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Effect of Dissolved Inorganic Carbon on β-Carotene and Fatty Acid Production in *Dunaliella* sp

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Abstract This study aimed to explore the effect of sodium bicarbonate (0–200 mM) on the production of β-carotene and lipid content in *Dunaliella salina* and *Dunaliella bardawil*. Total carotenoid and chlorophyll content were determined at regular intervals by a UV–VIS spectrophotometer. The β-carotene and lipid contents were analyzed using high-performance liquid chromatography (HPLC) and gas chromatography coupled with mass spectrometry (GC-MS). The HPLC results revealed a twofold increase of β-carotene in *D. salina* and *D. bardawil* cultures grown with sodium bicarbonate. Moreover, total fatty acid profiles from GC-MS indicated a maximum relative percentage of saturated fatty acids (tetradecanoic acid, 10,13-diethyl, methyl ester and methyl 16-methyl-heptadecanoate) compared to polyunsaturated fatty acids in both algae. Our results indicate that the optimum concentration of bicarbonate (100 to 150 mM) was required to stimulate a positive effect on β-carotene production as well as the lipid profile in *Dunaliella* sp.

Keywords β-Carotene · Chlorophyll · *Dunaliella salina* · *Dunaliella bardawil* · Bicarbonate · Saturated fatty acids

Introduction

Nutritional modifications such as limitation, depletion, and supplementation in culture media, plays a major role in enhancing the biochemical constituents of marine microalgae. Microalgae have the ability to adapt their cellular and physiological patterns with respect to nutrient availability by altering the metabolic pathways resulting in the production of several metabolites such as lipids, carbohydrates, and proteins. The ability of algae to survive under extreme conditions causes unusual changes in the level of intracellular components and lipid content [34]. Since algae have the ability to survive under extreme conditions and also produce acyl and nonacyl lipids (carotenoids), further modification in the media components might result in

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the improvement of the quality of nutritionally valuable lipids [22]. However, this is not always advisable because the high lipid content in the cell when produced under stress condition often results in significantly low biomass content. There are few algal species, including *Chlorella* sp. [16, 26], *Dunaliella* sp. [5, 10, 11, 34, 37], and *Nannochloris* sp. [38], which have been reported to have the capacity to accumulate a high quantity of carotenoids and lipids under favorable conditions [15, 24, 36]. The physiology and growth of marine microalgae not only depended on factors such as nutrient depletion, osmotic stress, temperature, and light, but is also influenced by the carbon source that is available in the medium. Inorganic carbon sources primarily can be considered as one of the main factors that help enhance the lipid and carotenoid content in cells by improving photosynthetic efficiency and growth rate [9, 19]. Carbon dioxide can be taken up and utilized by microalgae in two forms, namely inorganic carbon (sodium bicarbonate) and as gaseous carbon-dioxide. Only few algae have the ability to uptake direct carbon dioxide as a source for its growth, while others convert gaseous carbon into bicarbonate as a chemical disequilibrium and utilize this for photosynthesis. Moreover it also acts to reduce the contamination of other indigenous microorganisms and as a buffering agent to maintain the growth at elevated pH [13, 31, 32]. As sodium bicarbonate is convenient to transport, cost effective, and feasible, it can be considered as an excellent supplement for the growth of algae when compared with gaseous carbon source.

However, there are no reports on the induction of β-carotene production in *Dunaliella* sp. by the addition of sodium bicarbonate. Thus, the study aims to enhance the production of biomass and intracellular biochemical components of marine algae *Dunaliella salina* and *Dunaliella bardawil* by the addition of dissolved inorganic carbon (DIC) source.

Materials and Methods

Chemicals

Chemicals used for media preparation and HPLC-grade solvents were purchased from Himedia Laboratories, Mumbai, India. Standard β-carotene was obtained from Sigma-Aldrich (India). All other chemicals and solvents used were of analytical grade.

