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Carotenoid Profile in *Prochlorococcus* sp. and Enrichment of Lutein Using Different Nitrogen Sources

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Abstract Various carotenoids of the cyanobacterium *Prochlorococcus* sp. are identified using chromatographic/spectroscopic techniques and quantified using HPLC-DAD. In the present study, β-apo-8′-carotenal was used as internal standard. Identification of carotenoids was carried out by comparing the retention time, absorption spectra, and mass spectra of unknown peaks with reference standards. All-*trans*-lutein was found to be the major carotenoid in this cyanobacterium, and, therefore, algal productivity and the potential for lutein accumulation were analyzed as a function of different nitrogen sources such as nitrate, nitrite, ammonia, and urea for cultivation. Among them, urea clearly led to the best lutein accumulation. According to the experimental evidence, lutein increased from 2.54 to 3.34 mg g^{-1} in the cyanobacteria when urea was used as the nitrogen source.

Keywords Lutein · Carotenoids · Microalgae · Extraction · *Prochlorococcus* sp. · HPLC-DAD

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Introduction

Among the photosynthetic pigments, carotenoids contain a C_{30} methyl-branched hydrocarbon backbone (Fernández-Sevilla et al. 2010). They are responsible for giving yellow, red, and orange colors in higher plants, algae, bacteria, fungi, and some animals. They are essential in light harvesting and photoprotection in all photosynthetic organisms. Moreover, carotenoids are valuable compounds used as food colorants, pharmaceuticals, and health-promoting compounds. For this reason, their worldwide demand has been growing markedly (Zhang et al. 2014). Unfortunately, there is no exact and definite extraction and saponification procedure for carotenoids as the food matrix might vary. Many organic solvents have been used and the selection of the appropriate one is not an easy task. Moreover, carotenoids are relatively stable in the matrix, whereas they are sensitive to light, heat, oxygen, and acid when they are in solution (Amorim-Carrilho et al. 2014). For these reasons, each carotenoid-containing matrix should be treated independently.

Carotenoids are lipid-soluble pigments and they may be divided into two groups: xanthophylls and carotenes. Many carotenoids have significant therapeutic properties and lutein is only one of them. Lutein is also a xanthophyllic compound, and its role in the prevention of chronic diseases and health-promoting mechanisms has been well documented. Lutein is a very effective antioxidant that also prevents and reduces the damage caused by free radicals (Richmond 1990; Le Marchand et al. 1993). It is recommended for the prevention of some types of cancer (Astorg 1997; Demmig-Adams and Adams 2002; Heber and Lu 2002), cardiovascular diseases (Dwyer et al. 2001), and retinal degeneration (Granado et al. 2003). The human body is unable to

produce lutein; thus, this compound can only be supplied through our diet. As lutein is yellow, it efficiently absorbs the blue light part of the spectrum. Blue light can damage the retina by inducing photo-oxidative decay. Lutein acts as blue light filter in the eye, and when sufficient levels of lutein are present in the macula, blue light is absorbed and photo-oxidation is minimized (Bendich and Olson 1989; Krinsky et al. 2003). It is also referred to as the macular pigment. Intake of lutein has been strongly correlated with decreased risks of cataracts and age-related retinal degeneration (Moeller et al. 2000; Olmedilla et al. 2001; Delcourt et al. 2006; Arnal et al. 2009).

Naturally occurring lutein is produced mainly in higher plants and algae. Compared with higher plants, algae have an advantage due to the possibility of cultivation in bioreactors on a large scale and thus providing a continuous and reliable source of the product (Shi et al. 1997, 1999; Borowitzka 2010). The current commercial source of pure lutein is marigold (*Tagetes erecta* L.). However, the lutein content of marigold flowers is low which makes alternative lutein-rich sources interesting. Microalgae have faster growth rates and more free lutein than marigold flowers, the current source of lutein. It has been reported that the lutein production rate of microalgae is three to six times higher when compared to the lutein production rate of marigold flowers. Furthermore, in order to produce 1 kg of pure lutein, marigolds need more land and water, but require less nutrients (N, P, K) and less energy than microalgae (Lin et al. 2015). Several microalgae are considered to be potential sources of lutein as they have high lutein (0.5–1.2 % dry weight) content (Fernández-Sevilla et al. 2010; Manke Natchigal et al. 2010).

