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# Detection and Optimization of $\beta$ - Carotene Production in *Hapalosiphon Fontinalis*

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**ABSTRACT:** Carotenoids are extensively found pigments in nature, with diverse industrial applications such as in pharmacology, cosmetics, and the food sector. To achieve large-scale production using microalgae, it is essential to identify strains that excel in growth and yield of desired compounds. *Hapalosiphon fontinalis* demonstrates a remarkable ability to adapt to fluctuating and unfavorable environmental conditions. This study aimed to assess the potential of *H. fontinalis* under optimized conditions for enhancing beta-carotene production. The findings show that beta-carotene production increases when subjected to stress conditions like UV exposure, varied photoperiods, salinity, carbon sources, and pH levels.

**KEYWORDS:**  $\beta$  – carotene, growth, *Hapalosiphon fontinalis*, optimization, stress

## I. INTRODUCTION

Microalgae offer several benefits as natural carotenoid sources, including adaptability to environmental changes, simple and cost-effective production, and rapid growth, particularly in species like Chlorophyceae. Numerous studies have shown that environmental and culture conditions significantly influence carotenoid accumulation, with factors like light, temperature, and the addition of metal ions and salts being frequently examined [1]. Cyanobacteria, known for their photosynthetic capabilities, possess pigment complexes for light absorption, which include chlorophylls, phycobiliproteins, and carotenoids. In these organisms, while phycobiliproteins primarily absorb light, carotenoids play a critical role in non-photochemical quenching (NPQ), protecting against excessive light and reactive oxygen species (ROS) [2]. Carotenoids are essential terpenoid pigments across photosynthetic organisms. Among over 600 identified carotenoids, two main groups exist: carotenes (such as  $\alpha$ - and  $\beta$ -carotene) and xanthophylls like zeaxanthin and echinenone [3, 4]. Carotenoids not only fulfill vital biological roles but also hold significant industrial interest due to their antioxidant, anti-inflammatory, and anti-tumor properties [3, 4, 5]. The carotenoid market exceeded \$1.5 billion USD between 2016 and 2019, with  $\beta$ -carotene, astaxanthin, and lutein accounting for 60% of the market share [6, 7]. Established bioprocesses for carotenoid production include *Dunaliella salina* for  $\beta$ -carotene, *Haematococcus pluvialis* for astaxanthin, and *Tagetes erecta* for lutein. Recent advances suggest growing interest in cyanobacterial carotenoids, potentially leading to their increased market presence. Key strategies include enhancing carotenoid content in cyanobacteria through optimized growth conditions, stress stimuli, or genetic engineering; efficient biomass utilization; and exploring bioactive potential for applications in nutraceuticals, cosmetics, and feed [8]. The current study investigates *H. fontinalis* as a potential source for improving beta-carotene yields under various optimized conditions.

## II. MATERIAL AND METHODS

### 2.1 Culture conditions

The cells were grown in culture medium with 2000  $\pm$  200 lux light intensity, 12:12 light/dark regime, 29  $\pm$  1°C temperature and pH 7.4. The cultured flasks were swirled twice a day manually. Respective biomass was harvested through filtration, washed twice with distilled water to remove adhered salts and stored at -20°C for biochemical analysis. Each experiment was conducted in triplicates. All reagents and chemicals used were of analytical grade, obtained from Merck, Himedia and Sigma Aldrich.

### 2.2 Extraction and detection of $\beta$ -carotene

To estimate the  $\beta$  – carotene 10 mg biomass was taken in test tube and homogenized in 10 mL of KOH (5%) in MeOH (30%). Mixture was then heated in a water bath (Scientific, India) at 70°C for 5 min followed by centrifugation at 10,000rpm/4°C for 10 minutes. 5mL acetone (90%) was added to the pellet obtained by centrifugation and kept in waterbath for 5 mins. Collected the extract and again added 5mL acetone (90%) to the pellet and kept in waterbath. This

step was repeated until pellet became colourless. Then absorbance of collected extract was read at 429, 452, 478 nm for determination of  $\beta$ - carotene. 90% Acetone was used as blank [8].

