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Blocking of Sterol Biosynthesis by Statin Enhances Carotenoid Production in *Dunaliella*

Kotamballi N. Chidambara Murthy • Chandrappa Dayananda • Gokare Aswathanarayana Ravishankar*

Plant Cell Biotechnology Department, Central Food Technological Research Institute, Mysore 570 020, India

Corresponding author: * **pcbt@cftri.res.in**

ABSTRACT

In order to meet the demand for β-carotene, which is gaining commercial significance due to its health attributes, the algae *Dunaliella* is being commercially exploited. The current study was aimed at finding methods for enhanced production of β-carotene and also to understand the relationship between other pathways which were interlinked. The unicellular marine micro algal strains of *Dunaliella* species namely *Dunaliella salina* and *Dunaliella bardawil* were studied to understand the effect of atorvastatin, a well-known drug and HMG-CoA reductase inhibitor which inhibits cholesterol biosynthesis. Different concentrations of atorvastatin (5.0, 10.0 and 25.0 mg L^{-1}) were employed at different time intervals of growth (added on 3rd, 6th and 9th day). The algae were harvested and analyzed for yield of fresh biomass, and contents of β-carotene and squalene. Squalene content of the treated groups was analyzed and no traces were found whereas the control cultures of *D. bardawil* and *D. salina* contained 0.6 μg g^{-1} and 0.53 μg g^{-1} of squalene, respectively. Subsequently SDS-PAGE analysis showed an increase in the contents of lycopene cyclase (43.0 KD) in the treated cultures which was also further confirmed by analysis of lycopene cyclase activity. The total carotenoids content was found to be enhanced by 1.9- and 1.4-fold after 16 days in *D. bardawil* treated with 10 mg L^{-1} and 25.0 mg L^{-1} of statin, respectively while in *D. salina* this content was enhanced 1.8- and 2-fold, respectively in the same time period. *D. salina* accumulated 3.15 mg L^{-1} of carotenoids in the 10 mg L^{-1} atorvastatin treatment. Correspondingly, *D. bardawil* accumulated 5.6 mg L^{-1} of carotenoids in the 10 mg L^{-1} atorvastatin treatment. This is the first report on the effect of atorvastatin on the microalgae *Dunaliella*.

Keywords: atorvastatin, *Dunaliella salina*, *Dunaliella bardawil*, microalgae, squalene
Abbreviations: DOXP, 1,deoxy-D-xylulose-5 phosphate; **GC-MS**, gas chromatography-mass spectroscopy; **IPP**, isopentyl pyrophosphate; **MVA**, mevalonic acid pathway

INTRODUCTION

Carotenoids are an important class of biological compounds, which are widely distributed in foods (van Poppel 1996) and provide medical and health benefits, including the possible prevention and treatment of skin cancer and cardiovascular disease (Bast *et al*. 1996). β-carotene is a precursor of vitamin-A, and is an effective antioxidant, due to the presence of a long chain of carbon-carbon double bonds (Yen and Chen 1995; Everett *et al*. 1996; Gouveia *et al*. 2003).

The unicellular microalgae *Dunaliella* naturally occurs in saline habitats and is known to produce 3-5% (w/w) of β-carotene under favorable conditions (Ben-Amotz and Avron 1983). In the present study an attempt has been made to inhibit sterol biosynthesis using statins because both sterol and carotenoids pathways utilize common precursors. Statins, a class of fungal secondary metabolites, have become the focus of great attention due to their ability to influence the *de novo* synthesis of endogenous cholesterol by lowering the ratios of cholesterol precursors like squalene, D-cholesterol, desmosterol and lathosterol. Several *Monascus* and *Aspergillus* strains are known to produce statins and these statins are mostly found in both the mycelium and culture filtrate (Uusitupa *et al*. 1992; Manzoni *et al.* 1999). There is currently little information available with respect to the occurrence, fate, and properties of statins in the aquatic environment (Miao and Metcalfe 2003; Lam *et al*. 2004). They are powerful inhibitors of cholesterol synthesis so are prescribed in the treatment of hypercholesterolemia, and are found to be competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme-A reductase (HMGR). Statins are highly specific inhibitors of HMGR in higher plants (Bach and Lichtenthaler 1982), the key enzyme regulating the mevalonic acid pathway (MVA) of isoprenoid biosynthesis. Statins have displayed phytotoxicity in radish and *Lemna*, as well as Tobacco Bright Yellow-2 (TBY-2) cells (Brain *et al*. 2006). No reports are available regarding the effects of statins on the eukaryotic algae *Dunaliella* so an experiment was designed to know their possible effects.

