticancer activities. One of the most important aspects of growing halophilic bacteria to produce maximum amount of carotenoidis the formulation of the appropriate culture medium to be employed in the industrial process.

Media optimization for *Haloferox volcanii* with five different media was performed and halophilic media optimization for *Halobacterium salinarium* with six different media with changes in its components was also performed. After ten days of incubation in the different media, the cultures were measured for their colour intensity and cell viability at 494nm and 600nm respectively (Fig. 1 and 2). *Haloferox volcanii* grown in media (Id) showed high optical density of 1.48 and 1.167 at 494nm and 600nm respectively, while the others ranged from 0.08 to 0.4. On the other hand, *Halobacterium salinarium* grown in media (IIe) showed high optical density of 1.59 and 1.16 at 494nm and 600nm respectively, while the others ranged from 0.3 to 1.0.

Thin Layer ChromatoFig.y is a chromatoFig.ic method to separate the components in a mixture of compounds. In the current study, this technique was used to validate the extraction of carotenoids by comparing the profile of the extract with a standard (Beta carotene). Carotenoids were obtained using hexane extraction. The dried extract was weighed and their maximum yield is shown in table 5 and table 6.

Table 1. Five different Halophilic media used for *Haloferox volcanii*

Components g/100ml	Media Ia	Media Ib	Media Ic
$MgSO_4$	2g	2g	2g
K_2SO_4	0.5g	0.5g	0.5g
$CaCl_2.2H_2O$	0.1g	0.1g	0.1g
Sucrose	-	0.5g	0.5g
Yeast extract	0.5g	0.5g	-
Beef extract	-	-	0.5g
$MgCl_2$	-	-	-
NaCl	12g	12g	12g
Ampicillin	75µg/ml	75µg/ml	75µg/ml

Table 2. Five different Halophilic media used for *Haloferox volcanii*

Components g/100ml	Media Id	Media Ie
$MgSO_4$	2g	2g
K_2SO_4	0.5g	0.5g
$CaCl_2.2H_2O$	0.1g	0.1g
Sucrose	0.5g	0.5g
Yeast extract	-	0.5g
Beef extract	0.5g	-
$MgCl_2$	0.75	0.75
NaCl	12g	12g
Ampicillin	75µg/ml	75µg/ml

Maximum yield of 4g/l was obtained from *Haloferox volcanii* media Id and 3g/l of carotenoid was extracted from *Halobacterium salinarium* media IIe. Therefore, the media that showed maximum yield from both the cultures were considered for Beta carotenoid confirmation using TLC (Fig. 5). The final optimization was selected based upon the yield of the carotenoid pigments.

Carotenoids have also known to be strong antioxidants and their primary sources are the fruits and vegetables consumed by humans[17]. Free radical scavenging property of the isolated carotenoids were analysed by the DPPH assay. Antioxidant activity from *Haloferox volcanii* carotenoid extract (4g/l) was performed with three different concentrations (50µg, 100µg and 150µg) using DPPH assay. Fig. was plotted for the control BHT with carotenoid extracted from *Haloferox.* Percentage of inhibition increased with increase in sample concentration as 32.5%, 55% and 77% respectively (Fig. 3). Results revealed the strong anti-oxidant potentials of the carotenoids. The carotenoid extracted from the *Haloferox* strain showed considerable activity, which increased with increasing concentrations of the pigment, comparable with the standard BHT.

Further, the cytotoxicity of the carotenoids on human liver cancer cell lines, HepG2 was analysed. MTT assay, as described[15], is a technique of analyzing the cytotoxicity of substances, based on the conversion of the yellow MTT reagent into purple insoluble formazon crystals, by the enzymatic action of mitochondrial dehydrogenase in live cells. Cytotoxicity of the carotenoid extract from *Haloferox volcanii* was estimated using MTT assay. Cells were treated with 25µg, 50µg and 100µg of the carotenoid sample and the cytotoxicity was estimated as 27.2%, 39.15% and 53.52% respectively as shown in Fig. 4. The results of the assay on the cancer cell lines showed dose-dependent increase in cytotoxicity of the carotenoids on these cells, suggesting the probable potent anti-cancer property of the extracted carotenoids.