$$\beta - \text{carotene (g)} = \frac{A_y}{\text{Ex. Coeff.} \times 100}$$

$\beta - \text{carotene Ex. Coeff.} = 2273$   
 $A = \text{Absorbance}$   
 $y = \text{volume of solvent}$

### 2.3 Pigment Extraction and Sample Preparation for HPLC confirmation

Lyophilized biomass (25mg) was homogenized in the solvent mixture (2-mL aliquots) of dichloromethane and methanol (25:75, v/v). The mixture was then separated by centrifugation at 10000g for 5 min, and the supernatant containing pigments was collected. The extraction procedure was repeated at least three times until the cell debris was almost colorless, and 10 mL of pigment extract was obtained. The total pigment extracts were centrifuged again at 10000g for 15 min and stored at -20 °C for subsequent saponification and HPLC separation. All of the above processes were conducted in darkness [9].

#### 2.3.1 Saponification

One mL NaOH (0.05M) dissolved in methanol (freshly prepared), was added to 5 mL pigment extract solution under a nitrogen atmosphere. The mixture (6 mL) was evaporated and concentrated to 5 mL under nitrogen and then kept overnight or for at least 8 h under nitrogen in darkness at room temperature for complete saponification. The resulting product was directly separated and determined by HPLC.

#### 2.3.2 HPLC Spectra

HPLC was conducted on a Waters liquid chromatograph equipped with UV-VIS detector. The saponified pigment extract solution were separated and analyzed (20- $\mu$ L aliquots) by using a 250 $\times$ 4.6 Phenomenex C18 (5  $\mu$ m) column at 25 °C. The mobile phase consisted of solvent A (dichloromethane/methanol/acetonitrile/water, 5.0:85.0:5.5:4.5, v/v) and solvent B (dichloromethane/methanol/acetonitrile/water, 22.0:28.0:45.5:4.5, v/v). The flow rate was 1.0 mL min<sup>-1</sup>. The chromatogram was recorded at 480 nm. Chromatographic peaks were identified by comparing retention times and spectra against known standards or by comparing their spectra (by UV VIS Detector).

### 2.4 Optimization for $\beta$ -carotene and Astaxanthin under different growth conditions as follows-

Test strain was grown separately under conditions discussed below to see the effect of different growth conditions on the progressive growth and  $\beta$ - carotene content. Growth was measured in terms of culture biomass weight.

UV irradiance: The effect of UV on  $\beta$ - carotene content in was observed in *H. fontinalis*. 0, 5, 10, 15, 20, 25, 30 min/day UV exposure was given to the cultures till 20 day.

Light/Dark Regime: For the estimation of  $\beta$ - carotene under different light/dark regime, the *H. fontinalis* were exposed to 0:24, 4:20, 8:16, 12:12, 16:8, 20:4, 24:0 light/dark regime in BOD cabinet.

Salinity: The effect of salt stress on  $\beta$ - carotene was observed by treating *H. fontinalis* with different concentrations of Sodium chloride (0, 10, 50, 100, 150, 200mM).

Carbon Source: During present study effect of Sodium acetate as carbon source (0, 10, 20, 30, 40, 50mM) was observed on growth and  $\beta$ - carotene

pH: The effect of pH on  $\beta$ - carotene of *H. fontinalis* was examined by growing the organism at different pH (pH4 to pH10). The different pH values were adjusted by adding 1N NaOH or 1N HCl.

### 2.5 Statistical analysis

The results were shown as the mean  $\pm$ standrd error mean (SEM) and two way ANNOVA was performed for statistical analysis using GraphPad Prism version- 6.0 (Graph Pad Software, San Diago, CA, USA)". All p-values of < 0.05 are considered significant.

III. RESULTS

**3.1 Spectrophotometric Detection of  $\beta$ -carotene:** 8.9796  $\mu\text{g mg}^{-1}$   $\beta$ -carotene was found in *H. fontinalis* on 20<sup>th</sup> day by the spectrophotometric detection.

**3.2 Confirmation of  $\beta$ -carotene By HPLC:** Carotenoid sample when run on RP-HPLC (C18 column) with dichloromethane/methanol/acetonitrile/water solvent gradient system showed multiple peaks. Peak at time 54.733 min was the major peak and was identified as  $\beta$ - carotene (Fig 1b).  $\beta$ - carotene identified with the help of standard peak (Fig. 1a). Other peaks represented different types of carotenoids present in the sample which were not identified at that moment.

