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Research Article

EVALUATION OF CHEMICAL INDUCED CAROTENOGENESIS IN *TETRADESMUS SP*. NTAI04

Rajendiran Krishnan[a]., Jayabalan Jayaprakash[b]., Chandrasekaran Swaminathan[c]., D.John Milton[d] and A. Ilavarasi*

[a,b,c,d]Department of Microbiology, St. Joseph's College of Arts and Science (Autonomous), Cuddalore 607 001, Tamil Nadu, India
*Department of Microbiology, Indira Gandhi College of Arts and Science, Indira Nagar, Puducherry

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ABSTRACT

Carotenoids are tetraterpenoid group of pigments which represent a large group of red, orange, and yellow natural metabolites mainly involved in regulation of many metabolic processes. Microalgae are known to have carotenoid pigment along with chlorophyll pigments. Carotenoid induction in microalgae using physical methods were extensively studied, hence, the current study describes the chemical induced carotenogenesis viz., salinity stress, pH variation, sodium acetate and different solvents. Algal cultures (*Tetradesmus* sp. NTAI04, Tetradesmus sp. NTAI05, *Tetraselmis* sp. NTAI08, *Chroococcus* sp. NTAI11, *Phormidium* sp. NTAI12, *Oscillatoria* sp. NTAI13) were screened on the basis of biomass accumulation and total carotenoid content. In this, *Tetradesmus* sp. NTAI04, a green unicellular microalgae showed highest yield of carotenoids than the other algal species. It was mass cultivated and grown under chemical mediated stress such as salinity, pH, sodium acetate and solvents were used to induce the process of carotenogenesis. In salinity stress, it showed higher rate of carotenoids when compared to other stress such as pH and sodium acetate.

INTRODUCTION

In recent years, increasing evidences for toxicological effects of synthetic colors have prompted regulatory agencies, world over to drastically prune the list of permitted synthetic food colors. As synthetic colors are creating several health problems, people around worldwide are trusted to use natural coloring agents. Carotenoids are responsible for the red, yellow, and orange colors, widely distributed in fruits, flowers, roots, seaweeds, invertebrates, fishes, crustacean shells, skin, birds, bacteria, fungi, and yeasts (Johnson and Schroeder, 1995; Negro and Garrido-Fernandez, 2000; Gudin, 2003). Apart from these organisms, microalgae are considered to be the amazing source of carotenoids. Carotenoids act as a photosynthesis aid and also utilized for the photoprotection of their hosts.

Actually, a large number of green microalgae were known to accumulate secondary carotenoid (SC) under stress condition: *Euglena* sp. (Tischer, 1936), *Trachelomonas volvocina* (Green, 1963), *Ankistrodesmus* (Burzyk, 1987), *Botryococcus braunii* (Grung *et al*., 1989), *Eremosphera viridis* (Vechtel *et al*., 1992), *Chlamydomonas nivalis* (Bidigare *et al*., 1993), *Chlorococcum* sp. (Brown *et al*., 1967; Zhang and Lee, 2001), *Coelatrella striolata* var. *multistriata* (Hanagata *et al.*, 1996), *Chlorella* sp. (Orosa *et al*., 2000), *Protosiphon botryoides* (Orosa *et al.*, 2000), *Neochloris wimmeri*, *Scotiellopsis oocystiformis* (Orosa *et al*., 2000), *Tetracystis intermedium* (Del Campo *et al*., 2000), *Dunaliella* sp. (Ben-Amotz *et al*., 1989; Coesel *et al*., 2008), *H. pluvialis* (Park *et al*., 2009), *Chloromonas nivalis* (Remias *et al*. 2009) and *Scenedesmus* sp. (Hanagata *et al*., 1996; Orosa *et al.*, 2000, Pirastru *et al*., 2011). Among these taxa, *D. salina*, *H. pluvialis* and *C. zofingiensis* are the only extensively studied taxa (BenAmotz *et al*., 1989) because they are used in commercial production of β-carotene and/or astaxanthin in medium and large scale cultures (Lorenz and Cysewski, 2000; Olaizola, 2000; Olaizola and Huntley, 2003).