Table 3. Six different Halophilic media prepared for *Halobacterium salinarium*

Components g/100ml	Media Ia	Media Ib	Media Ic
$MgSO_4$	2g	2g	2g
$NaHCO_3$	0.02g	0.02g	0.02g
$CaCl_2.2H_2O$	0.11g	0.11g	0.11g
Glucose		0.5g	
Yeast extract	0.5g	0.5g	-
Beef extract	-	-	0.5g
$MgCl_2$	1.95g	1.95g	1.95g
NaCl	25g	25g	25g
Ampicillin	75µg/ml	75µg/ml	75µg/ml

Table 4. Six different Halophilic media prepared for *Halobacterium salinarium*

Components g/100ml	Media Ia	Media Ib	Media Ic
$MgSO_4$	2g	2g	2g
$NaHCO_3$	0.02g	0.02g	0.02g
$CaCl_2.2H_2O$	0.11g	0.11g	0.11g
Glucose	0.5g	0.5g	0.5g
Yeast extract	-	0.5g	-
Beef extract	0.5	-	0.5
$MgCl_2$	1.95g	2.5g	2.5g
NaCl	25g	25g	25g
Ampicillin	75µg/ml	75µg/ml	75µg/ml

Table 5. Carotenoids extraction using Hexane from *Haloferox volcanii*

Media I	Dry Weight of Carotenoids (G/L)
a	1.2
b	0.9
c	1.4
d	4
e	1.4

Table 6. Carotenoids extraction using Hexane from *Halobacterium salinarium*

Media I	Dry Weight of Carotenoids (G/L)
a	1.6
b	0.2
c	1.8
d	0.6
e	3
f	1

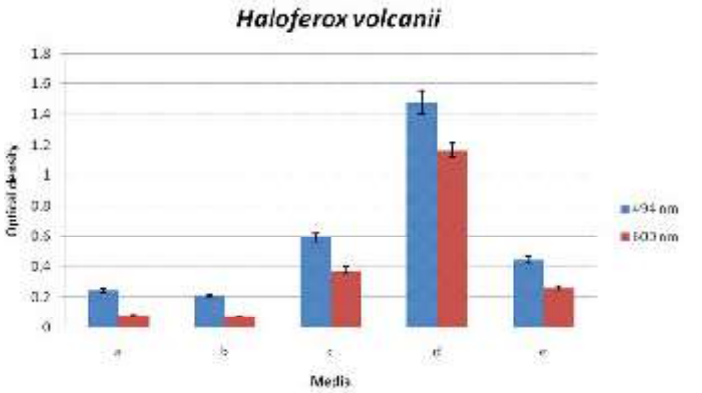

Fig. 1. Media Optimization for Cell Density and Pigmentation for *Haloferox volcanii*

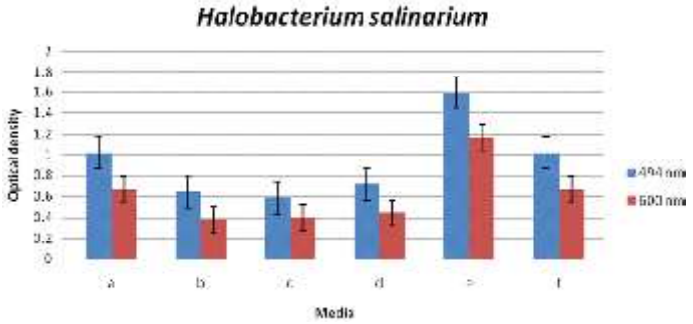

Fig. 2. Media Optimization for Cell Density and Pigmentation for *Halobacterium salinarium*

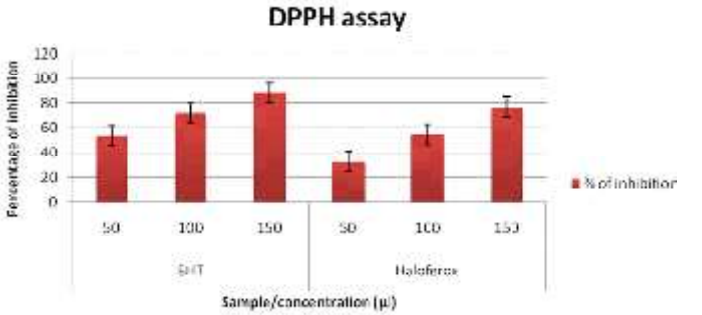

Fig. 3. DPPH assay for Carotenoids extracted from *Haloferox volcanii*

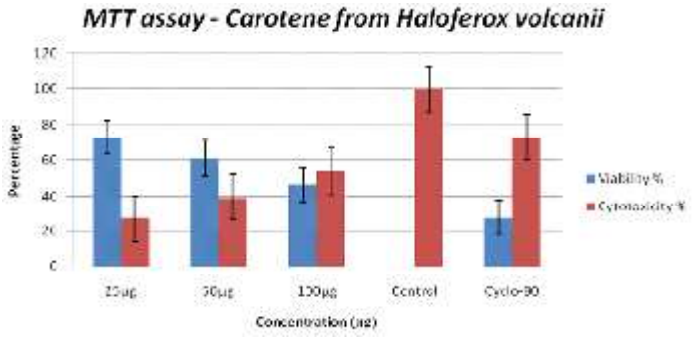

Fig. 4. *In vitro* Cytotoxicity of Carotenoids from *Haloferox volcanii* on HepG2 cell lines

