

Research Article

Assessment of optimal growth conditions for specific carotenoids production by *Chlorella vulgaris*

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Abstract

Exploration of regional microalgae for carotenoids production under optimized cultural conditions is a sustainable economic and technical perspective. This study details comprehensive research on the influence of growth conditions on microalgal carotenoids. Carotenoid triggering factors were optimized to identify suitable growth conditions to produce specific carotenoids by *Chlorella vulgaris*. Media optimization and cultivation conditions were the factors considered and the results revealed the optimum growth conditions for carotenoid production by *C. vulgaris* was pH 8, 35°C temperature, 0.04 M salinity and 160 $\mu\text{E}/\text{m}^2/\text{sec}$ light intensity. Among the nutrient sources, potassium nitrate and potassium phosphate were suitable for nitrogen and phosphorous supplements. The results indicated optimizing the culture conditions and nutrient sources help to attain desirable carotenoid production by *C. vulgaris*. Specific carotenoids were extracted from the algal extract and were identified by high-performance liquid chromatography in which lutein (8.8%) was present as major carotenoid followed by astaxanthin (4.6%) and β -carotene (3.9%). This study revealed that that carotenoid production by *C. vulgaris* could be enhanced by manipulating culture conditions thereby attain desirable carotenoid production.

Keywords: Astaxanthin, β -carotene, *Chlorella vulgaris*, Carotenoids, Lutein

INTRODUCTION

Carotenoids are naturally occurring pigments which have been attributed in the prevention of cardiovascular, neurodegenerative and inflammatory diseases (Chuyen and Eun, 2017; Raposo *et al.*, 2015). Among the photosynthetic organisms, microalgae are the richest producers of carotenoids. Some of the carotenoids such as astaxanthin are rarely found in other organisms which makes microalgae as a versatile source of carotenoids. Microalgae accumulate carotenoids to harvest light, regulate photo protecting process and stabilizing the structure of photosynthetic pigment-protein complexes (Mimuro and Akimoto, 2003; Mulders *et al.*, 2014). Some microalgae have the ability to produce carotenoids under unfavourable conditions. However, the carotenoid production by microalgae is low under native environmental conditions and it

varies between species. There are two possible ways to recover carotenoid from microalgae. One being the favouring of carotenoid production by facilitating the growth environment and another way of doing is adapting a suitable recovery process. Extraction of carotenoid from microorganisms by breaking down the cell membranes and isolation is a crucial step in the recovery process.

Among the various carotenoids produced by microalgae, β -carotene, astaxanthin and lutein have the highest market potential. β -carotene is related to provitamin A supply and colouring agent with industrial importance. Astaxanthin has antioxidant potential and anti-inflammatory effects, thereby benefitting human health (Li *et al.*, 2011; Guerin *et al.* 2003). Lutein is the predominant pigment in the macula and plays a significant role in eye health (Manayi *et al.*, 2015). Lutein

has been widely used for the pigmentation of various products (Shi and Chen, 1997). Carotenoids extracted from *Chlorella* species inhibited cancer cells (Cha *et al.*, 2008). Considering the abundancy and multipotential nature, this study focused on *Chlorella vulgaris* to evaluate its ability to produce carotenoids. To achieve the objective, this study focused on triggering factors influencing carotenoids produced by *C. vulgaris*. Media optimization and cultivation conditions were the factors considered, and the results were compared with previously reported literature to consider the present results as fruitful findings to apply in commercial production.

MATERIALS AND METHODS

Algae strain: *C. vulgaris* isolated from the sewerage treatment plant in the previous study (Mokashi *et al.*, 2016) was cultured in flat panel photobioreactor with a working volume of 5 litres. The culture medium was modified Bold's basal medium with an initial cell density of 30×10^5 per ml.

Chemicals: Methanol, methyl tert-butyl ether (MTBE), ethanol and β -carotene were obtained from Sigma-Aldrich (India).