Algal Strains and Culture Conditions

Algal strains *D. salina* and *D. bardawil* were acquired from the Centre for Advance Science Botany, Madras University, Chennai. Axenic cultures were maintained in *De Walne's* medium [28] which was composed of (sterile sea water per liter) 125.06 g NaCl, 779.22 mg $FeCl_3$, 360 mg $MnCl_2{\cdot}4H_2O$, 33.3 mg H_3BO_3, 44.652 mg $C_{10}H_{14}N_2Na_2O_8{\cdot}2H_2O$, 20.28 mg NaH_2PO_4, 100.3 mg $NaNO_3$, 20.829 mg $ZnCl_2$, 9 mg $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 19.04 mg $CoCl_2{\cdot}6H_2O$, and 20 mg $CuSO_4{\cdot}5H_2O$. Both strains were incubated at 24±1 °C in a growth chamber with illumination of 22 μmol m^{-2} s^{-1} under a 16:8-h photoperiod. The cultures were shaken manually on a day-to-day basis.

Experimental Conditions

The effect of changes in the level of carotenoid and lipid content by the addition of sodium bicarbonate, ranging from (50 mM to 200 mM) were examined in De Walne's medium. At regular intervals, chlorophyll and total carotenoids were measured, and on the 28th day,

cultures were harvested and used for estimating β-carotene content using HPLC and fatty acid by GC-MS.

Growth, Biomass, and pH of the Medium

The microalgal concentration and pH of the medium were determined at regular intervals by measuring optical density at 680 nm using a UV–vis spectrophotometer, GeneQuant 1300 (GE Healthcare), and EcoTestr pH1 (Thermo Scientific), respectively. Biomass concentration was determined by measuring dry cell weight. Ten milliliters of cultures were harvested and the pellets were dried in an oven at 80 °C until a consistent weight was attained [4].

Chlorophyll and Carotenoid Measurement

Dunaliella cells were extracted with 100 % acetone by incubation in the dark for 30 min and the supernatant was separated. Chlorophyll and carotenoid content present in the supernatants was measured spectrophotometrically using the GeneQuant 1300 UV spectrophotometer (GE Healthcare) [25].

Extraction and Determination of Total Lipid

Lipids were extracted from lyophilized algal cells by adding a methanol/chloroform (1:1) solvent mixture. The chloroform layer was separated and the remaining solids were re-extracted with the solvent mixture. The solvent layer was then passed through sodium sulfate using Whatman No. 1 filter paper and allowed to evaporate at 40 °C. Total lipid content was measured by the gravimetric weight of the extracted lipid [33].

Analysis of β-Carotene by HPLC

Freeze-dried algal biomass was extracted with acetone and analyzed using HPLC. Chromatographic analysis was performed on a reverse phase silica C_{18} column using Shimadzu LC 20A system equipped with a double pump (LC-20AT) and UV–vis detector (SPD-20A), using isocratic solvent acetonitrile/methanol/dichloromethane (70:10:20) at a flow rate of 1.0 ml and detected at 467 nm [20].

Analysis of Fatty Acid by GC-MS

Fatty acid methyl esters (FAMEs) were obtained from crude lipids as described in [33]. FAME composition was determined using Perkin Elmer Clarus 680 (GC-MS) and an Elite-wax (30.0 m, 0.25 mm ID, 0.25 μm df). The column was held at an initial temperature of 60 °C for 1 min and ramped to 300 °C at 10 °C/min, and it was then held for 6 min. The transfer line between GC-MS was kept at 240 °C.

Statistical Analysis

All the experiments were performed in triplicates. The analysis of variance (two-way ANOVA) between the nitrogen-depleted group and the sodium bicarbonate-supplemented groups was carried out using Tukey's multiple comparisons test at $p<0.05$ in GraphPad Prism (Version 6.0).

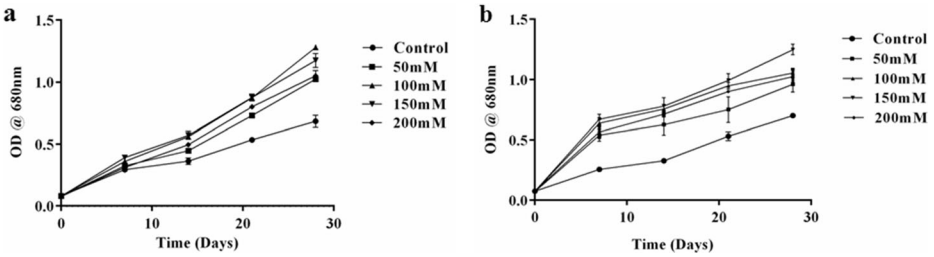

Fig. 1 Growth of *Dunaliella* sp. at various concentrations of sodium bicarbonate. **a** Growth of *D. salina*; **b** growth of *D. bardawil*. Values are expressed as mean±SD (n=3)

