Research Article

Influence of agriculture fertilizer for the enhanced growth and astaxanthin production from *Haematococcus lacustris* RRGK isolated from Himachal Pradesh, India

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Abstract

In the present study, *Haematococcus lacustris* RRGK which is the former name of *H. lacustris* HPI-001 isolated from Himachal Pradesh, India, and another strain *H. lacustris* SAG-19a retrieved from Gottingen Culture Collection, Germany, were used. The *H. lacustris* SAG-19a served as a control. The both strains were grown in a Bold basal medium in which the components of $NaNO_3$, $K_2HPO_4{\cdot}3H_2O$ and KH_2PO_4 were replaced with agriculture fertilizers such as NPK (17:17:17), urea (CH_4N_2O) and DAP (diammonium phosphate; $(NH_4)_2HPO_4$ + potash (K_2CO_3) in different concentrations. The isolate of *H. lacustris* HPI-001 showed maximum growth at 1.2 mM NPK, while the *H. lacustris* SAG-19a showed maximum growth at 1.5 mM. However, both the strains showed maximum astaxanthin content at 0.6 mM NPK. The *H. lacustris* HPI-001 showed a maximum growth and astaxanthin content with 3.3 mM and 4.9 mM of urea, respectively, while *H. lacustris* SAG-19a showed maximum growth and astaxanthin accumulation with 3.3 mM and 6.6 mM of urea, respectively. The *H. lacustris* HPI-001 showed maximum growth at 250 mg/L $(NH_4)_2HPO_4$ + 200 mg/L K_2CO_3 and maximum astaxanthin content at 150 mg/L $(NH_4)_2HPO_4$ + 100 mg/L K_2CO_3, while the maximum growth in *H. lacustris* SAG-19a was observed at 200 mg/L $(NH_4)_2HPO_4$ + 150 mg/L K_2CO_3, and the maximum astaxanthin content at 150 mg/L $(NH_4)_2HPO_4$ + 100 mg/L K_2CO_3. The basal medium was also amended with the addition of commercial $NaHCO_3$ wherein the maximum growth and content of astaxanthin in *H. lacustris* HPI-001 was at 0.6 mM and 1.5 mM of $NaHCO_3$, respectively. The *H. lacustris* SAG-19a showed maximum growth and astaxanthin content at 1.5 mM and 0.6 mM, respectively. Based on the investigation, a medium was formulated and named as modified HPI-001 medium. The results of the present study suggest that commercial agricultural fertilizers may be used as excellent substitutes to enhance cell growth and astaxanthin production in *H. lacustris*.

Keywords *Haematococcus lacustris* · Carotenoids · Astaxanthin · Agriculture fertilizer

1 Introduction

Microalgae are photosynthetic microorganisms that can synthesize various organic biomolecules such as proteins, carbohydrates, lipids, pigments and vitamins from carbon dioxide (CO_2) [1]. These microalgae products have attracted intensive academic and industrial application including cosmetics, nutraceuticals, pharmaceuticals, aquaculture, food and biofuels [2–4]. The natural astaxanthin can be produced from microalgae, yeast, bacteria and various seafood including salmon, lobster, trout, red sea bream, arctic shrimp, crawfish, krill and fish eggs [5, 6]. Various microorganisms such as *Haematococcus lacustris*, *Chromochloris zofingiensis*, red yeast *Phaffia rhodozyma*

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and marine bacterium *Agrobacterium aurantiacum* can produce astaxanthin [7]. The *H. lacustris* can accumulate a maximum astaxanthin of up to 4% dry weight under unfavorable environmental stress conditions [8–10].

The *H. lacustris* (Girod-Chantrans) Rostafinski 1875 is a green, motile, biflagellate microalga found in freshwater environments. It belongs to class Chlorophyceae, order Volvocales and family Haematococcaseae. Its life cycle consists of four stages such as macrozooids or zoospore, microzooids, palmella and aplanospore stages [11]. The macrozooid cells are between 8 and 20 µm long with a distinct gelatinous extracellular gelatinous matrix of variable thickness, and these may be divided into 2–32 daughter cells by mitosis [9]. They form amorphous multilayered structures in the inner regions of the extracellular matrix or the primary cell wall as they develop into non-motile palmella and become resting vegetative cells [12]. When the culture is exposed to stress conditions such as the decrease in nutrients and the increase in light intensity and high salinity, the palmella stage transforms into the hematocyst aplanospore stage [9, 13]. These microalgae can be usually found in temperate regions around the world and has been isolated from Europe, Africa, North America and Himachal Pradesh in India [14, 15] and also reported from Arctic region [16]. It is also found across various environmental and climatic conditions such as brackish water and on the rocks on seashore [16]. The astaxanthin-rich non-motile aplanospore coccoid cells have an exceptional tolerance to a wide range of adverse conditions [17–19]. The highly complex and dynamic composition of cell wall, photosynthetic apparatus reduction and cell dehydration allows *H. lacustris* to survive in hostile environment, but on the other hand presents an issue when *H. lacustris* biomass has to be processed to extract valuable intracellular compounds such as astaxanthin [20].

The choice of culture medium for microalgae growth depends on the nutritional requirements that contribute toward the efficient growth and biomass production [21, 22]. The alternative sources of nutrients in the composition of culture media are urea, ammonium sulfate, water plants, swine manure and inorganic fertilizers (NPK) [23]. Nutrients are crucial in the growth and development of microalgae, influencing the physiological adaptation and biochemical composition of microalgae [15]. Nitrogen and phosphorus are the main elements that limit growth of microalgae, which usually depends on the physiological requirements of each nutrient [24]. The low CO_2 concentration in the air is also limiting factor of photosynthesis in plants and algae [25]. There are several types of basic elements for growth of microalgae such as carbon, hydrogen, nitrogen, oxygen, phosphorus, magnesium, iron, sulfur and trace elements [26]. Potassium is a macroelement which is required for growth-related metabolic activities [27]. The inorganic fertilizer is simple and may be used with alternative medium for microalgae, since they are widely available, dissolve easily, have a defined composition, high nitrogen and phosphorus rate, and maintain moderate pH in the medium [28, 29]. The inorganic fertilizers such as NPK result in an efficient alternative medium for large-scale laboratory growth of Chlorophyceaen members [30].

Astaxanthin ($C_{40}H_{52}O_4$, 3, 3′-dihydroxy-*β*, *β*-carotene-4,4′-dione), a keto-carotenoid, is considered as a "super antioxidant" which spans the cell membrane bilayer and significantly reduces the free radicals and oxidative stress in human body, thereby helping to maintain a healthy state [5, 31, 32]. It has the most effective natural antioxidant activity and is known to be 10–65 times higher than that of β-carotene, canthaxanthin, zeaxanthin, lutein and vitamin C, and is 100 times more effective than α-tocopherol [11, 33]. It has wide range of applications in cosmetics, foods, nutraceuticals, aquaculture and pharmaceuticals industries [11, 34, 35]. Astaxanthin is also used as a promising therapeutic agent against atherosclerosis, cancers, hypertension, diabetes, cardiovascular diseases and several neurological diseases (e.g., Alzheimer's disease) because it is known to cross the blood–brain barrier and enables to provide antioxidant benefits beyond that barrier [36, 37]. Astaxanthin is also currently used in the prevention and control of many pathological conditions involving low oxidation and inflammation [38, 39].

In the present study, a new strain of *H. lacustris* HPI-001 was isolated from Himachal Pradesh, India, and another strain *H. lacustris* SAG-19a culture was obtained from Sammlung von Kulturen, Pflanzen Physiologisches Institut, University of Gottingen, Gottingen, Germany. The latter was treated as control, and its adaptation to laboratory conditions was investigated. The two strains were grown in Bold basal medium (BBM) [40] amended with the addition of commercial fertilizers such as N:P:K (17:17:17), CH_4N_2O and $(NH_4)_2HPO_4 + K_2CO_3$ and its influence on growth and astaxanthin production were evaluated.

