EXTRACTION OF BETA CAROTENE FROM HALO TOLERANT MICROALGAE

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Abstract: Microalga are currently cultivated commercially for human nutritional products around the world in small to medium scale production systems, with total commercial algal biomass production estimated at about 10,000 tons per year. Microalgae produce vast array of natural products including proteins, enzymes, bioactive compounds and carotenoids. Three genera: *Chlorella*, *Spirulina*, and *Dunaliella* represent 85% of the worldwide production. Pigments and metabolites produced by microalgae are highly common in nature and has high industrial potential. One of the metabolite produced by microalgae is Beta carotene. Carotenoids are known to be crucial for normal vision and have been associated with reducing the risk of several degenerative diseases including cancer. There are more than 400 carotenoids found in nature and beta carotene is perhaps the most important one. In the present investigation, experiment was conducted to study and evaluate the effect of environmental stress for accumulation of Beta-carotene. Main stress factor which was considered for analysis included high salinity as for more carotene production high saline conditions are required. The effect of inorganic compounds i.e. NH_4Cl, $NaPO_4$, NaCl on growth and carotene accumulation by halotolerant microalgae was studied. Beta carotene was extracted using solvent extraction method. Maximum Beta carotene accumulation of 2.974mg/L was observed in Bold's Basal medium supplied with 5% NaCl after 45days of incubation. When grown under different concentration of NaCl (1%, 3%, 9%) and NH_3Cl, $NaPO_4$ in BB medium, decreased carotene production was observed. The presence of Beta-Carotene was confirmed using HPLC.

Keywords: Halotolerant microalgae, Beta-Carotene , Salinity, Solvent extraction

I. INTRODUCTION

Microalgae are among the fastest growing autotrophs on the earth, which utilize commonly available material for growth (Pisal and Lele-2005). The main algae currently cultivated photosynthetically (e.g. with light energy) for various nutritional products are *Spirulina*, *Chlorella*, *Dunaliella* and *Haematococcus* (Campo et al., 2007; Benemann, 2008). *Dunaliella* is a unicellular, biflagellated, naked green alga (Chlorophyceae, Dunaliellales), and the type species of this genus, *Dunaliella salina* (Dunal) is often found in natural hypersaline waters where it colours the brines red (Teodoresco, 1905). This algal species was first recognised as containing high intracellular concentrations of β-carotene.

Beta-carotene belongs to the group of pigments called carotenoids. Carotenoids are a class of natural fat-soluble pig1ments found principally in plants, algae, and photosynthetic bacteria, where they play a critical role in the photosynthetic process. In human beings, carotenoids can serve several important functions. The most widely studied and well-understood nutritional role for carotenoids is their Provitamin-A activity. Beta-carotene is converted to Vitamin A in the body. Good sources of beta-carotene include dark green and orange-yellow vegetables, such as carrots, sweet potatoes, squash, spinach, broccoli, romaine lettuce, apricots, and green peppers. *Dunaliella salina*, a marine microalga is a rich source of beta-carotene, alpha-carotene, cryptoxanthin, zeaxanthin, lutein and lycopene. cells of *D.salina* are green under optimal growth conditions, but they are orange when exposed to environmental stresses such as high salinity owing to the accumulation of β-carotene (Polle, Jürgen,2008). The average concentration of carotenoids in most algae is only 0.1–2%, but *Dunaliella* when grown under the right conditions of high salinity and light intensity will produce up to 14% beta-carotene.

Administration of algal Beta-carotene to mouse and humans has been shown that it has protective effect against atherosclerosis. For instance, administration of *Dunaliella* rich in Beta-carotene inhibits low density

lipoprotein (LDL) oxidation and influences plasma triglycerides, cholesterol and high density lipoprotein (HDL) levels in mouse and humans (Wan Loy chu, 2012). The present study was aimed to isolate Halotolerant algae from saline sample and optimization of its growth conditions to achieve maximum production of β-carotene.

II. MATERIALS AND METHODS

Collection of sample

The saline water sample was collected from different salt pans located at Bhayander, Naigaon in clean glass bottle.