Growth conditions for carotenoid production: Carotenoid production by *C. vulgaris* was optimized by varying pH (5, 6, 7, 8 and 9), temperature (25°, 30°, 35°, 40° and 45°C), nitrogen sources (NaNO₃, KNO₃, Urea; 3–48 mM), and phosphate (K₂HPO₄; KH₂PO₄, Ca₃(PO₄)₂ 0.4–6.4 mM) sources, salinity (0.04–0.64 mM) of selected medium and by changing the intensity of light (50 -300 $\mu\text{E}\text{m}^{-2}\text{sec}$).

Extraction and quantification of carotenoids: Total carotenoids from the microalgae were estimated by centrifuging the cells at 5000 x g for 10 mins followed by dissolving the pellet in acetone (20 ml). The solvent biomass mixture was incubated at 50°C in a water bath for 2 hrs followed by centrifugation at 5000x g. The absorbance of the supernatant was read at 660, 645 and 470 nm using UV-Vis spectrophotometer and the total carotenoids content were determined using following equations (Eq.1, Eq.2 and Eq.3) (Lichtenthaler and Buschmann, 2001).

$$\text{Chlorophyll a } (\mu\text{g}/\text{ml}^{-1}) = (11.24 A_{660}) - (2.04 A_{645}) \quad \dots\dots (\text{Eq. 1})$$

$$\text{Chlorophyll b } (\mu\text{g}/\text{ml}^{-1}) = (20.13 A_{645}) - (4.19 A_{660}) \quad \dots\dots (\text{Eq. 2})$$

$$\text{Carotenoids} = \frac{1000 \times (A_{645} - 1.90 \text{ Chl a} - 63.14 \text{ Chl b})}{214} \quad \dots\dots (\text{Eq. 3})$$

Where A₆₆₀, A₆₄₅ and A₄₇₀ represent absorbance at 660 nm, 645 nm and 470 nm, respectively.

Optimum conditions for carotenoid production: Based on highest production of carotenoids by *C. vulgaris* in various parameters and nutrient sources, the final growth medium and conditions used were as follows: potassium nitrate as a nitrogen source, potassium phosphate as phosphorous source with 0.04 M

salinity. The concentration of KNO₃ was 6 mM whereas it was 0.8 mM and 1.6 mM for K₂HPO₄ and KH₂PO₄ respectively. The optimal pH was 8 and the microalgae were cultured at 35°C with a light intensity of 160 $\mu\text{E}\text{m}^{-2}\text{sec}$ for a period of 12 days.

HPLC conditions and peak identification: High-performance liquid chromatography (HPLC) analysis was performed using 1260 infinity series LC system installed with a G1311C pump, a G1329B autosampler and a G13166 column compartment and G4212B DAD detector. Chromatographic separation was carried out on a Zorbax Eclipse Plus C18 Analytical column (4.6 × 150 mm, 5 μ ; Agilent Technologies). The mobile phase used was methanol: MTBE (60:40 v/v) with the flow rate of 0.6 ml/min in the column at ambient temperature with an infusion volume of 20 μl in each experiment. Injection volume was 20 μl and detection was by UV absorbance at 450 nm. Stock standard solutions were prepared by accurately adding 20 mg/l each of β -carotene, lutein, lycopene in MTBE and methanol. A calibration curve in the range 0-10 mg/l was set up, where the sample was diluted in the mobile phase. The samples from *C. vulgaris* was analyzed directly by dissolving in ethanol: MTBE (40:60). Various carotenoids were identified by comparing the retention times and absorption spectra of unknown peaks with reference standards. For calibration, a stock solution of β -carotene, lutein and astaxanthin were prepared by dissolving 10 mg in 33.3 ml of MTBE, 1 mg in 3.3 ml of MTBE and 10 mg in 33.3 ml of DMSO, respectively.

β -carotene extraction from *C. vulgaris*: β -carotene from *C. vulgaris* was extracted by centrifuging the cells at 6000 rpm for 10 mins and the pellet was washed with distilled water followed by lyophilization. Dried biomass was suspended in 75% acetone, vortexed until a white precipitate appeared. The sample was mixed with KOH (100 μl), vortexed and centrifuged. The supernatant was diluted in acetone and the mixture was allowed to stand until the two phases had clearly separated. The upper layer containing β -carotene was collected, evaporated to dryness using nitrogen purging (Wellburn and Lichtenthaler, 1984).