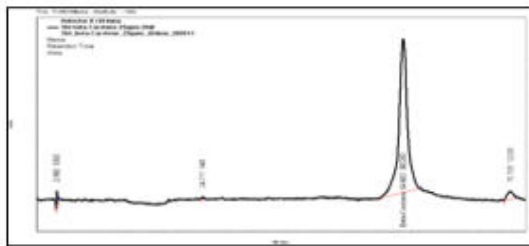


Fig. 1a: RP-HPLC of standard  $\beta$ -carotene

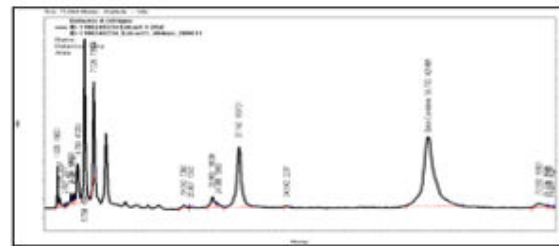


Fig. 1b: RP-HPLC of carotenoid extract of *H. fontinalis*

**3.3 Optimization for  $\beta$ -carotene:** *H. fontinalis* were exposed to various conditions viz UV irradiance, Light/Dark regime, Salinity (NaCl), Carbon source (Sodium acetate) and pH for finding out their effects on growth and  $\beta$ -carotene content. The findings are given below-

**UV irradiance:** Increase in the UV exposure time resulted gradual decrease in growth of the organism. Maximum reduction (45.3%,  $p < 0.001$ ) was observed on 20<sup>th</sup> day in 30 min exposed cultures. (Fig 2a) In general UV exposure resulted in increase in  $\beta$ -carotene with comparison to their respective controls (Fig 2b). Maximum  $\beta$ - carotene amount (13.46  $\mu\text{g mg}^{-1}$ ; 52.95%;  $p < 0.001$ ) was obtain in 25 min UV exposed samples on 8<sup>th</sup> day. However, this highest increased at 25 min exposure could not be maintained till 20<sup>th</sup> day. On 12<sup>th</sup>, 16<sup>th</sup> and 20<sup>th</sup> day 15 min UV exposure showed respective highest content.

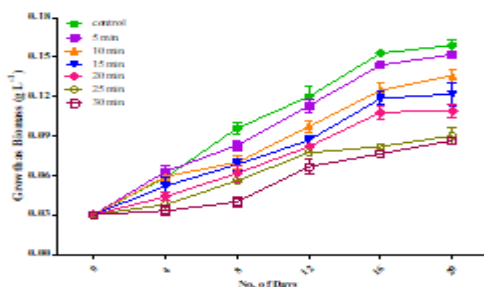


Fig2a. The effect of UV irradiance on growth (Biomass) of *H. fontinalis*.

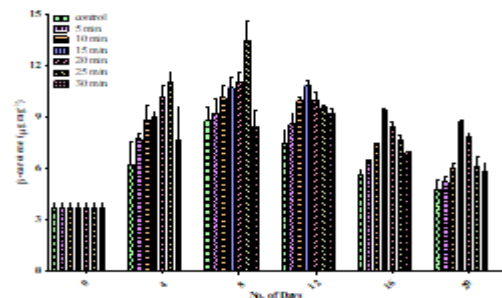


Fig 2b: The effect of UV irradiance on  $\beta$ -carotene of *H. fontinalis*.

**Light/dark regime:** Duration of light/dark regime had shown their remarkable effect on growth of *H. fontinalis*. Highest biomass yield (0.163  $\text{g L}^{-1}$ ) was obtained in 20:4 light/dark regime at 20<sup>th</sup> day (Fig 3a). However at 16:8 and 20:4 light/dark regime overall increase in biomass was recorded. Under low light conditions (0:24, 4:20, 8:16 light/dark regime) and high light (24:0 light/dark regime) growth was suppressed in comparison to their respective controls (12:12 light/dark regime). Like growth  $\beta$ - carotene also showed highest increase (29.63% and 43.24%;  $p < 0.0001$ ) were obtained in 20:4 light/dark regime at 8<sup>th</sup> day (Fig 3b). In 0:24 light/dark regime the  $\beta$ - carotene and was lowest on throughout the experiment (4<sup>th</sup>, 8<sup>th</sup>, 12<sup>th</sup>, 16<sup>th</sup> and 20<sup>th</sup> day).