Enrichment of halo tolerant algae

Collected saline sample was inoculated in various medias like Bold's Basal medium, Dewarln's medium (Dhanam and Dhandayuthapani 2013) and minimal medium (Pisal and Lele 2005) supplemented with 1% NaCl for enrichment of halo tolerant algae. Growth of algae was determined by observing green colour in Medias. Further confirmation was done by observing algal cells microscopically.

Optimization of medium for carotene production

Enriched algae were cultivated in Bold's Basal medium supplemented with NaCl for 15 days and such pre grown cells of density $1x10^6$cells/ml were inoculated under stress conditions for carotene production optimization. High salinity stress was maintained by addition of NaCl ranging from 1%-9% (0.17M-1.53M) into BB medium. Another stress condition was created by substituting the media components i.e. $NaNO_3$ and KH_2PO4 with NH_4Cl and Na_2PO4. The concentration of NH_4Cl used was 0.1mM, 1mM, 2mM and of Na_2PO4 was 0.1mM, 0.5mM, 2mM. The samples were withdrawn after every 48 hrs and assayed for biomass (cell number) and content of Beta carotene.

Extraction and estimation of Beta carotene

Extraction of beta carotene was carried out by solvent extraction method (Dhanam and Dhandayuthapani 2013). After determining the A453 value, the Beta carotene concentration was estimated using following formula (Tawfiq et.al 2010):

$C=A453x3.86xVe/Vt$

Where c=beta carotene concentration

Ve=volume of extract

Vt=Total volume

Correlation study of cell growth and carotene production

Study of growth phase at which optimum carotene production occurs was determined by inoculating $1x10^6$cells/ml in sterile BB medium supplemented with 1%, 3%, 5% and 9% NaCl. Inoculated flasks were incubated at RT for 45 days. At regular time interval cell suspension was removed for cell count using Hemocytometer as well as extraction and quantification of carotene was done. The graph was plotted of carotene concentration (mg/L) v/s Day interval to determine the growth phase at which optimum carotene production occurs.

Qualitative analysis of Beta carotene by HPLC

The presence of Beta-carotene in the extracted sample was confirmed by performing HPLC (High Performance Liquid Chromatography) technique. HPLC of Shimadzu LC 20 AD make, reverse phase column of Phenomex Luna 5u C18 with PDA detector was used in this analysis.

III. RESULTS

Enrichment of halo tolerant algae

The collected saline water sample was inoculated in different culture media such as Dewarln's medium, Minimal medium & Bolds Basal medium (BB medium) supplemented with 1% NaCl. The growth was not observed in Dewarln's medium and Minimal medium after 2 months of incubation but in BB medium

growth was observed (Figure 2) in 15 days, hence BB medium was used for further study. In the microscopic analysis oval structured and motile cells were seen (Figure 1). These characteristics were similar to the *Chlamydomonas* and *Dunaliella*, etc.

Optimization of medium for carotene production

BB medium was supplemented with 1%, 3%, 5% and 9% NaCl and also NH_4Cl along with Na_2PO_4 in concentration of 0.1mM to 2mM to optimise production of carotene by algae. Cell count was estimated after 7 days of inoculation and maximum cell yield was observed in 1% to 9% NaCl supplemented BB medium (Figure 3). The growth was also observed in BB media supplemented with 2mM NH_4Cl and 1mM Na_2PO_4 but compared to salt supplemented medium it was found to be less.

Correlation study of cell growth and carotene production

Sterile BB medium supplemented with 1%, 3%, 5% and 9% NaCl was inoculated with algae and incubated for 45 days. Cell count and carotene extraction with solvent extraction method was carried out after every 48 hours from BB medium supplemented with 1% to 9% NaCl. It was observed that carotene production was also increasing along with cell yield (Graph 1). Maximum beta carotene accumulation i.e, 29.74mg/L (Graph 2) was observed in BB medium supplemented with 5% NaCl after 45 days of incubation. Result shows that on 45th day of incubation algae enters in the stationary phase and it is the most suitable phase of algae to obtain maximum carotene production (Table 1).

