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Impact of cultivation parameters on astaxanthin accumulation in the green alga *Haematococcus lacustris* RRGK isolated from Himachal Pradesh, India

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ABSTRACT

The unicellular, freshwater microalga *Haematococcus lacustris* is one of the finest microbial sources of astaxanthin, but when cultivated with conventional media it exhibits low growth rates and final densities. In this study, the effect of media, pH, light intensities, and various inorganic salts such as $NaNO_3$, $K_2HPO_4 \bullet 3H_2O$, KH_2PO4, and NaCl were investigated on the vegetative cells of *Haematococcus lacustris* RRGK (formerly *H. lacustris* HPI-001) isolated from Himachal Pradesh in India and *H. lacustris* SAG-19a procured from Gottingen Culture Collection, Germany. The latter served as the control. The isolate of *H. lacustris* RRGK recorded a maximum of 26.48 mg/L astaxanthin in 3N-BBM+*V* medium, 23.16 mg/L at 40.0 $\mu Em^{-2}s^{-1}$ light intensity, 21.85 mg/L at pH 7.5, 21.84 mg/L in 6.0 mM of $NaNO_3$, 18.69 mg/L in 0.45 mM of $K_2HPO_4 \bullet 3H_2O$, 23.86 mg/L in 1.1 mM of KH_2PO_4 and 22.82 mg/L in 0.80 mM of NaCl. Based on the above-mentioned findings, a Modified HPI- 001A medium was formulated. A maximum production of algal biomass corresponding to a cell number of 53×10^4cells/mL and an astaxanthin content of 27.16 mg/L were documented in this novel media developed for improving growth and production of astaxanthin in *H. lacustris* RRGK under laboratory conditions.

1. Introduction

Haematococcus lacustris is one of the abundant natural reservoirs of the carotenoid astaxanthin which finds a wide realm of applications in nutraceutical, pharmaceutical, aquaculture, and food industries [1, 2]. Astaxanthin may be produced by various microorganisms such as the marine bacterium *Agrobacterium aurantiacum, Chromochloris zofingiensis*, and the red yeast *Phaffia rhodozyma* apart from *Haematococcus lacustris* [3]. The *H. lacustris* is capable of producing a maximum astaxanthin of up to 4% of its total dry weight when exposed to extreme stress conditions such as nutrients deficit, hyper salinity, excessive radiation and illumination, addition of acetate *etc.* [4, 5].

The *H. lacustris* (Girod-Chantrans) Rostafinski 1875 (*Haematococcus pluvialis* Flotow) is a freshwater, motile, biflagellate, unicellular green microalga belonging to the class Chlorophyceae [6], popular for its high production of the antioxidant carotenoid astaxanthin, utilized as pigment and/or as a nutritional constituent in foods and aquaculture [7]. In the current scenario, there is a rising concern over the use of the chemically synthesized astaxanthin as colorant in aquaculture of salmon and hence there is a growing interest in deriving astaxanthin from natural sources. The life cycle of *H. lacustris* consists of four stages *viz.*, macrozooid (zoospore), microzooid, palmella, and haematocyst (aplanospore) [8, 2]. The favorable environment supports the prevalence of the flagellated macrozooids, while on shifting to an adverse environmental condition, the macrozooids lose flagella, increase their cell size and form non-motile palmella. The palmella further turn into red-colored resting haematocysts with prolonged stress conditions such as high light irradiation and nutrient deficiency.

The *H. lacustris*is primarily occurs in small temporal freshwater bodies such as the natural and man-made ponds, temporary rain pools, and birdbaths and are widely distributed in many ecosystems around the world [9]. It is commonly found in temperate regions of the world and

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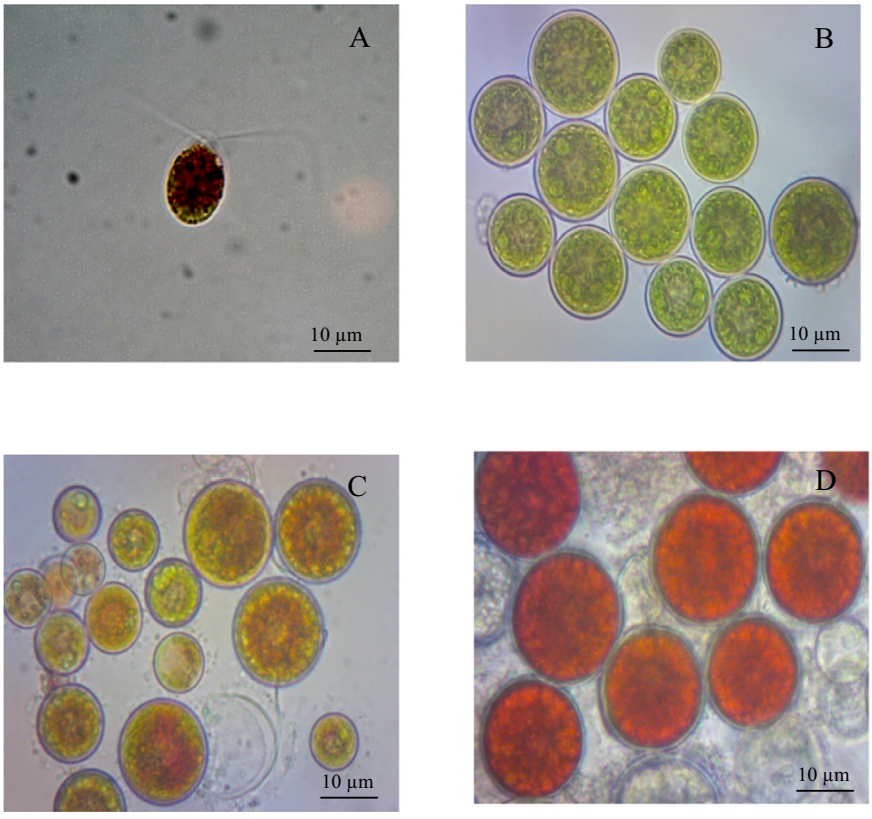

Fig. 1. Different growth stages of *Haematococcus lacustris* HPI- 001 (A) Bi-flagellate motile cells (B) Non-motile green vegetative cells (C) Astaxanthin accumulating palmella cells transition to aplanospores; (D) Astaxanthin accumulated aplanospores. Scale bar: 10 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

have been reported from Europe, North America, Africa, and Himachal Pradesh in India [10]. Among the four different stages of this microalga, the non-motile aplanospore coccoid with abundant astaxanthin has been found to be extremely tolerant to the changing adverse conditions [11].

Growth enhancement strategies can be successful in a suitable culture medium. The content of chlorophylls [12], carotenoids [13], fatty acids, and soluble carbohydrates [14] within cells vary significantly with a change in the concentration of nutrients in the medium for algal cultivation. The process of microalgal cultivation demands the expense of energy and involves the consumption of macro and micronutrient through the use of light. In addition, the efficiency of the utilization of various nutrients varies with increased production of biomass [15]. The processes of carotenogenesis as well as the encystment and germination in the life cycle of H. *lacustris* require light [16]. Higher light intensity favored haematocyst germination but not the astaxanthin production [17]. Microalgal growth depends on its physiological requirement for nutrients essentially nitrogen and phosphorus as well as the type of species [18, 19]. The macronutrient potassium is essential for the metabolic activities related to microalgal growth [20]. Tam et al. [21] reported the effect of different NaCl concentrations on the growth and production of astaxanthin in *H. lacustris*. An increase in NaCl concentration reduced growth and increased carotenoids content per cell observed under salinity stress.

Astaxanthin, chemically called 3, 3′-dihydroxy-β-carotene-4, 4′-dione is a ketocarotenoid with an extraordinarily high antioxidant activity than any other carotenoid or vitamin E [22, 1]. It is 54 times more potent than β-carotene, 65 times more powerful than vitamin C, 100 times effective than tocopherol [23, 24]. In addition, most studies indicated that astaxanthin aids in promotion of health by treatment and/or prevention of innumerable diseases for instance, human cardiovascular diseases, cancers *etc*. [25]. The antioxidative ability of astaxanthin enhances immune response and the health of heart, joints, skin, and prostate thereby protecting against inflammation, UV-light photo-oxidation, cancers, age-related macular degeneration, and aging [26].

In the present investigation, the isolate of *Haematococcus lacustris* RRGK which is the former name of *H. lacustris* HPI-001 collected from Himachal Pradesh, India and another strain *H. lacustris* SAG-19a obtained from Gottingen Culture Collection, Germany were used. The Gottingen strain served as a reference control. The latter's adaptation to the laboratory conditions was investigated. The two strains were grown in the 3N-BBM+*V* medium amended with sodium nitrate ($NaNO_3$), dipotassium hydrogen phosphate ($K_2HPO_4 \bullet 3H_2O$), potassium dihydrogen phosphate (KH_2PO_4), and sodium chloride (NaCl) under laboratory conditions to study the impact of different physical parameters and media modification on the growth and production of astaxanthin.

2. Materials and methods

2.1. *Isolation of* H. lacustris *HPI-001*

The water samples were collected from a town called Palampur (32°N latitude; 76°E longitude) in the Indian state of Himachal Pradesh during the month of March 2013. Palampur has an average annual temperature of 19 °C and an average annual rainfall of ≥250 cm. The samples from each aquatic source was collected and stored in plastic vials. Further, the samples were inoculated in Bold Basal Medium (BBM) at 25±1 °C, with light irradiations under 30$\mu Em^{-2}s^{-1}$ and a photoperiod of 12/12 (light/dark). The cultures were thoroughly mixed manually twice a day. The unialgal culture of *H. lacustris* HPI-001 was prepared by isolation and serial dilution followed by streak plate preparation on 2% agar in BBM. Based on morphological and molecular analysis, the cells were identified. *H. lacustris* SAG-19a was procured from Gottingen Culture Collection, Germany.