Interestingly, increased accumulation of carotenoids in algae has been reported as an oxidative (stress) response and therefore it can be up-regulated under different culture conditions. For example, stress-induced astaxanthin accumulation has been observed in green alga *Haematococcus pluvialis* in highlight, high-salt, high-temperature, and nutrient-deficiency environments, (Steinbrenner and Linden, 2001) as well as under elevated temperature and irradiance conditions in *Scenedesmus obliquus* (Qin *et al*.,2008). However, the large-scale production of astaxanthin by *Haematococcus* sp. is hampered by several disadvantageous characteristics of this alga such as a relatively

*✉ ***Corresponding author:*Dr. A.Ilavarasi**
Department of Microbiology, Indira Gandhi College of Arts and Science, Indira Nagar, Puducherry

slow growth rate, low growth temperature and low culture density.

Microalgae are considered to be one of the tremendous sources for carotenoid production and it has the ability to fulfill the world wide requirement. The simple modification in their culture conditions viz., high light intensity, high salinity and nutrient deprivation may enhance the production of carotenoids in microalgae. The photo induced carotenoid production is highly investigated in microalgal carotenogenesis. As a result, the present work mainly concentrated on chemical induced carotenogenesis. The onset of carotenogenesis in *Tetradesmus* sp. NTAI04 under various chemical stresses such as salinity stress, pH variation, sodium acetate and different solvents were studied. This is the first time of using solvent as a microalgal carotenogenic factor in *Tetradesmus* sp. NTAI04. The present study deals with the variation of carotenoid content as a function of time. Moreover, changes in the stress enzymes were also studied.

MATERIALS AND METHODS

Algae and culture conditions

A set of algal cultures such *Tetradesmus* sp. NTAI04, *Tetradesmus* sp. NTAI05, *Tetraselmis* sp. NTAI08, *Chroococcus* sp. NTAI11, *Phormidium* sp. NTAI12, *Oscillatoria* sp. NTAI13 used in the present study were obtained from Microalgal Repository, Division of Microbial Diversity and Bioenergy Laboratory, Department of Microbiology, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India. Algal cultures were maintained on Chu 10 broth for fresh water microalgae and Conway medium for marine microalgae at 26±2℃ under 100.5µmol- $1m^2s^{-1}$ light intensity with 16:8 hours, light: dark period.

Screening for efficient carotenoid producer

The algal cultures were screened for carotenoid pigment based on two aspects: Biomass accumulation (chlorophyll measurement), Carotenoid productivity (Total carotenoid analysis)**.** The cells were harvested at a regular interval of 3 days and the amount of pigment was estimated based on the equations proposed by Lichtenthaler (Lichtenthaler *et al*., 1987).

Inoculum

The selected microalga, *Tetradesmus* sp. NTAI04 was mass cultivated in plastic tank (25 litres capacity) maintained at 26±2℃ under 100.5µmol- $1m^2s^{-1}$ light intensity with 16:8 hours, light: dark period with aeration. The algae were harvested at their stationary phase and used for the chemical mediated carotenogenesis study.

Induction of chemical mediated carotenogenesis

In this experiment, 4 types of chemicals were introduced to the culture medium in varying concentrations to induce the carotenogenesis. For that, salinity (10ppt, 20ppt, 30ppt, 40ppt, 50ppt), pH (5,6,8,9), sodium acetate (30mM, 60mM, 90mm, 120mM, 150mM) and solvents (Hexane, Chloroform, Methanol, Ethyl acetate) stresses were used to induce the carotenoid production. *Tetradesmus* sp. NTAI04 biomass (5 ml) was harvested at a regular interval of 3 days in each and every stress individually. The pigments were extracted and were estimated according to Lichtenthaler.

Stress enzymes

The stress enzymes like superoxide dismutase (Marklund and Marklund, 1974) and catalase activity (Aebi *et al*., 1984) were measured.

18S rDNA characterization

Genomic DNA of *Tetradesmus* sp. NTAI04 was extracted according to the methods described by Moncalvo *et al*., (1995) with some modifications. The extracted genomic DNA of *Tetradesmus* sp. NTAI04 was amplified using the following primers: 5' -AGCTCGTAGTTGGATTTCTG-3' (18F1) and 5″-AGTCAAATTAAGCCGCAGGC-3″ (18R1). Amplified products were sequenced and submitted to GenBank via BankIt submission tool. The similarity sequences were analyzed using BLAST.