Results

Growth, Biomass, and pH in the Medium Added with Sodium Bicarbonate

Growth, biomass, and pH of the algal cultures were monitored till 28 days with different concentrations of sodium bicarbonate. We observed that there was an increase in the biomass of microalgae grown on media with sodium bicarbonate in compared to control. Maximum growth and biomass was attained at 100 mM concentration of bicarbonate. At 150 mM and above, there was a marked decrease in the growth and biomass of microalgae was observed which is presented in Figs. 1 and 2. The optimum level of sodium bicarbonate for the growth of *Dunaliella* sp. was in the range of 100 to 150 mM concentration. Excess addition of sodium bicarbonate did not cause much change on the pH of the medium during growth of the microalgae. The final pH in both algal cultures was recorded as 9.1 (Fig. 3). This indicates that *D. salina* and *D. bardawil* have the ability to withstand a high concentrations of bicarbonate.

Effect of DIC on Chlorophyll and Total Carotenoid Content of Microalgae

Spectrophotometric analysis indicates that the level of chlorophyll and carotenoid content of algae increases at various levels of sodium bicarbonate concentrations ranging from 0 to 200 mM of *D. salina* (Fig. 4) and *D. bardawil* (Fig. 5). The highest yield of chlorophyll and carotenoid was observed in the range of 50–150 mM concentration between the 14th and 21st day of incubation in both microalgae. The maximum amount of chlorophyll a and b was 4.12±

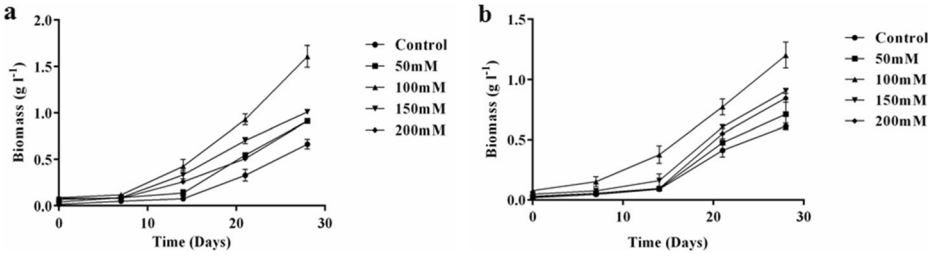

Fig. 2 Biomass concentration of *Dunaliella* sp. at various concentrations of sodium bicarbonate. **a** Biomass concentration of *D. salina*; **b** biomass concentration of *D. bardawil*. Values are expressed as mean±SD (n=3)

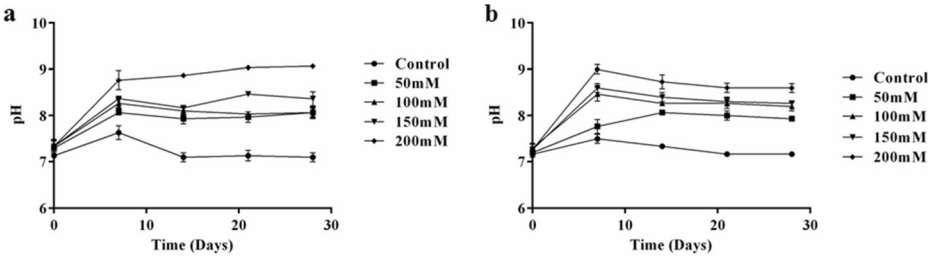

Fig. 3 pH variation during the growth of *Dunaliella* sp. at various concentrations of sodium bicarbonate. **a** pH variation during the growth of *D. salina*; **b** pH variation during the growth of *D. bardawil*. Values are expressed as mean±SD (n=3)

0.07 and 1.85±0.01 μg ml^{-1} in *D. salina* (Fig. 4a and b) and 2.80±0.06 and 1.80±0.06 μg ml^{-1} in *D. bardawil* (Fig. 5a and b), respectively. In *D. salina*, the highest amounts of carotenoid were obtained at a concentration of 150 mM (7.10±0.08 μg ml^{-1}) in comparison to the control (Fig. 4c), whereas in *D. bardawil*, the level of carotenoids was found to be relatively lesser in the corresponding concentration in comparison with *D. salina* (Fig. 5c) along with a substantial decrease in growth parameters such as the chlorophyll content of the algae as the concentration of sodium bicarbonate increases.