In addition, the use of microalgae in biotechnology has gained a significant attention since they produce a great variety of metabolites that are essential to human health (Borowitzka 2013). These metabolites include proteins, vitamins, minerals, enzymes, fatty acids, xanthophylls, and carotenoids produced during normal growth phase and/or when exposed to different environmental factors (Jin et al. 2003).

Microalgae exhibit a great metabolic plasticity due to changes in response to environmental conditions, sometimes so-called stress factors. Among these, the most important factors that affect lutein content are illumination, temperature, nitrogen availability and source, salinity, the presence of oxidizing substances, and the growth rate. It should be emphasized that any of these factors can affect either lutein content or biomass productivity in opposite ways (Fernández-Sevilla et al. 2010). Therefore, it is important that the microalga should also have a high growth rate, even if exposed to stress conditions in order to induce the accumulation of lutein in order to achieve high lutein productivity.

The interest in lutein from microalgae is still relatively new. Several microalgae have been proposed as potentially useful to produce lutein, such as *Muriellopsis* sp. (Del Campo et al. 2001; Blanco et al. 2007), *Chlorella zofingiensis* (=*Chromochloris zofingiensis*) (Del Campo et al. 2001), *Chlorella protothecoides* (=*Auxenochlorella protothecoides*) (Wei et al. 2008), or '*Scenedesmus almeriensis*' (Sánchez et al. 2008). Most of these studies have shown how the culture conditions and biomass productivity were optimized to increase the amount of lutein. Apart from this, it should be noted that solvent extraction conditions may also be a consideration as each microalgal species have different morphologies. There is no exact procedure for the determination of carotenoids or a specific type of carotenoid in microalgae. For this reason, new studies must be directed towards its accurate identification and quantification.

The aims of this study were to investigate the carotenoid composition of a novel cyanobacterium, *Prochlorococcus* sp., by using chromatographic/spectroscopic techniques and enhance its lutein content by altering the source of nitrogen in culture media.

Materials and methods

Chemicals

All carotenoid standards were obtained from CaroteNature and *trans*-β-apo-8′-carotenal and pyrogallol were purchased from Sigma-Aldrich. All the solvents used were LC grade and obtained from Sigma-Aldrich.

Instrumentation

An Agilent 1200 Series HPLC-DAD system was used for the determination of carotenoids. Prior to use, all HPLC-grade mobile phases were degassed using an (Elmasonic S80H) ultrasonic bath.

The reversed phase column selected for use was a YMC carotenoid C_{30}, 250 mm × 4.6 mm, 5 μm (Waters, USA). The

Table 1 Gradient program optimized for the analysis of carotenoids by [a]HPLC-DAD

Solvents containing 0.1 % TEA			
Time (min)	MeOH	MTBE	H_2O
0	70	25	5
5	60	35	5
10	45	55	0
15	25	75	0

[a] Detection was done at 446 nm on C_{30} column with a flow rate of 1.0 mL min^{-1}

mobile phase consisted of methanol, methyl-*tert*-butyl ether, and water containing 0.1 % TEA and 0.01 % pyrogallol with a gradient program at a flow rate of 1.0 mL min^{-1}. The injection volume was 20.0 μL, and detection was by diode array detector with quantitation at 446 nm. The column temperature was set at 25.0 °C. The spectrum from 300 to 600 nm was recorded and stored for tentative identification of other carotenoids. The details of the gradient program are presented in Table 1.

Extraction of carotenoids was performed by using ultrasonic bath. The solvents were removed by rotary evaporator (Heidolph Hei-VAP Advantage).

Cultivation and preparation of *Prochlorococcus* sp.

Prochlorococcus sp. was obtained from the Ege University Microalgae Culture Collection, Ege-MACC (coded with EGEMACC73 http://www.egemacc.com/cultures.php) with National Center for Biotechnology Information (NCBI) accession numbers JQ726703.1, JQ726702.1, and JQ726701.1. It was isolated from Burdur Lake, Turkey. It was cultivated in Bold's basal medium (BBM) (Andersen et al. 2005) (Table S1). The cells in 2.0-L bottles were illuminated with continuous light (27 μmol photons m^{-2} s^{-1}; cool white fluorescent lamps, Philips, 18 W/54) at 25.0 °C. Air was supplied to the culture at a flow rate of 1 L min^{-1} (1.25 vvm). Four-day-old culture (in exponential cell growth phase) was used as inoculum at 10 % volume for all experiments. All glassware and the medium were autoclaved at 121.0 °C for 20.0 min prior to use. When this medium was used, the specific growth rate was 0.203 day^{-1} calculated according to Becker (1993) (Eq. 1) using the data obtained by the absorbance values taken at 450 nm.