RESULTS AND DISCUSSION

Carotenoids are tetraterpenoid pigments which have been well known for its antioxidant (Edge *et al*., 1997), anticancer (Hennekens, 1997) properties and as immune response stimulants (Emodi, 1978). Because of these reasons commercial production of the carotenoids using microalgae has been attempted worldwide. Microalgae were highly efficient in carotenoid production since, they are easily manipulated in the processing schemes. It may be induced to enhance the production of carotenoid for the realistic applications.

Efficient carotenoid producing microalgae was screened based on biomass accumulation and total carotenoid content. At the stationary phase, *Tetradesmus* sp. NTAI04 showed highest carotenoid production than the other algae used in the study. Total carotenoid content from the obtained algae at 21st day was given in Table.1.

Table.1. Total carotenoid content from the obtained algae

Algae	Total carotenoid (µg/ml)
Tetradesmus sp. NTAI04	3.626
Tetradesmus sp. NTAI05	1.910
Tetraselmis sp. NTAI08	1.044
Chroococcus sp. NTAI11	0.552
Phormidium sp. NTAI12	0.522
Oscillatoria sp. NTAI13	0.893

Effect of salinity

Although *Tetradesmus* sp. NTAI04 is a fresh water microalga, it has been well adapted to wide range of saline conditions (10, 20, 30, 40 and 50 ppm). At the salinity of 10 and 20 ppm, the microalga *Tetradesmus* sp. NTAI04 showed luxurious growth which has been known from its chlorophyll content. On 18th day it reached stationary phase and remained constant till 21st day similar to control. At the stationary growth phase in 10 ppm, the amount of chlorophyll and carotenoid was found to be 2.714 µg/ml and 1.794 µg/ml respectively, whereas in 20 ppm it was estimated to be 2.586 µg/5ml and 2.168 µg/5ml. Subsequently, at 21st day a slight decrease in their chlorophyll content revealed that the culture reached decline phase. At 21st day, in 30 and 40 ppm, the amount of chlorophyll was greater than carotenoid (1.375 µg/ml ≥ 0.915 µg/ml) and (0.642 µg/ml ≥ 1.171 µg/ml)

respectively. Hart *et al*., 1991 showed the reduced growth at higher salinities due to decrease in photosynthetic rate. The present study agrees with earlier reports on their growth rate. Previous studies showed that the decreased yield of biomass as were probably due to non adaptability of the organism to higher salinity (Vazquez-Duhalt and Arredondo-Vega, 1991 and Ben-Amotz *et al*., 1985). Garcia *et al*., 2007 suggested that such different growth is due to the fact that these algae do not adapt to a specific saline condition but can tolerate a wide range of salinities. In the case of 50ppt, a gradual decrease in chlorophyll content was observed from 3rd day till 21st day (0.553µg/ml) and at the same time significant increase in the total carotenoid content (1.345 µg/ml) was also noted. The total carotenoid/chlorophyll ratio was higher in 50ppt than the control and this indicated that higher salinity induces carotenoid production.

Effect of pH

Tetradesmus sp. NTAI04 is a neutrophilic microalga which presents the similar tolerance to grow at acidic and basic pH values. At pH 5, 6, 8, 9 microalga showed chlorophyll content of about 3.1 µg/ml, 3.6 µg/ml, 2.9 µg/ml, 3.4 µg/ml and decreased amount of carotenoid value (3 µg/ml, 2.4 µg/ml, 2.6 µg/ml, and 2.5 µg/ml) as somewhat equal to control. Similar findings on the effect of pH on carotenoid content were studied on *Dunaliella* sp. (Celekli and Donmez, 2006). The effect of varying pH on carotenoid accumulation was studied on *Chlorococcum* sp. and it was reported that between pH values 5-8 the carotenoid accumulation was optimum whereas at pH 4 the rate of carotenoid accumulation was low (Liu and Lee, 2000) The biomass yield registered an increase with wide range of different pH. Decrease in carotenoid content was recorded with increase in chlorophyll content. It was denoted that both acidic and basic environment provide good biomass production and in this isolate pH between 5-9 is not a limiting factor for the carotenoid production.