Effect of Sodium Bicarbonate on β-Carotene of Microalgae

The crude acetone extracts obtained from the cultures grown with different concentrations of sodium bicarbonate (Fig. 6) was used for HPLC analysis, and it was found that the concentration of β-carotene in *D. salina* and *D. bardawil* was found to increase substantially up to 150 mM concentration of bicarbonate-supplemented cultures (Table 1). Among the two species, *D. salina* (180.06±0.07 μg 100 mg^{-1}) showed an increase in the amount of β-carotene in comparison with *D. bardawil* (8.34±0.02 μg 100 mg^{-1}). This shows that sodium bicarbonate supplementation significantly enhances β-carotene content in *D. salina*.

Effect of Sodium Bicarbonate on Lipid Content of Microalgae

The total lipid content of the cultures when analyzed gravimetrically (triplicate) revealed that the total amount of cellular lipid was significantly higher in the culture added with bicarbonate compared to the control. In both microalgae species, high lipid content was noticed at 100 mM concentration of bicarbonate. *D. salina* has attained a maximum content of lipid (1.15±0.01 g l^{-1}) than *D. bardawil* (0.95±0.01 g l^{-1}). This result indicates that sodium bicarbonate is found to be an enhancer of total lipids in both the strains under study (Fig. 7).

Effect of DIC on the Relative Fatty Acid Composition of *D. salina* and *D. bardawil*

Since the total lipid content of the cultures upon addition with sodium bicarbonate was significantly higher in comparison with the control, the relative fatty acid composition was studied using FAME analysis. From the results, we could find a remarkable increase of FAME percentage with the abundance of two major fatty acids, namely tetradecanoic acid, 10,13-diethyl, methyl ester (27.55 %) at 150 mM and a steady increase of methyl 16-methyl-heptadecanoate (29.69 %) at 200 mM (Table 2). *D. bardawil* also showed maximum relative

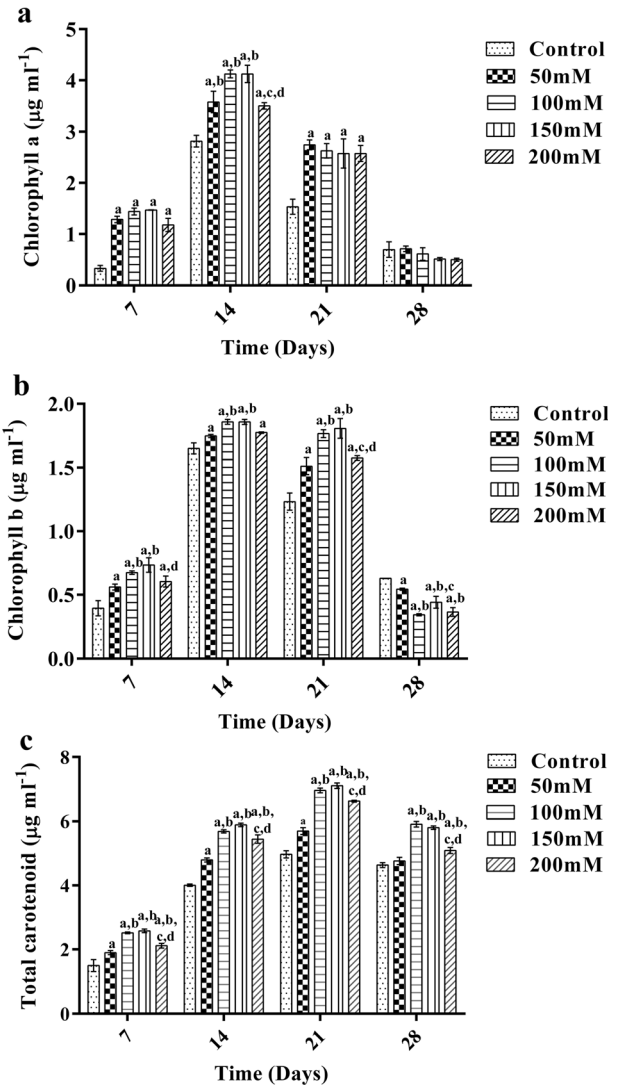

Fig. 4 Effect of sodium bicarbonate on cellular pigments in *D. salina*. **a** Chlorophyll a content; **b** chlorophyll b content; **c** total carotenoids. Values are expressed as mean±SD (n=3). *Letters a–d* represent significant values between 0, 50, 100, 150, and 200 mM (p<0.05)

