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Enhancement of carotenoids by mutation and stress induced carotenogenic genes in *Haematococcus pluvialis* mutants

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ABSTRACT

Growing culture of green alga *Haematococcus* was exposed to mutagens such as UV, ethyl methane sulphonate (EMS) and 1-methyl 3-nitro 1-nitrosoguanidine (NTG), and further screened over herbicide - glufosinate. The survival rate of cells decreased with increasing concentration of mutagens and herbicides. The mutants exhibited 23–59% increase in total carotenoid and astaxanthin contents. The NTG treated glufosinate resistant mutant showed increased (2.2% to 3.8% w/w) astaxanthin content. The transcript levels of phytoene synthase, phytoene desaturase, lycopene cyclase, β-carotene ketolase and β-carotene hydroxylase enzymes in the mutant cultures were found to be 13-18, 14-17, 3, 3-22 and 6-20 fold higher respectively compared to wild type. The mutant obtained by UV irradiation showed highest lycopene cyclase activity (458 nmole β-carotene formed/mg protein/h) followed by NTG mutant (315 nmole β-carotene formed/mg protein/h) when compared to that of parent strain (105 nmole β-carotene formed/mg protein/h). Expression analysis of carotenoid biosynthetic genes in the mutants exhibited increase in transcript levels compared to wild type.

1. Introduction

Haematococcus, the green alga, is known for its ability to accumulate keto carotenoid, astaxanthin in high concentrations under stress conditions. Astaxanthin (3, 3'- dihydroxy- β, β-carotene- 4, 4'-dione), owing to its high antioxidant activity, has received much attention for pharmaceutical and nutraceutical applications besides its use as pigmentation source in aquaculture (Guerin et al., 2003; Higuera-Ciapara et al., 2006; Sandesh et al., in press). *Haematococcus* has two distinct phases in its life cycle, viz.—green flagellated motile phase and non-motile non-flagellated cyst phase formed to combat stress conditions (Sarada et al., 2002).

Algal strain with improved growth rate and enhanced carotenoid accumulation makes the commercial process of astaxanthin production more feasible. Induction and selection of mutants has been widely employed technique for strain improvement as well as for studying mechanisms of metabolic processes (Fischer, 1998). Tjahjono et al. (1994) reported astaxanthin formation using norflurazon and floridone in *H. pluvialis* cultures. Mutants of *Phaffia rhodozyma* have been obtained by UV exposure and by EMS or NTG treatment for hyperproduction of astaxanthin (Chumpolkulwong et al., 1997). Tripathi et al. (2001b) and Chen et al. (2003) reported mutation of *Haematococcus* by UV and EMS followed by selection of mutants resistant to compactin, nicotine, diphenylamine, fluridone, or norflurazon. However, the information on astaxanthin hyper producing mutants is limited. In the present study, *H. pluvialis* cells were exposed to chemical and physical mutagens at different levels, subsequently screened them using a broad spectrum herbicide to obtain high yielding mutants. Furthermore biochemical properties like photosynthetic activity, chlorophyll fluorescence, lycopene cyclase activity, carotenoid gene expression were studied.

2. Methods

2.1. Algal culture and growth conditions

Haematococcus pluvialis was obtained from Sammlung Algenkulturen, Pflanzen Physiologisches Institüt, Universität Göttingen, Göttingen, Germany. The stock cultures were maintained as described by Tripathi et al. (1999) in Bold's basal medium (BBM), under continuous light intensity of 2.2058×10^7 erg m^{-2} s^{-1} at 25±1 °C temperature.

2.2. Isolation of Haematococcus mutants

2.2.1. Mutation with UV

Five ml culture of the *H. pluvialis* with cell count of 1×10^6 cells/ml in logarithmic growth phase was exposed to UV irradiation (254 nm) in an open petri dish using UV lamp (CAMAG, Germany). Two set of cells, one for 15 and another for 30 min duration, were irradiated from a distance of 10 cm.

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2.2.2. Mutation with ethyl methane sulphonate (EMS)

One ml culture of *H. pluvialis* (cell number of 1×10^6 cells/ml) was washed with 0.2 M phosphate buffer and treated with EMS in the concentration range of 0.1 M to 0.2 M level for 30 min.

2.2.3. Mutation with 1-methyl 3-nitro 1-nitrosoguanidine (NTG)

One ml culture of *H. pluvialis* (cell number of 1×10^6 cells/ml) was washed with 0.2 M phosphate buffer and treated with NTG at 0.1 mM to 0.6 M concentration for 60 min.