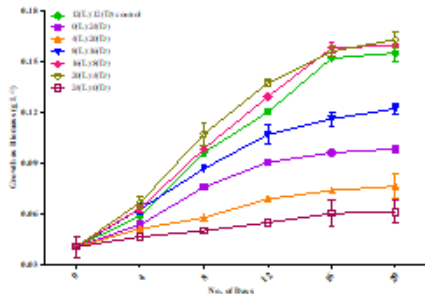


Fig 3a: The effect of light/dark regime on growth (Biomass) of *H. fontinalis*.

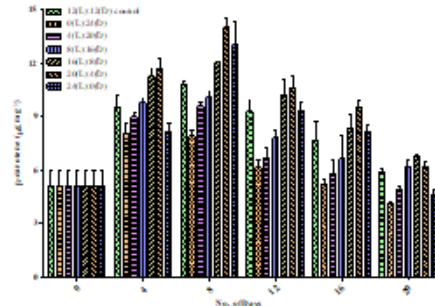


Fig 3b: The effect of light/dark regime on  $\beta$ -carotene of *H. fontinalis*.

Salinity: Present experiment proved the necessity of salt (NaCl) in the growth of *H. fontinalis*. However higher doses of salt were inhibitory. In 10mM of NaCl in each day of observation (4<sup>th</sup>, 8<sup>th</sup>, 12<sup>th</sup>, 16<sup>th</sup> and 20<sup>th</sup> day) growth was higher than their respective controls and maximum growth (0.108 g L<sup>-1</sup>) was observed on 20th day. (Fig 4a). The highest content of  $\beta$ - carotene (12.95  $\mu\text{g mg}^{-1}$ , 61.42%;  $p < 0.0001$ ) was observed on 8<sup>th</sup> day under at 10mM NaCl (Fig 4b). At 10 and 50mM concentration  $\beta$ - carotene and increase till 20th day in comparison to their respective controls but in 100mM increase were observed till 8<sup>th</sup> day and then decreased. In 150 and 200mM  $\beta$ - carotene inhibition was observed from the very beginning (4<sup>th</sup> day).

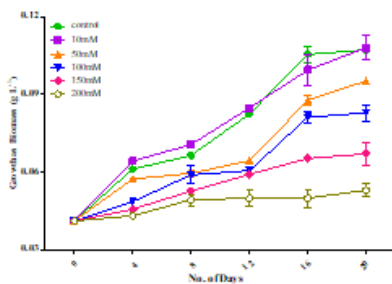


Fig 4a: The effect of NaCl on growth of *H. fontinalis*.

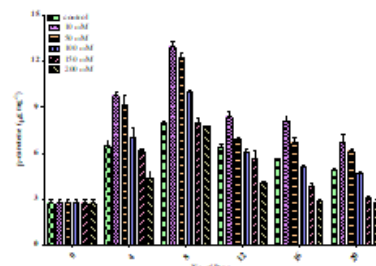


Fig 4b: The effect of NaCl on  $\beta$ -carotene of *H. fontinalis*.

Carbon Source: *H. fontinalis* was grown with sodium acetate for giving mixotrophic condition. In general sodium acetate enhanced the growth in concentration and time dependent manner. Highest growth (0.118 g L<sup>-1</sup>) was observed on 20th day in presence of 50mM sodium acetate (Fig 5a). Remarkable increase was observed in  $\beta$ - carotene in dose and time dependent manner in Sodium acetate treated cultures (Fig 5b). The highest increase in  $\beta$ - carotene (10.34  $\mu\text{g mg}^{-1}$ , 51.03%;  $p < 0.0001$ ) content was observed on 4<sup>th</sup> day at 50mM Sodium acetate.