2 Materials and methods

2.1 Isolation of *H. lacustris* HPI-001

The water samples were collected from Palampur in March 2013. Palampur (32°N latitude; 76°E longitude) is a town in the state of Himachal Pradesh in India and is 1325 ft. above sea level with average annual temperature of 19 °C and average annual rainfall of 250 cm and above. The water sample from each aquatic site was collected and stored in plastic bottles. The samples were inoculated in BBM at 25 ± 1 °C, under 30 µE m^{-2} s^{-1} light irradiations and a photoperiod of 12/12 (light/dark). The

cultures were thoroughly mixed manually twice a day, and the experiment was conducted at laboratory conditions. The cells of *H. lacustris* HPI-001 were isolated and prepared unialgal cultures by serial dilution followed by streak plate technique on 2% agar in BBM. The cells were identified based on morphological and molecular studies. *H. lacustris* SAG-19a was retrieved from Gottingen Culture Collection, Germany.

2.2 Composition of culture medium

The two strains of *H. lacustris* were cultured in the BBM in which the components, sodium nitrate ($NaNO_3$), dibasic potassium phosphate trihydrate ($K_2HPO_4{\cdot}3H_2O$) and potassium phosphate monobasic (KH_2PO_4), were replaced with different concentrations of commercial fertilizers such as N:P:K (17:17:17), urea (CH_4N_2O), diammonium phosphate ($(NH_4)_2HPO_4$) and potassium carbonate (K_2CO_3) (Shri Sai Gopal Agrotech Pvt. Ltd., Karnataka, India). The other chemicals of the medium were obtained from Sisco Research Laboratories Pvt. Ltd., Mumbai (Table 3). The growth and astaxanthin production in two test organisms were also recorded in basal medium added with different concentrations (0.3, 0.6, 0.9, 1.2, 1.5 and 1.8 mM) of commercial sodium bicarbonate ($NaHCO_3$) fertilizers (Table 1). The growth and astaxanthin production of *H. lacustris* HPI-001 and the *H. lacustris* SAG-19a in modified Bold basal medium (3 N-BBM + V) and formulated modified HPI-001 medium was compared (Table 2).

Table 1 Medium composition

Chemical components	mg/L
N:P:K (17:17:17)[a]	**2500**
CH_4N_2O[a]	**13,000**
$CaCl_2{\cdot}3H_2O$	2500
$MgSO_4{\cdot}7H_2O$	2500
$NaHCO_3$[a]	**2500**
$(NH_4)_2HPO_4$[a]	**5000**
K_2CO_3[a]	**7500**
NaCl	2500
EDTA (with Na_2)	750
$FeCl_3{\cdot}6H_2O$	97
$MnCl_2{\cdot}4H_2O$	41
$ZnCl_2{\cdot}6H_2O$	5
$CoCl_2{\cdot}6H_2O$	2
$Na_2MoO_4{\cdot}2H_2O$	4

All the medium components dissolved in 1 L of distilled water

pH of the medium is 7.5

[a]Replaced commercial fertilizers in basal medium (3N-BBM + V)

Table 2 Formulated commercial modified HPI-001 medium

Chemical components	mg/L
N:P:K (17:17:17)	50
CH_4N_2O	300
$CaCl_2{\cdot}3H_2O$	2500
$MgSO_4{\cdot}7H_2O$	2500
$NaHCO_3$	125
$(NH_4)_2HPO_4$	150
K_2CO_3	100
NaCl	2500
EDTA (with Na_2)	750
$FeCl_3{\cdot}6H_2O$	97
$MnCl_2{\cdot}4H_2O$	41
$ZnCl_2{\cdot}6H_2O$	5
$CoCl_2{\cdot}6H_2O$	2
$Na_2MoO_4{\cdot}2H_2O$	4

All the medium components dissolved in 1 L of distilled water

pH of the medium is 7.5

2.3 Maintenance and study of algal culture

The two strains of *H. lacustris* were grown under autotrophic condition, in a photoperiod of 12/12 h (light/dark) in liquid BBM and maintained at 25 ± 1 °C, under 30 µEm-2 s-1 light intensity provided by warm white Philips set of lamps (36 W; 4ft Philips Trulite, made in India), the light intensity was measured by using lux meter (TES 1331, Taiwan), and the culture parameters periodically were measured for 30 days. The experiments were conducted with sterilized 250-mL Erlenmeyer conical flasks. Ten milliliters each of pure culture was taken at exponential growth phase (7 days), and this initial culture density of 2.0×10^4 cells/mL was inoculated into 90 mL of sterilized BBM medium under aseptic condition. The growth parameters such as cell number, pigments such as chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*), total carotenoids and astaxanthin contents were recorded at every 5-day interval during the study period. Monoalgal culture of the algae was used in the following experiments.

2.3.1 Cell count

"Neubauer" hemocytometer (REF: 0303 212, Neubauer Improved Bright-Line, HBG, Germany) was used for the purpose. The mean of the cell numbers recorded in four chambers was calculated and expressed as multiples of 10^4 cells/mL.

2.3.2 Growth curve

Growth curves were plotted with cell count values (multiples of 10^4 cells/mL) against respective days on which the cell count was measured.

2.3.3 Determination of specific growth rate (μ), division rate (K) and generation time (DT)

The specific growth rate implies the number of generation or the number of doublings that occur per unit of time in an exponential growth culture. The specific growth rate was determined using the following equation [41]: $\mu = \ln(Nt/N0)/(T-t)$; Nt = no of cells at the end of the log phase; $N0$ = no of cells at the start of log phase; T = final day of log phase; t = starting day of log phase, if T expressed in days from the growth rate (μ) can be converted to $K = \mu/1n$ (2).

2.3.4 Extraction and estimation of pigments

Five milliliters of culture was centrifuged at 5000 rpm for 10 min, and the supernatant was discarded. The algal pellet was added with 5 mL of 100% acetone and macerated using pestle and mortar wrapped with black paper and kept overnight at 4 °C. The sample was centrifuged (R-8C; Remi Instruments Ltd, Mumbai, India) at 5000 rpm for 10 min, supernatant was collected, and the absorbance was measured at 661.6 nm, 644.8 nm, 470 nm and 490 nm wavelengths in Ultrospec 1100 pro UV–visible spectrophotometer (Amersham Bioscience, Germany) in standard quartz cuvette (190–2500 nm), path length 10 mm. The chlorophyll and total carotenoid contents were calculated using Lichtenthaler equations [42]. The amount of astaxanthin was determined from the acetone extract measured at 490 nm. The per unit volume of astaxanthin concentration (mg/L) was calculated by using the methods [43].

$$\text{Chl } a(\text{mg/L}) = 11.24 \times A_{661.6} - 2.404 \times A_{644.8}$$

$$\text{Chl } b(\text{mg/L}) = 20.13 \times A_{644.8} - 4.19 \times A_{661.6}$$

$$\text{Total carotenoids (mg/L)} = \frac{1000 \times A_{470} - 1.9 \times \text{Chl } a - 63.14 \times \text{Chl } b}{214}$$

$$\text{Astaxanthin (mg/L)} = 4.5 \times A_{490} \times \text{Va/Vb}$$

where A = absorbance; Va = volume of extracts; Vb = volume of culture sample.

2.3.5 Statistical analysis

All the experiments were carried out in triplicate and expressed as mean ± standard errors. The graphs were prepared by Graph Pad Prism 6 software.

3 Results

3.1 Identification of isolates

The different growth phase of the microalga *H. lacustris* HPI-001 was identified through microscopic examination and observed the cell size and shapes such as spherical, ellipsoidal or pear shape, in addition to which the cells clearly showed two flagella of equal length emerging from the anterior end, and a cup-shaped chloroplast with numerous, scattered pyrenoids. Further the culture was confirmed through molecular studies (18S rRNA) and identified as *H. lacustris* HPI-001, and the molecular data were submitted in GenBank and received accession number (KT285940). Under unfavorable conditions, the macrozooids get transition into the coccoid vegetative cell state by loosing flagella (LABOMED VISION 2000, 40× magnifications). When the cultures were exposed to stress conditions, the volume of the cell increased with a diameter of over 40 µm and the cell wall became resistant to the harsh conditions in which it was present. The maturation cyst aplanospore cells were accompanied by the enhancement of carotenoids biosynthesis and a gradual change in cell color to red. During optimal growth conditions, the daughter cells were released from the cystic cells and then vegetative cells regenerated from the daughter cells. Both the strains were maintained at Algal Culture Collection, Centre for Advanced Studies in Botany, University of Madras, Tamil Nadu, India (Figs. 1, 2).