After the treatment with chemical mutagens, the cells were washed thrice with 0.2 M phosphate buffer. Both the irradiated and mutagen treated cells were stored overnight at 7 °C. Later the cultures were grown in BBM for 3 days under 2.2058×10^7 erg m^{-2} s^{-1} light intensity. The cells grown were plated on BBM medium incorporated with 28 μM herbicide glufosinate [2-Amino 4(hydroxy methyl phosphinyl) butanoic acid]. The cultures were incubated at 25 ±1 °C under 2.2058×10^7 erg m^{-2} s^{-1} light intensity.

The colonies grown in petri dishes were transferred onto the BBM slants based on colony characteristics and colour. After the growth for 15 days in slant cultures, cells were transferred to liquid BBM. After two weeks of growth, one set of cultures were harvested for cell count and biomass estimation while the second set were incubated further for two weeks under high light (5.1468×10^7 erg m^{-2} s^{-1}) for carotenoid accumulation.

2.3. Evaluation of growth of mutants

Growth of the mutants and wild type was monitored by growing the cultures in BBM in two tier flasks facilitating 2% (v/v) CO_2 atmosphere as described by Tripathi et al. (2001a). The two-tier vessel consisting of two 250 ml narrow-neck Erlenmeyer flasks was used for enriching carbon dioxide in the culture environment. The lower chamber of the flask contained 100 ml of 3 M buffer mixture ($KHCO_3/K_2CO_3$) at specific ratio, which generated a partial pressure of CO_2 at 2% in the two-tier flask (Tripathi et al., 2001a). The upper chamber contained 40 ml of medium with 10 ml of inoculum. After two weeks of growth, one set of flasks were harvested for cell count and biomass estimation while second set of flasks were incubated further for two weeks under (high light) intensity of 5.1468×10^7 erg m^{-2} s^{-1} for carotenoid accumulation.

2.4. Extraction and analysis of carotenoids

Known volume of wild type and mutant culture was centrifuged and the lyophilized biomass was taken for extraction. The cells were homogenized and carotenoids were extracted with acetone. Total carotenoid content in the extract was calculated using the equations of the Lichtenthaler (1987) by measuring the absorbance at 470, 645 and 661.5 nm (Shimadzu UV–Visible spectrophotometer UV 160-A, Japan). Astaxanthin content was determined at 480 nm by using an extinction coefficient of 2500 at 1% level (Davies, 1976). The content of total carotenoid and astaxanthin were expressed in terms of percent dry weight. The carotenoid extracts were subjected to HPLC analysis in Shimadzu LC-10AT liquid chromatograph using reversed phase C18 column (Supelco, 25 cm × 4.6 mm). The following solvents were used at a flow rate of 1.25 ml min^{-1}: (A) acetone and (B) 90% methanol. The separation of carotenoids was achieved by a gradient between solvent A and B for 40 min as follows: B was run at 80 to 20% for 25 min, 20% for 10 min and 20 to 80% for 5 min. The separated carotenoids were identified by comparing retention times and spectra against known standards. Astaxanthin esters (mono and di) were identified using a photodiode array detector (SPD-M10AVP, Shimadzu) by comparing their spectra and retention time with published data (Vidhyavathi et al., 2007).

2.5. Analysis of carotenoid profile under normal and stress conditions

The mutants were grown in Bold's basal medium in 150 ml conical flask (culture volume −50 ml) under normal growth conditions. After 15 days growth, one set of cultures were subjected to stress by supplementing with 42 mM NaCl and incubated under light intensity of 5.1468×10^7 erg m^{-2} s^{-1}. At the end of 15 days, carotenoid content was analyzed and compared with that of wild type. The other set was maintained on BBM for experimentation.

2.6. Measurement of photosynthetic activity

Photosynthetic activity was determined according to the method modified from Schwelitz et al. (1972). To 5 ml of the algal suspension (equivalent to 25 μg chlorophyll), 2 ml of dye solution [0.1 mM of the dye 2, 6-dichlorophenol-indophenol and 0.01 M KCl in 0.4 M potassium phosphate buffer (pH 6.5)] was added. This suspension was exposed to cool white fluorescent light (2.2058×10^7 erg m^{-2} s^{-1} light intensity) and absorbance was recorded at 620 nm every min upto 12 min.

2.7. Fluorescence profile in presence of herbicide

The effect of herbicide glufosinate on the chlorophyll fluorescence of the mutants was tested. The chlorophyll content in the growing culture of mutants was estimated, centrifuged and normalized to get 25 μg of chlorophyll per ml of culture. To this culture (3 ml) herbicide glufosinate was added as to get final concentration of 200 μM. Excitation wavelength was set at 400 nm and emission was recorded in the range of 400–800 nm up to 40 min using spectrofluorophotometer (Shimadzu RF-5301PC). The variable fluorescence (Fv) was calculated according to Constant et al. (1997), using the equation Fv= (Fmax-Fo)/Fo where Fmax is fluorescence intensity at particular time interval and Fo is initial fluorescence intensity. Fv of 1.6 was considered as 100% for calculations.

