Study of the changes in the growth, protein, and bioactive profile of *Chlorella emersonii* KJ725233 in response to sodium and ammonium nitrate

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ABSTRACT

Chlorella emersonii KJ725233 is a non-fastidious microalga isolated from the western regions of Maharashtra, India. The alterations in its chlorophyll, protein, and antioxidant content in response to cationic stress were studied. Chlorella emersonii KJ725233 (CEK) was subjected to $NaNO_3$ and $NH_4 (NO_3)^2$ at concentrations equivalent to 0.9 g/l (1×) and 1.8 g/l (2×) in the cultivation media. Qualitative alterations in the bioactives of the microalga were identified by gas chromatography–high-resolution mass spectrometry (GC-HRMS), while the protein, chlorophyll, and antioxidant manipulations were spectrophotometrically quantified. Doubling of the protein content was observed when CEK was grown in 1× NH_4^+, whereas in 2× NH_4^+, chlorosis was significant. 2× NH_4^+ also induced oxidative stress on CEK as evident from the 85.72% ± 6.72%, 197.47% ± 7.01%, 22.24% ± 1.78%, and 187.37% ± 1.88% increase in antioxidant potential, ferric reducing capacity, radical scavenging potential, and total phenolic content, respectively, as compsared to CEK grown in 1× Na^+. These alterations as indicated from the GC-HRMS data correlate to the bioactive inductions/variations of/in Vitamin E, phytol, and its isomer in CEK grown in 2× NH_4^+. The study thus indicates that manipulations of nitrate salts in the media significantly induce, as well as alter the concentrations of commercially significant compounds like Vitamin E, phytol, etc.

1. INTRODUCTION

Cultural conditions such as nutrient composition, as well as their concentrations, temperature, light intensity, photoperiod, and pH are known to affect the growth and induce changes in the biochemical composition of microalgae [1]. This is mainly due to the stressed altered metabolic pathways leading to the induction of bioactives that eventually enable it in its survival and adaptation to the coarse growth environments [2]. Therefore, in order to explore the commercial potential of the microalga, there is a necessity to manipulate the growth conditions so as to alter the synthesis of biologically active microalgal compounds [3]. The amount of these bioactives is, however, largely species-specific and is not only reliant on growth conditions but also on the age of the culture [2].

Nitrogen, the growth limiting factor, is incorporated in the algal culture media in the form of a variety of organic (amino acids and urea) or inorganic (nitrates, nitrites, and ammonium salts) nitrogen sources [4]. Alterations in the type and concentration of the nitrogen sources are known to affect the growth, as well as the lipid content of microalgae [3,5–11]. These nitrogen sources are usually first reduced to ammonium, which is directly taken up by facilitated diffusion and assimilated into amino acids [5,12]. It is also known to affect the protein synthesis by increasing the functional activity of the ribosomes and hence, the nutritional content [13]. Nitrate-N is the most widely used nitrogen source for microalgae since it is not known to evaporate on autoclaving of the media [14]. Also, unlike Ammonium-N, Nitrate-N is less likely to induce an acidic pH shift of the medium. Ammonium assimilation leads to a drop in pH, thus reducing the growth rate, whereas a rise in pH can be observed in Nitrate-N growing culture with an increased growth rate [5]. Contrarily, sodium plays an important role in the nutrient transport system, especially in nitrate transport, as well as maintaining osmoregulation and photosynthesis [15]. The advantages and the role of nitrates along with sodium,

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therefore, make sodium nitrate the most preferred nitrate salt in the algal culture medium. The pH shifts induced may, in turn, alter the antioxidant status of the alga by stimulating the pH-dependent reactive oxygen species (ROS) generating enzymes all of which mainly produce H_2O_2 [16].