3.2 Effect of different concentrations of commercial N:P:K (17:17:17)

The two strains of *H. lacustris* were grown at different concentrations of commercial NPK (17:17:17). The *H. lacustris* HPI-001 showed maximum cell number of 47×10^4 cells/mL in 1.2 mM of commercial NPK on 15th day of culture. At 1.2 mM, a specific growth rate of 0.117 day^{-1}, division rate of 0.169 day^{-1} and generation time of 5.931 day were recorded. The maximum concentration of Chl *a* (7.13 mg/L) and Chl *b* (3.57 mg/L) was recorded on 20th day at 1.2 mM NPK. The *H. lacustris* SAG-19a showed a maximum cell number of 42×10^4 cells/mL, and a maximum concentration of Chl *a* (6.25 mg/L) and Chl *b* (3.13 mg/L) on 20th day of 1.5 mM NPK. The increase in growth was 11%, and pigments Chl *a* 14% and Chl *b* were 10% which was more than that of *H. lacustris* SAG-19a. The alga *H. lacustris* HPI-001 accumulated maximum total carotenoids (22.33 mg/L) and astaxanthin content (17.94 mg/L) in 0.6 mM NPK on 30th day (Fig. 3). The *H. lacustris* SAG-19a exhibited maximum content of total

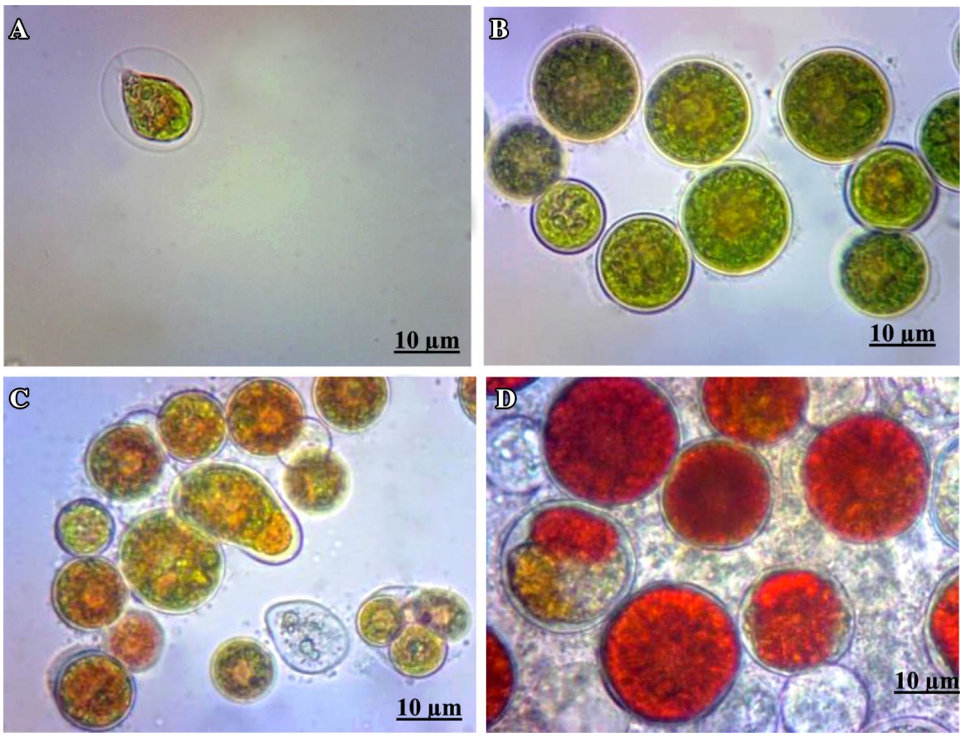

Fig. 1 Light microscopic image of isolate *H. lacustris* HPI-001 cells in life cycle in laboratory conditions. **a** Biflagellated motile cells (macrozooids); **b** non-motile green vegetative cells (palmella); **c** astaxanthin accumulating palmella cell in transition to aplanospores; **d** astaxanthin accumulated aplanospore cell. Scale bar: 10 µm

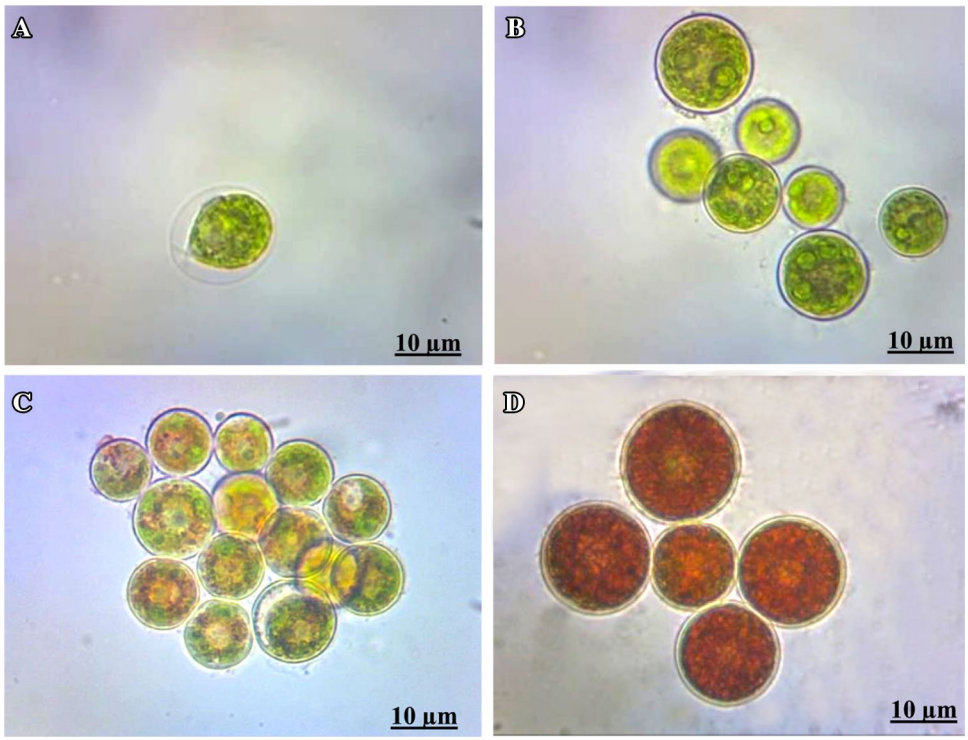

Fig. 2 Light microscopic image of *H. lacustris* SAG-19a cells in life cycle in laboratory conditions. **a** Biflagellated motile cells (macrozooids); **b** non-motile green vegetative cells (palmella); **c** astaxanthin accumulating palmella cell in transition to aplanospores; **d** astaxanthin accumulated aplanospore cell. Scale bar: 10 µm

carotenoids (20.60 mg/L) and astaxanthin (15.37 mg/L) in 0.6 mM of commercial NPK on 30th day (supplementary data S1). In the *H. lacustris* HPI-001, the increase in content of total carotenoids (10%) and astaxanthin (16%) was more than that of *H. lacustris* SAG-19a.