2.8. Determination of lycopene cyclase activity

Lycopene cyclase activity in the mutant cells was determined by the method of Schnurr et al. (1996). *H. pluvialis* cells grown for 8 days (in the vegetative phase) and 15 days old stress induced [Stress induction by NaCl (42 mM) and light intensity of 5.1468 $x10^7$ erg m^{-2} s^{-1} for a week] were harvested and cell extract was used for enzyme assay. The culture was centrifuged and the wet biomass was extracted with 0.2 M Tris-Maleate buffer (pH 6.8). The extract was centrifuged and supernatant was used for enzyme assay. The reaction mixture (final volume 0.5 ml) contained 50 μl of 5 mM NADH and 150 μl of lycopene in soybean oil (as substrate) and 300 μl of enzyme. The incubation was done in dark at 30 °C for 4 h. Reaction was terminated by adding methanolic KOH. The mixture was extracted with diethyl ether and petroleum ether (1:1) mixture. Reaction products were analyzed by HPLC as mentioned earlier. Lycopene cyclase activity was calculated in terms of β-carotene formed and expressed as nmoles β-carotene formed/mg protein/h. Protein content in the cell free extract was estimated by the method of Lowry et al. (1951).

2.9. Expression analysis of carotenoid biosynthetic genes

To induce secondary carotenogenesis in *H. pluvialis* cells, green motile cells of wild strain and mutants were exposed to sodium chloride (34 mM) and sodium acetate (4.4 mM) containing medium and incubated under continuous light intensity of 5.1468×10^7 erg m^{-2} s^{-1} for 48 h. Then the cells (1×10^8) from each treatment were harvested, frozen under liquid nitrogen

and subsequently powdered. RNA isolation and Reverse transcription-polymerase chain reaction (RT-PCR) was carried out as described by Vidhyavathi et al. (2007). Total RNA was extracted using RNAqueous® kit according to instruction manual (Ambion, Austin, TX). Possible contaminant genomic DNA in RNA extract was removed using TURBO DNA-free™ kit (Ambion, Austin, TX). The gene specific primers for phytoene synthase (PSY), phytoene desaturase (PDS), lycopene cyclase (LCY), β-carotene ketolase (BKT), β-carotene hydroxylase (CHY) and actin (ACT) were used (Vidhyavathi et al., 2008). First-strand cDNAs were synthesized from 0.2 μg of total RNA in 20 μl final volume, using M-MuLV reverse transcriptase and oligo-dT (18-mer) primer (Fermentas GmbH, Germany).

PCR amplifications were performed routinely using PCR mixture (15 μl) which contained 1 μl of RT reaction product as template, 1× PCR buffer (Bangalore Genei, Bangalore, India), 200 μM dNTPs (Fermentas GmbH, Germany), 1 unit (U) of Taq DNA polymerase (Bangalore Genei, Bangalore, India), 0.1 μM of each primer depending on the gene. PCR was performed at initial denaturation at 94 °C for 4 min, 30 or 22 cycles (1 min at 94 °C; 1 min at 55 or 60 °C or 20 s at 61 °C; 1 min at 72 °C) and final elongation (10 min at 72 °C) using a thermal cycler (Eppendorf Thermal cycler, Germany). The PCR products obtained were separated on 1.8% agarose gel, stained with ethidium bromide (0.001%) and documented in a gel documentation system (Herolab GmbH Laborgerate, Germany). The size of the amplification products was estimated from 100 bp DNA ladder (Fermentas GmbH, Germany). The band intensity of each gel was checked using the Herolab E.A.S.Y Win32 software (Herolab GmbH Laborgerate, Germany). Transcript levels of each gene in control cells were taken for comparison in calculating the transcript abundance of respective genes of mutants under stress conditions. To normalize the RT-PCR data, each gene was compared with actin transcript.

3. Results

3.1. Isolation of H. pluvialis mutant

The appropriate level of UV, EMS and NTG was determined by treating the algal cells in a wide concentration range. The treated cells were selected further by plating in the medium containing herbicide. Green colonies of *H. pluvialis* were observed after 9 days of incubation. The survival rate was found to be concentration dependent in all the mutants and the survival rate decreased with increasing concentration of mutagens. A wide range of concentration of herbicide was first tested to examine its inhibitory levels. Lower concentration of mutagen (<0.1 M EMS and <0.1 mM NTG) and herbicide often produced large number of colonies (> 10,000) with the low possibility of producing mutant. Hence suitable concentration of mutagen as well as herbicide was standardized to obtain satisfactory survival rate. Exposure to UV for 15 minutes resulted in survival rate of 11.8% while exposure to 30 minutes reduced the survival rate to 5.9%. Similarly treatment with NTG at 0.1 mM showed 33.6% survival rate while 0.2 mM reduced it to 18.7%. EMS treatment at 0.1 M and 0.2 M concentration produced 17.3% and 15.4% survival rate respectively.