In plants, isoprenoids play important biochemical functions like electron transport chains (prenyl side chain of quinones), components of lipid membranes (sterols), sub-cellular targeting and regulation (prenylation of proteins), photosynthetic pigments (carotenoids and prenyl side chain of chlorophylls), hormones (gibberellins, brassinosteroids, abscisic acid, cytokinins), and defense compounds as well as pollinator attractants (monoterpenes, sequiterpenes, and diterpenes) (Kasahara *et al*. 2002; Rupasinghe *et al*. 2003). Two independent pathways exist in plants for synthesizing isopentyl diphosphate (IPP) and its isomer dimethylallyl phosphate (DMAPP), the universal precursors for isoprenoid biosynthesis; a cytosolic mevalonate (MVA) pathway of eukaryotic origin and a chloroplastic (2-C-methyl-D-erythritol 4-phosphate (MEP) pathway of prokaryotic origin, also known as the non-mevalonate pathway. In the mevalonate pathway the formation of IPP and DMAPP proceeds via the intermediate mevalonate from acetyl-CoA, which under normal physiological conditions, is responsible for the formation of sterols and ubiquinone (Lichtenthaler *et al*. 1997). Whereas in non-mevolonate pathway, formation of IPP and DMAPP proceeds through the condensation of pyruvate and glyceraldehyde-3-phosphate via a 1-deoxy-D-xylulose 5-phosphate intermediate and is used to form isoprene, carotenoids, abscisic acid, and the side chains of

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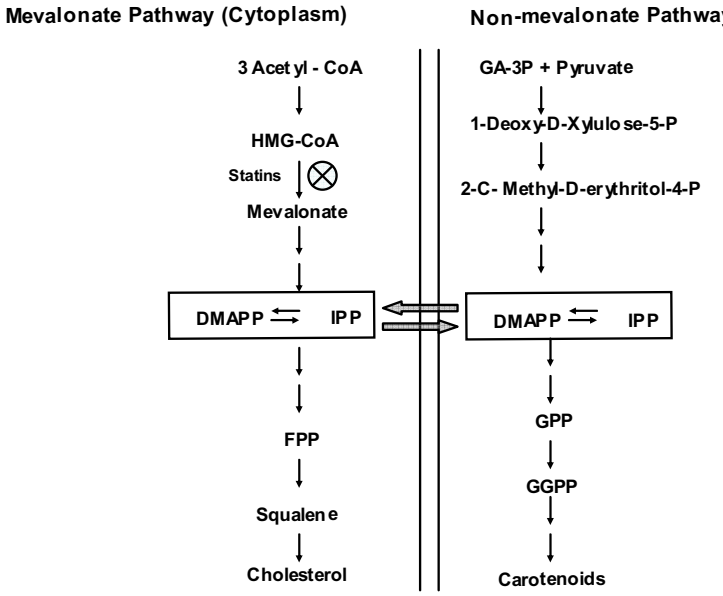

Fig. 1 Sterol biosynthetic pathway and its inhibitors.

chlorophylls and plastoquinone. However both pathways operate in parallel (Lam *et al*. 2004) and sharing of intermediates does occur generally through the transfer of IPP, DMAPP, geranylgeranyl diphosphate and farnesyl diphosphate (Laule *et al*. 2003; Brain *et al*. 2006). Although metabolite sharing appears to operate more readily from the chloroplast to the cytosol and complementary compensation has been shown for both the pathways in the face of inhibitors (statin) or mutants defective in the early steps of IPP biosynthesis (Laule *et al*. 2003) (**Fig. 1**).