content values of tetradecanoic acid, 10,13-diethyl, methyl ester (22.01 %) at 150 mM and methyl 16-methyl-heptadecanoate (18.94 %) at 100 mM (Table 3), respectively.

Discussion

Dunaliella sp. is a well-known photoautotroph and high salt-tolerant unicellular marine alga which grows at extreme environmental conditions. Being a photoautotrophic organism, it requires an adequate supply of inorganic carbon source for its regular growth and metabolism.

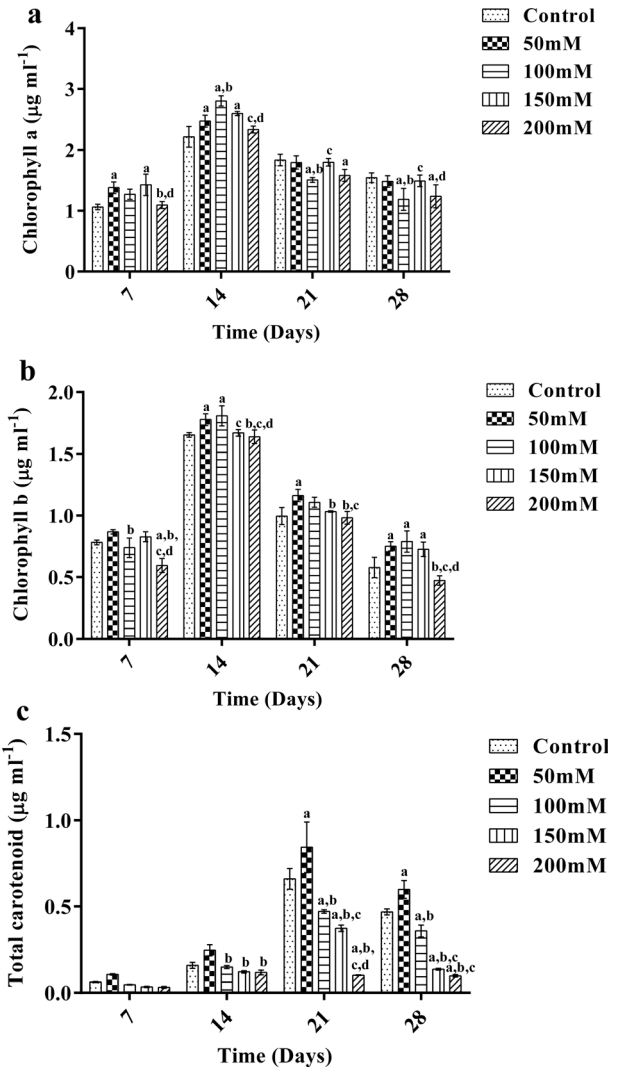

Fig. 5 Effect of sodium bicarbonate on cellular pigment in *D. bardawil*. **a** Chlorophyll a content; **b** chlorophyll b content; **c** total carotenoids. Values are expressed as mean±SD (n=3). *Letters a–d* represent significant values between 0, 50, 100, 150, and 200 mM (p<0.05)

This can be achieved by either in the form of gaseous or bicarbonate source. Being convenient to transport and cost-effective, inorganic carbon source is an alternative way for commercial usage. Many green algae can tolerate high sodium ion concentration but not more than 0.2 M concentration. Profound studies reported that *Dunaliella* strains were able to tolerate high pH and can be selected for bicarbonate-based integrated carbon capture and algae production systems (BICCAPS) [6]. In order to prove these findings, we carried out a study on the effect of sodium bicarbonate on carotenoid and fatty acid content in *Dunaliella* sp.