More than 50 algal colonies from each treatment, grown in the petri dishes, were selected randomly based on the colour and colony characteristics. The selected colonies were transferred onto the herbicide free BBM slants and allowed to grow under normal growth condition. Those colonies which are survived on the slants were transferred to autotrophic growth medium and finally those which survived and showed moderate to high growth rates were selected and taken for further studies.

3.2. Growth and astaxanthin production by mutants

The mutants isolated after 15 min exposure to UV were designated as U1 and U2 and those exposed for 30 min were designated as U3, U4 and U5. Growth profile of these mutants is shown in Fig. 1a. Out of these five UV mutants, biomass yield of four of the mutants was comparable with wild strain (Fig. 1a). However, their carotenoid content and production was found to be less compared to wild strain (Fig. 1a and Fig. 2a). The mutant U1 showed maximum astaxanthin content of 2.6% (w/w) which is almost 23% higher than that of wild strain. However, this increase has not been reflected in astaxanthin production since the biomass yield of this mutant was less (1.95 g/L) in comparison to wild strain (2.72 g/L).

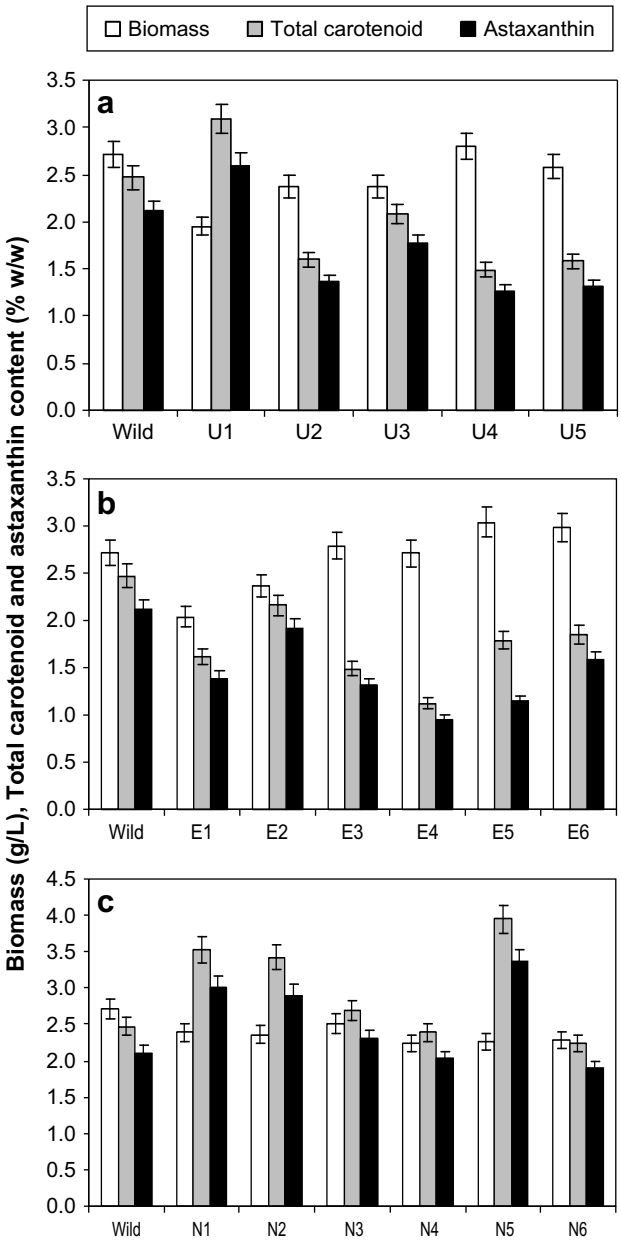

Fig. 1. Biomass, total carotenoid and astaxanthin contents of *H. pluvialis* mutants obtained with (a) UV irradiation, (b) EMS and (c) NTG treatments. U1, U2 - UV mutants obtained for 15 min and U3, U4, U5 for 30 min exposure. E1, E2 - mutants obtained with 0.1 M and E3, E4, E5, E6 with 0.2 M EMS. N1, N2, N3, N4, N5 - mutants with 0.1 mM and N6 with 0.2 mM NTG.