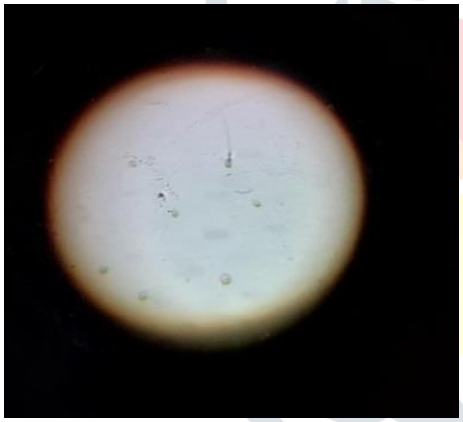
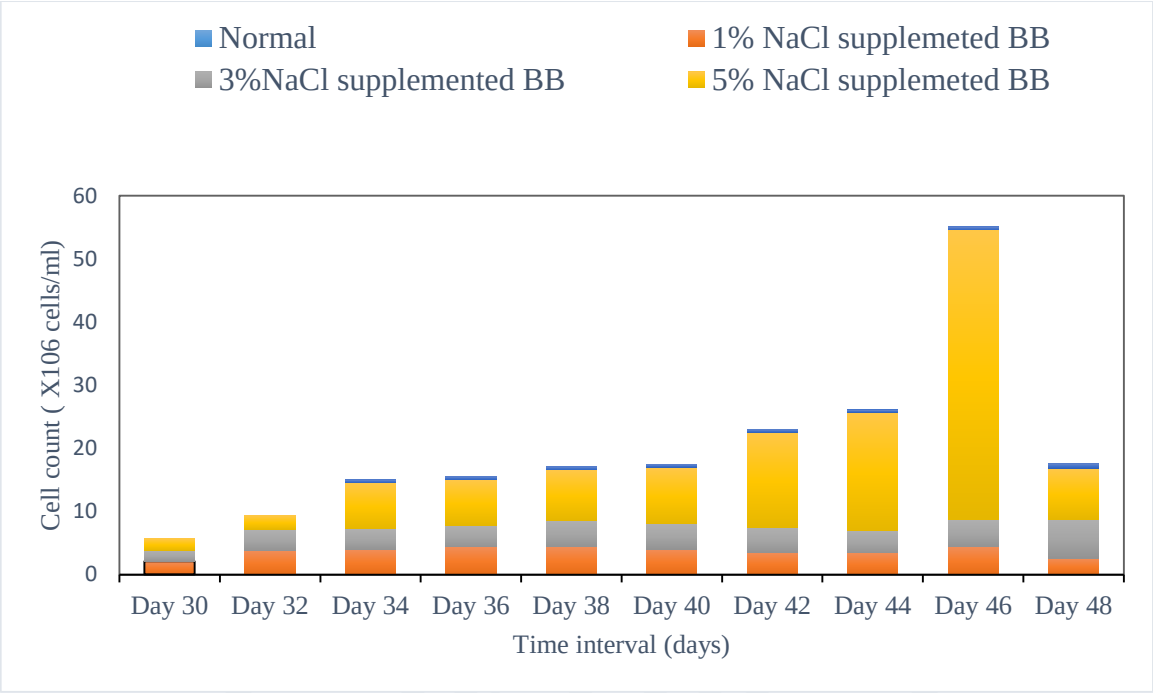