Atorvastatin, a well-known drug that inhibits cholesterol biosynthesis (an HMG-CoA reductase inhibitor) was used in the present work to study its effect on the carotenoid content of the alga *Dunaliella salina* and *D. bardawil* at different levels and under different culture conditions. Carotenoid levels have been estimated because both the pathways share common precursors such as DMAPP and IPP. The present communication is based on this scientific information which could be used to enhance the levels of carotenoids in microalgae. Since statins are known to reduce sterol biosynthesis we analyzed samples for the presence of squalene as is one of the major intermediates in cholesterol biosynthesis and is also a precursor for many metabolites (steroids, terpenoids and some of the flavanoids) in plants and animals. In addition, there is a possibility of increasing the levels of non-sterol compounds like carotenoids so an attempt was also made to measure the key enzyme *lycopene cyclase* responsible for the production β-carotene, a major carotenoid of the algae *Dunaliella*. This is the first report on the effect of atorvastatin on the microalgae *Dunaliella*.

MATERIALS AND METHODS

Algal strains

Dunaliella salina (No 19-3) was obtained from Sammulung von Algen Kulturen, Pflanzen, Physiologische Institute, Universitat Gottingen, Gottingen, Germany. *Dunaliella bardawil* was locally isolated from saltpans of Sambar lakes of Rajasthan, India and further authenticated at our laboratory. Both the strains were maintained on agar slants and in liquid medium using AS-100 media (Vonshak 1986) composition containing 1.5 M sodium chloride and incubated at 18 ± 2°C under 18 ± 2 µmol/m^2/s light intensity by sub-culturing (inoculum 20% v/v) at two week intervals.

Stock cultures of both algal strains were inoculated into modified Ben-Amotz medium (Ben Amotz and Avron 1983) and the two weeks-old cultures were sub-cultured at least twice in modified Ben-Amotz medium and used for all the experiments.

Effect of atorvastatin on *Dunaliella*

The experiment was carried out for both *Dunaliella* strains in 250 mL Erlenmeyer flasks containing 80 mL of modified Ben-Amotz medium for a period of 16 days. The culture flasks were uniformly inoculated (25% v/v) with two-weeks-old *Dunaliella* culture and incubated at 18 ± 2°C under 18 ± 2 µmol/m^2/s light intensity using florescent light 24 h a day. Different concentrations (5, 10 and 25 mg L^{-1}) of atorvastatin (purchased from local pharmacy, manufactured by Ranbaxy India Pvt. Ltd.) from the tablet a stock of 10 mg mL^{-1} and the solution was filter sterilized and added to the culture media containing algae on the 3rd, 6th and 9th day of algal growth. Cultures were drawn on the 4th, 8th, 12th and 16th day of culture and estimated for total carotenoid content. The cultures were harvested after 16 days of growth and estimated for fresh biomass, carotenoids, β-carotene, squalene and lycopene cyclase. All experiments were carried out in triplicate.

Biomass estimation

The cultures were harvested after centrifuging at 800 × *g* for 10 min at 4-6°C. The supernatant was decanted and the pellet was washed at least twice with isotonic solution (0.9% sodium chloride solution) to remove excess salts. The fresh weight of the algal pellet was determined gravimetrically and growth was expressed in terms of g L^{-1} fresh biomass weight. The fresh biomass was used for the estimation of β-carotene, squalene and lycopene cyclase assay.

Estimation of total carotenoids

A known volume of culture was centrifuged at 800 × *g* for 10 min and the pellet was treated with *n*-hexane: isopropyl alcohol (1:1) for the extraction of carotenoids and quantified spectrophotometrically using the formula (Carotenoids (mg/vol.) = (OD 450 × volume of the sample taken)/2500) given by Devis (1976) by measuring the optical density at 450.0 nm and further confirmed by reverse phase gradient HPLC as explained below.

Analysis of carotenoids by HPLC

The HPLC system used for carotenoid analysis consisted of Hewlett Packard HPLC (Palo Alto, CA) equipped with a quaternary pump fitted with a zorbax C_{18} (Hewlett Packard) analytical column (25 cm x 4.6 nm I.D 5 µm particle size). Detection was done by an HP 1250 series with a variable wavelength detector at 450.0 nm. The gradient mobile phase consisted of acetonitrile (A) and chloroform (B) with a flow rate of 1.0 mL min^{-1}. The elution program involved a linear gradient from 80 to 20% of A for 0-5 min followed B from 20 to 80% for 5-15 min and again 80% of A for 15-20 min followed by 5 min equilibrium. The total programme

time was 25 min. The carotenoid content was calculated in comparison to the peak area of all *trans* β-carotene (ACN Chemicals). The compounds were quantified using HP Chemstation software. Samples were dissolved in the mobile phase and 10 µL volumes were injected (Murthy *et al*. 2005b).