Effect of solvents

Effect of solvents on carotenoid production in *Tetradesmus* sp., different solvents (hexane, chloroform, methanol and ethyl acetate) in equal proportions (10%) was used. The photosynthetic pigments were notably influenced by solvents **.**The cells were unable to tolerate the harsh environmental conditions exposed by chloroform, ethyl acetate and hexane (Table-2); thereby gradually it started to decline its growth from 3rd day onwards. Chloroform and ethyl acetate totally arrest the photosynthetic pigments and its metabolic activity. It also leads to cell wall lysis (data not shown) reduction in their chlorophyll content was due. In addition to that, the stress condition mediated by those solvents does not support carotenoid accumulation too. Hexane showed increased carotenoid productions in the earlier days. Rapidly, it reached the decline phase. In methanol, the chlorophyll value was decreased constantly (0.2 µg/ml) and the carotenoid values (0.9 µg/ml) were increased continuously. The addition of solvents such as ethanol, methanol, isopropanol, and ethylene glycol to the culture medium has been reported to stimulate microbial carotenogenesis (Daraseliya and Daushvili, 1982). The supplementation of ethanol (2%, v/v) was reported to stimulate β-carotene and torulene formation in *Rhodotorula glutinis* but torularhodin formation was suppressed (Margalith and Meydav, 1968). The total carotenoid/chlorophyll ratio was found to be higher in methanol added cultures. Similar to the reports of Daraseliya and Daushvili, 1982, the carotenoid production was positively influenced by methanol. The increase in the amount of carotenoid in methanol added cultures proved that methanol was successfully acted as carotenoid inducer in *Tetradesmus* sp. NTAI04. Moreover, this is the first report of using methanol as carotenoid inducer in microalgae.

Table 2 Chlorophyll to carotenoid ratio for each chemical stress

Solvents (10%)		**Sodium acetate (mM)**		**Salinity (ppt)**		**pH**	
Hexane	-	30	0.52	10	0.46	5	0.59
Methanol	3.578	60	0.543	20	0.54	6	0.45
Chloroform	-	90	0.548	30	0.51	8	0.55
Ethyl acetate	-	120	0.500	40	1.19	9	0.44
		150	0.513	50	1.58		

Effect of Sodium acetate

Exposure of *Tetradesmus* sp. NTAI04 to different concentrations (30mM, 60mM, 90mM, 120mM and 150 mM) of sodium acetate for 21 days affected the photosynthetic activity of microalgae. In control, *Tetradesmus* sp. NTAI04 reached early stationary phase at 18th day and it remained constant till 21st day with maximum accumulation of biomass i.e. evident from their increased chlorophyll values. On the other hand, in sodium acetate added cultures, the chlorophyll content was found to decrease with increase in sodium acetate concentration. Similarly, the decrease in total chlorophyll content was demonstrated in sodium acetate exposed *Scenedesmus* sp. cultures and they also stated that the decrease in their chlorophyll content may be due to the alteration in the functional properties of photosynthetic system (Pirastru *et al.,* 2012). Moreover, it may also indicate important structural reorganization of the photosynthetic apparatus (Kim *et al*., 1993). Earlier report stated that exposure of *Haematococcus* sp. to 10mM sodium acetate showed increase in their carotenoid content (Hagen *et al*., 2001) whereas in *Scenedesmus* sp. the addition of 120mM sodium acetate showed maximum carotenoid accumulation (Pirastru *et al*., 2012). In contrast, in the present study, addition of 150mM sodium acetate did not show much difference in the carotenoid accumulation. So, it was clear from previous reports that the induction of carotenoid accumulation may vary to organism to organism based on the concentration of sodium acetate. It was stated that the high concentrations of acetate are needed to induce carotenoid biosynthesis could be that acetate can be used in some microalgae as a carbon source for the formation of isoprenoids as precursor of carotenoids (Ladygin, 2000). Furthermore, the carotenoid/chlorophyll ratio was lower in this stress when compared to the other chemical stress.