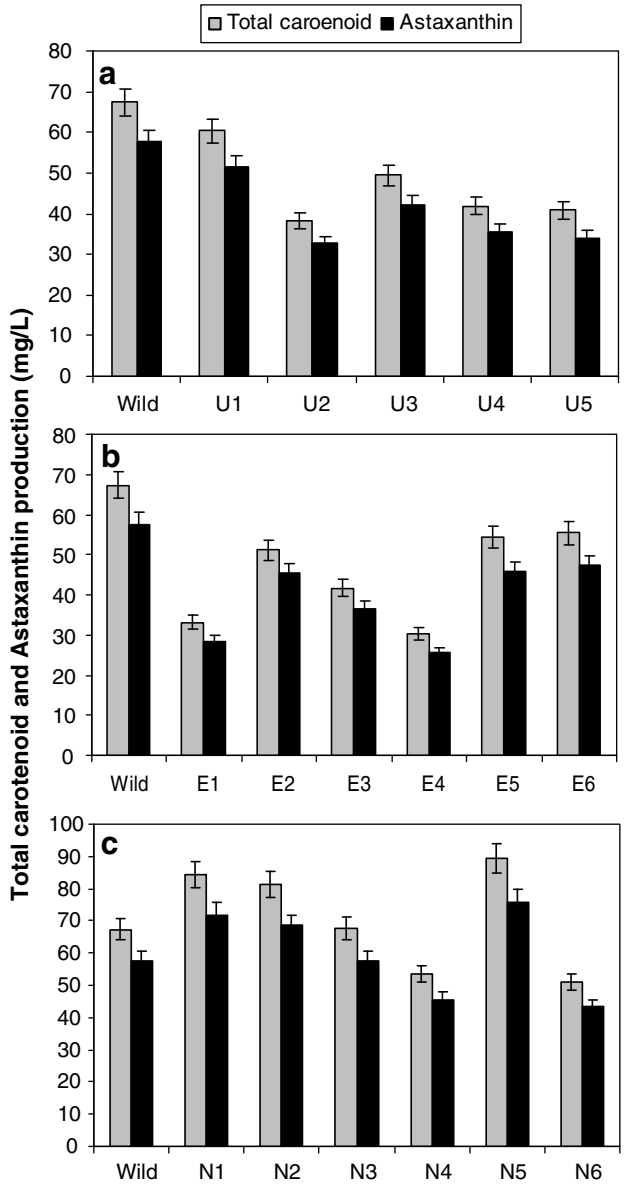

Fig. 2. Total carotenoid and astaxanthin production in *H. pluvialis* mutants obtained with (a) UV irradiation, (b) EMS and (c) NTG treatment. U1, U2 - UV mutants obtained for 15 min and U3, U4, U5 for 30 min exposure. E1, E2 - mutants obtained with 0.1 M EMS and E3, E4, E5, E6 with 0.2 M EMS. N1, N2, N3, N4, N5 - mutants with 0.1 mM NTG and N6 with 0.2 mM NTG.

Mutants isolated after treatment with 0.1 M EMS were designated as E1, E2 and 0.2 M EMS were designated as E3, E4, E5, E6. The growth profile of EMS mutants is shown in Fig. 1b. The mutants E5 and E6 produced maximum biomass yield of 3.04 and 2.99 g/L respectively (Fig. 1b). EMS treatment had significant effect on carotenoid biosynthesis as indicated by lower amounts of total carotenoid and astaxanthin content (Fig. 1b). Total carotenoid production was in the range of 30–55 mg/L which was lower than that of wild type (67.3 mg/L) (Fig. 2b).

Mutants isolated after treatment with 0.1 mM NTG were designated as N1, N2, N3, N4, N5 and 0.2 mM NTG were designated as N6. The growth profile of NTG mutants is shown in Fig. 1c. The biomass yield of these mutants was in the range of 2.24–2.51 g/L (Fig. 1c). Mutation with NTG has resulted in significant increase of 42–59% in the carotenoid content (Fig. 1c). Out of the six mutants tested for growth and carotenoid production, the mutant N5 produced a maximum of 3.95% (w/w) total carotenoid content and 89.2 mg/L of total carotenoid production (Fig. 1c and Fig. 2c).

3.3. Analysis of carotenoid profile

The HPLC profile of carotenoid extracts from wild and mutants did not exhibit significant variation on qualitative basis. This indicates the mutagens-UV, EMS and NTG have not affected the carotenoid biosynthetic pathway to that extent which would result in major shift in carotenoid profile.

3.4. Analysis of carotenoid profile under normal and stress condition

The effect of salinity stress (42 mM) and light stress (5.1468×10^7 erg m^{-2} s^{-1}) on mutants was studied and compared with the normal growth conditions without stress and also with wild type. Under normal growth conditions, the total carotenoid and astaxanthin content of mutants was comparable with the wild strain. The salinity and light stress had a significant effect on the carotenoid content of the mutants. The total carotenoid content of wild type was found to be 2.55% with the astaxanthin content of 2.18%. While mutants have shown an increase of 23–59% in total carotenoid and astaxanthin content in comparison with wild type except the mutant E3 which produced comparable carotenoid and astaxanthin contents. The mutant N5 produced a maximum total carotenoid content of 4.08% (w/w) and astaxanthin content of 3.51% (w/w).