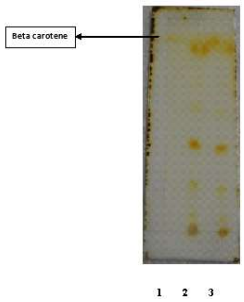

Fig. 5. Thin layer Chromatography: Lane 1: Beta carotenoid – standard, Lane 2: carotenoid – *Haloferox volcanii*, Lane 3: carotenoid – *Halobacterium salinarium*

CONCLUSION

This study comes in the light of the current commercial exploitation of the halophilic strains[18]. Media optimization was performed to generate a better yield of carotenoids from two strains of halophiles *H. salinarium* and *H. volcanii.* The extracted carotenoids displayed potent antioxidant and anticancer activities. The current study is a preliminary attempt at elucidating the medical applications of these halophilic microorganisms.

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REFERENCES

1. American cancer society (AMC) Vitamin-A and Beta- Carotenoid. 2012 Available at: http://www.cancer.org/treatment/treatmentsandsideeffects/complementaryand alternative medicine/herbs vitamins and minerals/vitamin-a-and-beta-carotenoid
2. Delgado.V.F, Jimenez.A.R, Paredes.L.O. Natural pigments: carotenoids, anthocyanins, and betalains characteristics, biosynthesis, processing and stability. *Crit Rev Food Sci Nutr* 2000; **40**(3):173-289. PMID:15150.
3. Sies.H and Stahl.W. Vitamins E and C, beta-carotenoid, and other carotenoids as antioxidants. *Am J ClinNutr* l995;**62**(suppl): l3 15S- 215.
4. Burri BJ . Carotenoids and gene expression. *Nutrition;* 2000: **16**(7-8):577-8. PMID:15140.
5. Bjelakovic. G, NikolovaD,Gluud.L.L, Simonetti. R.G, Gluud.C. Mortality in randomized trials of antioxidant supplements for primary and secondary prevention: systematic review and meta-analysis. *JAMA* 2007; **297**: 842-57.
6. European Commission. Opinion of the Scientific Committee on Food on beta-carotenoid from *Blakesleatrispora* (SCF/CS/ADD/COL) 2000; 158.
7. Wailap V., Sean P.K., Gregory G. M., Brian B.M.P., Hem D.S., Stephen R. *et al*., Genome sequence of *Halobacterium salinarium* species NRC-1. *PNAS*, 2000; 97 u no. 22.
8. Hartmann.R, Sickinger.H.D, and Oesterhelt.D. Anaerobic growth of halobacteria. *Proc Natl Acad Sci. U S A*. 1980; **77**(7): 3821–3825.
9. Chu, H., Sheng, W., Gan, D. C., & Kuznetz, L. Exobiology: The survival ability of halophiles under martian conditions. Retrieved April 20, 2009, from University of California at Berkeley.
10. Mullakhanbhai.M. F., and Larsen.H. *H. volcanii* sp. nov., a Dead Sea halobacterium with a moderate NaCl requirement. *Arch. Microbiol*. 1975;104:207– 214.
11. Barry, P. L. Secrets of a NaCly survivor: A microbe that grows in the Dead Sea is teaching scientists about the art of DNA repair. Retrieved April 11, 2004, from NASA.
12. Asker D, Ohta Y. Production of canthaxanthin by extremely halophilic bacteria. *J BiosciBioeng*. 1999; **88**(6):617-21.
13. Blois, M. S. Antioxidant determinations by the use of a stable free radical. *Nature,* 1958: **181**, 1199-1200.
14. Yen.G.C and Duh. P.D. Scavenging eûect of methanolic extracts of peanut hulls on free-radical and active-oxygen species. *Agric. Food Chem.,* 1994; **42**: 629– 632.
15. Mossman. T. Rapid colorimetric assay for cellular growth and survival - application to proliferation and cytotoxicity assays. *J. Immunol. Methods*, 1983; **65**: 55-63.
16. Maria de L.M, Cristina S.P, María T.G and Encarnació.M. Microbial Carotenoids from Bacteria and Microalgae. *Methods Mol Biol.* 2012; **892**: 207-217
17. Lars.M and Volker.B. Antioxidant Activity of ²-Carotenoid Compounds in *Different in Vitro Assays. Molecules*, 2011; **16**: 1055-1069.
18. Oren A. Diversity of halophilic microorganisms: Environments, phylogeny, physiology, and applications. *J Ind. Microbiol. Biotechnol.,* 2002; **28**: 56-63.