3.3 Effect of different concentrations of CH_4N_2O

The alga *H. lacustris* HPI-001 showed maximum cell number of 41×10^4 cells/mL on 15th day in 3.3 mM of CH_4N_2O. At 3.3 mM of CH_4N_2O, the specific growth rate, division rate

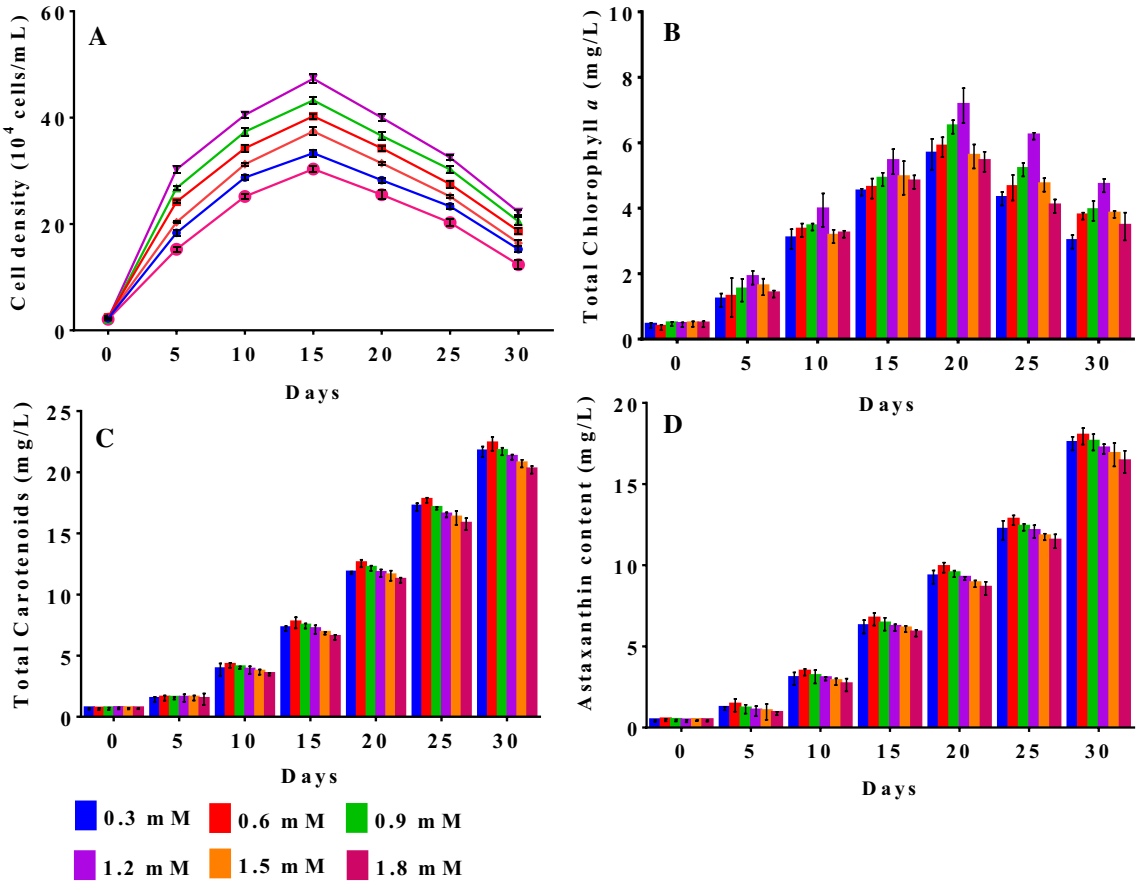

Fig. 3 Effect of different concentrations of commercial agriculture fertilizer N:P:K (17:17:17) (0.3, 0.6, 0.9, 1.2, 1.5 and 1.8 mM) on a cell number (**a**), chlorophyll a (**b**), total carotenoids (**c**) and astaxanthin content (**d**) isolate of *H. lacustris* HPI-001 at different intervals in laboratory conditions

and generation time were 0.197 day^{-1}, 0.284 day^{-1} and 3.515 day, respectively. The maximum synthesis of pigments Chl *a* (8.26 mg/L) and Chl *b* (4.13 mg/L) was on 15th day in 3.3 mM CH_4N_2O. The *H. lacustris* SAG-19a exhibited maximum growth of 32×10^4 cells/mL in 3.3 mM CH_4N_2O on 15th day. The increase in growth (28%), pigments Chl *a* (14%) and Chl *b* (13%) was more than that of *H. lacustris* SAG-19a. The maximum accumulation of total carotenoids in *H. lacustris* HPI-001 was 18.50 mg/L, and astaxanthin content was 15.19 mg/mL at 4.9 mM of CH_4N_2O on 25th day (Fig. 4). The *H. lacustris* SAG-19a showed a maximum concentration of 15.97 mg/L total carotenoids and 12.84 mg/L astaxanthin content at 6.6 mM CH_4N_2O on 25th day (supplementary data S2). The increment of total carotenoids (15%) and astaxanthin content (18%) was more than *H. lacustris* SAG-19a.

3.4 Effect of different concentrations of $(NH_4)_2HPO_4$ and K_2CO_3

Under different concentrations of $(NH_4)_2HPO_4 + K_2CO_3$, the alga *H. lacustris* HPI-001 exhibited the maximum cell number of 35×10^4 cells/mL in the medium with 250 mg/L $(NH_4)_2HPO_4$ + 200 mg/L K_2CO_3 on 20th day of culture. At 250 mg/L $(NH_4)_2HPO_4$ + 200 mg/L K_2CO_3, a specific growth rate of 0.178 day^{-1}, division rate of 0.257 day^{-1} and generation time of 3.893 day were observed. The maximum synthesis of pigments recorded was 7.11 mg/L Chl *a* and 3.56 mg/L Chl *b* on 20th day at 250 mg/L $(NH_4)_2HPO_4$ + 200 mg/L K_2CO_3. The maximum growth in *H. lacustris* SAG-19a was recorded as 30×10^4 cells/mL in the medium with 200 mg/L $(NH_4)_2HPO_4$ + 150 mg/L K_2CO_3 on 20th day. Similarly, the maximum concentration of Chl *a* was 6.76 mg/L and Chl *b* was 3.39 mg/L at 200 mg/L $(NH_4)_2HPO_4$ + 150 mg/L K_2CO_3 on 20th day. The increase in growth (16%) and pigments Chl *a* (5%) and Chl *b* (5%) was more than that of *H. lacustris* SAG-19a. The *H. lacustris* HPI-001 accumulated a maximum total carotenoids of 15.99 mg/L and astaxanthin content of 12.69 mg/L at 150 mg/L $(NH_4)_2HPO_4$ + 100 mg/L K_2CO_3 on 25th day (Fig. 5), while the *H. lacustris* SAG-19a accumulated a maximum total carotenoids of 13.88 mg/L and astaxanthin content of 11.99 mg/L at 150 mg/L $(NH_4)_2HPO_4$ + 100 mg/L