Chlorella strains due to their cosmopolitan occurrence are naturally designed to counteract inconsistent environmental hostilities by synthesizing a variety of metabolites [4,17,18]. *Chlorella emersonii* KJ725233—a novel, fast growing, non-fastidious microalga with inherently higher antioxidant content was isolated from the western regions of Maharashtra, India [19]. Though the effect of nitrate sources and its concentrations have been studied on the growth and lipid productivity of a few *Chlorella* species, its effect on the metabolic alterations and hence, antioxidant responses under such stressed conditions are still untapped. In order to exploit the potential of *C. emersonii* KJ725233 as a source of nutraceuticals and/or cosmeceuticals, the present study was designed to determine the effect of Na^+, NH_4^+ ($NaNO_3$, NH_4NO_3) and their concentration on its growth (chlorophyll, carotenoids, and biomass), protein content, alterations in the bioactives, and hence, its antioxidant potential.

2. MATERIALS AND METHODS

2.1. Microalgal Strain and Culturing

Chlorella emersonii KJ725233 isolated from the western regions of Maharashtra, India [19], was cultured in BG-11 medium with a 12 hours photoperiod and 24 hours of aeration at 28°C ± 2°C till the logarithmic phase. After 15 days, this seed culture was inoculated into four different variants of BG-11 medium: (A) 1× $NaNO_3$ (1.5 g/l), (B) 2× $NaNO_3$ (3.0 g/l), (C) 1× NH_4NO_3 (1.4 g/l), and (D) 2× NH_4NO_3 (2.8 g/l); such that the nitrate concentration in 1× NO_3^- salt was 0.9 g/l, whereas in 2× it was 1.8 g/l. Since BG-11 medium, in general, contains 1.5 g/l of $NaNO_3$, 1× $NaNO_3$ was considered as a control. The alga was grown in the nitrate variants until the late stationary phase was reached.

2.2. Measurement of Growth

After every 5 days, 10 ml of the culture was taken in a pre-weighed tube and centrifuged at 2,800 rcf for 15 minutes. The biomass was weighed and extracted in methanol on a shaker at 60°C for 2 hours. These suspensions were centrifuged and the absorbance of the supernatant was read on a Thermo Fischer MultiScan Go at 665, 652, and 470 nm. The chlorophyll, as well as carotenoid concentration in terms of µg chlorophyll/carotenoid content per gram fresh weight of chlorella emersonii KJ725233 (CEK) (µg/gfw) was determined by the following formulas [20,21]:

$$\text{Chlorophyll a}_{(\mu g/ml)} = 16.72 * \text{Absorbance}_{(665)} - 9.16 * \text{Absorbance}_{(652)}$$

$$\text{Chlorophyll b}_{(\mu g/ml)} = 34.09 * \text{Absorbance}_{(652)} - 15.28 * \text{Absorbance}_{(665)}$$

$$\text{Carotenoids}_{(\mu g/ml)} = \frac{(1,000 * \text{Absorbance}_{(470)} - 1.63_{\text{Chlorophyll a}} - 104.96_{\text{Chlorophyll b}})}{221}$$

After the late stationary phase was reached, the cultures were centrifuged at 2,800 rcf for 15 minutes and the biomass yield (g/l) was determined. The biomass was rinsed with distilled water

and dried at 60°C for 48 hours. The dried biomass was then used to determine the protein content, antioxidant potential, phenolic content, and the metabolite alterations by gas chromatography–high-resolution mass spectrometry (GC-HRMS).

2.3. Estimation of the Protein Content

In order to determine the protein content, 0.2 g of dried biomass was extracted in 1 N NaOH. The extraction was carried out thrice and the supernatants were pooled together. The protein content was estimated by Bradford's method [23,24].

2.4. Preparation of *Chlorella Emersonii* KJ725233 Extracts

The dried biomass of *C. emersonii* KJ725233 was suspended in methanol at a concentration of 0.1 g/ml. These suspensions were sonicated for 30 minutes in ice in a bath sonicator. The suspensions were centrifuged at 2,800 rcf for 15 minutes and the supernatants were transferred to a fresh plate. The extraction was carried out thrice and the supernatants were pooled together. One microliter of these extracts was taken up for the bioactives identification by GC-HRMS, whereas the remaining were allowed to dry at room temperature (30°C ± 2°C) and then reconstituted in absolute dimethyl sulfoxide. These dimethyl sulfoxide extracts were further used to determine the antioxidant, as well as phenolic content.