$$\mu = \frac{\ln x_2 - \ln x_1}{\Delta t} \quad (1)$$

where μ = specific growth rate, x_2 = cell concentration at time t_2, x_1 = cell concentration at time t_1, and $\Delta t = t_2 - t_1$.

The *Prochlorococcus* sp. cultures were filtered by Whatman No. 1 filter paper and washed with deionized water to remove the growing medium. The harvested cells were stored at −20.0 °C and lyophilized prior to extraction of carotenoids.

Identification of *Prochlorococcus* sp.

For the identification of the cyanobacteria, optical image was obtained by using a trinocular light microscope (Olympus CH40) and SEM image was provided using Philips XL-30S FEG (Fig. 1). The elemental composition of *Prochlorococcus* sp. was determined with an LECO-932 elemental analyzer, and according to the results, it mainly consists of 47.06 % C, 6.92 % N, 7.05 % H, and 0.64 % S by mass.

Preparation of standard carotenoid solutions

As carotenoids are light sensitive, standard solutions were prepared under yellow light (Philips lamp TLD 36 W/16 yellow, light transmission at 500–750 nm) at room temperature within the shortest possible time. All stock carotenoid standards were accurately weighed and dissolved in 10.0 mL dichloromethane, whereas 10.0 mg of *trans*-β-apo-8′-carotenal (internal standard) was dissolved in 10.0 mL chloroform (stabilized with 1 % ethanol) to produce stock solutions which were then diluted to obtain appropriate intermediate standard solutions of 100.0 mg L^{-1}. Calibration standards were prepared containing a constant concentration of the internal standard and a variable concentration of carotenoid for the construction of calibration curves. All standard and sample solutions were kept in amber-colored volumetric flasks wrapped with aluminum foil.

Different concentrations (0.010–5.0 mg L^{-1}) of available carotenoid standards were injected into the HPLC, and the linear regression equations for each calibration curve were acquired by plotting the quantity of standard compound injected against their ratio of analyte peak area to the internal standard peak area. Measurement of the magnitude of analytical background

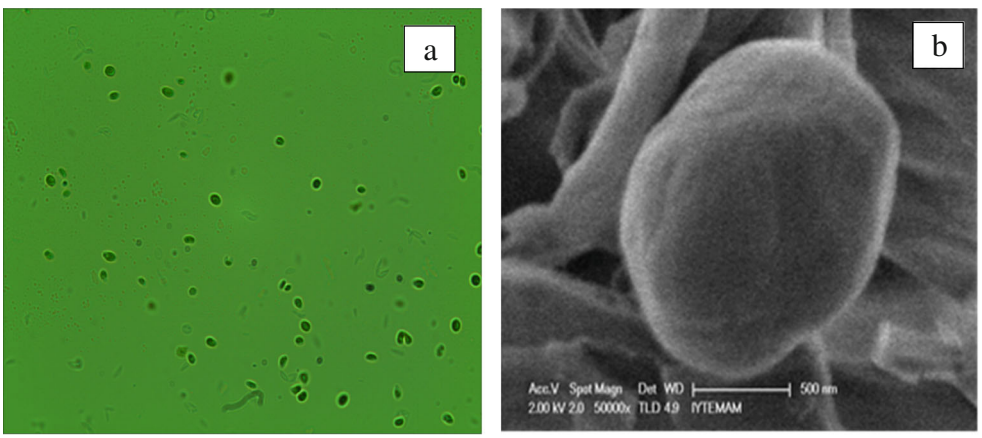

Fig. 1 **a** Optical microscope image (×40) and **b** SEM image (×50,000) for *Prochlorococcus* sp.