Quantitative analysis of Enzyme activity

Superoxide dismutase

The SOD activities remained slightly higher than the control whereas, salinity and combined stress (methanol, salinity) showed trivial increase in the enzyme activity and the value

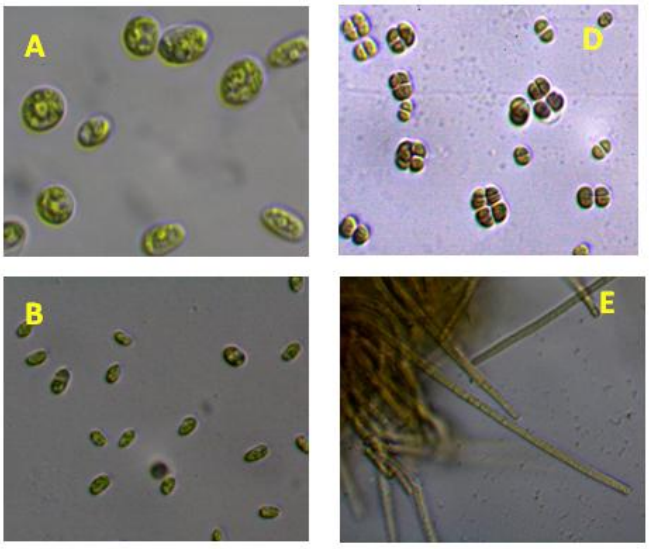
reached (0.0014 U/mg cells and 0.0019 U/mg cells) when compared with control (0.001 U/mg cells). activity (0.002 U/mg cells) than the control. A main protective role against free radicals is recognized to SOD in catalyzing and dismutation of superoxide anions to O_2 and H_2O_2 (Sreenivasulu *et al*., 2000). This response corresponds to the primary enzymatic defense response that plays a critical role upon onset of stress and during the transition of green vegetative cells to cyst formation (Wang *et al*., 2003). The increase of SOD in our experiments showed higher amount of enzyme present under the stress conditions, suggesting an oxidative stress was established in the cells.

Catalase

The activity of CAT was significantly increased under stress condition when compared with the control. The presence of enzyme was measured in control (0.0002 µmol/mg cells) was determined, whereas in salinity and methanol showed similar amount (0.0004 µmol/mg cells) of enzyme present in the cells and it was higher than the control. The activity of CAT was remarkably increased in combined (methanol, salinity) and age-old cells were (0.0005 µmol/mg cells and 0.0006 µmol/mg cells) quantified. Free radicals induced by chemical mediated stress had a response of increased amount of CAT. Then the scavenging and detoxification of H_2O_2 produced by scavenging enzymes such as CAT, which therefore increased in a stressed cell. The increase of CAT in our experiments showed higher amount of enzyme present under the stress conditions, suggesting an oxidative stress was established in the cells.

Molecular characterization

PCR amplification followed by DNA sequencing allowed the determination of approximately 1000 bp of the 18SrDNA gene. *Tetradesmus* sp. NTAI04 showed maximum 18SrDNA sequence similarity to the genus *Tetradesmus* sp. belonging to the family Scenedesmaceae. The sequence was submitted to available public database, NCBI with accession number JQ796862. The microphotograph of the different algal species was shown in the fig.-1



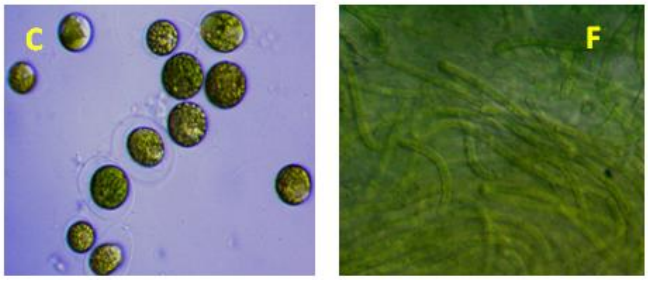


Fig. 4 Microphotograph for obtained algal species *Tetradesmus* sp. NTAI04, B. *Tetradesmus* sp. NTAI05,C. *Tetraselmis* sp. NTAI08, D. *Chroococcus* sp. NTAI11, E. *Phormidius*sp. NTAI12, F. *Oscillatoria* sp. NTAI13)

CONCLUSION

Microalgae are considered to be one of the potential microorganisms applied for the commercial production of carotenoid. The present work concluded that the chemical mediated carotenogenesis is not alone sufficient for increased amount of carotenoid content. When the chemical and physical carotenogenic factors given in combination, it may induce higher carotenoid content in *Tetradesmus* sp. NTAI04. Moreover, the present work is the first report to induce the carotenoid production by solvents as the carotenogenic factor.

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