Lutein extraction from *C. vulgaris*: Lutein was extracted by performing consecutive extraction cycles using ethanol and the total lutein content was gravimetrically quantified after the complete removal of the solvent (Molino *et al.*, 2020). After evaporating the solvent completely, saponification was carried out, adding 0.05 M NaOH solution in methanol (1 ml) to the algal extract (5 ml). This solution was left in the dark in an inert atmosphere for 7 h and the sample was neutralized using 0.05 M NH₄Cl solution in methanol (3 ml).

Astaxanthin extraction from *C. vulgaris*: Astaxanthin in the microalgae was extracted by centrifuging the cells and the pellet was disrupted in mortar and pestle. Dichloromethane (10 ml) was added to the disrupted cells and the extraction was repeated until the cells were colourless. The solvent was evaporated in a

rotary evaporator and saponified (Taucher *et al.*, 2016) using acetone (2.25 ml), methanol (0.25 ml) and 0.05 M NaOH in methanol (0.5 ml). This was followed by the addition of petroleum ether (3 ml), washing with 10% NaCl (3 ml) and centrifugation for 2 mins at 5500 rpm. The upper phase was washed twice with NaCl and the organic phase was evaporated. Finally, the extracted astaxanthin was dissolved in 3 ml of solvent (methanol, MTBE, water, 8:89:3, v/v).

Quantification of carotenoids: The extracts were eluted at a flow rate of 1 ml/min, and the carotenoids content was detected by measuring absorbance at the wavelength range of 220–750 nm. The maximal absorbance was chosen for quantification of carotenoid extracts (Berman *et al.*, 2015; Harasym and Oledzki, 2014; Zhang *et al.*, 2015; Gayathri *et al.*, 2016). Accordingly, the extinction coefficient ($E^{1\%}_{1cm}$) was 2,273 at 450 nm, 3450 at 476 nm and 2589 at 450 nm for β -carotene, astaxanthin and lutein, respectively (Eq.4).

$$A \times V \text{ (ml)} \times \text{dilution factor}$$

$$\text{Concentration of carotenoids} = \frac{\text{-----}}{\epsilon \times W \text{ (g)}} \text{ (}\mu\text{g/g of sample)} \text{(Eq. 4)}$$

where A = absorbance, V = volume of extract in milliliters, ϵ = extinction coefficient, and W = dry weight of sample.

RESULTS AND DISCUSSION

Carotenoids are essential nutrients for human health in which β -carotene, lutein and astaxanthin derived

from microalgae are reported to have potential biological activities. β -carotene serves as a precursor for vitamin A and reduces cardiovascular disease and certain cancers (Johnson, 2002). Lutein is the important pigment in the retina (Roberts *et al.*, 2009) and astaxanthin is the nutrient supplement (Ambati *et al.*, 2014). Reports are available, suggesting that culture conditions affect carotenoid production in chlorophyceae species (Benavente-Vald *et al.*, 2016; Begum *et al.*, 2016; Khan *et al.*, 2018). Huang *et al.* (2018) characterized *Chlorella zofingiensis* to accumulate high amounts of carotenoid under various culture conditions. In this study, Culturing temperature, pH, salinity, luminosity and nutrient type influenced *C. vulgaris* metabolism to accumulate carotenoids. The present results of carotenoid production under varying pH, temperature, salinity and light intensity are depicted in Fig.1 (A-D). Carotenoid content was highest at 35°C with 7.11 $\mu\text{g/ml}$ and was 7.39 $\mu\text{g/ml}$ at pH 8. Carotenoid biosynthesis enzymes are controlled by temperature and high temperatures increase the nutrient uptake and carotenoid accumulation (Bhosale *et al.*, 2004; Fernandez-Sevilla *et al.*, 2010) while Ben-Amotz (1996) reported lower temperatures as favourable for the production of carotenoids in *Haematococcus*, *Dunaliella*, *Murielopsis* and *Scenedesmus* species.