3.5. Effect of herbicide on photosynthetic activity of mutants

The effect of herbicide –glufosinate (250 μM) on actively growing *Haematococcus* wild and mutant strains was evaluated by measuring photosynthetic activity using the dye 2, 6-dichlorophenol indophenol (DCPIP). As shown in Fig. 3, the mutants N5 and E3 were resistant to herbicide action and continued active photosynthesis as indicated by the reduction in the dye DCPIP within 5–10 min. The photosynthesis was inhibited in the wild strain as shown by the absorbance at 620 nm. In case of the mutant U1, the extent of photosynthetic inhibition by glufosinate was lesser than that of wild strain.

3.6. Effect of herbicide on chlorophyll fluorescence profile of mutants

The effect of herbicide glufosinate (200 μM) on the chlorophyll fluorescence characteristics of the mutants was evaluated. The

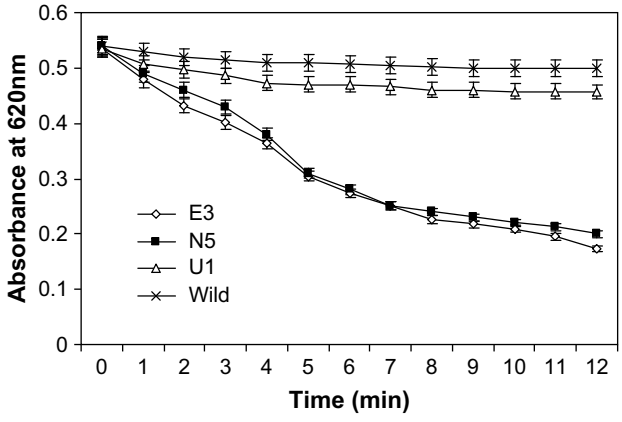

Fig. 3. Photosynthetic activity in mutants of *H. pluvialis* in presence of herbicide - glufosinate (250 μM).

variable chlorophyll fluorescence (Fv) of the mutants in comparison with the wild strain is shown in the Fig. 4. The *H. pluvialis* mutants N5 and E3 have shown higher Fv (variable fluorescence) in comparison to the wild strain. After 20 and 30 min of excitation at 400 nm, the Fv of the mutants N5 and E3 was almost 1.5 to 2.0-fold higher than that of wild type. The mutant U1 did not show significant difference in the chlorophyll fluorescence emission pattern.

3.7. Lycopene cyclase activity of H. pluvialis mutants

Formation of β-carotene from lycopene is a crucial step and it is mediated by the enzyme lycopene cyclase. Hence mutants obtained after treatment with mutagen were evaluated for lycopene cyclase activity. The mutants in the vegetative phase of growth and after stress induction were used to determine the enzyme activity. Lycopene cyclase activity of tested mutants is shown in Table 1.

In the vegetative phase, the lycopene cyclase activity was highest in the mutant U1 which was 458 nmoles β-carotene formed/mg protein/h which is almost 4-fold higher than that of wild strain. After the stress induction, the mutant N5 showed the maximum enzyme activity of 172 nmoles β-carotene formed/mg protein/h.

3.8. Expression analysis of carotenoid biosynthetic genes

The stable mutants in terms of growth and carotenoid contents from UV, EMS and NTG treatments were selected for carotenogenic genes expression studies. The expression levels of genes associated with general carotenogenesis and specific astaxanthin biosynthesis in mutants and wild strain were quantified by reverse transcription polymerase chain reaction (RT-PCR) and compared. These genes included phytoene synthase (PSY, the first committed step in the carotenoid pathway), phytoene desaturase (PDS, which converts phytoene to lycopene), lycopene cyclase (LCY, which converts lycopene to β-carotene), BKT (specific to astaxanthin biosynthesis, which converts β-carotene to echinenone and to canthaxanthin), and CHY (which convert canthaxanthin to astaxanthin and α-carotene to lutein and other xanthophylls). The transcript levels of these enzymes were analysed after 48 h of stress induction to wild and mutant strains. The transcript levels of all the genes were found to be higher in mutants than wild type (Fig. 5). The mutants differed in the extent of transcript levels for each carotenogenic genes studied. The transcript levels of PSY, PDS, BKT and CHY were 15 to 20-fold high in E3 mutant while N5 exhibited 12-fold increase in PSY, PDS and only 3-fold increase in LCY and BKT. Mutant U1 showed only 3 to 5-fold increase in transcripts of LCY, BKT and CHY genes. The lycopene cyclase enzyme activity of the mutants E3 and N5, as shown in the Table 1, correlates with lycopene cyclase gene expression.