2.2. *Growth and culture experiments*

Both the strains of *H. lacustris* were grown autotrophically in liquid BBM maintained at 25±1 °C under 30µEm^{-2}s^{-1} light intensity (warm white Philips lamps; 36 W; 4 ft Philips Trulite, India) for a photoperiod

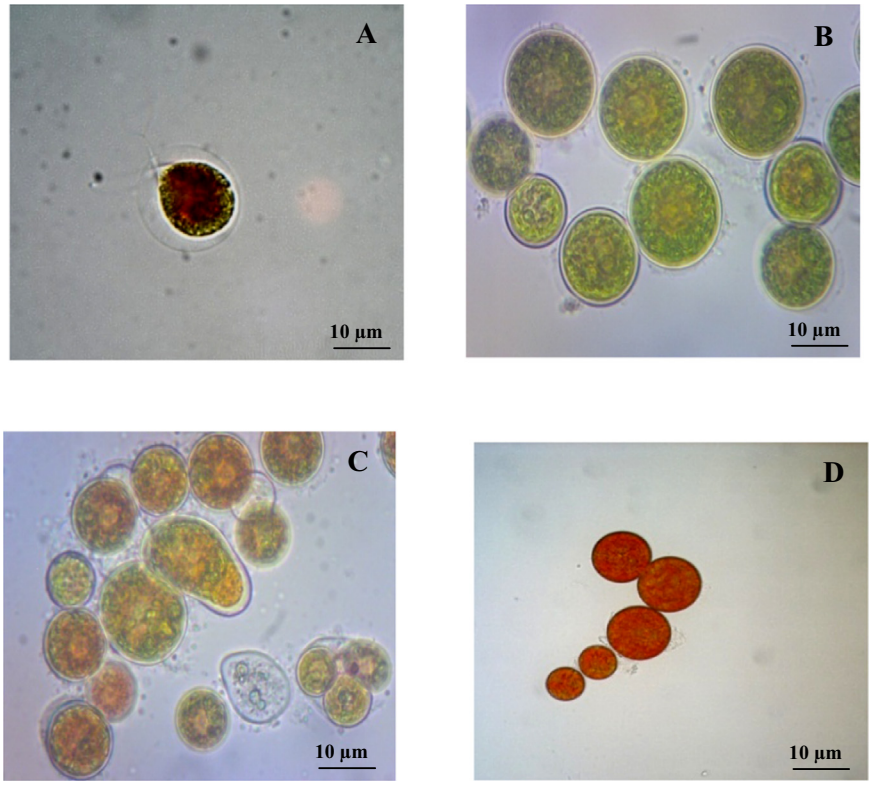

Fig. 2. Different growth stages of *Haematococcus lacustris* SAG- 19a (A) Bi-flagellate motile cells (B) Non-motile green vegetative cells (C) Astaxanthin accumulating palmella cells transition to aplanospores; (D) Astaxanthin accumulated aplanospore s. Scale bar: 10 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of 12/12 h (light/dark). The pure culture (10 mL) with a cell density of 2.0×10^4 cells mL^{-1} was taken from the 7th day culture (exponential growth phase) and was inoculated into 90 mL of sterilized BBM aseptically. The lux meter (TES 1331, Taiwan) was used to measure light intensity. The culture growth parameters like the number of cells, content of pigments *viz*., Chlorophyll *a* (Chl *a*), Chlorophyll *b* (Chl b), total carotenoids, and astaxanthin were measured periodically at every 5 days interval for a period of 30 days. The monoalgal culture developed so was used in the following studies.

2.3. Maintenance of microalgae

The strains of *H. lacustris* were grown autotrophically in liquid Bold Basal medium and maintained at 25±1 °C, 30µEm^{-2}s^{-1} light intensity and 12/12 h (light/dark) photoperiod.

2.4. Culture and induction medium composition

The alga was grown in different media such as BBM, 3N-BBM+*V*, BG-11, and modified CHU-13 (**Table 1**), light intensities such as 10.0, 20.0, 30.0 (control) and 40.0 µEm^{-2}s^{-1}, and various pH such as 6.0, 6.5, 7.0 (control), 7.5, 7.8 and 8.0. The basal medium (3N-BBM+*V*) minus sodium nitrate ($NaNO_3$) was amended with different concentrations of $NaNO_3$ such as 3.0, 6.0, 9.0 (control), 12.0, and 15.0 mM. The basal medium minus dipotassium hydrogen phosphate ($K_2HPO_4 \bullet 3H_2O$) was added with different concentrations of $K_2HPO_4 \bullet 3H_2O$ such as 0.15, 0.30, 0.45 (control), 0.60, and 0.75 mM. The basal medium minus potassium dihydrogen phosphate (KH_2PO_4) was added with different concentrations of KH_2PO_4 such as 0.9, 1.1, 1.3 (control), 1.5, and 1.7 mM, and the basal medium minus sodium chloride (NaCl) was added with different concentrations of NaCl such as 0.08, 0.25, 0.45 (control), 0.60, and 0.80 mM. All these modified media were used to culture *H. lacustris* and the cell number, total chlorophylls (Chl *a* and Chl b), total carotenoids, and astaxanthin were recorded at an interval of five days.

2.4.1. Cell count and growth curve

The 'Neubauer' hemocytometer was used for this purpose (REF: 0303 212, Neubauer Improved Bright-Line, HBG, Germany). The cell count was calculated as the mean of the cell numbers recorded in four chambers and expressed as multiples of 10^4cells mL^{-1}. Growth curves were plotted with the cell count against the respective days on which it was recorded.

2.4.2. Specific growth rate, division per day, generation time

The specific growth rate implies the number of generations or doubling in an exponential growth culture per unit time. The following formula was used to determine this [27]. $\mu = \ln (N_t/N_0)/(T\text{-}t)$; where N_t and N_0 are the number of cells at the end and the beginning of log phase respectively, 'T' and 't' implies the final day and the initial day of log phase respectively. When 'T' is expressed in days, it is possible to convert the growth rate (µ) to the division level as $K = \mu/1n$ (2).

2.4.3. Estimation of carotenoids and chlorophylls

The different photosynthetic pigments such as Chl *a*, Chl b, and total carotenoids of *Haematococcus* samples were extracted by following the methods [28]. The culture (5 mL) was centrifuged (R-8C; Remi Instruments Ltd, Mumbai, India) at 5000 rpm for 10 min and discarded the supernatant. Five milliliters of 100% acetone was added to the pellet and macerated using mortar and pestle. The ground pellet along with the mortar was wrapped in black paper and stored at 4 °C overnight. Further, the sample was centrifuged at 5000 rpm for 10 min to collect the supernatant. The absorbance of the supernatant was recorded at wavelengths 661.6 nm, 644.8 nm, 470 nm, and 490 nm in Hitachi U2900 UV–Vis spectrophotometer (Hitachi, Japan) in standard quartz cuvette (190 – 2500 nm), and 10 mm path length. The astaxanthin content was measured from the acetone extract at 490 nm. The astaxanthin per unit volume was calculated using the method [29].

$$\text{Chl } a \text{ (mg } L^{-1}) = 11.24 \times A_{661.6} - 2.404 \times {}_{644.8}$$

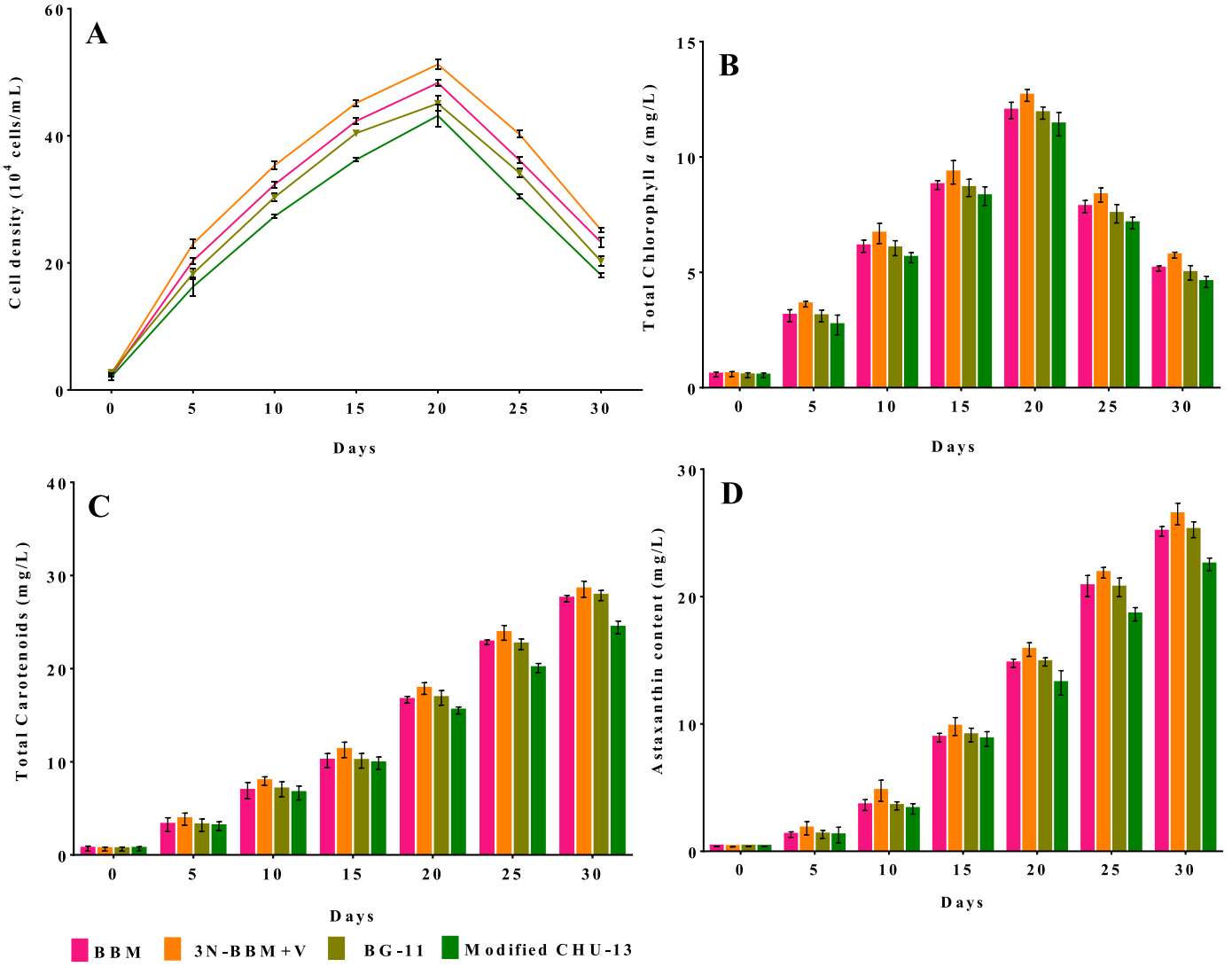

Fig. 3. Effect of different medium on a cell number (A), Chlorophyll *a* (B), total carotenoids (C) and astaxanthin content (D) isolate of *H. lacustris* HPI-001 at different intervals in laboratory conditions.

$$\text{Chl b (mg } L^{-1}) = 20.13 \times A_{644.8} - 4.19 \times {}_{661.6}$$

$$\frac{\text{Totalcarotenoids(mg L}^{-1}) = 1000 \times A_{470} - -1.9 \times \text{Chl } a - -63.14 \times \text{Chl b}}{214}$$

$$\text{Astaxanthin (mg } L^{-1}) = 4.5 \times A_{490} \times \text{Va/Vb}$$

Where, *A* = absorbance, Va = volume of extracts, Vb = volume of culture sample

2.5. Statistical analysis

The experiments were carried out in triplicate and presented as Mean ± Standard errors and Graph Pad Prism 6 software was used in the construction of the graphs.