Analysis of squalene by GC-MS

Lyophilized biomass of *D. bardawil* and *D. salina* were homogenized (using mortar and pestle) in the ratio of 1: 50 (w/v) in hexane. The hexane extract was purified by column chromatography on silica gel by eluting with hexane. The sample was analyzed using ELITE -5 capillary columns (0.5 x 30.0 m). The oven temperature was kept initially at 130°C for 5 min and then increased to 200°C at a rate of 8°C/min, at which point the temperature of the column was maintained for 2 min and then increased up to 280°C at a rate of 5°C/min, at which point the temperature of the column was maintained for 15 min. The injector port and the detector temperatures were 240°C and 250°C, respectively. The mass spectra were recorded under electron impact ionization at 70 eV electron energy with a mass range from 40-600 at a rate of one scan sec^{-1}. Squalene was estimated and identified in comparison with the fragmentation pattern of the authentic squalene standard (Sigma) and also with the NIST library.

Assay of lycopene cyclase

Lycopene cyclase was estimated quantitatively by SDS-PAGE and quantified by monitoring the amount of lycopene converted to β-carotene and the same were monitored by HPLC as explained by Schnurr *et al*. (1996). Protein concentrations in different fractions of ammonium sulphate were estimated by the Bradford method (1976) method. Protein concentrations were also estimated from SDS-PAGE in comparison with the staining intensity of standard markers form Gene-I (Biotechnology Ltd., Bangalore, India). The relative amount of expressed lycopene cyclase band on staining SDS-polyacrylamide gels was determined by using a flatbed scanner with densitometry software. Samples were prepared in soybean lipids containing 20% of L-α-phosphotidylcholine. The lipid was first dissolved in chloroform and solvent was removed by stream of nitrogen gas. 50 mM of Tris/maleate buffer, pH 6.5 was added and the suspension was produced by sonication for 30 minutes in the dark at 0°C to which lycopene was added.

The assay mixture contained 150 µL of lipid suspension including substrate and 5 mM NADP and enzymes were added (~25 µg of protein) in 59 mM Tris/maleate buffer (pH 6.5) and incubated in the dark at 30°C for 5 h. The reaction was terminated by adding 1.5 mL of 6% methanolic KOH and heated at 60°C for 20 min. The remaining substrate and the product formed were extracted from the aqueous incubation mixture with diethyl ether/petrol (1:9 v/v). The reaction product was analyzed by HPLC in a C18 column (5 µm) using acetonitrile: methanol: propanol (85:10:5) using a UV detector at 440.0 nm (Schnurr *et al*. 1996).

STATISTICAL TREAMENT

Results were expressed as the mean ± the standard deviation (SD) of three replicate analyses. The difference between the groups was statistically analyzed by using one-way ANOVA.

RESULTS

Dunaliella salina and *D. bardawil* were evaluated for growth and metabolite production. Both strains of *Dunaliella* showed a decrease in the yield of biomass as atorvastatin concentration increased (**Fig. 2**) while, in contrast an increased production of carotenoids was observed in both the strains. The color of *Dunaliella* cultures treated with atorvastatin was also changed as seen on the 12th day of their growth period (**Fig. 3**). It is also evident from **Fig. 3** that there is an early (after 12 days) onset of carotenoids production in the treated cultures. The total carotenoid content was found to be enhanced 1.9- and 1.4-fold after 16 days in *D. bardawil* treated with 10 mg L^{-1} and 25.0 mg L^{-1} of statin, respectively. The same was found to be enhanced 1.8- and 2-fold in case of *D. salina* when measured on the 16th day (**Figs. 4, 5**). *D. salina* had accumulated 3.15 mg L^{-1} of carotenoids at 10 mg L^{-1} of statin treatment on the 16th day (**Fig. 4**). Similarly *D. bardawil* cultures treated with 10 mg L^{-1} of statin had accumulated 5.6 mg L^{-1} (as observed on the 16th day) of carotenoids (**Fig. 5**).