The salinity of 0.04 mM has resulted in highest carotenoid accumulation (6.95 $\mu\text{g/ml}$) and the optimum light intensity was 160 $\mu\text{E/m}^2\text{/sec}$. Salt stress significantly

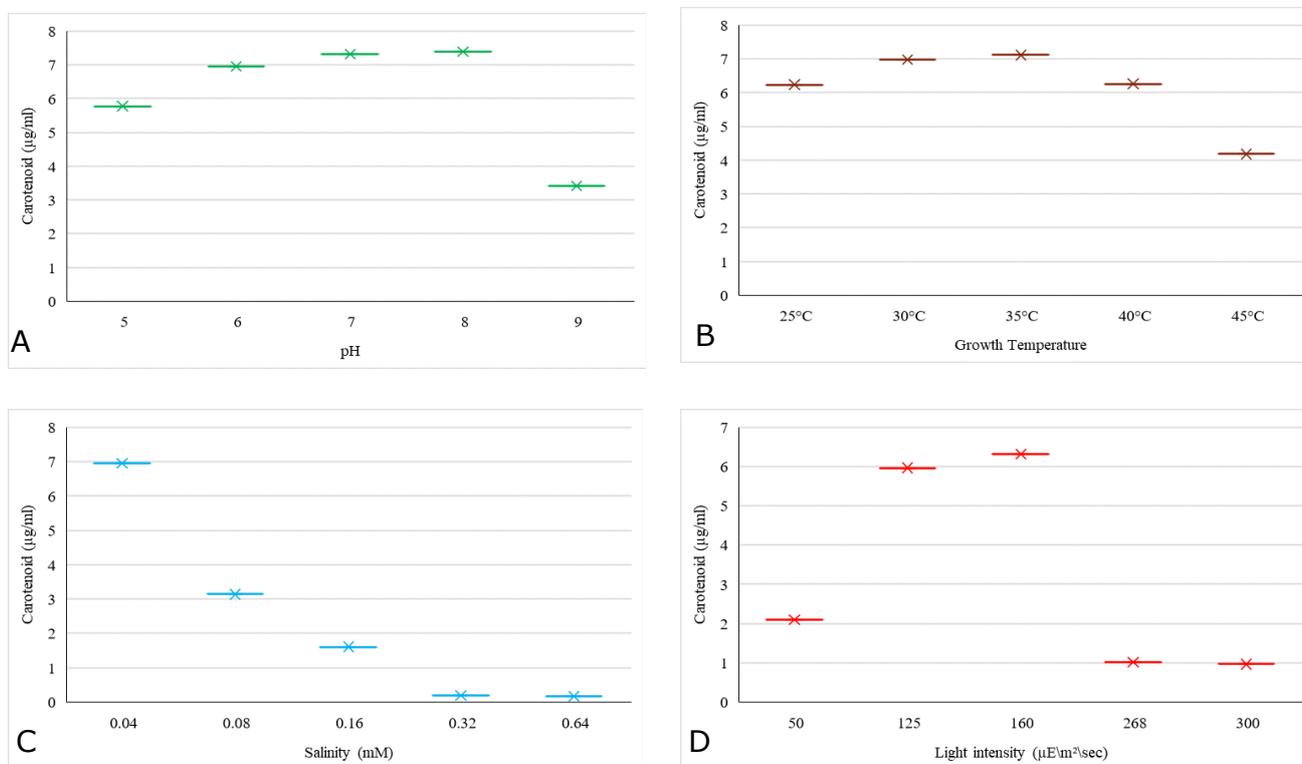


Fig. 1. Influence of Growth conditions on carotenoid content of *C. vulgaris* (A) pH (B) Temperature (C) Salinity (D) Light intensity.