3. Results

3.1. Identification and morphological features of H. lacustris HPI-001

Haematococcus lacustris which is related to most of the unicellular green volvocalean algae has four stages in its life cycle such as macrozooid, microzooid, palmella, and haematocysts. The vegetative cells are spherical to ovoid with a length of 20–30 μm, diameter of 10–20 μm, and biflagellate with thin cell walls detached from the protoplast by mucilaginous material filled broad spaces. Cytoplasmic fibers are typically branched, distributed evenly across the protoplasm binding the protoplast to the cell wall. *H. lacustris* has a single, cup-shaped, parietal chloroplast occupying 1/3 of the protoplast and 2 to 6 scattered pyrenoids. There are two anterior isokont flagella equal to the size of a cell and surrounded by short divergent tubing between the protoplast and the envelope. There is a central nucleus. Cysts are characterized by the absence of flagella, a gradual increase in cell size and thick cell wall growth. The resting cells contain high levels of astaxanthin. Reproduction takes place via the development of zoospores, which contain 2 to 4 cells per sporangium. Sexual reproduction is sometimes observed in certain cells under stressed conditions resulting in quadriflagellated motile zygotes, followed by dropping of flagella and formation of thickened walls (LABOMED VISION 2000, 40 X magnifications). The strain was confirmed through molecular studies (18S rRNA) and recognized as *H. lacustris* HPI-001 and the molecular data was submitted in GenBank (KT285940). The strains were maintained at the Algal Culture Collection, center for Advanced Studies in Botany, University of Madras, Tamil Nadu, India (Figs. 1& 2).

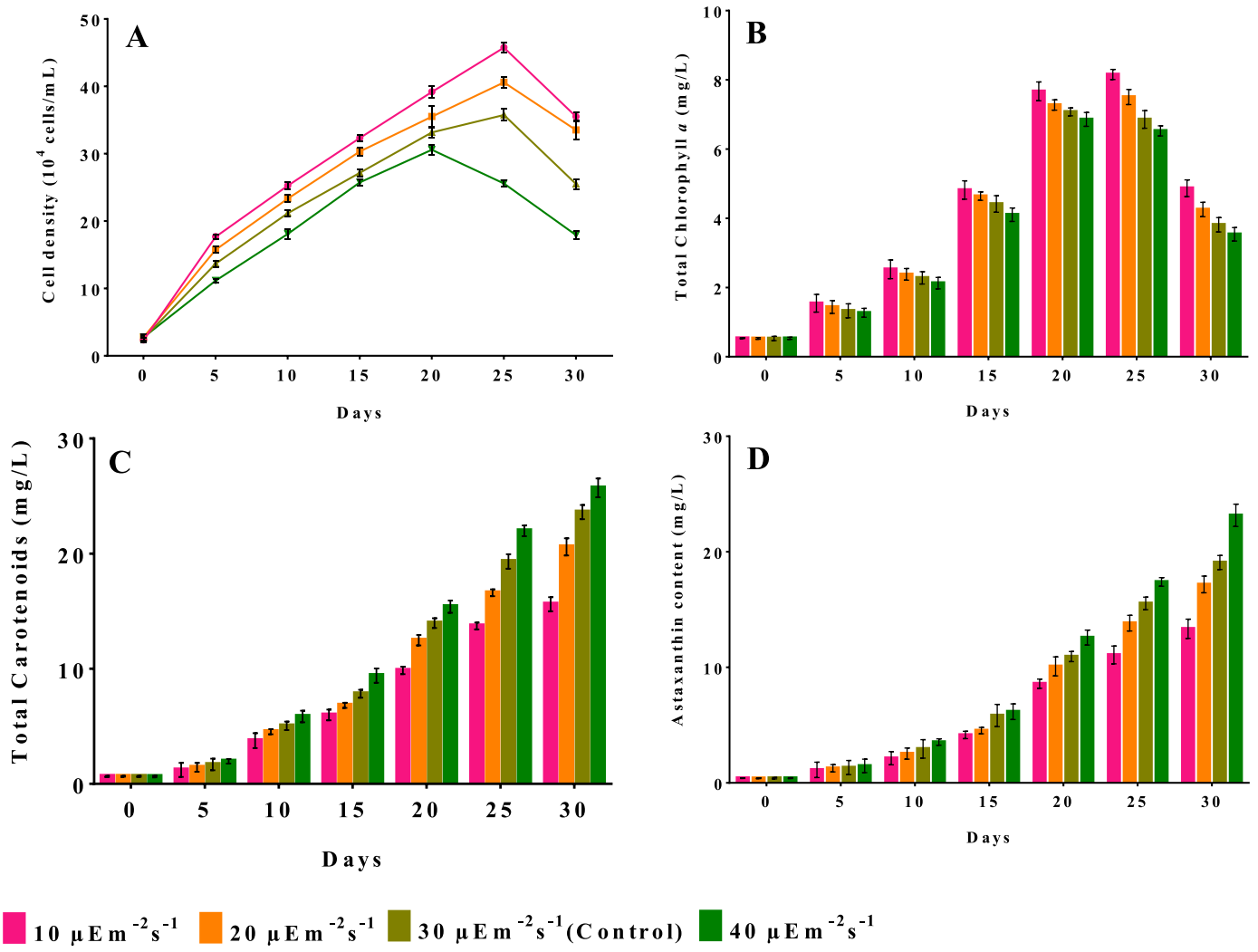

Fig. 4. Effect of different light intensities on a cell number (A), Chlorophyll *a* (B), total carotenoids (C) and astaxanthin content (D) isolate of *H. lacustris* HPI-001 at different intervals in laboratory conditions.

Table 1
Chemical and medium composition.

Components	BBM (mg/L)	3N-BBM+*V* (mg/L)	BG-11 (mg/L)	Modified CHU-13 (mg/L)
$NaNO_3$	25,000	25,000	1500	–
$CaCl_2.3H_2O$	2500	2500	36	107
$MgSO_4.7H_2O$	7500	7500	75	200
$K_2HPO_4.3H_2O$	7500	7500	40	80
KH_2PO_4	17,500	17,500	–	–
NaCl	2500	2500	–	–
KnO_3	–	–	–	371
$C_6H_5FeO_7$	–	–	–	20
$C_6H_8O_7$	–	–	6	100
$C_6H_8FeNO_7$	–	–	6	–
EDTA (with Na_2)	750	750	10	–
Na_2CO_3	–	–	20	–
$FeCl_3.6H_2O$	97	97	–	–
$MnCl_2.4H_2O$	41	41	109	181
$ZnCl_2.6H_2O$	5	5	–	–
$CoCl_2.6H_2O$	2	2		–
$Na_2MoO_4.2H_2O$	4	4	39	39
H_3BO_3	–	–	289	289
$ZnSO_4.7H_2O$	–	–	22	22
$CuSO_4.5H_2O$	–	–	79	8
$Co(No_3)_2.6H_2O$	–	–	50	5
H_2SO_4	–	–	–	1mL
Glass distilled H_2O	1000 mL	1000 mL	1000 mL	1000 mL
pH	7.0	7.5–7.8	7.5	7.5

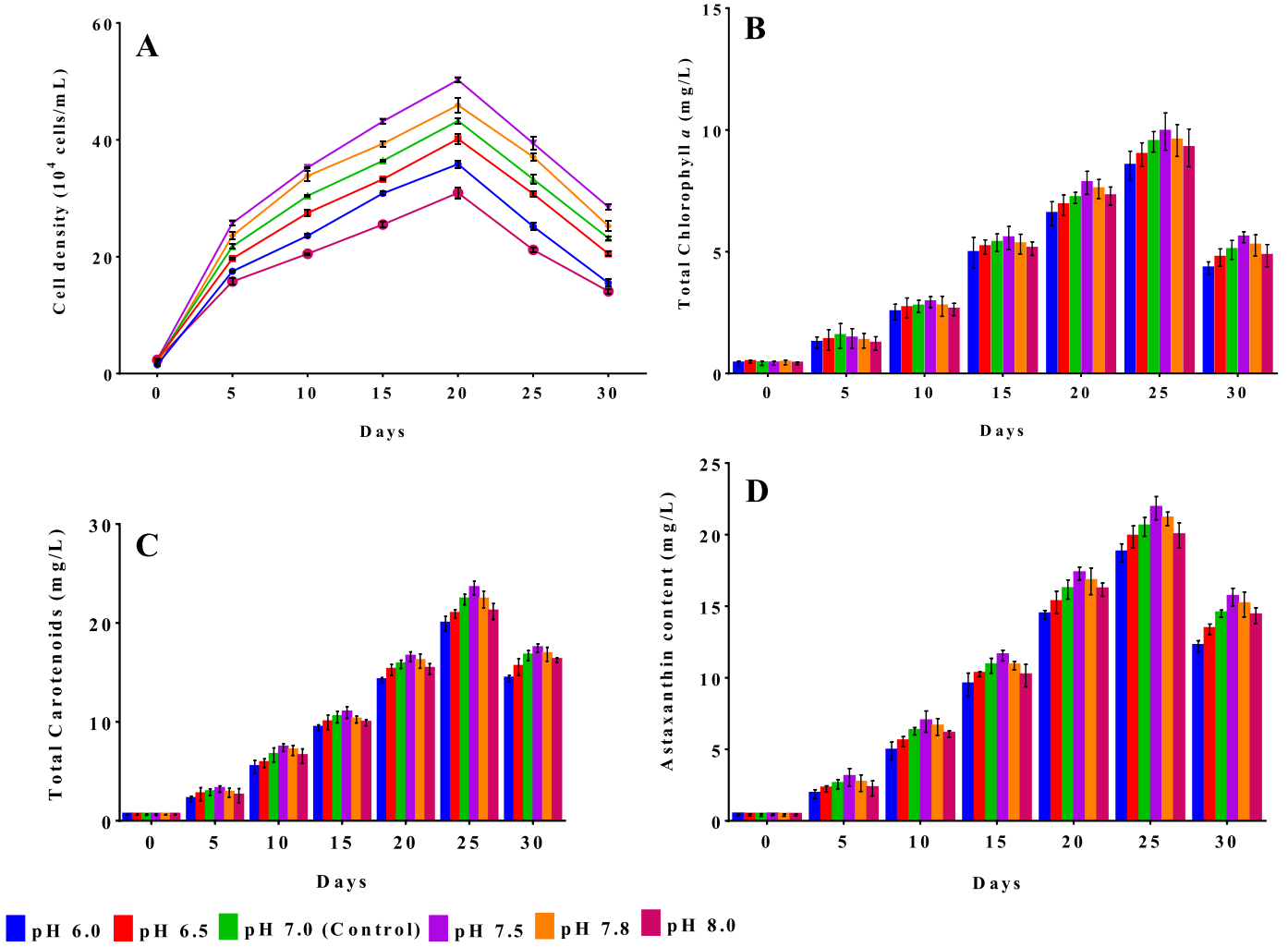

Fig. 5. Effect of different pH on a cell number (A), Chlorophyll *a* (B), total carotenoids (C) and astaxanthin content (D) isolate of *H. lacustris* HPI-001 at different intervals in laboratory conditions.