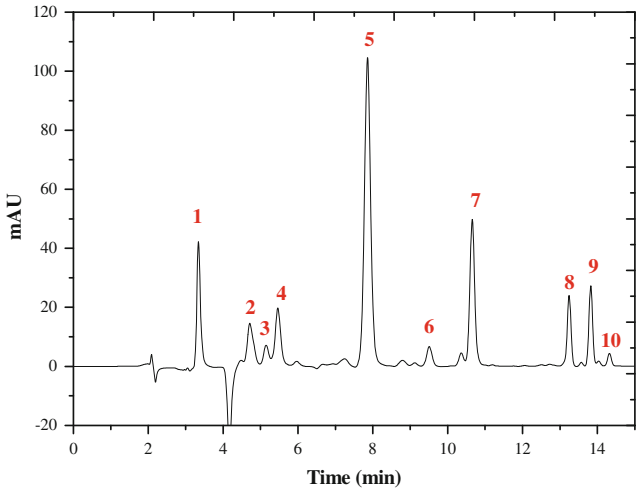

Fig. 2 HPLC chromatogram for *Prochlorococcus* sp. obtained at 446 nm: (*1*) degraded chlorophyll, (2) all-*trans*-neoxanthin, (*3*) all-*trans*-violaxanthin, (*4*) 9-*cis*-violaxanthin, (*5*) all-*trans*-lutein, (*6*) 9- or 9′-*cis*-lutein, (*7*) *trans*-β-apo-8′-carotenal, (*8*) all-*trans*-β,ε-carotene, (*9*) all-*trans*-β,β-carotene, (*10*) 9-*cis*-β,β-carotene

response was performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses. Ten repeats of blank sample were tested. Using the standard deviation of the blank sample, the detection and quantification limits were set. The mean of the ten repeats, plus three times the standard deviation, is the detection limit, while ten times the standard deviation is the quantification limit.

Extraction and saponification of carotenoids from *Prochlorococcus* sp.

The extraction and saponification of carotenoids from *Prochlorococcus* sp. were performed according to the procedure reported in our previous study in which the method validation was also performed (Erdoğan et al. 2015).

In brief, 0.25 g of dry microalgal biomass was treated with 10.0 mL of tetrahydrofuran:dichloromethane (1:1 *v*/*v*) solution for the extraction of carotenoids using an ultrasonic bath (37 kHz) to disrupt the algal cells. The saponification procedure was performed (10 % methanolic potassium hydroxide) for 2.0 h in order to remove the unwanted lipids and the chlorophylls. The solvent of the carotenoid solution was removed by rotary evaporation. Finally, it was dissolved in the mobile phase prior to HPLC analysis.

The same methodology was followed for the identification and quantification of carotenoids in *Prochlorococcus* sp.

Effect of different nitrogen sources on lutein content

In this study, different nitrogen sources were provided such as nitrite, ammonium, and urea as some literature studies have implied that nitrogen content increases with increased lutein availability and growth rate (Shi et al. 2000). Fernández-Sevilla et al. (2010) have pointed out that nitrogen availability and source are one of the most important factors that affect the lutein content in microalgae. Apart from nitrate, nitrite, ammonium, and urea have been selected as the different nitrogen sources in order to change the lutein productivity of both microalgae used in this study. An equivalent nitrogen concentration was used in the culture media for the comparison of nitrogen sources with the nitrate.

Results and discussion

Identification of and determination of carotenoids in *Prochlorococcus* sp.

The gradient system developed by Erdoğan et al. (2015) was applied for the separation of carotenoids in the selected

Table 2 UV-visible absorption spectra for tentative identification of carotenoids in *Prochlorococcus* sp.

Peak no	Compound	λ (reported)[a]			λ (observed)			λ (standards)			% III/II[c]
2	All-*trans*-neoxanthin	418	442	471	418	440	471	418	440	471	85.1
3	All-*trans*-violaxanthin	419	440	470	417	440	470	418	440	470	94.0
4	9-*cis*-Violaxanthin	414	436	464	418	440	470				96.2
5	All-*trans*-lutein	422	445	474	423	446	474	424	446	474	64.3
6	9- or 9′-*cis*-lutein	420	442	470	418	440	470				67.9
7	Internal standard[b]		458			456			456		
8	All-*trans*-β,ε-carotene	423	444	473	423	446	474	423	446	474	59.2
9	All-*trans*-β,β-carotene	428	450	478		452	478		452	478	22.4
10	9-*cis*-β,β-Carotene	422	448	474	423	446	474	423	446	474	30.9

[a] Britton et al. 2004

[b] *trans*-β-apo-8'-Carotenal

[c] Spectral fine structure: ratio of the longest wavelength absorption peak (designated as III) and that of the middle absorption peak (designated as II)

Table 3 LC-APCI-MS (positive mode) data of carotenoids in *Prochlorococcus* sp.