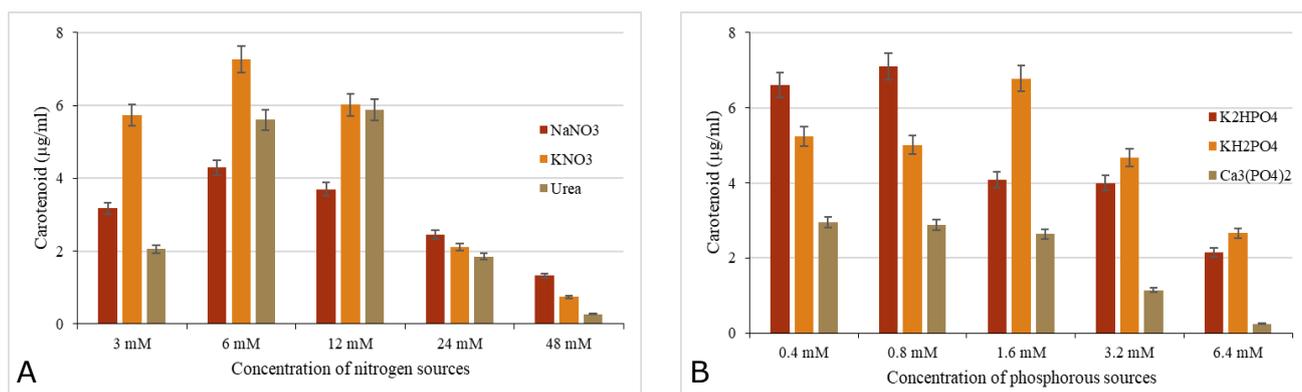


Fig. 2. Influence of nutrient sources on carotenoid content of *C. vulgaris*. (A) Nitrogen sources, (B) Phosphorous sources.

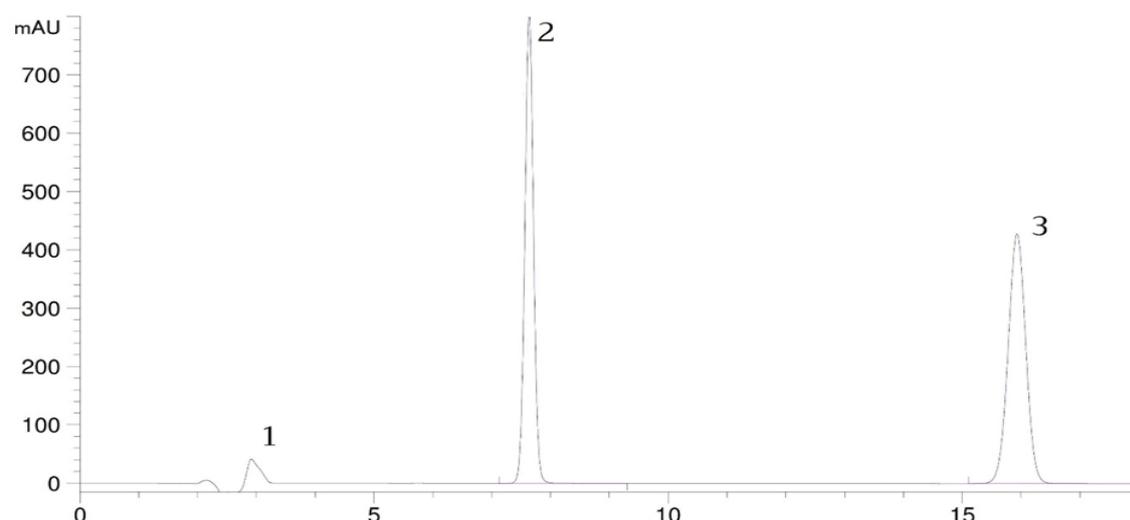


Fig. 3. HPLC profile of carotenoid standards. Peaks were identified as astaxanthin (1), lutein (2) and β -carotene (3).

increases the accumulation of secondary carotenoids in *C. zofingiensis* (Pelah *et al.*, 2004). Another important factor affecting carotenoid production in microalgae is light intensity (Xie *et al.*, 2013) as cell division slows down and cell lysis occurs under strong light. Photosynthetic machinery damages when there is excess light whereas insufficient light supply limits photosynthesis. Gayathri *et al.*, (2020) indicated $200 \mu\text{mol m}^{-2}\text{s}^{-1}$ was suitable for lutein production by *C. salina*.