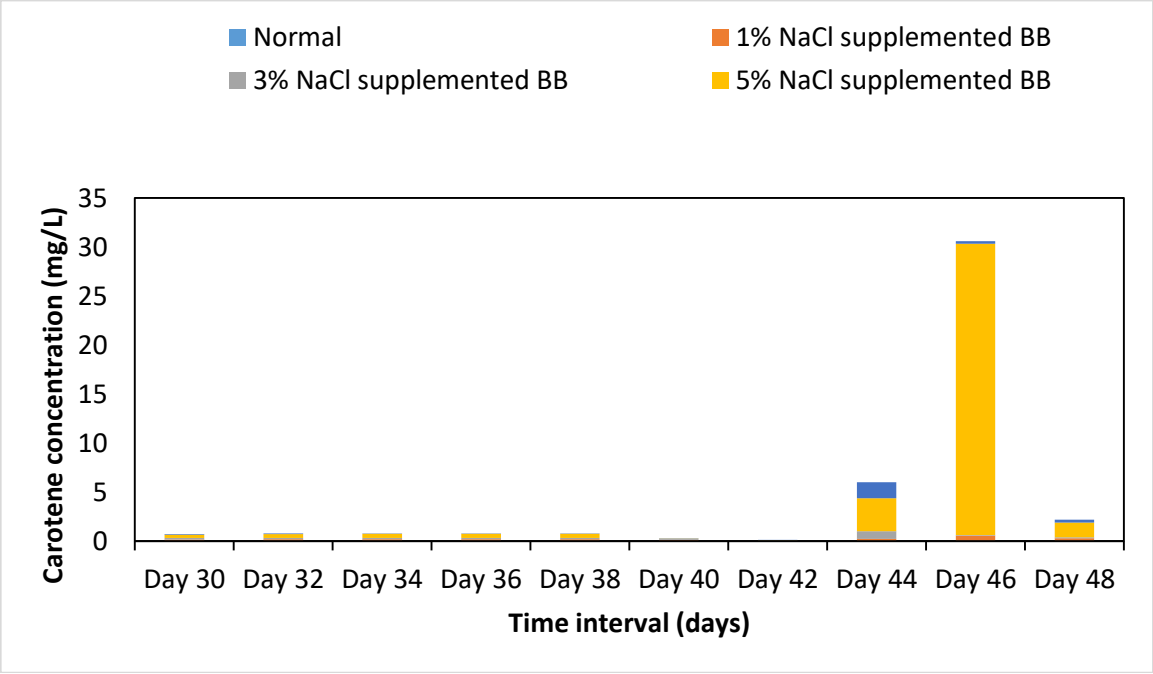
Figure 1. Microscopic characterisation of algae

Figure 2. Growth of algae in BB medium supplemented with 1% NaCl

Figure 3. Growth of algae in BB medium supplemented with various concentration of NaCl



Graph 1. Algal cell count in various concentration of NaCl supplemented BB medium



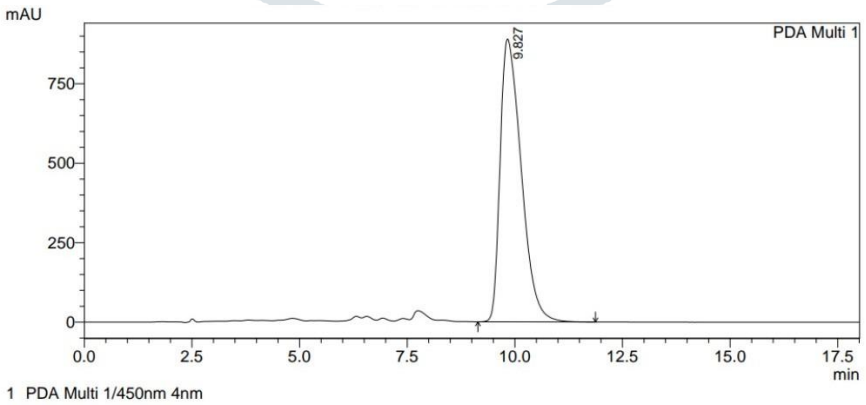
Graph 2. Effect of various NaCl concentrations on carotene production by algae

Table 1. Correlation study of cell yield and carotene production by Halotolerant microalgae

Days	Cell count (per ml)					Carotene concentration (mg/L)				
	Normal	1%	3%	5%	9%	Normal	1%	3%	5%	9%
Day 30	0.02492x 10^6	2.065x 10^6	1.755x 10^6	1.917x 10^6	0.0025x 10^6	0.00452	0.139	0.16	0.35	0.039
Day 32	0.03040x 10^6	3.760x 10^6	3.367x 10^6	2.225x 10^6	0.003x 10^6	0.005326	0.169	0.146	0.394	0.046
Day 34	0.0471x 10^6	3.86x 10^6	3.3x10^6	7.37x10^6	0.44x10^6	0.0054	0.17	0.15	0.4	0.046
Day 36	0.047x10^6	4.35x 10^6	3.39x10^6	7.25x10^6	0.517x 10^6	0.0054	0.17	0.15	0.4	0.047
Day 38	0.0535x 10^6	4.35x 10^6	4.2x10^6	8x10^6	0.5x10^6	0.0054	0.17	0.151	0.41	0.047
Day 40	0.02880x 10^6	4x10^6	4x10^6	9x10^6	0.420x 10^6	0.005	0.12	0.1	0.038	0.03
Day 42	0.024x10^6	3.5x 10^6	3.9x10^6	15.1x10^6	0.48x10^6	0.000514	0.03	0.0069	0.039	0.01
Day 44	0.027x10^6	3.38x 10^6	3.54x10^6	18.8x10^6	0.37x10^6	0.000501	0.2	0.787	3.38	1.62
Day 46	0.0213x 10^6	4.36x 10^6	4.39x10^6	45.8x10^6	0.52x10^6	0.0088	0.52	0.0733	29.74	0.270
Day 48	0.0188x 10^6	2.58x 10^6	6.19x10^6	8.10x10^6	0.7x10^6	0.0041	0.27	0.096	1.6	0.29