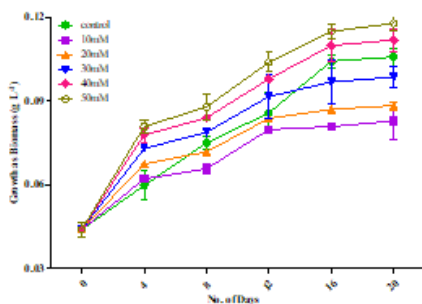


Fig 5a: The effect of sodium acetate on growth of *H. fontinalis*.

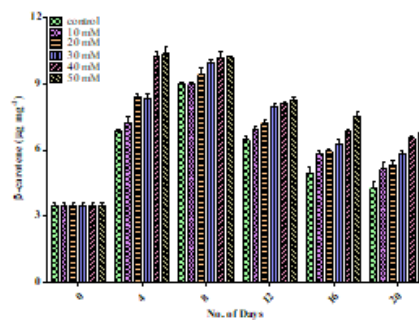


Fig 5b: The effect of sodium acetate on  $\beta$ -carotene of *H. fontinalis*.

pH: Growth was enhanced under alkaline pH 8 and pH 9 as compared to control till 12th day. However acidic (pH 4, 5, 6) and high basic (pH 10) pH have not favoured the growth of *H. fontinalis* (Fig 6a). pH 8 and 9 showed enhancement of  $\beta$ - carotene and (Fig 6b). The highest content of  $\beta$ - carotene ( $10.6 \mu\text{g mg}^{-1}$ , 89%;  $p < 0.0001$ ) was observed on 8th day under pH 8. In acidic pH (4, 5 and 6) and basic pH (10)  $\beta$ - carotene content was decrease.

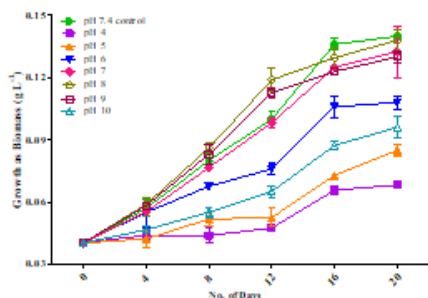


Fig 6a: The effect of pH on growth of *H. fontinalis*.

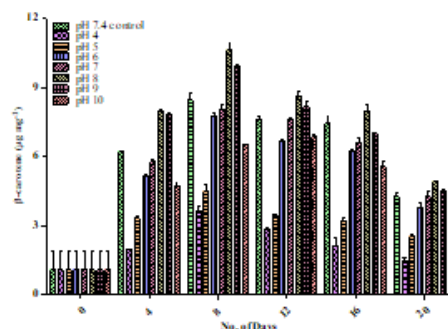


Fig 6b: The effect of pH on  $\beta$ -carotene of *H. fontinalis*.

#### IV. DISCUSSION

Carotenoids are a vital group of natural pigments with a wide range of applications, including their use as colorants, feed additives, and in nutraceuticals. They also have roles in medical, cosmetic, and biotechnological fields. Among the various naturally occurring and synthetic carotenoids, a few like  $\beta$ -carotene, lycopene, astaxanthin, canthaxanthin, lutein, and capxanthin have been commercialized [11]. Although over 600 carotenoids have been identified in carotenogenic microorganisms [12],  $\beta$ -carotene, lycopene, and astaxanthin are the primary compounds produced commercially by microorganisms. These carotenoids have several biological functions, such as coloration, light absorption, photoprotection, antioxidant activity, and serving as hormone precursors. Dietary carotenoids are known to provide health benefits, potentially delaying the progression of diseases like arteriosclerosis, cataracts, age-related macular degeneration, multiple sclerosis, cardiovascular diseases, and certain cancers [13]. Given these benefits, the demand for carotenoids, especially  $\beta$ -carotene, has surged, with the  $\beta$ -carotene market projected to reach USD 520 million by 2025 [14, 15]. This study focuses on  $\beta$ -carotene and investigates the effects of varying cultural conditions on its yield in the strain *H. fontinalis*.