3.2. Effect of media

The strains of *H. lacustris* were grown in different media. *H. lacustris* HPI-001 exhibited a maximum cell number of 51×10^4cells mL^{-1} in 3N-BBM+*V* medium on the 20th day of culture. In 3N-BBM+*V* medium, the alga showed a specific growth rate of 0.149 day^{-1}, the division rate of 0.214 day^{-1} and generation time of 4.666 days. The *H. lacustris* SAG-19a exhibited the maximum cell number of 45×10^4cells mL^{-1} on the 20th day in the 3N-BBM+*V* medium. The algae showed a specific growth rate of 0.149 day^{-1}, a division rate of 0.213 day^{-1} and a generation time of 4.698 days. The increase in growth of 13% was more than that of *H. lacustris* SAG-19a. The alga *H. lacustris* HPI-001 produced a maximum concentration of pigments such as Chl *a* (12.67 mg/L), Chl b (6.36 mg/L), total carotenoids (28.52 mg/L), and astaxanthin (26.48 mg/L) in 3N-BBM+*V* on 20th, 20th, 30th and 30thday, respectively (Fig. 3).

While *H. lacustris* SAG-19a showed a maximum of Chl *a* (10.83 mg/L),Chl b (5.42 mg/L), total carotenoids (25.49 mg/L), and astaxanthin (22.77 mg/L) in 3N-BBM+*V* on 20th, 20th, 30th and 30th-day, respectively **(Supplementary Fig. (S1)**. Among the strain, *H. lacustris* HPI- 001 showed high values of different media when compared to control, *H. lacustris* SAG- 19a. The increase of Chl *a* by 17%, Chl b by 17%, total carotenoids by 13%, and astaxanthin content by 16% was noted when compared to *H. lacustris* SAG- 19a.

3.3. Effect of light intensities

When grown in different light intensities, *H. lacustris* HPI-001 showed a maximum cell number of 46×10^4cells/mL under 10μEm^{-2}s^{-1} on the 25th day. At 10μEm^{-2}s^{-1}, a specific growth rate of 0.129 day^{-1}, a division rate of 0.187 day^{-1} and a generation time of 5.353 days was observed. The *H. lacustris* SAG-19a showed a maximum cell number at 40×10^4cells/mL under 10μEm^{-2}s^{-1} on the 25th day. At 10μEm^{-2}s^{-1}, the algae showed a specific growth rate of 0.116 day^{-1}, a division rate of 0.168 day^{-1} and a generation time of 5.970 days. The increase in growth of 15% was more than that of *H. lacustris* SAG-19a. The *H. lacustris* HPI-001 had a maximum concentration of Chl *a* (8.16 mg/L) and Chl b (4.09 mg/L) on the 25th day of 10μEm^{-2} s^{-1} light intensity. The *H. lacustris* SAG-19a had a maximum concentration of Chl *a* (7.39 mg/L) and Chl b (3.70 mg/L) in 10μEm^{-2} s^{-1} light intensity on 25th day. The *H. lacustris* HPI-001 had 10% increase in pigments Chl *a* and Chl b compared to *H. lacustris* SAG-19a. In *H. lacustris* HPI-001, maximum accumulation of total carotenoids (25.71 mg/L) and astaxanthin (23.16 mg/L) was at 40μEm^{-2}s^{-1} on 30th day (Fig. 4). In *H. lacustris* SAG-19a, maximum synthesis of total carotenoids of 23.13 mg/L and astaxanthin content of 20.82 mg/L was at 40μEm^{-2}s^{-1} on 30th day **(S2)**. The former exhibited an increase in total carotenoids (11%) and astaxanthin content (11%) when compared to control.

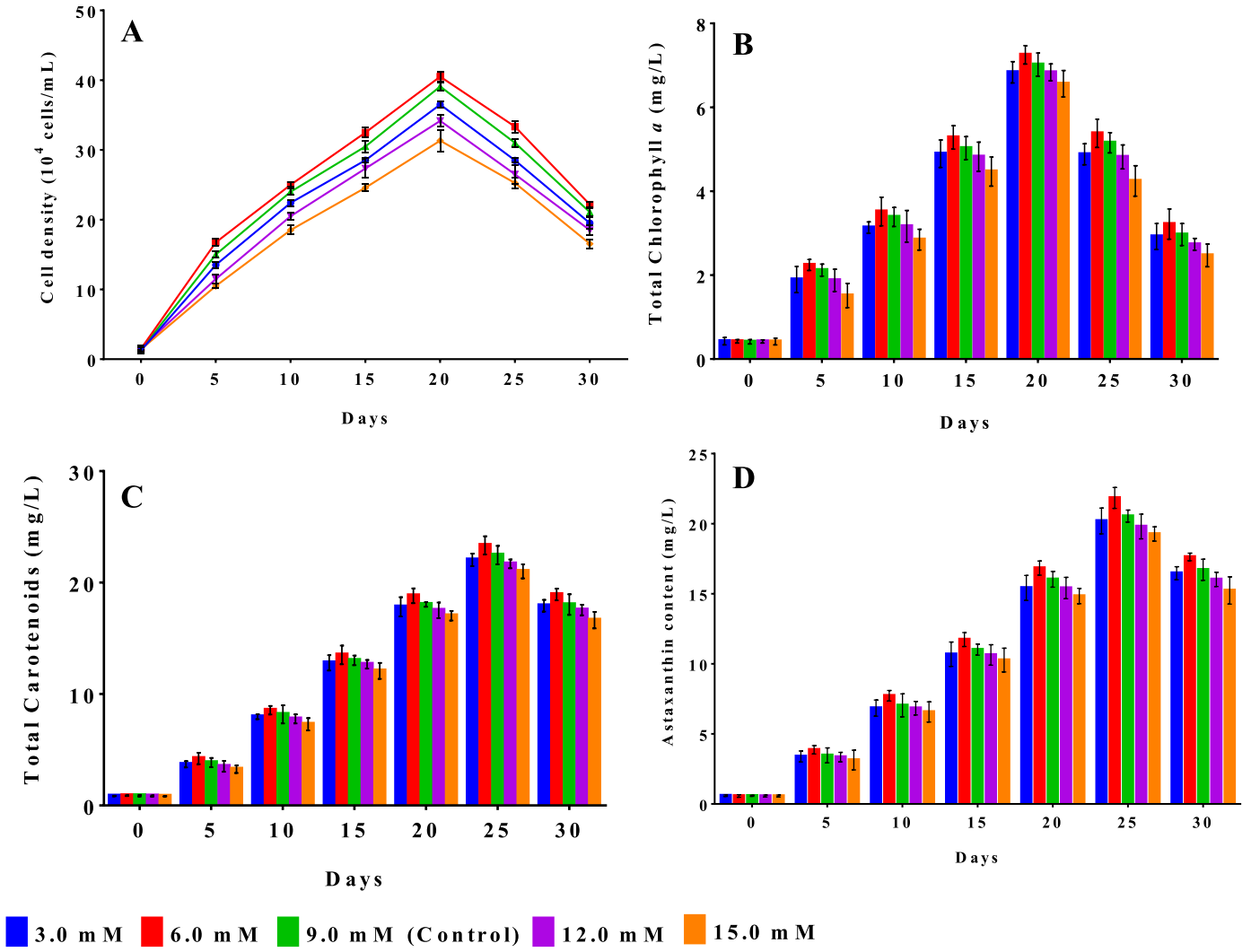

Fig. 6. Effect of different concentration of $NaNO_3$ on a cell number (A), Chlorophyll *a* (B), total carotenoids (C) and astaxanthin content (D) isolate of *H. lacustris* HPI- at different intervals in laboratory conditions.

3.4. Effect of initial pH

The maximum cell number of 50 × 10^4cells/mL in *H. lacustris* HPI-001 was at pH 7.5 on the 20th day. At pH 7.5, the alga showed a specific growth rate of 0.130 day^{-1}, a division rate of 0.188 day^{-1} and a generation time of 5.328 days. In *H. lacustris* SAG-19a, the maximum cell number of 40 × 10^4cells/mL was at pH 7.5 on the 20th day. At pH 7.5, a specific growth rate of 0.153 day^{-1}, the division rate of 0.221 day^{-1} and generation time of 4.521 days was observed. The increase in growth by 25% was more than that of control. The maximum synthesis of pigments Chl *a* (9.93 mg/L) and Chl b (4.96 mg/L) in *H. lacustris* HPI-001 was at pH 7.5 on the 25th day. While *H. lacustris* SAG-19a showed a maximum concentration of Chl *a* (7.88 mg/L) and Chl b (3.94 mg/L) at pH 7.0 on 20th day culture. The increase of Chl *a* by 26% and Chl b by 25% was more compared to *H. lacustris* SAG-19a. The *H. lacustris* HPI-001 showed maximum accumulation of total carotenoids (23.52 mg/L) and astaxanthin (21.85 mg/L) at pH 7.5 on 25th day of culture (**Fig. 5**). The *H. lacustris* SAG-19a showed maximum accumulation of total carotenoids (21.36 mg/L) and astaxanthin content (18.81 mg/L) at pH 7.0 on 25th day **(S3)**. The former accumulated more amount of total carotenoids (10%) and astaxanthin content (16%) when compared to control.