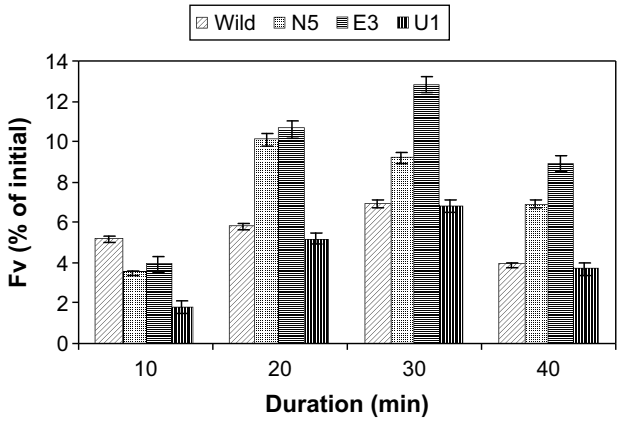

Fig. 4. Variable fluorescence (Fv) exhibited by mutants in presence of herbicide-glufosinate (200 μM).

Table 1
Lycopene cyclase activity of *H. pluvialis* mutants

Mutant	Vegetative cell	Stress induced cells[a]
	nmoles β-carotene formed/mg protein/h	
Wild	106 ± 5.30	92 ± 4.10
U1	458 ± 16.30	6 ± 0.30
E3	0.8 ± 0.04	51 ± 1.25
N2	315 ± 15.14	75 ± 3.12
N5	40 ± 2.11	172 ± 8.14

Data are mean ± SD of three replicates.
[a] Stress induction by NaCl (42 mM) under high light (5.1468×10^7 erg m^{-2} s^{-1}) for 60 h.

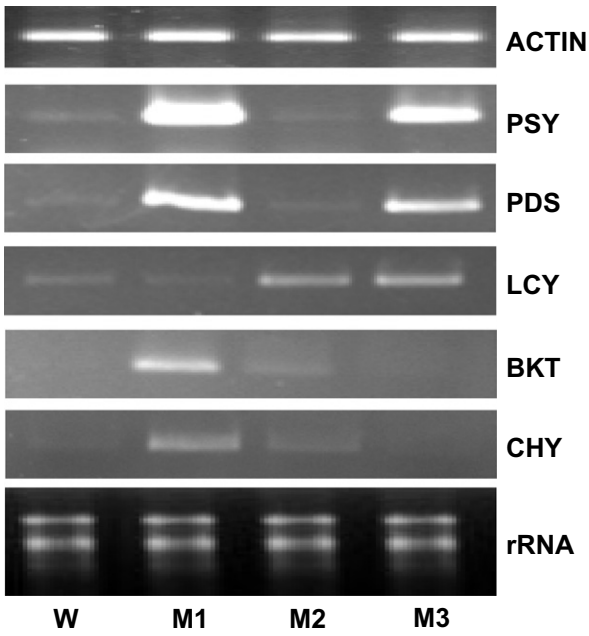

Fig. 5. Expression of carotenoid biosynthetic genes in *H. pluvialis* mutants. Wild and mutant strains were exposed to 34 mM NaCl and 4.4 mM sodium acetate for 48 h. RNA was isolated and RT-PCR was performed as described in materials and methods. The PCR products were analysed by agarose gel electrophoresis. For comparison, total RNA was stained with ethidium bromide (lower panel).

4. Discussion

The mutagens EMS and NTG produces lesions such as alkylphosphotriesters which are slowly removed from DNA (Goth-Goldstein, 1980). Growth of algal colony after treatment with mutagen is believed to be the cell's capacity to repair DNA damage produced by that agent (Eckardt et al., 1980). In the current study, these mutagens were used to isolate high yielding *H. pluvialis* mutants and the results reveal the satisfactory outcome of mutagenesis. The isolated mutants exhibited an increase of 23–59% in total carotenoid and astaxanthin content (Fig. 1) compared to wild type. The mutants also showed distinct differences from wild type in terms of photosynthetic activity, chlorophyll fluorescence profile, lycopene cyclase enzyme activity and expression of carotenoid biosynthetic genes.

The combination of an efficient method for generating random mutation and a strategy that permits high throughput screening is essential for successful mutagenesis approach. In the present

study, mutagenesis with chemical and physical agents was attempted to increase the microalgal biomass and carotenoid production in *H. pluvialis*. It was anticipated that the target enzyme would be altered by the action of mutagen. Number of trials was carried out to obtain satisfactory survival rate after the treatment with mutagens. The degree of mutagenesis was controlled by changing the parameters such as concentration of EMS or NTG, incubation time or exposure time of UV. Although, on screening, glufosinate containing medium generated acceptable number of colonies, the efforts using other herbicides like glyphosate, atrazine, diphenylamine and diquat were not useful. The appropriate concentration of glufosinate required for screening the mutants was determined after checking the minimum inhibitory concentration by growing the wild type *H. pluvialis* strain in wide concentration range.