2.5. Antioxidant Potential

2.5.1. Total antioxidant capacity

The total antioxidant capacity (TAC) was determined by the phosphomolybdenum method described earlier [24]. Three hundred microliters of the TAC reagent (0.6 mmol sulphuric acid, 28 mmol sodium sulphate, and 4 mmol ammonium molybdate) was added to 30 µl of the extracts and incubated at 95°C for 90 minutes. After incubation, the absorbance was measured at 695 nm in a Thermo Fishcer MultiScan Go. Ascorbic acid was used as a standard and antioxidant capacity was expressed as mg ascorbic acid equivalence per g dried biomass (mg/g AAE DW).

2.5.2. Ferric reducing antioxidant potential

The ferric reducing antioxidant potential (FRAP) was determined by the potassium ferricyanide method as reported [25]. To 50 µl of the extracts, 125 µl of 0.1% potassium ferricyanide was added and incubated at 50°C for 20 minutes. After incubation, 125 µl of 10% trichloroacetic acid was added. One hundred microliters of these reaction mixtures were then transferred to fresh wells with an equal volume of distilled water. Finally, 20 µl of 0.1% ferric chloride was added and the absorbance was immediately measured at 700 nm in a Thermo Fishcer MultiScan Go. Ascorbic acid was used as a standard and the FRAP was expressed as mg AAE per g dried biomass (mg/g AAE DW).

2.5.3. DPPH radical scavenging potential

The radical scavenging potential was determined by 2,2 – diphenyl- 1-picrylhydrazyl (DPPH) radical scavenging assay as previously described [26]. One hundred and fifty microliters of 0.2 mM methanolic DPPH was added to an equal volume of various concentrations of the extracts (2–10 mg/ml) and incubated in the

dark at room temperature for 30 minutes. After incubation, the absorbance was measured at 517 nm. Ascorbic acid was used as a standard. Percent inhibition was calculated by the following formula:

$$\frac{Absorbance_{(blank)} - Absorbance_{(test)}}{Absorbance_{(blank)}} * 100 = \% \text{ inhibition}$$

Absorbance $_{(blank)}$ − Absorbance of blank

Absorbance $_{(test)}$ − Absorbance of test

A graph of extract concentration versus percent inhibition was plotted and inhibition concentration $(IC)_{50}$ (mg/ml) was determined.

2.6. Total Phenolic Content

The total phenolic content (TPC) was determined by the Folin's Ciocalteau method [27]. Twenty-five microliters of 1 N Folin's Ciocalteau reagent was added to 50 µl of the extracts and incubated for 5 minutes at room temperature in the dark. After 5 minutes, 125 µl of 20% Na_2CO_3 was added and further incubated at room temperature in the dark. The absorbance was measured at 765 nm in a Thermo Fishcer MultiScan Go. Gallic acid was used as a standard and the TPC was expressed as mg gallic acid equivalence per g dried biomass (mg/g GAE DW).

2.7. Identification of Bioactives by GC-HRMS

GC-HRMS analysis of all the methanolic extracts was carried out using GC (Agilent Technologies, USA) equipped with Accutoff mass spectrometry (MS). Compounds were separated on HP-5 MS capillary column having 5% phenyl polysiloxane as stationary phase, column length 30 m, internal diameter 0.32 mm, and film thickness 0.25 µm. One microliter of the methanolic extracts was injected in the split ratio of 10:1, the injector and transfer line temperatures were 250°C and 260°C while the ion source temperature was 200°C. Oven temperature was programmed from 80°C to 280°C at 10°C minute^{-1}; flow rate of carrier gas helium was 1 ml minute^{-1}. Compounds were identified comparing their retention times and mass fragmentation patterns with the data of standards at the National Institute of Standards and Technology library [26].

2.8. Statistical Analysis

All experiments are performed in triplicates. Data are represented as mean ± standard deviation. Data were analyzed using one-way analysis of variance, and a p-value less than 0.05 was considered to be statistically significant.