3.5. Effect of sodium nitrate

The alga *H. lacustris* HPI-001 showed the highest cell number of 41 × 10^4cells/mL in the medium with 6.0 mM $NaNO_3$ on the 20th day. At 6.0 mM of $NaNO_3$ the specific growth rate of 0.161 day^{-1}, the division rate of 0.233 day^{-1} and generation time of 4.300 days were recorded. While in case of *H. lacustris* SAG-19a, the highest cell number of 35 × 10^4cells/mL at 12.0 mM of $NaNO_3$ on the 20th day was observed. At 12.0 mM of $NaNO_3$ a specific growth rate of 0.147 day^{-1}, a division rate of 0.212 day^{-1} and a generation rate of 4.712 days were observed. The alga increased in cell number by 17% compared to control. The alga *H. lacustris* HPI-001 showed a maximum synthesis of Chl *a* (7.25 mg/L) and Chl b (3.62 mg/L) at 6.0 mM of $NaNO_3$ in the 20th day. In control alga, the amount of Chl *a* (6.56 mg/L) and Chl b (3.29 mg/L) was the highest at 12.0 mM of $NaNO_3$ on 25th day culture. Thus the former strain has an increase in pigments Chl *a* (10%) and Chl b (10%) than the latter. In *H. lacustris* HPI-001, accumulation of total carotenoids (23.35 mg/L) and astaxanthin content (21.84 mg/L) was the maximum at 6.0 mM of $NaNO_3$ in 25th day culture (**Fig. 6**). In control *H. lacustris* SAG-19a, a synthesis of total carotenoids (21.91 mg/L) and astaxanthin (19.56 mg/L) was the maximum at 12.0 mM of $NaNO_3$ on 30th day

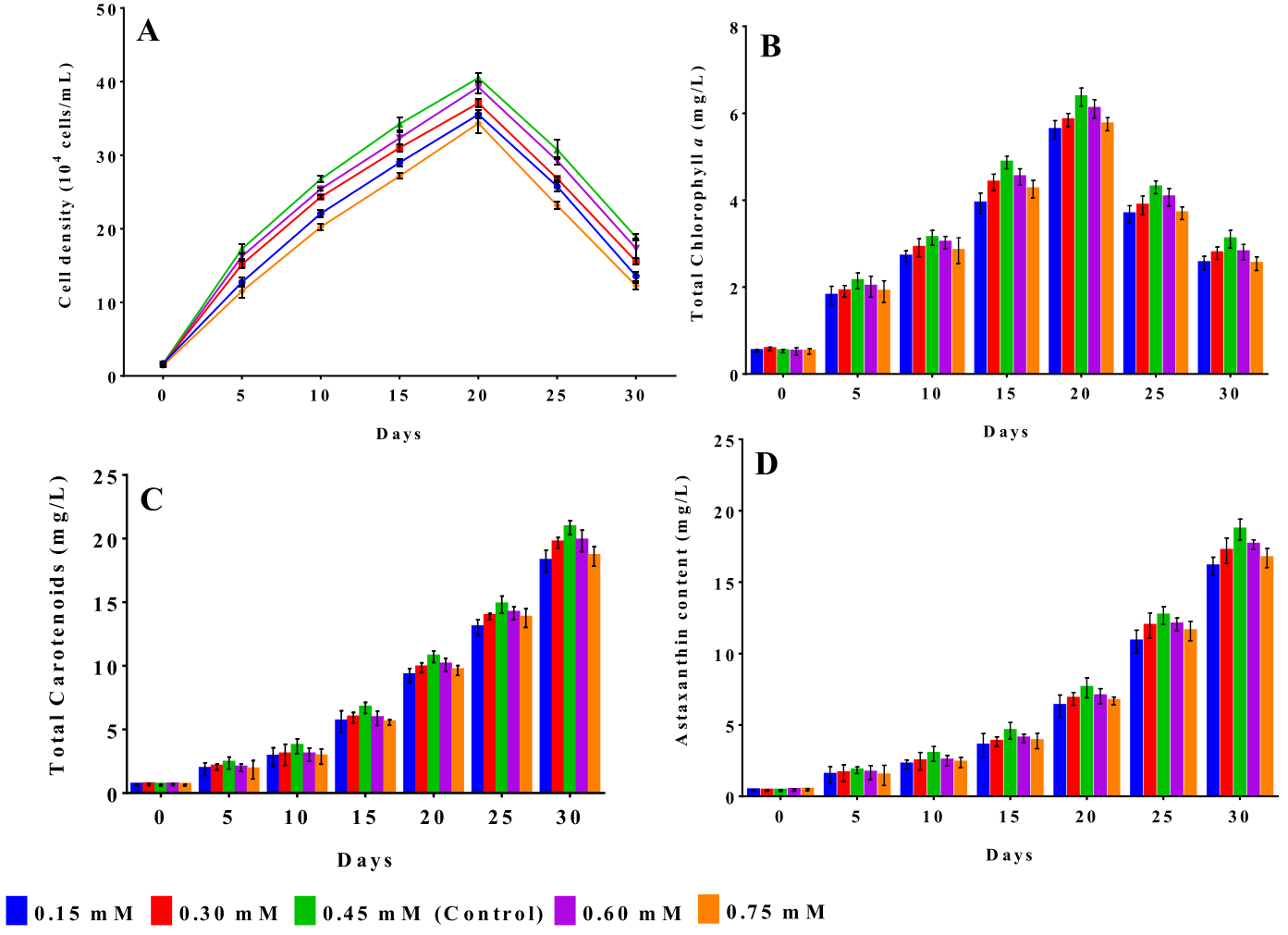

Fig. 7. Effect of different concentration of $K_2HPO_4 \bullet 3H_2O$ on a cell number (A), Chlorophyll *a* (B), total carotenoids (C) and astaxanthin content (D) isolate of *H. lacustris* HPI-001 at different intervals in laboratory conditions.

(S4). The increase in total carotenoids (7%) and astaxanthin (11%) in the former was more compared to *H. lacustris* SAG-19a.

3.6. Effect of dipotassium phosphate

In case of *H. lacustris* HPI-001 the highest cell number of 41 × 10^4cells/mL at 0.45 mM of $K_2HPO_4 \bullet 3H_2O$ on the 20th day of culture was observed. At 0.45 mM of $K_2HPO_4 \bullet 3H_2O$, a specific growth rate of 0.156 day^{-1}, a division rate of 0.226 day^{-1} and a generation time of 4.433 days were observed. Whereas the *H. lacustris* SAG-19a had the highest cell number of 36 × 10^4cells/mL at 0.45 mM of $K_2HPO_4 \bullet 3H_2O$ on the 20th day. At 0.45 mM $K_2HPO_4 \bullet 3H_2O$, the specific growth rate of 0.182 day^{-1}, the division rate of 0.263 day^{-1} and generation time of 3.800 days were observed. The isolate increased in cell number by 13% in comparison to the control. The *H. lacustris* HPI-001 showed a maximum accumulation of Chl *a* (6.37 mg/L) and Chl b (3.18 mg/L) while the *H. lacustris* SAG-19a exhibited a maximum synthesis of Chl *a* (5.72 mg/L) and Chl b (2.86 mg/L) at 0.45 mM $K_2HPO_4 \bullet 3H_2O$ in 20th day culture. The increase in pigment content, Chl *a* by 11% and Chl b by 11% was higher compared to control. Similarly, the maximum amount of total carotenoids (20.87 mg/L) and astaxanthin (18.69 mg/L) was observed in *H. lacustris* HPI-001 at 0.45 mM of $K_2HPO_4 \bullet 3H_2O$ in 30th day culture **(Fig. 7)**. In *H. lacustris* SAG-19a, the amount of total carotenoids (18.47 mg/L) and astaxanthin (16.67 mg/L) was maximum on the 30th day at 0.60 mM $K_2HPO_4 \bullet 3H_2O$ **(S5)**. The alga with an increase of total carotenoids by 13% and astaxanthin content by 12% was higher than in *H. lacustris* SAG-19a.

3.7. Effect of potassium dihydrogen phosphate

On using KH_2PO_4 in the medium, the highest cell number of 48 × 10^4cells/mL was noted in *H. lacustris* HPI-001 at 1.5 mM KH_2PO_4 on 20th day of culture. At 1.5 mM of KH_2PO_4, the specific growth rate of 0.179 day^{-1}, the division rate of 0.259 $day^{-1}$and generation time of 3.865 days were observed. The *H. lacustris* SAG-19a reached a maximum cell number of 38 × 10^4cells/mL at 1.5 mM KH_2PO_4 on the 20th day. At 1.5 mM of KH_2PO_4, the specific growth rate of 0.168 day^{-1}, the division rate of 0.243 day^{-1} and generation time of 4.114 days were noted. The increase of cell number by 26% was observed when compared to control. In alga *H. lacustris* HPI-001, synthesis of pigments Chl *a* and Chl b was recorded at 9.85 mg/L and 4.97 mg/L, respectively at 1.5 mM of KH_2PO_4 on 20th day. The maximum Chl *a* (7.83 mg/L) and Chl b (3.91 mg/L) in *H. lacustris* SAG-19a was at 1.5 mM KH_2PO_4 in the 20th day of culture. The increase in content of Chl *a* by 25% and Chl b by 27% in *H. lacustris* HPI-001 was higher in comparison to *H. lacustris* SAG-19a. Whereas the total carotenoids (25.74 mg/L) and astaxanthin (23.86 mg/L) in *H. lacustris* HPI-001 was maximum at 1.1 mM of KH_2PO_4 on 30th day **(Fig. 8)** and in *H. lacustris* SAG-19a the total carotenoids (23.56 mg/L) and astaxanthin (20.75 mg/L) was maximum at 1.5 mM KH_2PO_4 on 30th day **(S6)**. The isolate had increased total