Peak No	Carotenoid	*m/z* (observed)	*m/z* (standards)	*m/z* (reported)[a]
2	All-*trans*-neoxanthin	601.5 $[M+H]^+$, 583.5 $[M+H\text{-}H_2O]^+$, 565.4 $[M+H\text{-}2H_2O]^+$	601.5, 583.5, 565.4	601 $[M+H]^+$, 583 $[M+H\text{-}H_2O]^+$, 565 $[M+H\text{-}2H_2O]^+$
3	All-*trans*-violaxanthin	601.5 $[M+H]^+$, 583.5 $[M+H\text{-}H_2O]^+$, 509.4 $[M+H\text{-}92]^+$	601.5, 583.5, 509.4	601 $[M+H]^+$, 583 $[M+H\text{-}H_2O]^+$, 509 $[M+H\text{-}92]^+$
4	9-*cis*-Violaxanthin	601.5 $[M+H]^+$		601 $[M+H]^+$
5	All-*trans*-lutein	569.5 $[M+H]^+$, 551.5 $[M+H\text{-}H_2O]^+$	569.5, 551.5	569 $[M+H]^+$, 551 $[M+H\text{-}H_2O]^+$
6	9- or 9′-*cis*-lutein	569.5 $[M+H]^+$		569 $[M+H]^+$
8	All-*trans*-β,ε-carotene	537.4 $[M+H]^+$, 519.4 $[M+H\text{-}H_2O]^+$	537.4, 519.4	537 $[M+H]^+$, 519 $[M+H\text{-}H_2O]^+$
9	All-*trans*-β,β-carotene	537.4 $[M+H]^+$, 519.4 $[M+H\text{-}H_2O]^+$	537.4, 519.4	538 $[M+H]^+$, 519 $[M+H\text{-}H_2O]^+$
10	9-*cis*-β,β-Carotene	537.4 $[M+H]^+$, 519.4 $[M+H\text{-}H_2O]^+$	537.4, 519.4	539 $[M+H]^+$, 519 $[M+H\text{-}H_2O]^+$

[a] Britton et al. 2004

cyanobacteria. Internal standard calibration method was used for the determination of carotenoids. This LC-DAD method is fast, specific, and precise for the determination of carotenoids. An HPLC chromatogram for *Prochlorococcus* sp. is shown in Fig. 2. All the carotenoids were separated within 15 min. The first peak in the chromatogram is considered to be a degradation product of chlorophyll that might stem from the saponification process. For this reason, the negative peak observed in the fourth minute could also be due to a chlorophyll degradation product.

For the identification of carotenoids, absorbance values and mass spectrometric data were combined. Figure S1 shows absorbance spectra for available carotenoid standards and carotenoids extracted from *Prochlorococcus* sp. UV-visible absorption spectra for tentative identification of carotenoids are presented in Table 2. In addition, spectral fine structural values (% II/II) obtained from this study agree well with the values in the literature (Britton et al. 2004). LC-APCI-MS (positive mode) data of carotenoids in *Prochlorococcus* sp. are also summarized in Table 3.

Finally, Table 4 presents the validation parameters regarding the proposed LC method for *Prochlorococcus* sp.

Improving the lutein content using different nitrogen sources

A number of different nitrogen sources, such as ammonia, nitrate, nitrite and urea, can be used as nitrogen source for growing microalgae (Becker 2004). Among these, urea ($CO(NH_2)_2$) is a low molecular weight, polar, and relatively lipid-insoluble organic compound which can be considered as a combined source of nitrogen and carbon. In the literature, several examples can be found where urea has been shown to be an effective combined source of N and C for the production of *Arthrospira platensis*, *Neochloris oleoabundans*, and *Chlorella* sp. under different cultivation modes (Becker 2004; Rangel-Yagui et al. 2004; Sánchez-Luna et al. 2004; Soletto et al. 2005; Li et al. 2008; Hsieh and Wu 2009).