Qualitative analysis of Beta carotene by HPLC

Confirmation of the presence of Beta carotene in the extracted sample was done by HPLC. Retention time of extracted sample was found to be nearly same as that of standard Beta carotene (Graph 3 and 4).

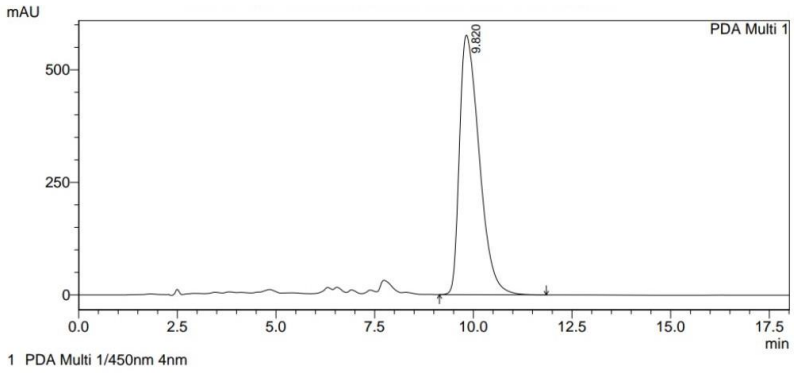


PeakTable

PDA Ch1 450nm 4nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	9.827	30314627	889993	100.000	100.000
Total		30314627	889993	100.000	100.000

Graph 3. HPLC chromatogram of standard Beta carotene



PeakTable

PDA Ch1 450nm 4nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	9.820	20050848	576586	100.000	100.000
Total		20050848	576586	100.000	100.000

Graph 4. HPLC chromatogram of extracted sample

IV. CONCLUSION

Use of synthetic pigments is a boon to society as it can cause many chronic diseases. Thus, natural pigments which are obtained from natural resources should be preferred. Microalga are among the fastest growing autotrophs on earth which utilize commonly available nutrients. They produce vast array of natural products including proteins, enzymes and carotenoids. Beta carotene is a member of family of molecules known as carotenoids which has various properties like it is a good antioxidant agent. Due to various benefits offered by beta carotene, it's extraction from isolated microalgae is carried out in current research. The saline water sample was collected from different salt pans and enrichment was carried out in Bold's Basal medium, Dewarln's medium, Minimal medium. Halotolerant microalgae was cultivated at laboratory scale to increase the beta carotene content by using different stress conditions. In present study, carotene production was optimised by exposing algae to saline stress and nutrient content stress. The result of experiments conducted related to stress factors showed that the BB medium supplemented with NaCl concentration ranging from 1%-9%(0.17M-1.53M) had maximum growth as well as maximum carotene production. The BB medium containing 5% NaCl showed maximum growth, while the results obtained by Pisal and Lele (2005), maximum biomass was obtained in 2M NaCl supplemented Minimal medium. When extraction of beta carotene was done using solvent extraction method, there was increase in beta carotene with the increase in cell biomass. The results showed that BB medium supplemented with different concentration showed good beta carotene production as compared to BB medium with 2mM NH_4Cl and 1mM Na_2PO_4. Maximum beta carotene was obtained from BB containing 5% NaCl i.e 24.74 mg/L after 45 days of incubation. HPLC was performed for confirming the presence of Beta carotene in the extracted sample.The retention time for standard beta carotene and sample was found to be almost equal i.e 9.827 min and 9.820 min respectively. From all the results, it can be concluded that BB medium supplemented with 5% NaCl is the efficient medium for growth as well as production of beta carotene by isolated halotolerant microalgae.

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