Nutrient limitation leads to the accumulations of carotenoids as they affect the growth of microalgae and altering the photoprotective pigments. Among the nitrogen sources tested, both KNO_3 and urea increased carotenoid content in *C. vulgaris* when the concentration was increased up to 12 mM. However, the addition of KNO_3 to the growth medium at a concentration of 6 mM resulted in $7.26 \mu\text{g/ml}$ (Fig.2 A and B). Minyuk *et al.*, (2020) confirmed the replacement of nitrates with urea had increased astaxanthin by *C. zofingiensis*. Adding K_2HPO_4 and KH_2PO_4 increased carotenoid production while $\text{Ca}_3(\text{PO}_4)_2$ had little impact on carotenoid accumulation. Based on the highest carotenoid production under varying culture conditions, the growth media and conditions were optimized for *C. vulgaris* to improve carotenoids accumulation.

β -carotene, lutein and astaxanthin were extracted from *C. vulgaris* and identified by HPLC (Fig. 3 and 4) to utilize *C. vulgaris* for the production of carotenoids of biological importance. From the HPLC chromatogram, lutein (8.8%) was present in highest concentration followed by astaxanthin (4.6%) and β -carotene (3.9%). Microalgae are a good source of β -carotene and can be an alternate source for chemically synthesized β -carotene (Garcia-Gonzalez *et al.*, 2005; Kleinegriss *et al.*, 2011). *C. protothecoides* has high productivity of lutein (Wei *et al.*, 2008) while *C. emersonii* (Malis *et al.*, 1993) and *C. zofingiensis* (Pelah *et al.*, 2004) are able to astaxanthin.

Conclusion

Increasing demand for natural, health-protecting bioactive molecules has driven the research to explore alternate natural resources for carotenoid production. Exploration of regional microalgae for sustainable production of carotenoids is required as wild microalgae harvesting may have a negative ecological impact. Carotenoids derived microalgae are mostly from marine species, and this study attempted to produce β -carotene along with astaxanthin and lutein by freshwater *C. vulgaris*. Further, this study indicated that carot-

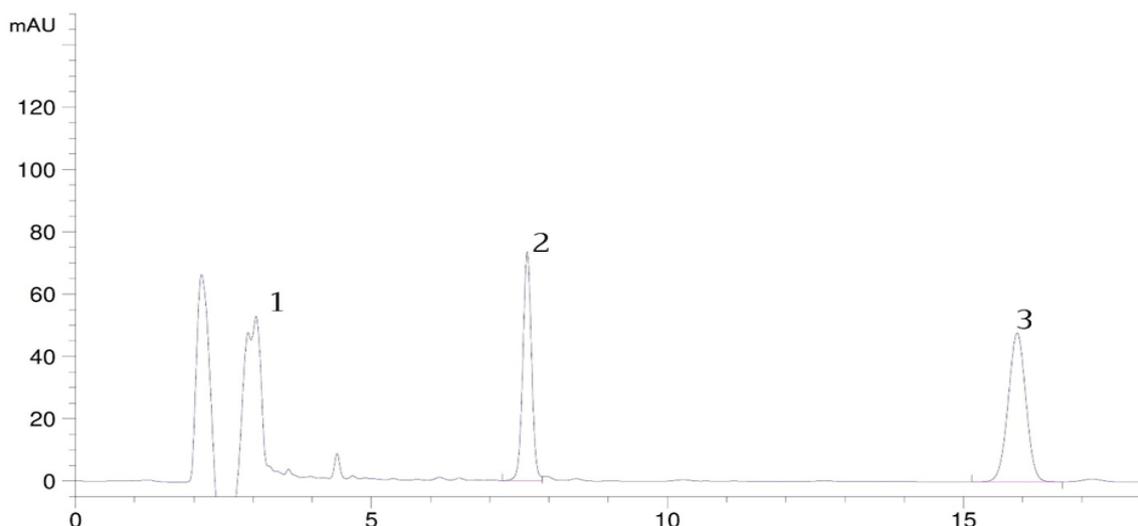


Fig. 4. HPLC profile of carotenoid samples from *C. vulgaris*.

enoid production by *C. vulgaris* could be enhanced by manipulating culture conditions, thereby attain desirable carotenoid production.

Conflict of interest

The authors declare that they have no conflict of interest.

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