3. RESULTS

3.1. Effect of Cations and its Concentration on Growth

A time course analysis was performed to determine the alterations in the growth in terms of the chlorophyll and carotenoid content of *C. emersonii* KJ725233 in response to the nitrate variants. At 30 days of incubation, the highest chlorophyll content of 1,322.79 ± 7.2 µg/g fresh weight was observed in *CEK* grown in 2× Na$^+$, whereas *CEK* grown in 2× NH$_4^+$ exhibited the lowest chlorophyll content of 663.69 ± 9.85 µg/g fresh weight (Fig. 1). After 35 days

of incubation, the highest biomass was obtained in 2× Na$^+$, whereas 2× NH$_4^+$ severely affected the growth of the microalga (Table 1). A 12.81% ± 0.13% increase in the biomass of *C. emersonii* KJ725233 in 2× Na$^+$ thus can be attributed to the increased chlorophyll content. Chlorosis induced by 2× NH$_4^+$ might have led to a 62.26% ± 0.45% decrease in its biomass (Fig. 1, Table 1).

3.2. Effect of Cations of Nitrate Salt on pH

The initial pH of all the four variants was equivalent; however, with the growth of *C. emersonii* KJ725233, the pH started becoming alkaline in case of Na$^+$ and acidic in case of NH$_4^+$. In Na$^+$, the pH increased from 7.2 to 9.2 ± 0.1, whereas in NH$_4^+$, the pH decreased from 7.2 to 6.2 ± 0.1 after 35 days of incubation (Fig. 2).

3.3. Effect of Cation of Nitrate Salt on Protein Content

The highest protein content of 56.41 ± 3.41 g% signifying an increase of 102.99% ± 6.66% was observed in CEK grown in the presence of 1× NH4+ as compared to CEK grown in the presence of 1× Na+. Also, a 10%–12% increase in protein content was observed in CEK grown in the presence of 2× Na+ (30.83 ± 1.37 g%) and 2× NH4+ (31.58 ± 0.38 g%) (Table 1).

3.4. Effect of Cation and its Concentration on Antioxidant Potential and Total Phenolic Content

The highest antioxidant potential of 30.9 ± 0.68 mg/g AAE was observed in 2× NH$_4^+$, whereas the lowest 8.55 ± 0.96 mg/g AAE
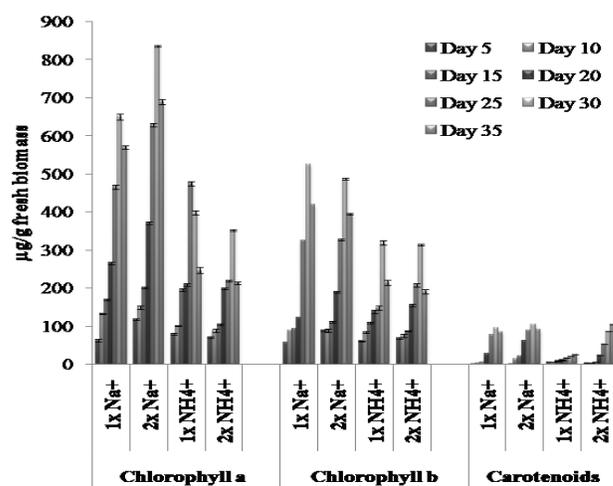

Figure 1: Effect of cations and their concentration on chlorophyll and carotenoid content of *C. emersonii* KJ725233 (p value < 0.05).

Table 1: Effect of cations on the biomass and protein content of *C. emersonii* KJ725233.

Cationic variants	Biomass (g/l)	Protein content (g%)
1× Na$^+$	11.794 ± 0.025	27.786 ± 1.265
2× Na$^+$	13.305 ± 0.128	30.833 ± 1.376
1× NH$_4^+$	8.599 ± 0.024	56.416 ± 3.412
2× NH$_4^+$	4.451 ± 0.117	31.25 ± 0.75

Values are expressed as mean ± SD. p value of less than 0.05 was considered to be significant.

was observed in $1\times$ NH_4^+ (Table 2). Significant alteration of 85.72% ± 6.72%, 197.47% ± 7.01%, and 187.37% ± 1.88% in total antioxidant potential, FRAP, and TPC was observed in $2\times$ NH_4^+ as compared to the control $1\times$ Na^+.