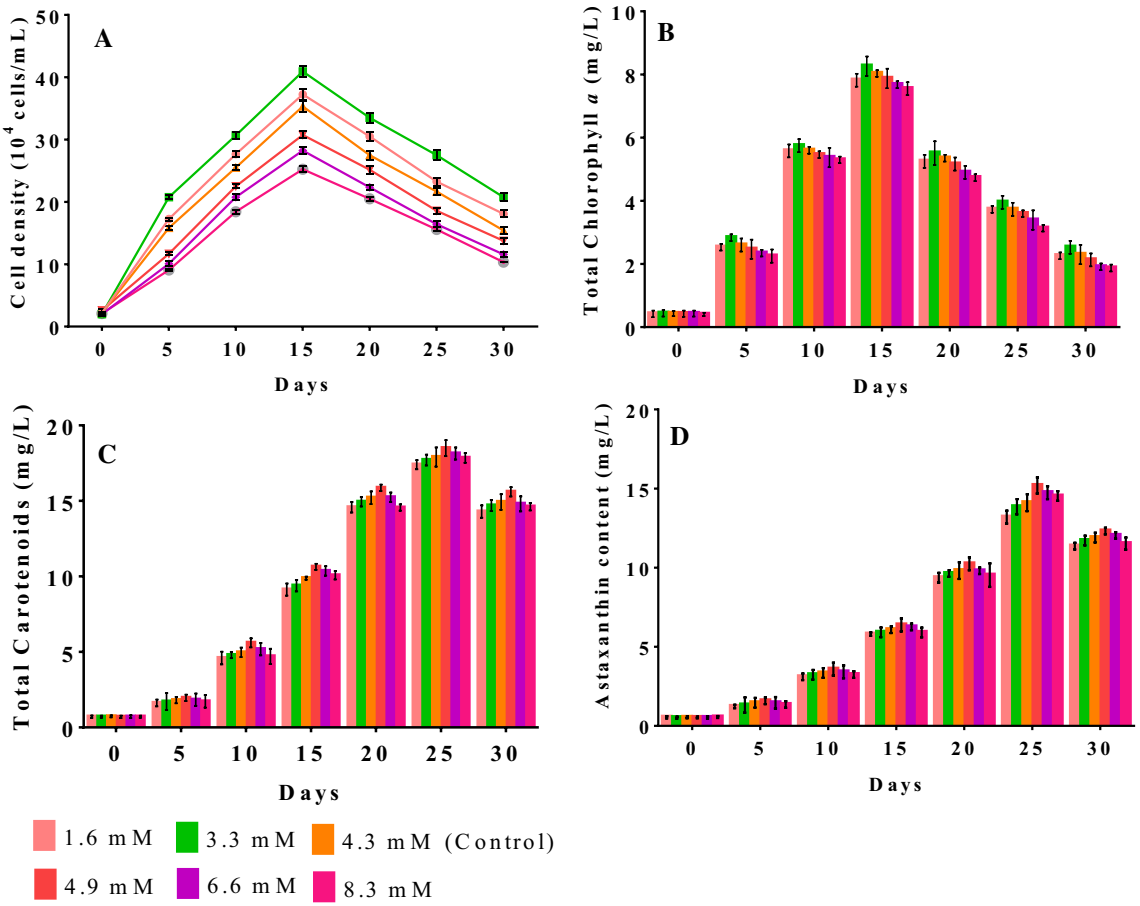

Fig. 4 Effect of different concentration of commercial agriculture fertilizers CH_4N_2O on a cell number (**a**), chlorophyll a (**b**), total carotenoids (**c**) and astaxanthin content (**d**) isolate of *H. lacustris* HPI-001 at different intervals in laboratory conditions

K_2CO_3 on 30th day (supplementary data S3). The increase in total carotenoids (15%) and astaxanthin content (10%) was more than control of *H. lacustris* SAG-19a.

3.5 Effect of different concentrations of $NaHCO_3$

The *H. lacustris* HPI-001 exhibited a maximum cell number of 51×10^4 cells/mL at 0.6 mM commercial $NaHCO_3$ in 20 days. At 0.6 mM of $NaHCO_3$, a specific growth rate of 0.184 day^{-1}, division rate of 0.266 day^{-1} and generation time of 3.759 day were observed. The maximum synthesis of pigments, Chl *a* (9.52 mg/L) and Chl *b* (4.76 mg/L), was in 0.6 mM of $NaHCO_3$ on 20th day. The *H. lacustris* SAG-19a showed a maximum growth of 45×10^4 cells/mL at 1.5 mM $NaHCO_3$ on 20th day. The maximum concentration of Chl *a* (8.81 mg/L) and Chl *b* (4.40 mg/L) was in 0.6 mM $NaHCO_3$ on 20th day. The increment of growth (13%) and pigments Chl *a* (8%) and Chl *b* (8%) was more than that of *H. lacustris* SAG-19a. The maximum accumulation of total carotenoids in the isolate of *H. lacustris* HPI-001 was 19.24 mg/L, and astaxanthin content was 15.53 mg/L in 1.5 mM of $NaHCO_3$ on 25th day of culture (Fig. 6). The strain *H. lacustris* SAG-19a synthesized maximum total carotenoids of 16.89 mg/L and astaxanthin content of 13.71 mg/L in 0.6 mM of $NaHCO_3$ on 30th day (supplementary data S4). The increase in total carotenoids (13%) and total astaxanthin (13%) was more compared to *H. lacustris* SAG-19a.

3.6 Comparative study of the two strains of *H. lacustris* grown in modified 3 N-BBM + V medium and formulated modified HPI-001 medium

The isolate *H. lacustris* HPI-001 grown in two different media tested showed a maximum growth of 43×10^4 cells/mL in formulated modified HPI-001 medium on 20th day. The alga grown in formulated modified HPI-001 medium showed a specific growth rate of 0.162 day^{-1}, division rate of 0.233 day^{-1} and generation time of 4.284 day. The alga synthesized 8.68 mg/L Chl *a* and 5.12 mg/L Chl *b* on 20th day, and 25.51 mg/L total carotenoids and 22.21 mg/L

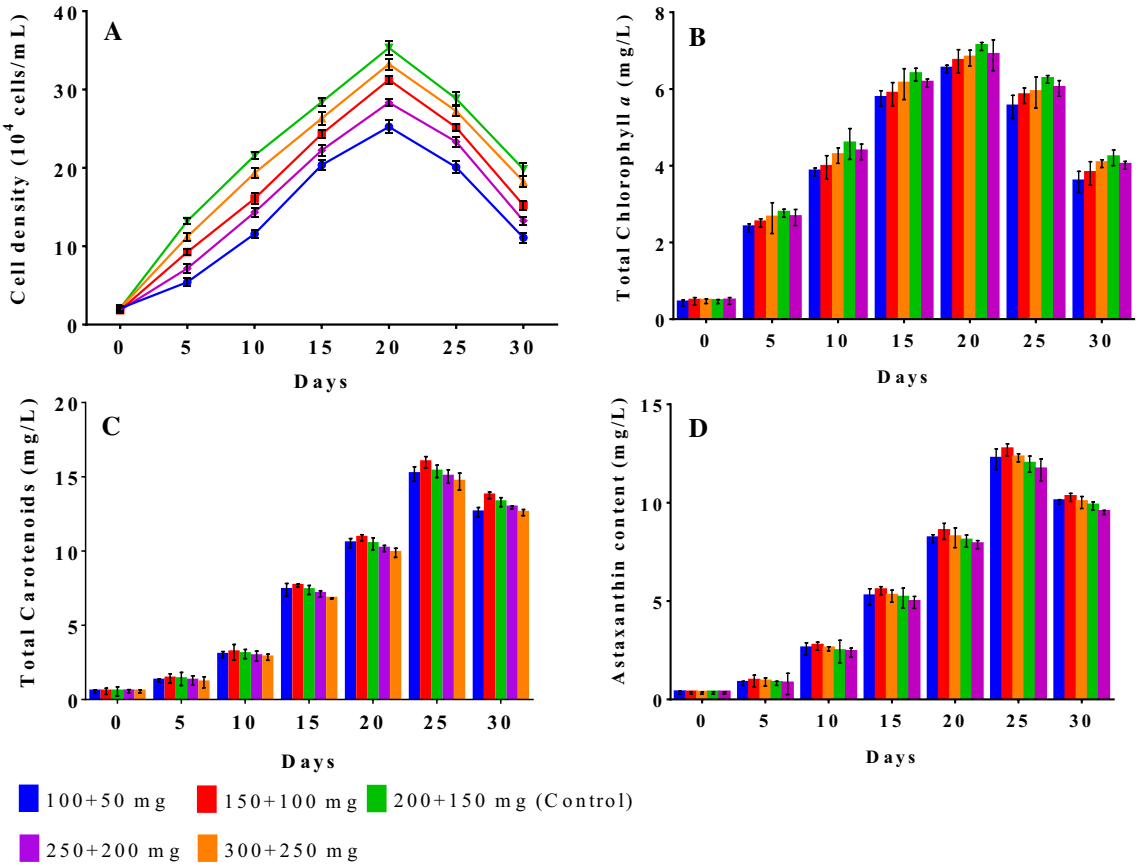

Fig. 5 Effect of different concentration of commercial agriculture fertilizers $(NH_4)_2HPO_4 + K_2CO_3$ on a cell number (**a**), chlorophyll a (**b**), total carotenoids (**c**) and astaxanthin content (**d**) isolate of *H. lacustris* HPI-001 at different intervals in laboratory conditions