This part of the study aimed at assessing the effect of different nitrogen sources on biomass productivity and lutein accumulation of *Prochlorococcus* sp. Table 5 shows the growth rates and lutein accumulation of cultures grown on different N sources. For an equivalent nitrogen

Table 4 Summarized validation parameters with proposed LC method (gradient elution with 70:25:5 methanol:methyl *tert*-butyl ether:water at the wavelength of interest, flow rate 1.0 mL min^{-1}) for *Prochlorococcus* sp.

Compound	LOD (mg L^{-1})	LOQ (mg L^{-1})	r^2	Peak purity %	Capacity factor (k')	Selectivity factor (α)	Content (mg g^{-1})
All-*trans*-neoxanthin	0.011	0.035	0.9997	99.3	1.43	1.87	0.45
All-*trans*-violaxanthin	0.014	0.045	0.9998	98.9	1.60	1.12	1.39
9-*cis*-Violaxanthin	No available standard			97.5	2.10	1.31	ND
All-*trans*-lutein	0.0032	0.011	0.9998	99.5	3.11	1.49	2.54
9- or 9′-*cis*-lutein	No available standard			97.5	3.91	1.27	ND
All-*trans*-β,ε-carotene	0.016	0.054	0.9997	98.6	5.67	1.27	0.24
All-*trans*-β-carotene	0.012	0.042	0.9995	99.1	5.99	1.06	0.30
9-*cis*-β,β-Carotene	0.015	0.051	0.9995	97.6	6.25	1.04	0.13

RSD values are <2 %

ND not detected

Table 5 Growth rates and lutein accumulation of *Prochlorococcus* sp. grown in different N sources

Nitrogen sources	Log phase (day)	μ_{max} (day^{-1})	[a]All-*trans*-lutein accumulated (mg g^{-1})
$NaNO_3$	4–8	0.419	2.54
$NaNO_2$	4–8	0.376	1.82
NH_4Cl	3–10	0.262	0.42
CH_4N_2O	4–8	0.232	3.34

RSD values are <2 %

[a] Experiments were performed three times

concentration, urea gave a higher yield for lutein. Some authors (Goldman 1977; Stengel and Soeder 1975) have reported that ammonium was an excellent nitrogen source for marine as well as freshwater algae. On the other hand, it has not been used as widely as nitrate as a nitrogen source for most algae such as *Chlorella protothecoides* (Shi et al. 2000) and *Dunaliella salina* (Borowitzka and Borowitzka 1988) which might be due to its inconvenience for sterilization, and the lethal effect on cells due the severe drop in pH after ammonium has been consumed. Urea as a nitrogen source was found to be superior in several respects to the commonly used nitrogen source, nitrate.

On the other hand, growth rates in cultures grown on urea were lower than the growth rates when other nitrogen sources are used. This might be due to the fact that urea is an organic source while the rest are inorganic sources of nitrogen. Probably, the accumulation of lutein due to utilization of organic source may not be the same due to metabolic pathways when inorganic sources are used. In addition, this organic compound can be considered as a combined source of nitrogen and carbon. The reason why urea gave higher yields for an equivalent nitrogen concentration might be due to the fact that it is usually hydrolyzed into ammonia and bicarbonate and therefore leads smaller pH fluctuations in the medium during the algal growth (Perez-Garcia et al. 2011).

Conclusion

In recent years, there has been particular emphasis on obtaining more accurate data on the types and concentrations of carotenoids in foods for various health and nutrition activities. On the other hand, significant attention has recently been drawn to the use of microalgae since they produce carotenes and xanthophylls in rich amounts during their normal growth or when exposed to stress conditions. For this reason, carotenoid production has become one of the most successful branches of biotechnology of microalgae. According to the experimental data obtained via chromatographic and spectroscopic analyses, the identification and quantification of carotenoids in *Prochlorococcus* sp. have been explored. It has been shown that lutein is the most abundant carotenoid in the selected microalga. In addition, availability of different nitrogen sources has significantly affected its lutein content. Urea as nitrogen source is advantageous for the accumulation of lutein as it is a cheap N source which could then be an interesting alternative to the traditional nitrate-based media for the microalgae. In conclusion, our work provides necessary information about the carotenoid profiles and the enhanced productivity for lutein from *Prochlorococcus* sp. using different types of nitrogen sources.

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