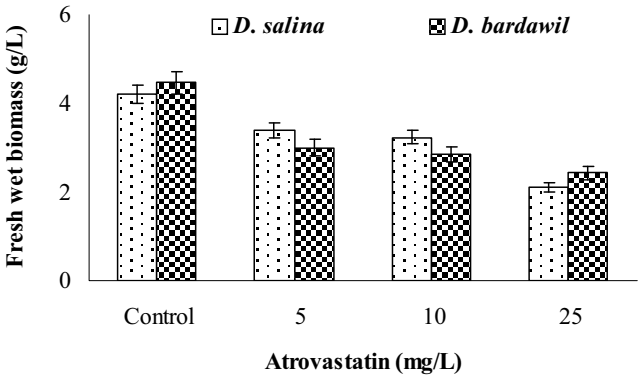

Fig. 2 Effect of atorvastatin on the biomass yields of *D. salina* and *D. bardawil*. Values are the mean of three measurements and expressed as the mean ± SD.

It is well established that statins are known to inhibit sterol biosynthesis so we analyzed for the contents of squalene, which is a precursor for many metabolites like steroids, terpenoids and for some of the flavanoids in plants. Squalene was identified by GC-MS. The contents of squalene were estimated and no traces were found at all treated concentrations of atorvastatin; in control cultures, in contrast it was found at 0.53 ± 0.25 µg g^{-1} and 0.62 ± 0.18 µg g^{-1} in *D. salina* and *D. bardawil* cultures, respectively.

In contrast to this there is a possibility of increasing the levels of non-sterol compounds like carotenoids, so an attempt was also made to measure the key enzyme *lycopene cyclase* (EC 1.14.-.-.), which is responsible for the production of β-carotene, a major *Dunaliella* carotenoid (Ben Amotz and Avron 1983). Ammonium sulphate fractions of *D. salina* and *D. bardawil* showed the presence of significant amounts of *lycopene cyclase* (43 kDa) (Sandmann 1994), which indicates the enhancement of carotenoid content as analyzed by SDS-PAGE (10.0%) (**Fig. 6**). HPLC was used to assess the β-carotene conversion in the assay. Ammonium sulphate (40%) fraction showed the maximum enzyme concentration, which was further confirmed by HPLC and quantified in terms of the amount of lycopene converted to cyclic β-carotene. The concentration of *lycopene cyclase* was calculated in terms of the amount of lycopene converted to β-carotene according to method of Schnurr *et al*. (1996). The amount of *lycopene cyclase* was magnified to 1.75 and 2.02 at 5.0 mg L^{-1} in the atorvastatin-treated group in the case of *D. salina* and *D. bardawil*, respectively when compared to the untreated group (as calculated by band intensity using Hero Lab Easy Win 3.2

Fig. 3 Effect of atorvastatin treatment on total carotenoid production in *D. bardawil* on 12th day of culture (A = control, B = 5.0 mg L^{-1}, C =10.0 mg L^{-1}, D = 25.0 mg L^{-1} atorvastatin).

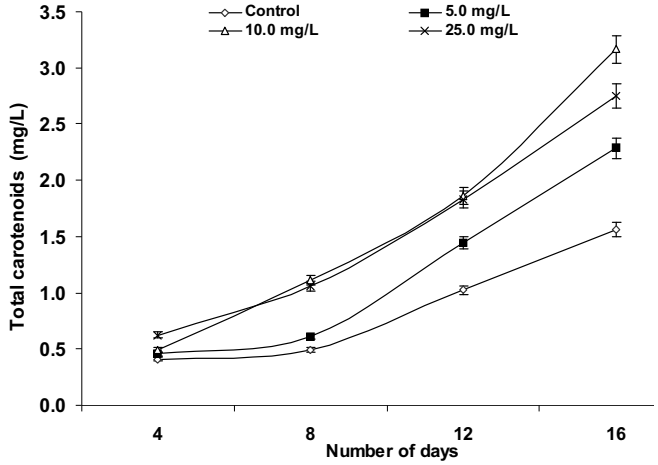

Fig. 4 Effect of atorvastatin treatment (0 (control), 5.0, 10.0, and 25.0 mg L^{-1}) on total carotenoid production in *D. salina*. Values are the mean of three measurements and expressed as the mean ± SD. Enhancement of carotenoid content were significant on the 12th day and highly significant on the 16th day in 5 and 10 mg L^{-1} atorvastatin treatment compared to the control without statin (P<0.05).