Sodium bicarbonate supplementation on algal biomass growth and enhanced production of carotenoid and lipid has been reported extremely in marine microalgae [29, 32, 37, 39]. So far,

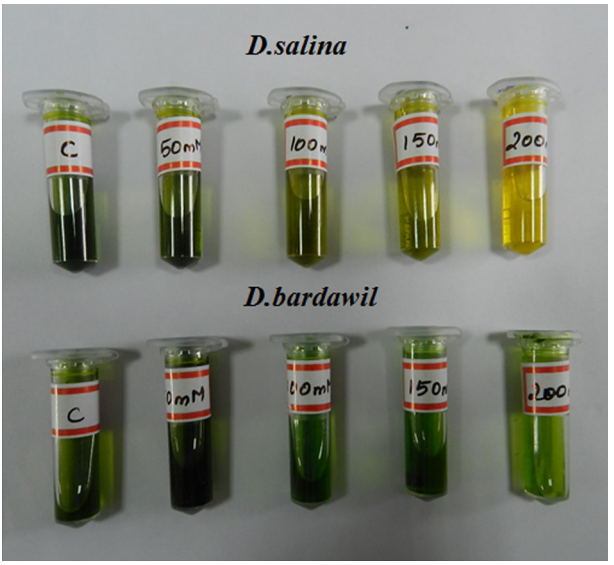

Fig. 6 Crude acetone extract of *Dunaliella* sp. for the quantification of β-carotene content

there are no extensive reports stating the effect of bicarbonate on the biochemical composition in *Dunaliella* sp. The results of the present study also revealed that the addition of dissolved bicarbonate can help to promote the growth and biomass of *Dunaliella* sp. In addition, we investigated the effect of bicarbonate on pH of the medium during the growth of algae and if pH can cause changes in the biochemical composition of the microalgae. In our study, there was an elevation in the level of pH during the growth which did not cause changes in the level of intracellular biochemical composition of microalgae. The result clearly indicated that sodium bicarbonate is a potential carbon source to enhance the production of biomass as well as growth.

Microalgae have two major divisions of intracellular pigments such as chlorophyll and carotenoids. In all photosynthetic organisms, carotenoids are highly masked by chlorophyll present in the cytoplast. These two pigments that coexist prevent the photodynamic sensitization of chlorophylls, which in their absence leads to the destruction of the chloroplast [12]. Chlorophyll a and b are prominent indicators for the growth of microalgae. Moreover, algal biomass can be indirectly quantified using chlorophyll content [8]. Especially, during high salt or nutrient-deficient condition, there was an arrest in the cell biomass and it leads to a decrease

Table 1 Effect of sodium bicarbonate on β-carotene in *D. salina* and *D. bardawil*

Sodium bicarbonate (mM)	*D. salina* (μg 100 mg^{-1})	*D. bardawil* (μg 100 mg^{-1})
0	76.2±0.04	4.66±0.05
50	134.3±0.10	8.34±0.02
100	180.6±0.07	1.81±0.03
150	180.5±0.08	0.89±0.01
200	37.20±0.06	0.05±0.01

Values are expressed in mean± SD (n=3)

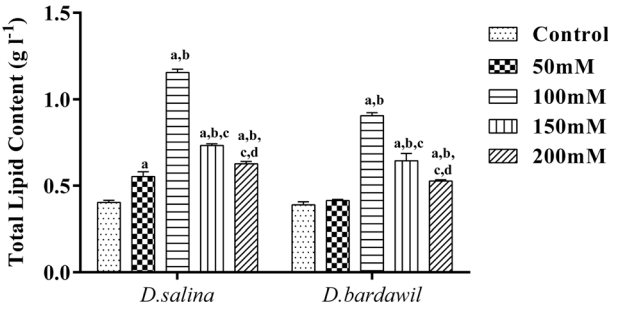

Fig. 7 Total lipid content of *D. salina* and *D. bardawil* was estimated gravimetrically at various concentrations of sodium bicarbonate. Values are expressed in mean±SD (n=3). *Letters a–d* represent significant values between 0, 50, 100, 150, and 200 mM (p<0.05)