The highest IC_{50} 7.58 ± 0.18 mg/ml was observed in $2\times$ Na^+, whereas the lowest 4.19 ± 0.07 mg/ml was observed for $2\times$ NH_4^+ (Fig. 3). A significant increase of 22.24% ± 1.78% was seen in the IC_{50} of $2\times$ NH_4^+ as compared to that of the control $1\times$ Na^+.

3.5. Metabolite Profiles of *Chlorella emersonii KJ725233* by GC-HRMS Analysis

In order to confirm that the quantitative alterations observed in the antioxidant potential could be due to a corresponding occurrence of any such modifications in the antioxidant metabolites, the methanolic extracts of $1\times$ Na^+ and $2\times$ NH_4^+ were subjected to GC-HRMS analysis. GC-HRMS analysis revealed qualitative, as well as quantitative alterations in the metabolites in response to the two cations (Table 3).

Phytol, its isomer 3,7,11,15-Tetramethyl-2-hexadecen-1-ol and 1–Docosene were found at higher concentrations of 17.79%, 17.94%, and 9.51%, respectively, in $2\times$ NH_4^+. Similarly, alterations were also observed in the induction of hydrocarbons, as well as the fatty acids in both Na^+ as well as NH_4^+. The sterols induced in the presence of sodium were not observed in $2\times$ NH_4^+; however, the lipid-soluble antioxidant Vitamin E marked its existence in the same.
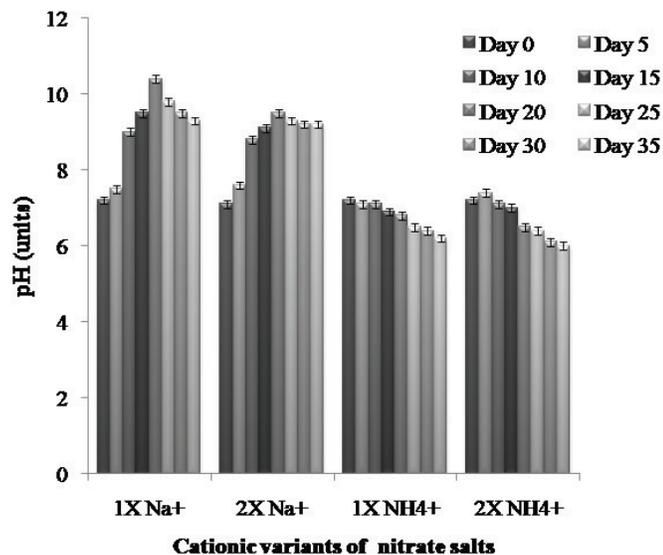
4. DISCUSSION

In order to determine the cationic effect of nitrate salts on the biomass as well as chlorophyll, carotenoid content of CEK, a time course analysis was performed. The study revealed though nitrate concentrations were kept constant, Na+ at a higher concentration stimulated growth in terms of both biomass productivity, as well as chlorophyll content, whereas NH4+ at a higher concentration repressed growth as evident by chlorosis and hence an increase in carotenoid content. Nitrogen manipulations are known to distress photosynthesis by reducing the proficiency of energy assemblage owing to chlorophyll loss and hence result in an increase in the non-photochemically active carotenoids [28].