astaxanthin content on 30th day in formulated modified HPI-001 medium (Fig. 7). The *H. lacustris* HPI-001 showed a maximum growth of 37×10^4 cells/mL in modified 3N-BBM + V medium on 20th day. The maximum synthesis of pigments Chl *a* (7.44 mg/L), Chl *b* (3.72 mg/L), total carotenoids (22.88 mg/L) and astaxanthin content (20.45 mg/L) was on 20th and 30th day in modified 3N-BBM + V medium. In case of *H. lacustris* SAG-19a, the maximum cell number of 40×10^4 cells/mL in formulated modified HPI-001 medium was on 20th day. The alga grown in formulated modified HPI-001 medium showed a specific growth rate of 0.184 day^{-1}, division rate of 0.266 day^{-1} and generation time of 3.763 day. The maximum concentration of pigments Chl *a* (7.22 mg/L) and Chl *b* (4.36 mg/L) was on 20th day, and maximum accumulation of total carotenoids (23.59 mg/L) and astaxanthin content (20.10 mg/L) was on 30th day in formulated modified HPI-001 medium (Table 3). The *H. lacustris* SAG-19a showed maximum growth of 35×10^4 cells/mL on 20th day of modified 3N-BBM + V medium. The maximum synthesis of Chl *a* (6.50 mg/L), Chl *b* (3.25 mg/L), total carotenoids (20.55 mg/L) and astaxanthin content (18.25 mg/L) was on 20th and 30th day in modified 3N-BBM + V medium (supplementary data S5). The increase in growth (8%), Chl *a* (20%), Chl *b* (17%), total carotenoids (8%) and astaxanthin content (10%) was more than that of *H. lacustris* SAG-19a.

4 Discussion

H. lacustris is a photosynthetic green microalga that can produce astaxanthin in cells. In general, astaxanthin production from *H. lacustris* is accomplished through a two-stage cultivation process including vegetative (green) and aplanospore (red) stages [44, 45]. The accumulation of astaxanthin is affected by environmental stress factors such as light, temperature, pH, salt concentration and nutritional stresses [11]. However, some factors limit outdoor production such as light intensity and temperature. Therefore, the induction of high astaxanthin accumulation with nutrition is a significant option. Though, including the neutral pH, neutral to the moderately basic range was recognized as the most favorable and suitable condition for the fungal contaminant to infect the algae cells [46], the present cultures were found to be devoid of any fungal

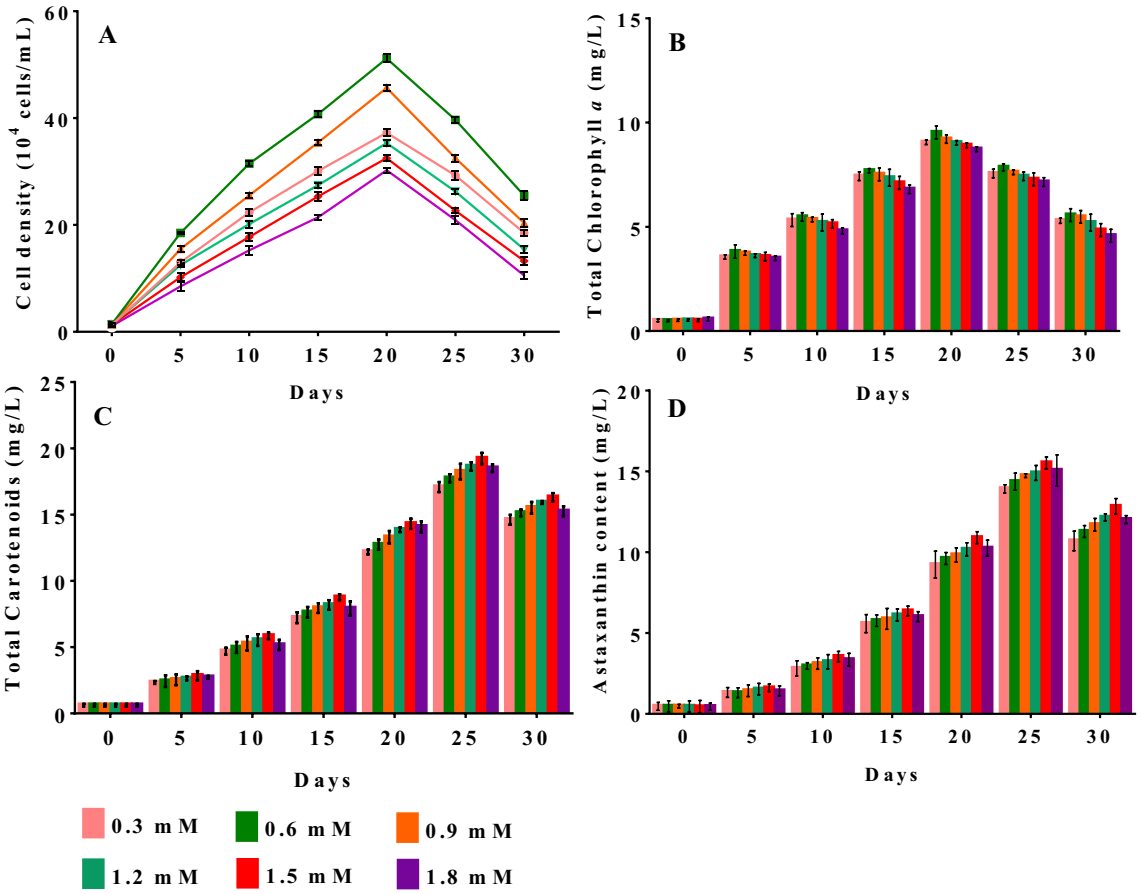

Fig. 6 Effect of different concentration of commercial $NaHCO_3$ on a cell number (**a**), chlorophyll a (**b**), total carotenoids (**c**) and astaxanthin content (**d**) isolate of *H. lacustris* HPI-001 at different intervals in laboratory conditions

contamination while grown at a pH of 7.5–7.8. In the present study, the commercial agriculture fertilizers such as NPK (17:17:17), CH_4N_2O, $(NH_4)_2HPO_4$, K_2CO_3 and $NaHCO_3$ were used for the enhancement of growth and astaxanthin production in two strains of *H. lacustris*.

In common, nitrogen has a marked positive effect on growth and a negative effect on lipid accumulation due to the fact that microalgae can assimilate several nitrogen sources like nitrate, nitrite, ammonium and urea [47]. However, the nitrogen is a crucial element for microalgae's growth development, reproduction and other physiological activities [48]. Dominguez-Bocanegra et al. [49] reported that the *H. lacustris* culture in test BBM obtained a maximum cell density of 3.5×10^4 cells/mL, whereas Goksan et al. [47] obtained a maximum cell density of 2.6×10^4 cells/mL with BG-11 medium plus different nitrogen sources. Similarly, Dalay et al. [21] reported that the commercial fertilizers (N:P:K 20:20:20) maximized cell concentration to 0.90 g/L with growth rate of 0.150 day^{-1}. Since the microalga *H. lacustris* grow better in culture media with low N:P, high phosphorus levels favored the accumulation of biomass content [50]. However, when compared to nitrogen, phosphorus concentration over 50% caused a decrease in growth of culture [51]. In fact, differences in N:P concentrations may reduce algal growth for its adaptation to stress conditions [52].