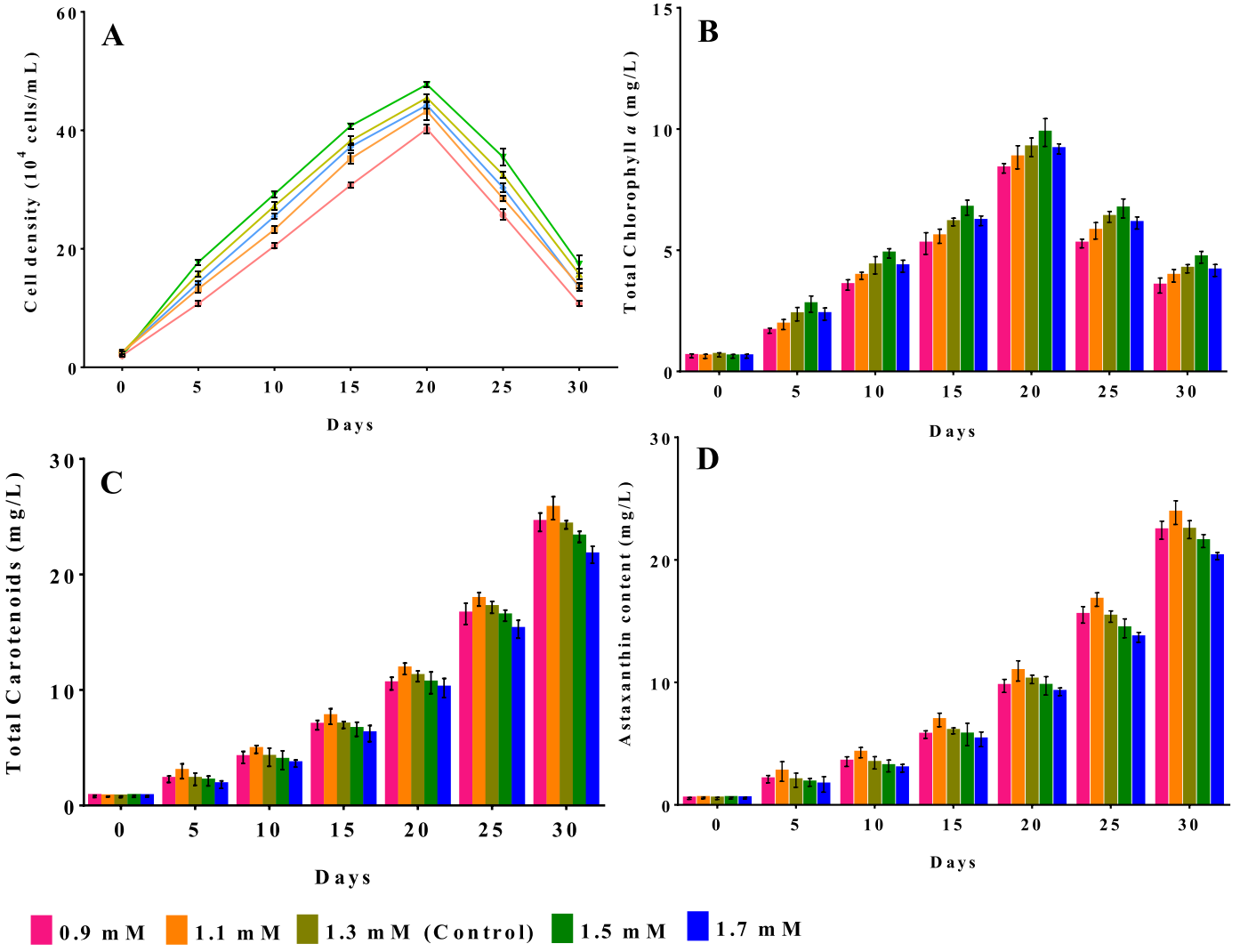

Fig. 8. Effect of different concentration of KH_2PO_4 on a cell number (A), Chlorophyll *a* (B), total carotenoids (C) and astaxanthin content (D) isolate of *H. lacustris* HPI-001 at different intervals in laboratory conditions.

carotenoids by 10% and astaxanthin by 15% when compared to *H. lacustris* SAG-19a.

3.8. Effect of sodium chloride

The *H. lacustris* HPI-001 was observed with the maximum cell number of 43 × 10^4cells/mL at 0.45 mM NaCl on the 20th day. At 0.45 mM of NaCl, the specific growth rate of 0.185 day^{-1}, the division rate of 0.267 day^{-1} and generation time of 3.743 days were noted. The maximum cell number of 37 × 10^4cells/mL in *H. lacustris* SAG-19a was at 0.60 mM NaCl on the 20th day. At 0.60 mM of NaCl, a specific growth rate of 0.174 day^{-1}, a division rate of 0.251 $day^{-1}$and a generation time of 3.977 days were recorded. The former strain with an increase of cell number by 16% was higher compared to *H. lacustris* SAG-19a. Further, the maximum amount of pigments Chl *a* (6.59 mg/L) and Chl b (3.29 mg/L) was noticed in *H. lacustris* HPI-001 at 0.45 mM NaCl on 20th day and in *H. lacustris* SAG-19a, Chl *a* and Chl b were maximum (5.80 mg/L and 2.91 mg/L respectively) at 0.60 mM NaCl on 20th day. The isolate with an increase of Chl *a* by14% and Chl b by 13% was higher compared to *H. lacustris* SAG-19a. The maximum total carotenoids and astaxanthin of 24.94 mg/L and 22.82 mg/L respectively was observed in *H. lacustris* HPI-001 at 0.80 mM of NaCl on 30th day (**Fig. 9**). While a maximum of total carotenoids and astaxanthin content of 21.53 mg/L and 18.87 mg/L respectively was noted in *H. lacustris* SAG-19a at 0.80 mM NaCl on 25th day **(S7)**. The isolate alga with an increase of total carotenoids by 16% and astaxanthin content by 21% were more compared to *H. lacustris* SAG-19a.

3.9. Comparative study of H. lacustris HPI-001 grown in modified 3N-BBM+V medium and modified HPI- 001A medium

H. lacustris HPI-001 grown in the Modified HPI-001A exhibited the highest cell number of 53 × 10^4cells/mL on the 20th day. A specific growth rate of 0.215 day^{-1}, a division rate of 0.310 day^{-1} and a generation time of 3.222 days was also observed. The amount of Chl *a* (9.24 mg/L) and Chl b (4.63 mg/L) was the maximum on the 20th day while that of total carotenoids (30.35 mg/L) and astaxanthin (27.16 mg/L) was on 30th day. On cultivating *H. lacustris* HPI-001 in Modified 3N- BBM+*V* medium, the highest cell number of 41 × 10^4 cells/mL was noted on 20th day which was 29% less than the alga grown in the Modified HPI-001A medium. The pigments, Chl *a* 7.40 mg/L, Chl b 3.70 mg/L were recorded the maximum on 20th day and the total carotenoids 27.88 mg/L and astaxanthin 25.38 mg/L were recorded the maximum on 30th day in the Modified 3N- BBM+*V* medium (**Fig. 10**) (**Table 1**). The above values were 25%, 25%, 10% and 8% less than the organism grown in the Modified HPI- 001A medium.

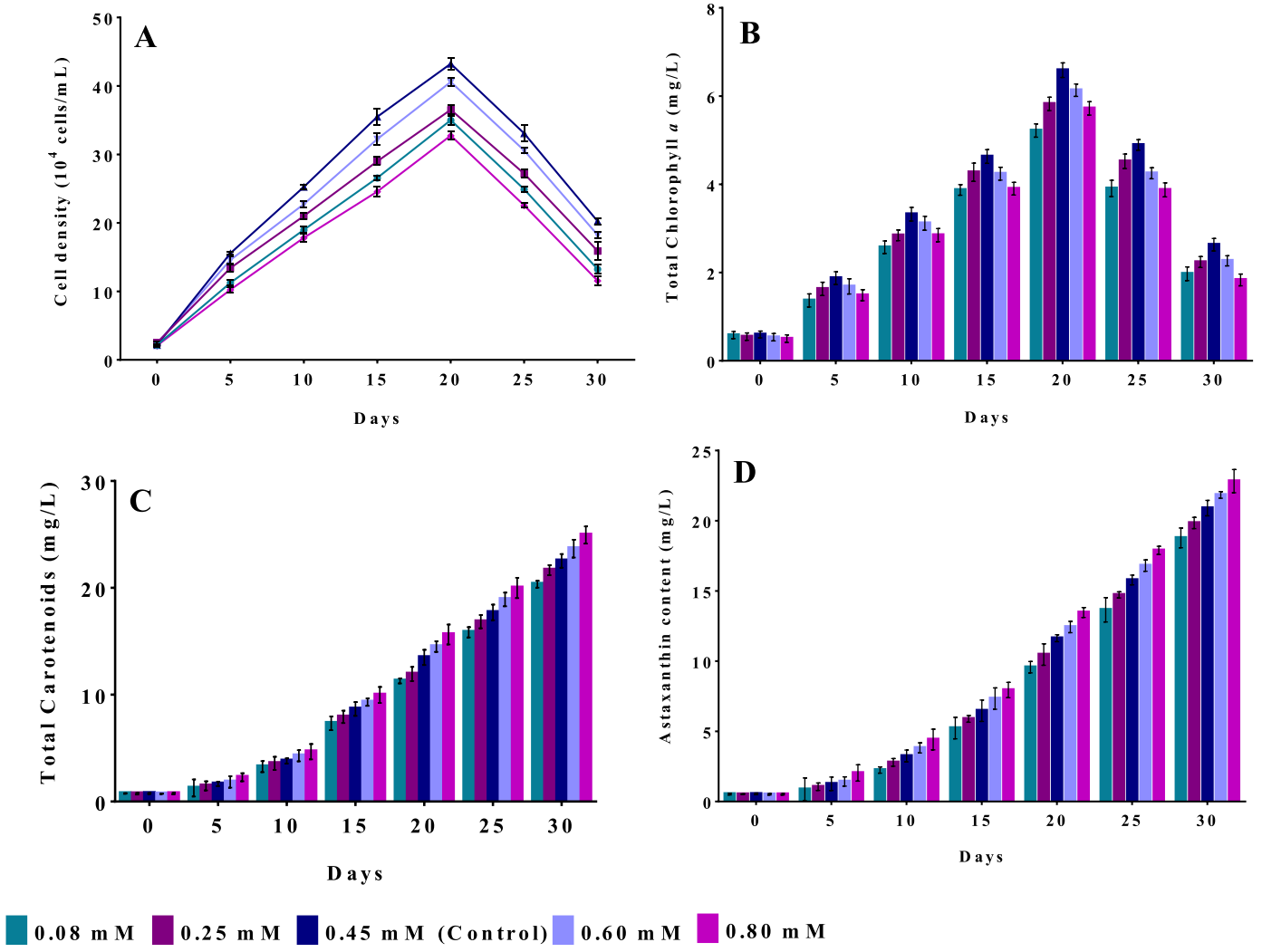

Fig. 9. Effect of different concentration of NaCl on a cell number (A), Chlorophyll *a* (B), total carotenoids (C) and astaxanthin content (D) isolate *H. lacustris* HPI-001 at different intervals in laboratory conditions.