##### 4.1 Spectrophotometric Detection of $\beta$ -carotene

The concentration of  $\beta$ -carotene in *H. fontinalis* was found to be  $8.97 \mu\text{g/mg}$ , or 0.897% w/w (Table 4.1), which is comparable to previously reported values in *Chlorella zofingiensis*, *Spirulina platensis*, and *Caulerpa taxifolia*, which accumulate  $\beta$ -carotene in the range of 0.1–2% dry weight [16]. Among microalgae, *Dunaliella* species are notable for their high  $\beta$ -carotene content, producing up to 13% dry weight [17].

##### 4.2 Confirmation of $\beta$ -carotene by HPLC

Further analysis of the total saponified extract from *H. fontinalis* was conducted using high-performance liquid chromatography (HPLC) with a C18 column and UV-VIS detector. The  $\beta$ -carotene peak was observed at a retention time of 54.733 minutes in the chromatogram, closely matching the standard (Fig. 1b), and in agreement with similar studies [18]. The same HPLC method, involving a PDA detector, has been used for separating carotenoids in various algae, including *Chlorella sorokiniana* and *Scenedesmus bijuga* [18].

##### 4.3 Optimization for $\beta$ -carotene

UV Irradiance: Exposure to UV light resulted in reduced biomass in *H. fontinalis* (0–30 minutes per day; Fig. 2a). This aligns with findings by Xue et al. [19], which showed that increasing UV-B radiation suppressed growth, damaged chloroplasts, and reduced photosynthetic efficiency in *Neoporphyra haitanensis*. Similarly, *Anabaena variabilis* and *Spirulina platensis* showed decreased photosynthetic yield and structural damage when exposed to UV radiation [20, 21]. In this study,  $\beta$ -carotene content increased gradually up to the 20th day, with the highest levels observed at 25 minutes

of UV exposure on the 4th and 8th days and at 15 minutes on the 12th, 16th, and 20th days (Fig. 2b). UV stress likely induces oxidative stress, leading to increased  $\beta$ -carotene production, as observed in other studies [22, 23, 24, 25].

**Light/Dark Regime:** Optimal growth was observed under light/dark cycles of 16:8 and 20:4 until the 20th day, whereas reduced growth occurred under 0:24, 4:20, 8:16, and 24:0 light/dark cycles (Fig. 3a). Proper light intensity enhances the efficiency of photosystem II (PSII) and thus boosts photosynthesis and growth [26], but excessive light can cause photoinhibition [27].  $\beta$ -carotene levels were highest under the 20:4 light/dark regime, likely due to stimulated carotenogenesis (Fig. 3b). Studies have shown that different lighting conditions significantly affect carotenoid synthesis, with blue and red light boosting  $\beta$ -carotene and astaxanthin production [28, 29, 30].

**Salinity:** Growth decreased under all salinity levels (50, 100, 150, and 200 mM NaCl) by the 20th day (Fig. 4a). Salinity affects algae by altering osmotic pressure, membrane transport, and metabolic rates [31, 32]. In this study,  $\beta$ -carotene content increased under 10, 50, and 100 mM NaCl on the 4th and 8th days, with further increases observed at 10 and 50 mM NaCl on the 12th, 16th, and 20th days (Fig. 4b), similar to findings in *Dunaliella tertiolecta* [33, 34].

**Carbon Source:** Growth was enhanced with 40 and 50 mM sodium acetate until the 20th day, while lower concentrations (10 and 20 mM) inhibited growth (Fig. 5a). Sodium acetate can promote carotenoid accumulation in mixotrophic conditions, as seen in *Haematococcus pluvialis* and *Chlorella vulgaris* [35, 36]. A dose-dependent increase in  $\beta$ -carotene was observed with sodium acetate (Fig. 5b), corroborating previous findings [37].

**pH:** Growth was optimal at alkaline pH levels of 8 and 9, while acidic and highly basic conditions (pH 4–6 and 10) suppressed growth (Fig. 6a). Algae typically prefer neutral pH, but some species can thrive in more extreme condition [38].  $\beta$ -carotene production was highest at pH 8 and 9 (Fig. 6b), consistent with other studies, such as in *Dunaliella salina* [39] and *Euglena* sp. [40].

## V. CONCLUSION

*H. fontinalis* is a good example of Biofertilizer. It can withstand under various stress conditions. It could be a good alternative source of  $\beta$ -carotene production while further work are required to do for purification and industrial purposes.

## ACKNOWLEDGEMENT

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