Since the uptake of ammonium is energy efficient, it appears to be a favorable nitrogen source; however, factors like light intensity, presence of organic carbon, and transport of other molecules result in alternative algal nitrogen preferences [14]. Also, ammonium at higher concentrations decreases the pH of the medium by releasing H^+ cells, thus becoming inhibitory to cell growth, as well as causing cell lysis [5]. Similar results are earlier reported wherein they reported that the maximal biomass productivity of *Scenedusmus bijugatus* was observed in the presence of sodium nitrate over ammonium nitrate [29]. In the culture medium, ammonium nitrate splits up into ammonium ions (charged ionic ammonia species) and nitrate. These charged hydrated ammonium ions are known to be transported into the cell via specific channels. Ammonium, however, acts as a weak acid and further splits into the unionized uncharged ammonia species and a proton [30]. An increase in the protein content of *Helianthus annus* L. var Mammoth Russian in the presence of both ammonium and nitrate has been reported [31]. Also, a seven-fold increase in ribosome number of Soybean mixotrophic callus was observed in the presence of ammonium at 10–20 mM [13]. In the present study, 17.653 mM NH_4^+, as well as the presence of nitrate in $1\times$ NH_4NO_3 could, therefore, have led to a doubling in the protein content. 7.6 mM is the average optimum ammonium concentration for the Chlorophycean algae (*Chlorella vulgaris*, *Chlorella protothecoides*, *Chlorella* sp.); however,
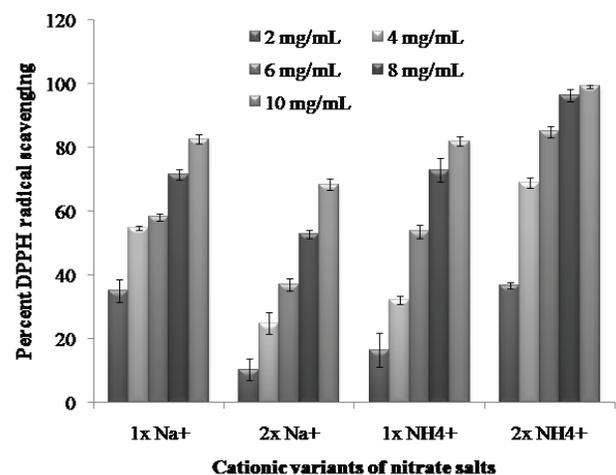
Figure 2: Effect of cations and their concentrations on pH of the culture media (*p* value < 0.05).

Table 2: Effect of cations on the TAC, FRAP, and the TPC.

	$1\times$ Na^+	$2\times$ Na^+	$1\times$ NH_4^+	$2\times$ NH_4^+
TAC (mg/g AAE)	16.65 ± 0.45	11.2 ± 0.086	8.55 ± 0.94	30.9 ± 0.69
FRAP (mg/g AAE)	6.25 ± 0.45	4.35 ± 0.18	2.82 ± 0.23	18.57 ± 0.80
TPC (mg/g GAE)	1.77 ± 0.06	1.79 ± 0.02	1.29 ± 0.03	5.08 ± 0.17

Values are represented as mean ± SD. *p* values less than 0.05 were considered to be significant.

Figure 3: Effect of cations and their concentration on the radical scavenging activity of *C. emersonii* KJ725233 (*p* value < 0.05).

Table 3: Effect of nitrate stress on compounds synthesized by *C. emersonii* KJ725233 as identified by GC-HRMS.

RT	1× NaNO$_3$		2× NH$_4$NO$_3$	
	Compound	Area (%)	Compound	Area (%)
17	Heptadecane	4.5	-	-
18.89	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	0.89	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	15.92
20.57	9,12-Octadecadienoic acid	3.577	-	-
20.70	cis, cis -7,10-Hexadecadienal	9.1	-	-
21.05	n-Hexadecanoic acid	13.1	n-Hexadecanoic acid	6.72
21.55	Phytol	0.34	-	-
23.46	9,12- Octadecadienoic acid methyl ester	0.88	-	-
23.57	9-Octadecanoic acid methyl ester	1.04	-	-
23.87	-	-	Phytol	17.79
24.33	9,12- Octadecadienoic acid	20.3	9,12 – Octadecadienoicacid	23.45
25.42	-	-	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	2.02
28.9	1-Docosene	4.77	1-Docosene	9.51
29.68	Ergosterol	4.54	-	-
31.05	Stigmasterol	20.95	13,14-Epoxytetradec-11-en-1-ol acetate	8.57
38.95	-	-	Vitamin E	4.89

RT = retention time in mins; Area % = Percentage area of the chromatogram.

they are able to tolerate ammonium at a concentration as high as 39 mM [32].