4. Comparative study of *H. lacustris* SAG-19a grown in modified 3N-BBM+*V* medium and modified SAG-a medium

The control strain of *H. lacustris* SAG- 19a exhibited the maximum cell number of 45 × 10^4 cells/mL while grown in the Modified SAG- A medium on the 20th day which was 28% more than the organism grown in the Modified 3N-BBM+*V* medium. The alga grown in the Modified SAG- A medium showed a specific growth rate of 0.168, a division rate of 0.243 and a generation time of 4.117. Maximum concentrations of Chl *a* 7.86 of mg/L, Chl b of 3.94 mg/L, total carotenoids of 27.78 mg/L, and astaxanthin of 25.67 mg/L recorded on 20th, 20th, 30th and 30th day, respectively, in Modified SAG- A medium were 21%, 21%, 11% and 10%, respectively, more than the alga grew in the Modified 3N-BBM+*V* medium **(S8) (Table 2).**

4.1. Discussion

The present study was conducted as an attempt to improve the production of total carotenoids and astaxanthin in the two strains of *H. lacustris* through modifications of the usually used 3N-BBM+*V* medium. *H. lacustris* is a photosynthetic microalga that accumulates astaxanthin in cells. The astaxanthin production in *H. lacustris* is achieved through a two-stage cultivation process involving the green vegetative and red aplanospore stages [30]. This is also influenced by several abiotic factors including light, pH, temperature, salt concentration, and nutritional stress [2].

Several experiments have been carried out on the growth of *Haematococcus* sp. in order to obtain the maximum growth rate [31]. A study on the batch culture of *Haematococcus* in optimal *Haematococcus* media (OHM) reported a cell number of 6.25 × 10^4cells/mL without astaxanthin production after 14 days of cultivation [32]. Whereas a study by Dominguez-Bocanegra et al. [33] reported a maximum growth of *H. lacustris* (3.5 × 10^4cells/mL) in BBM under continuous illumination (177 µmol photons $m^{-2}s^{-1}$) and aeration (1.5vvm). In the present study both strains were grown in four different media (BBM, 3N-BBM+*V*, BG-11 and modified CHU-13 medium). *H. lacustris* HPI-001 exhibited maximum cell number of 51 × 10^4 cells/mL with the specific growth rate of 0.149 d^{-1} on 20th day in the 3N-BBM+*V* medium. The cell number was higher than that of Zhang et al. [32] and Domìnguez-Bocanegra et al. [33]. In the red stage of macrozooids and palmelloids, Del Rio et al. [34] recorded astaxanthin content of 0.8%, which is 65% of the total carotenoids, following which Del Rio et al. [35] stated that single stage process resulted in 1.1% dry weight of astaxanthin representing 85% of the total carotenoids. In *H. lacustris* HPI-001, an increased production of Chl *a* and Chl b was observed on the 20th day of cultivation in 3N-BBM+*V* medium. The 3N-BBM+*V* have a maximum accumulation of total carotenoids and astaxanthin content on the 30th day. The increase of growth by 13%, Chl *a* and Chl b by 16% and 17% respectively, to-

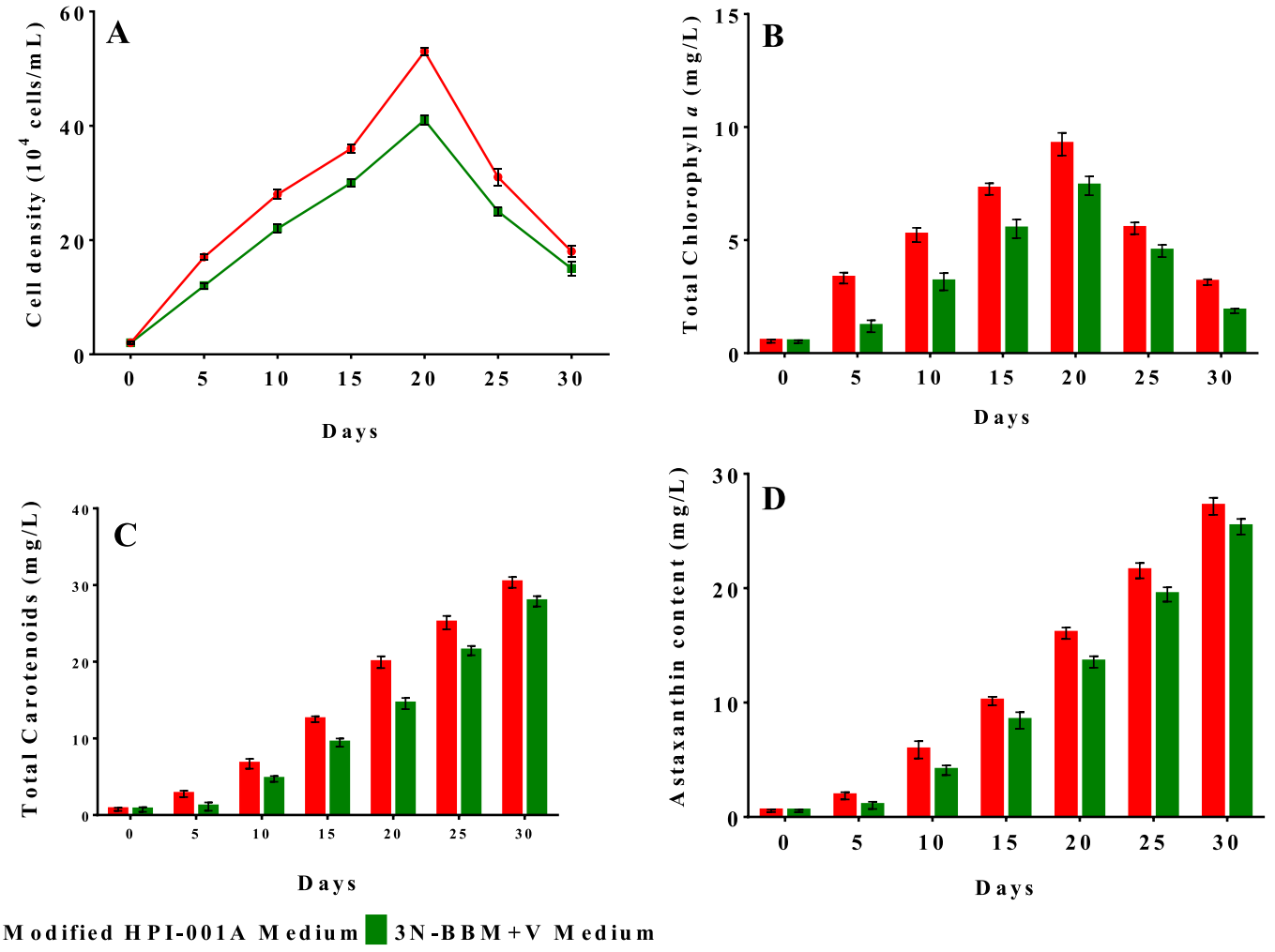

Fig. 10. Comparative study of algae grown in modified 3N-BBM+ V medium and formulated modified HPI-001A medium on a cell number (A), Chlorophyll *a* (B), total carotenoids (C) and astaxanthin content (D) isolate of *H. lacustris* HPI-001 at different intervals in laboratory conditions.

tal carotenoids by 11% and astaxanthin by 16% found in *H. lacustris* HPI-001 was notably higher compared to control.

The growth and other features of the autotrophic photosynthetic microalgae are influenced by many factors of which the light and nitrogen are the most significant factors While cultivating *H. lacustris* in flasks or columns, a light intensity range of as low as 30–60 $\mu molm^{-2}s^{-1}$ has been used in many studies [36]. *H. lacustris* contained as much as 36.58 mg/L of carotenoids while Cifuentes et al., [37] reported 25.0 mg/L. Light intensity was previously stated to be a limiting factor for successful germination [16]. In the current study, the isolate *H.lacustris* HPI-001 had the highest cell number of 46 × 10^4 cells/mL with a specific growth rate of 0.129 d^{-1} on 25th day and the concentrations of Chl *a* with 8.16 mg/L and Chl b with 4.09 mg/L on 25th day at the light intensity of 10.0 $\mu Em^{-2}s^{-1}$. An increase in light intensity increased the accumulation of total carotenoids and astaxanthin from 30th day, on the other hand, the amount of Chl *a* and Chl b decreased in the intensity of 40.0 $\mu Em^{-2}s^{-1}$. On the 30th day, the isolate algae *H. lacustris* HPI-001 contained 25.71 mg/L of total carotenoids and astaxanthin content 23.16 mg/L. The increase in algal growth (15%) and pigments (Chl *a* with 10% and Chl b with 10%) and total carotenoids with 11% and astaxanthin content with 11% were more than that of control strain of *H. lacustris* SAG-19a.

Although buffered systems were used in the current study to grow *H. lacustris*, the cell density of cultures was lower compared to the reports of Nagaraj et al. [38]. In this case, a notable difference was observed in cell density ($p > 0.05$) with the highest value at pH 6.3 on the 5th day of culture. Sarada et al. [39] and Nagaraj et al. [38] had also investigated the impact of pH in the vegetatively growing cells of *H. lacustris* without adding buffers. Sarada et al. [39] obtained the highest biomass in cultures with an initial pH of 7.0, with no growth in pH 5.0, while Nagaraj et al. [38] obtained the highest biomass of 6.44 × 10^6 cells ml^{-1} at a pH of 7.0 after 18 days of incubation.

Growth was observed in both the strains of *H. lacustris* in all the selected initial pH, in the BBM *viz.*, 6.0, 6.5, 7.0 (control), 7.5, 7.8, and 8.0. There was an increase in cell number of *H. lacustris* HPI-001 from pH 6.0 to 7.5. The growth increased up to 50 × 10^4 cells/mL with a specific growth rate of 0.130 d^{-1}at a pH 7.5 on the 20th day, which was more than 25% to that of control. The growth was higher than that reported by [39,38]. The abundance of chlorophyll at alkaline pH and reduction in acidic pH suggested that the chlorophyll formation was inhibited by the H^+ ion activity in media. In *H. lacustris* HPI-001, the amount of total carotenoids and astaxanthin were higher in pH 7.5 on the 25th day. The increase in pigments Chl *a* (26%), Chl b (25%), total carotenoids (10%), and astaxanthin (16%) was more than that of control strain. The content of astaxanthin as well as the carotenoids in total was very insignificant in the acidic pH.

The concentration of nitrate has a very significant impact on the rate of cell division and the astaxanthin accumulation in *H. lacustris* [40]. Nitrate was also found to be essential for the germination of haematocyst [41]. Fabregas et al. [41] reported that a decrease in nitrogen

Table 2
Comparative studies on cell number and astaxanthin content isolate of *H. lacustris* HPI-001 and *H. lacustris* SAG-19a.