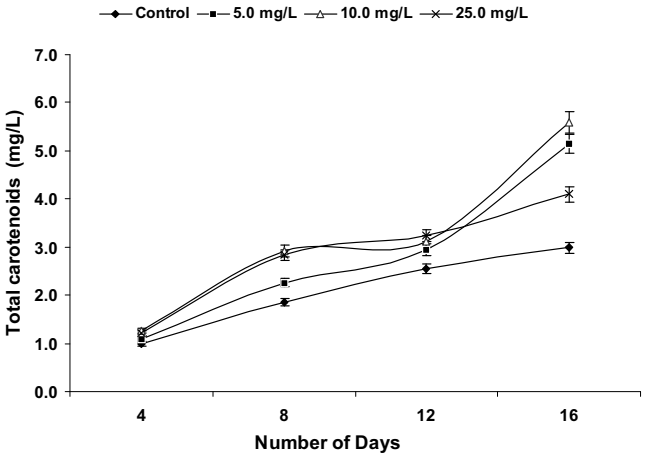

Fig. 5 Effect of atorvastatin treatment on carotenoid production in *D. bardawil*. Values are the mean of three measurements and expressed as the mean ± SD, Enhancement of carotenoid content were significant on the 12th day and highly significant on the 16th day compared to the control without atorvastatin treatment (P<0.05).

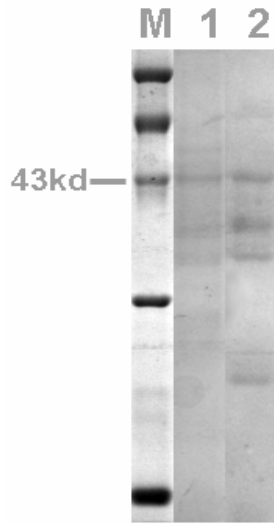

Fig. 6 SDS-PAGE of 40% ammonium sulphate fractions showing the presence of *lycopene cyclase* in (1) *D. salina* and (2) *D. bardawil*. (M) Marker proteins. Presence of the band at 43 kd indicates *lycopene cyclase* (as reported by Schnurr *et al*. 1996).

software). Maximum enzyme concentration was observed at 40% of ammonium sulphate. This fraction showed 1.534 nM conversion of lycopene to β-carotene in *D. bardawil* and the activity was 0.348 nM hr^{-1} as calculated from HPLC results. The amount of lycopene cyclase was 32.61 ± 0.15% of total protein in *D. bardawil* and 27.28 ± 0.25% in the case of *D. salina* in the 40% fraction.

These results clearly indicate the role of statin in enhancing the carotenoids content in *Dunaliella*, which in general will not accumulate carotenoids at low light (requires at least more than 120 μmol/m^2/s light intensity for induction of carotenoid accumulation) and without salt stress (Ben Amotz and Avron 1990). The present method helps in the production of carotenoids at a low light intensity, and can be done throughout the year in different places of the world were they receives lesser light intensities. There is an increasing demands for natural colorants, in particular β-carotene, due to their health benefits; for this reason increased productivity of carotenoids in *Dunaliella* using statin inhibitors is of great relevance.