Unlike ammonium, uncharged ammonia species is highly permeable to the biological membrane and, therefore, penetrates into the cell through simple diffusion, thus causing swelling eventually leading to osmotic lysis of the cells [30]. Free ammonia toxicity not only just leads to lower cell densities but also induces oxidative stress leading to a decline in chlorophyll content and hence an overproduction of ROS. Free ammonia is known to induce light-dependent photodamage of photosystem II, thereby affecting the photosynthetic machinery [33]. Due to the excessive photosynthetic light, the production of ROS is accelerated at both the photosystems I and II. To counteract this oxidative stress, chloroplasts promote ROS scavenging through alterations in the antioxidant compounds (water soluble such as ascorbate, phenols; lipid soluble such as α-tocopherol), as well as antioxidant enzymes (superoxide dismutase, ascorbate peroxidase, and catalase) [34].

Chlorosis induced by 2× NH$_4$$^+$ led to a corresponding increase in the chlorophyll degradation product phytol along with its isomer 3,7,11,15-Tetramethyl-2-hexadecen-1-ol. Phytol is reported as an antioxidant, anti-nociceptive, anti-inflammatory, anti-mycobacterial, and anti-microbial whereas its isomer is a known antioxidant, as well as anti-microbial [35–39]. Phytol when phosphorylated yields phytyl-phosphate and phytyl-diphoshphate, which subsequently would feed into the tocopherol biosynthesis pathway [40]. The induction of this potent lipophilic antioxidant Vitamin E might, therefore, be attributed to the higher availability of phytol and its isomer in 2× NH$_4$$^+$.

Apart from Vitamin E, 9,12-Octadecadienoic acid methyl ester was also identified in 2× NH$_4$$^+$ at an equivalent concentration of that of the essential fatty acid 9,12-Octadecadienoic acid in the control. 9,12-Octadecadienoic acid methyl ester is reported as an anticancer compound [41]. The unusual sterols of *Chlorella*,

i.e., Ergosterol and Stigmasterol induced in Na$^+$ but not in NH$_4$$^+$ are reported hypocholesterolemic agents [42,43]. Higher concentrations of NH$_4$$^+$ are known to hinder sterol synthesis and hence could have accounted for their disappearance in 2× NH$_4$$^+$ [44,45]. Sterols play a significant role in maintaining the algal cellular membrane fluidity and hence permeability. Moreover, they serve as phytohormonal precursors and are involved in signal transduction in these organisms [46]. The hindrance in sterol synthesis could possibly be a defense mechanism of the alga to combat the NH$_4$$^+$ induced stress.

The antioxidant potential due to non-enzymatic antioxidants such as ascorbic acid, phenols, α-tocopherols, carotenoids, and reduced glutathione was measured by the phosphomolybdenum method, whereas that of hydrophilic antioxidants such as phenols was evaluated by the FRAP method [47,48]. Therefore, the quantitative alterations observed in the TAC and the FRAP could be attributed to a corresponding increase in the synthesis and/or induction of the antioxidant compounds in 2× NH$_4$$^+$. The increase in radical scavenging activity due to the lipophilic antioxidants as evaluated by DPPH might be due to the induction of the lipid-soluble antioxidant Vitamin E in 2× NH$_4$$^+$. In addition, phenols are considered to be exhibiting strong antioxidant potential due to their ability to inactivate free radicals, as well as prevent decomposition of hydrogen peroxides into free radicals [35]. Thus, the boosting of these potent antioxidants could attribute to the increased antioxidant potential of 2× NH$_4$$^+$.

The non-fastidious, robustly growing *C. emersonii* KJ725233 when subjected to varying nitrate conditions exhibited an increased growth in media with 2× Na$^+$. Its growth was, however, repressed in the media with 1× NH$_4$$^+$ and chlorosis set in 2× NH$_4$$^+$. However, the protein content doubled when grown in media containing 1× NH$_4$$^+$ as compared to Na$^+$. Although at 2× NH$_4$$^+$, the biomass was severely compromised due to chlorosis, an overproduction of ROS could have led to a doubling of its antioxidant content because

of the induction of the potent lipophilic antioxidant—Vitamin E. Although the responses may be species-specific, an appropriate cationic salt of nitrate should be considered for mass culturing of the alga depending upon the final nutraceutical and cosmeceutical formulations.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

FUNDING

ETHICS STATEMENT

We give our consent to participate under the 'Ethics, consent, and permissions' heading as applicable.

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