Different parameters conditions	*H. lacustris* HPI-001 cell number (10^4cells/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)
Different medium				
BBM	48 ± 0.400	43 ± 0.435	25.11 ± 0.383	21.58 ± 0.404
3N-BBM+*V*	51 ± 0.419	45 ± 0.326	26.48 ± 0.448	22.77 ± 0.390
BG-11	45 ± 0.314	40 ± 0.364	25.25 ± 0.424	21.11 ± 0.407
Modified CHU-13	43 ± 0.381	38 ± 0.377	22.53 ± 0.428	19.58 ± 0.488
Different light intensities				
10 μ Em^{-2} s^{-1}	46 ± 0.482	40 ± 0.473	13.34 ± 0.331	12.34 ± 0.453
20 μ Em^{-2} s^{-1}	41 ± 0.363	38 ± 0.377	17.17 ± 0.321	16.70 ± 0.410
30 μ Em^{-2} s^{-1} Control)	36 ± 0.286	36 ± 0.429	19.08 ± 0.307	18.07 ± 0.361
40 μ Em^{-2} s^{-1}	26 ± 0.300	33 ± 0.320	23.16 ± 0.360	20.82 ± 0.260
Different pH				
pH 6.0	36 ± 0.363	28 ± 0.590	18.73 ± 0.227	16.57 ± 0.563
pH 6.5	40 ± 0.282	32 ± 0.335	19.83 ± 0.374	17.63 ± 0.328
pH 7.0 (Control)	43 ± 0.420	38 ± 0.334	20.54 ± 0.363	18.81 ± 0.427
pH 7.5	50 ± 0.382	40 ± 0.425	21.85 ± 0.416	18.25 ± 0.482
pH 7.8	46 ± 0.420	36 ± 0.566	21.10 ± 0.408	17.65 ± 0.571
pH 8.0	31 ± 0.400	24 ± 0.416	19.94 ± 0.481	16.55 ± 0.388
$NaNO_3$ (mM)				
3.0	37 ± 0.443	28 ± 0.302	20.19 ± 0.419	17.01 ± 0.292
6.0	41 ± 0.544	31 ± 0.442	21.84 ± 0.447	17.81 ± 0.475
9.0 (Control)	39 ± 0.244	33 ± 0.291	20.54 ± 0.427	18.63 ± 0.304
12.0	34 ± 0.333	35 ± 0.560	19.80 ± 0.381	19.56 ± 0.288
15.0	31 ± 0.422	27 ± 0.384	19.27 ± 0.409	18.54 ± 0.351
$K_2HPO_4 \bullet 3H_2O$ (mM)				
0.15	36 ± 0.483	31 ± 0.316	16.11 ± 0.338	13.85 ± 0.306
0.30	37 ± 0.446	33 ± 0.455	17.20 ± 0.392	14.51 ± 0.409
0.45 (Control)	41 ± 0.129	36 ± 0.546	18.69 ± 0.224	15.67 ± 0.432
0.60	39 ± 0.334	34 ± 0.429	17.63 ± 0.332	16.67 ± 0.333
0.75	34 ± 0.300	29 ± 0.381	16.69 ± 0.467	15.69 ± 0.335
KH_2PO_4 (mM)				
0.9	40 ± 0.300	32 ± 0.362	22.42 ± 0.240	17.58 ± 0.374
1.1	43 ± 0.443	34 ± 0.408	23.86 ± 0.467	18.68 ± 0.231
1.3 (Control)	46 ± 0.340	37 ± 0.387	22.47 ± 0.329	19.85 ± 0.273
1.5	48 ± 0.479	38 ± 0.391	21.53 ± 0.432	20.75 ± 0.353
1.7	44 ± 0.322	35 ± 0.305	20.30 ± 0.290	19.87 ± 0.429
NaCl (mM)				
0.08	35 ± 0.461	29 ± 0.546	18.78 ± 0.301	14.75 ± 0.394
0.25	37 ± 0.382	32 ± 0.545	19.84 ± 0.401	15.83 ± 0.344
0.45 (Control)	43 ± 0.487	35 ± 0.266	20.90 ± 0.447	16.86 ± 0.485
0.60	41 ± 0.383	37 ± 0.267	21.84 ± 0.234	17.90 ± 0.349
0.80	33 ± 0.529	34 ± 0.346	22.82 ± 0.329	18.87 ± 0.364
Modified HPI-001A	53 ± 0.449	45 ± 0.536	27.16 ± 0.497	25.67 ± 0.5 06
3N-BBM+*V* medium (Control)	41 ± 0.448	35 ± 0.443	25.38 ± 0.482	23.55 ± 0.451

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

along with an increase in high light intensity led to increased astaxanthin production of up to 49.52 μg/mL. Fabregas et al. [42] and Wang et al. [43] showed that higher nitrate concentrations stimulated growth in microalgae and a concentration of 17.6 mM nitrate was the most effective. It was also found that a higher nitrate concentration of 500 mM enhanced microalgal growth without any negative impact [38]. Tocquin et al. [44] reported that the algal growth can be stimulated through medium supplementation with phosphate of concentration greater than 3 mM.

Both strains of *H. lacustris* when grown in five concentrations of $NaNO_3$, the maximum growth and cell number (41×10^4cells/mL) in *H. lacustris* HPI-001 with a specific growth rate of 0.161 d^{-1} was at 6.0 mM $NaNO_3$ on 20th day and in control strain, a cell number of 35×10^4cells/mL with a specific growth rate of 0.147 $d^{-1}$was observed at 12.0 mM $NaNO_3$ on 20th day. The growth in *H. lacustris* HPI-001 was increased by 17% which was more than control. The increase in Chl *a* by 10% and Chl b by 10% was more than control strain. However, the accumulation of total carotenoids increased by 7% and astaxanthin content increased by 11% in *H. lacustris* HPI-001, more than control strain of *H. lacustris* SAG-19a maximum at low concentration of nitrogen, $NaNO_3$ at 6.0 mM.

Phosphorus is an essential growth nutrient for algae, participating in cell energy transfer, cell membrane formation, and production of nucleic acids. The algal growth medium optimization strategies involving the use of phosphate has been barely carried out and it is known to promote growth at low to moderate concentrations (± 0.5 mM) [42], although it may also promote carotenogenesis at a higher concentration (up to 0.9 mM) (Borowitzka et al., 1991). However, the carotenoids accumulation has been shown to decline with phosphate supply above 0.85 mM (up to 3.4 mM) in other studies [45]. Phosphate also plays an inevitable role in the synthesis of useful products such as astaxanthin and PUFAs. Further in *H. lacustris*, a high biomass of 3.5 gL^{-1} and astaxanthin of 15.0 mg g^{-1} was recorded by Brinda et al. [46] under phosphate deficiency. Tocquin et al. [44] stated that high phosphate is a key factor beneficial for the growth of *H. lacustris*.

The present study focused on the effect of two potassium sources $K_2HPO_4 \bullet 3H_2O$ and KH_2PO_4. The *H. lacustris* HPI-001 exhibited a maximum specific growth rate of 0.156 d^{-1} on 20th day at 0.45 mM

$K_2HPO_4 \bullet 3H_2O$. The low concentration of $K_2HPO_4 \bullet 3H_2O$ favored *H. lacustris* with a higher production of pigments [47]. In the current study, the basal medium amended with 0.45 mM favored the *H. lacustris* HPI-001 for the synthesis of maximum concentration of chlorophyll, carotenoids, and astaxanthin. *H. lacustris* HPI-001 exhibited a maximum cell number (48×10^4cells/mL) with a specific growth rate of 0.179 d^{-1} on 20th day at 1.5 mM of KH_2PO_4. The 1.1 mM KH_2PO_4 favored *H. lacustris* HPI-001 for the maximum accumulation of carotenoids and astaxanthin content on the 30th day. On the other hand, Ping et al. [48] had only reported 11.0 mg/L of carotenoid under conditions of phosphate deficiency.

The death of *H. lacustris* cells increases considerably with increase in salt concentration with only 55% of cells surviving at 0.8% NaCl (138 mM) [45, 49]. There was a lower astaxanthin production in 1% NaCl added cultures in accordance with the observation of Sarada et al. [50]. The study performed by Kobayashi et al. [17] reported an optimal NaCl concentration of 0.1% in *Haematococcus* standard cultures with the addition of another stress factor. Furthermore, a successful astaxanthin production was achieved by the addition of 0.25 to 0.5% NaCl to the mediums [2]. In the present attempt, the *H. lacustris* HPI-001 increased steadily up to a maximum of 43×10^4cells/mL on 20th day with a specific growth rate of 0.185 d^{-1}at 0.45 mM NaCl. The 16% increase in cell number was more compared to the control *H. lacustris* SAG-19a. Similarly, 0.45 mM of NaCl favored the maximum concentration of Chl *a* and Chl b which was more than 14% compared to control. On the 30th day, *H. lacustris* HPI-001 grown at 0.80 mM NaCl had maximum total carotenoids of 16% and astaxanthin content of 21% as compared to the control. The increase in salinity stress was found to enhance carotenoid production and accumulation in the algal cells.

In batch culturing, the optimal *Haematococcus* media (OHM) produced a cell number of 6.25×10^4cells/mL after 14 days of cultivation without accumulation of astaxanthin [32]. Dominguez-Bocanegra et al. [33] stated that the maximal growth of *H. lacustris* obtained was 3.5×10^4cells/mL in the BBM under continuous illumination (177 µmol photons $m^{-2}s^{-1}$) and aeration (1.5vvm). The maximum recorded was 2% DW when the green cells were re-suspended in nitrate and phosphate depleted BBM [46]. Del Rio et al. [34] recorded astaxanthin content of 0.8%, with astaxanthin being 65% of the carotenoids.

A new medium was formulated and designed as a Modified HPI-001A medium and compared to the basal medium (3N-BBM+*V*). The strain of *H. lacustris* HPI-001 exhibited a maximum cell number of 53×10^4 cells/mL with a specific growth rate of 0.215 d^{-1} on the 20th day in the Modified HPI-001A medium. The increase in the cell number was 29% compared to the alga grown in the basal medium (3N-BBM+*V*). *H. lacustris* HPI-001 synthesized maximum concentrations of Chl *a* of 9.24 mg/L, Chl b of 4.63 mg/L, total carotenoids of 30.35 mg/L and astaxanthin of 27.16 mg/L on 20th, 20th, 30th and 30th day, respectively, in the Modified HPI- 001A medium. The above values were 18%, 17%, 10%, and 10% Chl *a*, Chl b, total carotenoids and astaxanthin, respectively, compared to the control *H. lacustris* SAG- 19a.

5. Conclusion

The present study is evidence that the growth and astaxanthin content of indigenous isolate of *H. lacustris* HPI-001 increased in the formulated Modified HPI-001A medium when compared to the control *H. lacustris* SAG-19a. The Modified HPI-001A medium which is formulated in this study is cost-effective compared to the basal medium (3N-BBM+*V*) which served as the control medium and thus for the production of astaxanthin in the cosmetic and pharmaceuticals industries, the formulated Modified HPI-001A medium can be highly recommended.

Conflict of Interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Supplementary materials

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