Herbicides are known for their enzyme inhibitory actions, they disrupt basic metabolic processes essential for plant or algal cells. Resistance of the strain to inhibitor or herbicide has been generally used for screening the mutants with desired properties (Tjahjono et al., 1994; Erickson et al., 1989; Modi et al., 1991; Tripathi et al., 2001b; Lange et al., 2001). It has been reported that the herbicide –glufosinate efficiently inhibits the cell growth of *H. pluvialis*, induces astaxanthin accumulation by blocking the activity of enzyme glutamine synthetase (GS), a key enzyme in ammonia assimilation (Aflalo et al., 1999). Pigment mutants are unique tools to study the function of photosynthetic complexes and carotenoid pathways (Sun et al., 1998; Chitnis et al., 1997; Hoshino et al., 1994). Shaish et al. (1991) have isolated β-carotene rich mutants of *Dunaliella bardawil* and suggested that the mutants are affected in the regulatory path, which controls the β-carotene production. Astaxanthin overproducing mutant of *Phaffia* after treatment with NTG has been reported by Bon et al. (1997). Effect of UV irradiation on motility, pigmentation and several metabolic processes of algal system have been reported (Agrawal, 1994; Dohler, 1989). UV light exposure resulted in rapid inactivation of algal cells and consequently yields a low percentage of mutants among the survivors. Alonso et al. (1996) have reported the increase in the yield of eicosapentaenoic acid content in microalga *Phaeodactylum tricornutum* by UV-induced mutagenesis. Wachi et al. (1995) have reported the effect of UV-A on cyanobacteria- *Oscillatoria* where the chlorophyll biosynthesis was inhibited by the UV irradiation. Out of the 5 mutants isolated after UV treatment, four have shown less carotenoid content indicating the possible effect of UV irradiation on carotenoid biosynthesis pathway. The herbicide glufosinate, used as screening agent, did not influence the photosynthetic ability of the UV induced mutant as demonstrated by the unaltered photosynthetic activity (Fig. 3) and chlorophyll fluorescence (Fig. 4).

Numerous herbicide-resistant mutants of *Chlamydomonas* with different patterns of resistance to such herbicides have been reported (Erickson et al., 1989). Green algae can develop resistance to herbicide that block metabolic pathways like photosynthesis by competing with quinines in binding to DI, the 32-kD reaction-center protein of chloroplast photosystem II. The mutants obtained with different mutagen treatments and subsequent screening over herbicide glufosinate did not show any differences in the carotenoid profile as analysed by HPLC. However the mutants differed in growth (biomass yield) and total carotenoid content which may be due to possible changes in photosystem. The differences in the transcript levels of carotenogenic genes in response to stress in different mutants also substantiate the above statement.

The carotenoid production, photosynthetic activity and fluorescence of the mutant N5 was well correlated with the expression of carotenoid genes – PSY and PDS. The relative inhibition of photosynthesis by herbicide in this mutant was less compared to wild type (Fig. 3). Mutant N5 also showed almost 60% higher variable fluorescence (Fv; Fig. 4) and 12-fold higher carotenoid gene transcript abundance in comparison with wild type. These results provide insight into the regulation of carotenoid biosynthesis in the mutant N5. It can be contemplated that, by the action of mutagen NTG, the components of the photosynthetic electron transport has been affected, hence the carotenoid expression is dependent on the redox state. This view on photosynthetic regulation is also supported by the investigations by Steinbrenner and Linden (2003), that entire carotenoid biosynthesis in *H. pluvialis* is under photosynthetic redox control.

Though light is crucial and governing factor in both photosynthesis and carotenoid formation in *H. pluvialis*, maximum astaxanthin biosynthesis is the result of synergistic action by several other factors (Sarada et al., 2002; Wang et al., 2003; Lababpour et al., 2004). Therefore the above postulation does not hold good for the mutant U1 and E3 and the exact mechanism responsible for their differential expression remains to be elucidated.

The findings of this study reveal that the chemical mutagen and UV irradiation have altered certain biochemical characteristics of the wild type. In concurrence with the above reports, the altered biochemical properties of *H. pluvialis* mutants can be attributed to the changes at molecular level. This reasoning is also supported by the data on expression of carotenoid biosynthetic genes in mutants. These data provides scope for further studies on molecular aspects of *H. pluvialis* mutants.

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