DISCUSSION AND CONCLUSION

Carotenoids are gaining importance due to their pharmaceutical and nutraceutical applications. There are many approaches to enhance the content of β-carotene and other carotenoids (phytoene, phytofluene, α-carotene, lutein, violaxanthin, neoxanthin and zeaxanthin) in the microalga *Dunaliella*. *Dunaliella* accumulates large amounts of β-carotene when cultured under appropriate conditions. These includes stress induction through high light, salt tolerance, nutrient stress, like nitrate and phosphate and duration as reported by various researchers elsewhere (Ben-Amotz and Avron 1990; Ben-Amotz 1999, 2004; Garcia-Gonzalez *et al*. 2005). However there are limited reports available on approaches to modify the intermediates of carotenoid biosynthesis in algae. Enhancement of carotenoids is essential to meet the market requirement as well as to overcome the difficulties in their production, which generally requires much time and input by other routes. Rabbani *et al*. (1998) showed that the synthesis of β-carotene is dependent on the triacylglycerol deposition in *D. bardawil.* Upon blocking triacylglycerol production it was observed that overproduction of β-carotene was blocked indicating the inter-dependency. This also indicated that under normal growth conditions the carotenoid pathway is not maximally active and the same can be appreciably stimulated in the presence of sequestering structures, creating a plastid-localized sink for the end product of the carotenoid biosynthetic pathway. The present study is one such study, which involves utilization of statin – a well known and a highly specific inhibitor of HMGR in higher plants – to enhance the carotenoid content.

In general *Dunaliella* requires high light intensity (240-300 μmol/m^2/s) to accumulate carotenoids. In this study a treatment of statin induced the accumulation of carotenoids under normal white light (horizontal) of intensity 22-26 μmol/m^2/s. This implies better production of carotenoids under indoor conditions and under low light intensity, as enhancement of light intensity in a closed system is a major problem since the radiation produced will heat up the culture. It is evident that the majority of algae exhibit a temperature growth response curve wherein a pronounced inhibitory effect occurs at temperatures which exceed the optimum by only 2-3ºC. And an increase in temperature may eventually cause the breakdown of protein structures so that affinity for substrate and enzyme regulators will be affected (Richmond 1986). The conventional acetate mevalonate (Ac-MVA) pathway operates in the cytoplasm and in mitochondria to predominantly synthesize sterols, sequiterpenes and ubiquenones. The plastidic non-mevalonate pathway however, operates to synthesize hemi, mono, sesqui- and diterpenes, along with carotenoids and the phytol chain of chlorophyll is well established (Dubey *et al*. 2003). So, in this study the enhancement of carotenoid content may be mainly due to the formation of carotenoids via the non-MVA pathway. It is evident from the results that sterol synthesis is reduced (evidenced by the squalene contents in the atorvastatin-treated cultures) more than the control *Dunaliella* cultures. This was proved in other unicellular micro-

algae like *Chlorella* and *Scenedesmus* (Saakov 2003). This is the first report, which supports the enhancement of carotenoid biosynthesis by the possible involvement of DOXP or non-MVA pathway in halotolerant microalgae *D. bardawil* and *D. salina*. It is also the first attempt to increase the carotenoid biosynthesis in *Dunaliella* using statin as an HMGR inhibitor (**Fig. 1**). *Lycopene cyclase* is an enzyme involved in the conversion of lycopene to β-carotene (Sandmann 1994) and hence its concentrations were estimated; the results show that the content of *lycopene cyclase* increased in the atorvastatin-treated cultures than in the control cultures. It is correlated with a reduction in squalene, a precursor for steroid synthesis, and hence it could be concluded that statins can be used to increase the secondary metabolites like carotenoids under normal growth conditions as an alternative of various stress conditions like high light intensities, high salt concentration, nitrate or phosphate deficiencies and duration. These results indicate that an increase in β-carotene production may be due to the increased flux through the non-MVA pathway and also may be due to the reduced catabolism of carotenoids in response to inhibition of cytosolic MVA pathway. Rodríguez-Concepcion and Gruissem (1999) reported similar results in tomato fruits in which they showed an increase in the accumulation of the carotenoid lycopene in lovastatin-treated tomato fruits. Similar results were observed by Brain *et al*. (2006) who reported the concentration-dependent decrease of sterols with statin (atorvastatin and lovastatin) treatment in an aquatic plant *Lemna gibba*; accordingly an increase in the concentrations of end products of the parallel unaffected non-mevalonate pathway.

However, further experiments are required to fine-tune the mechanism and will help in commercialization of the technique for wider practical application. Statins are required at very low concentrations to enhance the productivity of carotenoids and more importantly they are not extracted along with the carotenoids, which is due to differences in solubility. Hence it can be safely used to increase the commercial production of the highly valuable and bioactive compound β-carotene.

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