NPK fertilizer is a low-cost nitrogen fertilizer and a relevant tool in microalgal culture, mainly for Chlorophyceae. In the current study, the Bold basal medium minus $NaNO_3$, $K_2HPO_4{\cdot}3H_2O$ and KH_2PO_4 was added with commercial agricultural fertilizer (N:P:K 17:17:17) and used to grow two different strains of *H. lacustris*. The isolate of *H. lacustris* HPI-001 showed a maximum growth of 47×10^4 cells/mL with specific growth rate of 0.117 day^{-1}. The growth attained here is higher than that reported by Dominguez-Bocanegra et al. [49] and Goksan et al. [47]. The pigment Chl *a* was increased to 7.13 mg/L at 1.2 mM of commercial (N:P:K 17:17:17) in 15th and 20th day cultures. At 0.6 mM (N:P:K 17:17:17), the total carotenoids increased to 22.35 mg/L and astaxanthin content to 17.94 mg/L on 30th day. In strain *H. lacustris* SAG-19a exhibited a maximum cell number increased up to 42×10^4 cells/mL specific growth of 0.122 day^{-1}. The

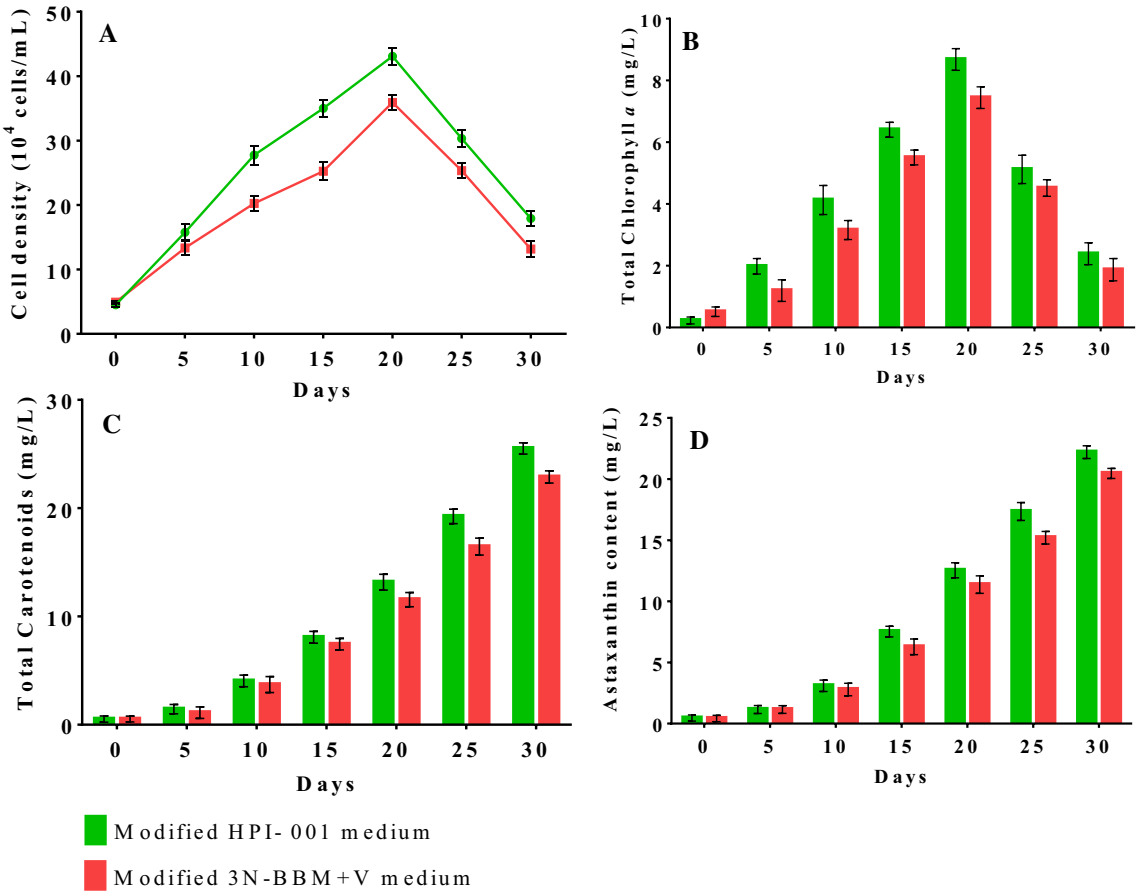

Fig. 7 Comparative study of algae grown in modified 3N-BBM+V medium and formulated commercial modified HPI-001 medium on a cell number (**a**), chlorophyll a (**b**), total carotenoids (**c**) and astaxanthin content (**d**) isolate of *H. lacustris* HPI-001 at different intervals in laboratory conditions

isolate *H. lacustris* HPI-001 increased in growth (11%), pigment Chl *a* (14%), Chl *b* (10%), total carotenoids (10) and astaxanthin content (16%) was more than control of *H. lacustris* SAG-19a.

It has been reported that nitrate is an important nutrient supplement, affecting the growth and biomass accumulation in the microalgae. It is also able to significantly change the rates of cell metabolism through the transformation among different forms [53, 54]. Moreover, nitrate was demonstrated to have a vital role for haematocyst germination in *H. lacustris* [55]. Nitrogen depletion in the culture medium is a stress factor of *H. lacustris*, while high phosphorus concentration foregrounds algal growth in the vegetative stage which normally lasts between 9 and 20 days according to the ratio between biomass and cell activity [56]. Chlorophyll as a nitrogen-rich compound can be utilized as an intracellular nitrogen supporter to continue the growth of cells once nitrogen sources are decreased in culture. However, chlorophyll degradation may occur due to the reduced level of chlorophylls a and b with reutilizing of nitrogen for cells [57]. These strategies are also employed in the *H. lacustris* cultures, and a remarkable enhancement of astaxanthin production is achieved due to nitrogen deficiency. For example, Fabregas et al. [55] reported the combined effects of light intensity and nutrient deficiency on astaxanthin synthesis by *H. lacustris*. In the present investigation, when grown in BBM devoid of $NaNO_3$ and added with the commercial fertilizer CH_4N_2O, the *H. lacustris* HPI-001 showed maximum growth of 41×10^4 cells/mL with specific growth rate of 0.197 day^{-1} and *H. lacustris* SAG-19a exhibited the maximum cell number of 32×10^4 cells/mL with specific growth rate of 0.205 day^{-1}. The increase in growth (28%) was higher than that of *H. lacustris* SAG-19a. *H. lacustris* HPI-001 produced maximum concentration of Chl *a* (8.26 mg/L) and Chl b (4.13 mg/L) at 3.3 mM CH_4N_2O on 15th day. The 4.9 mM of CH_4N_2O supported the highest accumulation of total carotenoids (18.49 mg/L) and astaxanthin (15.19 mg/L) on 25th day. The increase in pigments such as Chl *a* (14%), Chl b (13%), total carotenoids (15%) and astaxanthin content (18%) was higher compared to that of *H. lacustris* SAG-19a.

Table 3 Comparative study on growth and astaxanthin content of *H. lacustris* HPI-001 and *H. lacustris* SAG-19a using agriculture fertilizers

Different parameters conditions	H. lacustris HPI-001 cell number (10^4 cells/mL)	H. lacustris SAG-19a cell number (10^4 cells/mL)	H. lacustris HPI-001 Astaxanthin (mg/L)	H. lacustris SAG-19a Astaxanthin (mg/L)
N:P:K (17:17:17) (mM)				
0.3	33 ± 0.344	33 ± 0.444	17.49 ± .0.414	14.66 ± 0.410
0.6	40 ± 0.420	35 ± 0.350	17.94 ± 0.509	15.37 ± 0.455
0.9	43 ± 0.527	37 ± 0.325	17.57 ± 0.503	15.02 ± 0.326
1.2	47 ± 0.524	40 ± 0.515	17.15 ± 0.303	14.72 ± 0.510
1.5	37 ± 0.422	42 ± 0.520	16.81 ± 0.419	14.50 ± 0.420
1.8	30 ± 0.324	38 ± 0.210	16.36. ± 0.286	14.16 ± 0.222
CH_4N_2O (mM)				
1.6	37 ± 0.525	25 ± 0.447	13.20 ± 0.407	11.35 ± 0.467
3.3	41 ± 0.338	32 ± 0.557	13.85 ± 0.481	13.83 ± 0.448
4.3	35 ± 0.520	29 ± 0.407	14.11 ± 0.532	12.13 ± 0.435
4.9	31 ± 0.544	27 ± 0.348	15.19 ± 0.509	12.47 ± 0.539
6.6	28 ± 0.244	24 ± 0.532	14.74 ± 0.409	12.84 ± 0.285
8.3	25 ± 0.243	22 ± 0.546	14.53 ± 0.307	12.81 ± 0.372
$(NH_4)_2HPO_4 + K_2CO_3$ (mg)				
100 + 50	25 ± 0.419	22 ± 0.510	12.21 ± 0.528	11.54 ± 0.450
150 + 100	31 ± 0.533	26 ± 0.425	12.69 ± 0.307	11.99 ± 0.525
200 + 150	33 ± 0.300	30 ± 0.520	12.28 ± 0.206	11.76 ± 0.310
250 + 200	35 ± 0.267	28 ± 0.310	11.96 ± 0.406	11.51 ± 0.520
300 + 250	28 ± 0.164	24 ± 0.255	11.67 ± 0.553	11.33 ± 0.425
$NaHCO_3$ (mM)				
0.3	37 ± 0.444	31 ± 0.515	13.93 ± 0.374	9.85 ± 0.455
0.6	51 ± 0.342	34 ± 0.325	14.37 ± 0.521	12.71 ± 0.420
0.9	46 ± 0.536	36 ± 0.310	14.73 ± 0.345	10.10 ± 0.510
1.2	35 ± 0.233	41 ± 0.215	14.91 ± 0.463	9.84 ± 0.525
1.5	33 ± 0.144	45 ± 0.420	15.53 ± 0.370	9.73 ± 0.420
1.8	30 ± 0.443	39 ± 0.443	15.07 ± 0.455	9.51 ± 0.510
Modified HPI-001 medium	43 ± 0.351	40 ± 0.425	22.21 ± 0.357	20.10 ± 0.429
3N-BBM + V medium (Control)	37 ± 0.320	35 ± 0.442	20.45 ± 0.549	18.25 ± 0.425