in chlorophyll content and an increase in the amount of carotenoid have been reported. According to Ben-amotz and Avron [1], in *Dunaliella* sp., chlorophyll content was found to be inversely proportional to carotenoid production. In our study, microalgae underwent two stages during its growth period. In the initial stage, i.e., 0 to 14 days, there was an increase in the accumulation of total carotenoid and chlorophyll content. In the later stage, i.e., 18 days till the end of the period, there was an increase only in the carotenoid content and a gradual decline in the level of chlorophyll content. *Dunaliella* sp. is one of the best producers of β-carotene among marine microalgae [2, 3, 30], so we carried out a study on the effect of sodium bicarbonate on β-carotene through HPLC analysis. The HPLC analysis revealed that β-carotene content was increased twofold in the medium containing 100 mM of sodium bicarbonate as compared to the control. However, *D. salina* was a maximum producer of β-carotene than *D. bardawil*. There are lines of evidence of previous reports in accordance with our study where some *Dunaliella* sp. isolated from salt pans of Tamilnadu, India, were unable to produce carotene even at extreme conditions [17].

Biosynthesis of lipid and fatty acids in microalgae was highly dependent on various nutrients available. Several studies have reported that the addition of bicarbonate enhances the production of lipid content in many marine microalgae in response to cell division [14, 18, 21]. The present study reveals that the addition of bicarbonate results in a relative increase in

Table 2 Effect of bicarbonate on the relative percentage fatty acid content in *D. salina*

FAMEs	Sodium bicarbonate (mM)				
	0	50	100	150	200
Methyl 12-methyl-tridecanoate	1.50	1.48	1.02	1.17	1.30
Tetradecanoic acid, 10,13-diethyl, methyl ester	23.77	24.22	23.68	27.55	24.33
Methyl 10-*trans*, 12-*cis*-octadecadienoate	0.73	1.19	1.68	1.65	1.81
Methyl 7,11,14-eicosatrienoate	2.97	5.32	7.22	6.68	7.70
Methyl 16-methyl-heptadecanoate	25.27	26.61	27.84	28.97	29.69
Methyl 11,14-eicosadienoate	0.63	–	–	–	–
Heptacosanoic acid, 25-methyl, methyl ester	1.39	1.42	1.02	1.11	1.49

Values are represented in relative content% of FAMEs

Table 3 Effect of bicarbonate on relative percentage fatty acid content in *D. bardawil*

FAMEs	Sodium bicarbonate (mM)				
	0	50	100	150	200
Methyl 12-methyl-tridecanoate	0.90	1.02	0.90	0.83	0.83
Methyl 6,9,12,15,18-heneicosapentaenoate	1.10	5.22	2.19	1.09	1.11
Tetradecanoic acid, 10,13-diethyl, methyl ester	20.79	20.58	18.98	22.01	18.68
Methyl 10-*trans*, 12-*cis*-octadecadienoate	4.22	3.93	3.01	6.51	4.85
Methyl 7,11,14-eicosatrienoate	9.70	9.07	6.93	14.19	11.27
Methyl 13-octadecenoate	0.98	0.90	0.75	1.25	0.81
Methyl 16-methyl-heptadecanoate	13.70	17.80	18.94	18.32	17.66
Methyl 11,14-eicosadienoate	–	0.93	0.89	0.48	0.84
Heptacosanoic acid, 25-methyl, methyl ester	0.93	0.96	1.11	1.13	0.93

Values are represented in relative content% of FAMEs

total lipid content from 0.46±0.02 to 1.15±0.01 g l^{-1} in *D. salina* and 0.48±0.04 to 0.95±0.01 g l^{-1} in *D. bardawil*, respectively. The data obtained in the experimental results corroborate with those of other reports [7, 38]. Normally under limitation or starvation condition, microalgae are the main key factors that control the accumulation of oil associated with changes in fatty acid composition [23, 35, 40]. During this condition, saturated fatty acids are produced predominantly compared to polyunsaturated fatty acids. Our results proved that a major amount of saturated fatty acid is produced than monounsaturated and polyunsaturated fatty acids. This may be due to the adverse effect on desaturation and elongation of fatty acid synthesis during high sodium bicarbonate condition [27].

Our study concludes that the optimum concentration of bicarbonate was in the range from 100 to 150 mM to enhance the production of β-carotene and fatty acids in *Dunaliella* sp. Thus, sodium bicarbonate can be considered as a suitable carbon source to be considered for large-scale production to improve the yield of β-carotene and lipid content particularly in *Dunaliella* sp.

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