Each value is the means of three experiments with triplicate each (n = 3). Statistically the means of three experiments not significantly different ($P < 0.05$)

Phosphorus is an important nutrient for algal growth. It is responsible for the energy transfer of cells and the formation of cell membranes and nucleic acids. Besides being a structural element in nucleic acid and phospholipids, it plays crucial roles in various biological functions such as energy transformation, activation of metabolic intermediates, signal transduction cascades and regulation of enzymes [58, 59]. When compared to nitrate, phosphate has received less attention in the optimization approach and has long been considered to promote the growth at moderate or low concentration approximately 0.5 mM [60, 61], while at the same time it can promote the carotenogenesis at higher concentration up to 0.9 mM [60]. Harker et al. [62] reported that the carotenoids accumulation has been shown to be reduced when phosphate supply was increased above 0.85 mM. There are some few reports on *H. lacustris* growth in N/P ratio close or below 1, but in all cases it was identified that the low N/P conditions were favorable for growth [20, 21, 62]. Brinda et al. [63] reported that the high biomass and astaxanthin accumulation were achieved in *H. lacustris* under phosphorus deficiency conditions. In our study, the $K_2HPO_4.3H_2O$ and KH_2PO_4 in basal medium was replaced with commercial agricultural fertilizer $(NH4)_2HPO_4 + K_2CO_3$ and both the strains were grown in it. In the *H. lacustris* HPI-001 exhibited maximum cell number of 35×10^4 cells/mL with a specific growth rate of 0.178 day^{-1}, in which the growth (16%) was more than that of *H. lacustris* SAG-19a. Similarly, the photosynthetic pigment also increased as compared to the control on 20th days. The maximum accumulation of total carotenoids (15.99 mg/L) and astaxanthin content of (12.69 mg/L) in isolate *H. lacustris* HPI-001 was on 25th days. Ping et al.

[64] reported that only 11 mg/L carotenoids were obtained in phosphate deficiency conditions. In the isolate, the increase in carotenoids (15%) and astaxanthin content (10%) was more than that of *H. lacustris* SAG-19a.

The carbon rate characterizes algal nutrition rates and constitutes 40–50% of algal biomass, although biomass is 47–50% in *H. lacustris* [65]. Moreover, based on the recent study, carbon mass fraction in *H. lacustris* can range from 46 to 55% [66]. The enrichment of CO_2 is a requirement for achieving high microalgal culture productivity. At the same time, high CO_2 levels are frequently stressful to microalgae, particularly for their photosynthetic apparatus [67, 68]. The recent research reported that the cultivation of *H. lacustris* under high CO_2 demonstrated that increasing CO_2 percentage in the gas mixture used for the culture sparging to 5% is favorable for astaxanthin accumulation [69, 70]. Ding et al. [71] reported that under steady-state conditions, astaxanthin content in *H. lacustris* was 0.41%. The previous studies on the cultivation of *H. lacustris* have showed that acetate appears to be an important carbon source, enhancing both growth and carotenogenesis [45, 72, 73]. However, the effect of acetate was concentration dependent, higher concentrations inhibiting growth but markedly increasing astaxanthin content per cell [74]. Bicarbonate is widely used as a primary carbon source in suspended culture of photosynthetic microorganisms [68]. The present investigation used basal medium added with commercial $NaHCO_3$ to grow *H. lacustris* HPI-001 which showed a maximum growth of 51×10^4 cells/mL with specific growth rate of 0.184 day^{-1} and maximum chlorophyll production at 0.6 mM of $NaHCO_3$ on 20th day, whereas the increase in total carotenoids (13%) and astaxanthin content (13%) was at 1.5 mM $NaHCO_3$ on 25th day.

In batch culture processes, the optimal *Haematococcus* media produced a cell number of 6.25×10^4 cells/mL after 14 days of culture with no astaxanthin being accumulated [65]. A report by Domìnguez-Bocanegra et al. [49] states that the maximal growth of *H. lacustris* obtained was 3.5×10^4 cells/mL in the BBM medium under continuous illumination (177 µmol photons m^{-2} s^{-1}) with continuous aeration (1.5 vvm). Based on the above observations, a modified medium was formulated and designed, named as formulated commercial modified HPI-001 medium. The algal strains grown in the above medium were compared with the modified 3N-BBM + V. The isolate *H. lacustris* HPI-001 showed maximum growth of 43×10^4 cells/mL with a specific growth rate of 0.162 day^{-1}. The maximum synthesis of pigments was 8.68 mg/L Chl *a*, 5.12 mg/L Chl *b* on 20th day, while the maximum accumulation of total carotenoids (25.51 mg/L) and astaxanthin content (22.21 mg/L) was on 30th day in cultures using formulated modified HPI-001 medium. The *H. lacustris* SAG-19a culture serving as *H. lacustris* SAG-19a exhibited maximum growth of 40×10^4 cells/mL. The maximum concentration of pigments Chl *a* (7.22 mg/L) and Chl *b* (4.36 mg/L) was on 20th day, while the maximum accumulation of total carotenoids (23.59 mg/L) and astaxanthin content at (20.10 mg/L) was on 30th day, when cultured in the formulated modified HPI-001 medium. A comparatively high growth rate and pigment production was observed in the indigenously isolated *H. lacustris* HPI-001 than in the *H. lacustris* SAG-19a, when both were cultured using the formulated modified HPI-001 medium. This indicates that a promising alternative, cheaper and reproducible medium can be formulated using commercial agricultural fertilizers for achieving higher biomass and pigment astaxanthin production from *H. lacustris*.

5 Conclusion

From the present study, it is evident that the indigenous isolate of *H. lacustris* HPI-001 exhibited increased growth and astaxanthin production in the formulated modified HPI-001 medium when compared to the *H. lacustris* SAG-19a. This indicates that a promising alternative, cheaper and reproducible medium can be formulated using commercial agricultural fertilizers for achieving higher biomass and pigment astaxanthin production from *H. lacustris*. It is important that the modified HPI-001 medium which was formulated in this study for the first time is a very cost-effective medium compared to the control modified Bold basal medium (3N-BBM + V). Currently, the algal-based biotechnology companies and industries are looking for cost-effective solution for mass cultivation of commercially important microalga like *H. lacustris* for maximum biomass production. Hence, the present investigations recommend the formulated modified HPI-001 medium as a best source for maximum content of biomass and astaxanthin production. The outdoor studies are under progress with Bayir Extracts Pvt. Ltd., Bangalore, India.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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