Astaxanthin accumulation in *Haematococcus pluvialis* observed through Fourier-transform infrared microspectroscopy imaging

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**Abstract**

*Haematococcus pluvialis* has emerged as a promising microalga for its rapid accumulation of astaxanthin. However, the underlying mechanism for astaxanthin biosynthesis is still unclear and needs further exploring in terms of the chemical changes of algal cell during the carotenogenesis pathway. In this study, the chemical changes were monitored in the algal cells exposed to carotenogenesis inhibitors by Fourier-transform infrared (FTIR) microspectroscopy imaging. The significant increase of signals at 3010 and 2925 cm\(^{-1}\) suggests the accumulation of carotenoids during the algal cell encysting. In contrast, a relatively low level of carotenoids in the algal cells contacted to nicotine and diphenylamine were observed for the biosynthesis blocking of \(\beta\)-carotene and astaxanthin. The decrease of the absorbance at 1740 and 1650 cm\(^{-1}\) indicates that lipids, proteins and astaxanthin biosynthesis were also inhibited by nicotine and diphenylamine while the cell wall biosynthesis was not disturbed by the inhibitors for the strong absorbance band at 1035 cm\(^{-1}\) assigned to polysaccharides. This work demonstrates that FTIR microspectroscopy imaging is an effective and non-invasive tool to analyze chemical changes and the components location in the single algal cell with high spatial resolution and it facilitates the study of carotenoid biosynthetic pathway.

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1. Introduction

Astaxanthin (3,3′-dihydroxy-\(\beta\),\(\beta\)′-carotene-4,4′-dione), as one of the red secondary carotenoids, is widely used in the cosmeceutical, nutraceutical and aquacultural industries due to its higher scavenging effect on reactive oxygen species (ROS) compared to many other natural antioxidants such as \(\beta\)-carotene, zeaxanthin, lutein and vitamin E [1,2]. Human and animals cannot synthesize astaxanthin in vivo but have to obtain from the diet. *Haematococcus pluvialis* (*H. pluvialis*), a unicellular freshwater Chlorophyte microalga, in contrast, is known to be able to synthesize astaxanthin. Its production can reach up to 4% of the total cellular dry weight under stress conditions such as light illumination, nutrient deprivation, increased salinity, acetate addition, and radiation [3,4]. However, the industrial large-scale cultivation of *H. pluvialis* has encountered some technical obstacles that are associated to cell’s low growth rate, susceptibility to contamination, and low biomass concentration [5]. Studies have been conducted aiming to improve the astaxanthin production of *H. pluvialis* in terms of the understanding of the astaxanthin synthesis pathways and their associated carotenogenic key enzymes activity [6–8]. While progress has been made in describing the astaxanthin biosynthesis pathway by inhibiting the carotenogenic enzymes [9], the knowledge of the locations of the pathways deserves more study.

In this work, FTIR microspectroscopy imaging was used to investigate the inhibition of astaxanthin synthesis in *H. pluvialis* cells. Compared with other conventional methods such as gas or liquid chromatography, FTIR microspectroscopy imaging has the advantages for its spatially-resolved, fast, non-invasive and multiplex measurements [10,11], which allows to obtain qualitative information about the chemical changes of algal cells under carotenogenesis inhibitors of diphenylamine and nicotine at high spatial resolution in situ.

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2. Experimental

2.1. Microalgal strain and culture conditions

The *H. pluvialis* strain was obtained from the Freshwater Algae Culture Collection of the Chinese Academy of Sciences and cultivated in a Bold’s Basal Medium (BBM) [12]. The microalgae stock cultures were maintained in 250 ml Erlenmeyer flasks under a cycle of 12 h light/12 h dark illumination conditions at 25 ± 1 °C with low light intensity of 50 μmol photons m−2·s−1. Once the *H. pluvialis* cells grew sufficiently, they were exposed to high light irradiance of 150 μmol photons m−2·s−1 to induce secondary carotenogenesis [13]. In order to evaluate the activity of carotenogenic enzymes, the inhibitors of diphenylamine and nicotine for the biosynthesis blocking of β-carotene and astaxanthin were used.

2.2. Measurement of carotenogenesis inhibitory rate

The carotenogenesis inhibitory rate (diphenylamine and nicotine) was estimated by cell counting using a haemacytometer. Nicotine was added to the culture medium with final concentrations of 0.05, 0.10, 0.15, 0.20, 0.25 and 0.30 μmol l−1. Diphenylamine was dissolved in 70% v/v basic ethanol and prepared at final concentrations of 20, 40, 60, 80, 100 and 120 μmol l−1. The required concentration for the carotenogenesis inhibition was evaluated by the inhibitory rate.

2.3. FTIR microspectroscopy imaging measurements

Aliquots of 50 μl washed algal cells were dropped onto the BaF2 windows as substrates and dried at room temperature before spectral recording. The carotenoids (astaxanthin, β-carotene and lycopene) were mixed with completely dried potassium bromide (KBr) at a ratio of 1:100 and grounded in an agate mortar to obtain gill powder. The gill powder was then made into a clear transparent KBr disc of 15 mm diameter and 1 mm thickness by a tablet press machine for the FTIR measurement.

The FTIR microspectroscopy imaging measurements were carried out at the National Synchrotron Radiation Laboratory (NSRL, Hefei, China) using a FTIR spectrometer (Bruker IFS 66v/s) which was coupled with an infrared microscope Hyperion 3000 (Bruker Optics Ltd., Ettlingen, Germany) and a 64 × 64 focal plane array (FPA) detector. The spectra for imaging were recorded in transmission mode with a 4 cm−1 resolution, including the middle infrared region from 4000 to 900 cm−1 and scanning for 256 times [14]. The raw FTIR spectra were baseline-corrected and vector normalized before being integrated into chemical mapping using the OPUS 7.0 software provided by Bruker.

3. Results and discussion

3.1. Spectra of carotenoids in *H. pluvialis*

As shown in Fig. 1, carotenoids based on their molecular vibrations can be easily identified by FTIR spectroscopy. The spectrum of astaxanthin, β-carotene and lycopene exhibited peaks at 3010 cm−1 for the trans –CH=CH– [15], 2925 and 2855 cm−1 for asymmetric and symmetric stretching vibrations of the CH2 respectively, and 972 cm−1 for the trans conjugated alkene –CH=CH– out-of-plane deformation mode [16]. We also observed that a strong peak at 1650 cm−1 of pure astaxanthin which may be associated with the shift of C=O peak from 1740 cm−1 to low frequency due to its conjugated system and mainly to the presence of hydrogen bonds in the dimer form [17].

![Figure 1](image_url) **Fig. 1.** The FTIR spectra of pure astaxanthin, β-carotene and lycopene.

3.2. Effect of inhibitors on carotenogenesis in *H. pluvialis*

Various inhibitors can block carotenoid synthesis by inhibiting the activity of carotenogenesis key enzymes, resulting in further blocking the pathway of astaxanthin production in *H. pluvialis* as shown in Fig. 2. It has been reported that nicotine efficiently inhibits the cell growth of *H. pluvialis*, and induces lycopene accumulation by blocking the activity of enzyme lycopene cyclase, a key enzyme catalyzing the transformation from lycopene to β-carotene [18]. Diphenylamine is another inhibitor which could block astaxanthin synthesis by inhibiting β-carotene oxygenase and β-carotene hydroxylase [19]. These carotenoid synthesis inhibitors can be used to explore the underlying biochemical and molecular mechanisms of carotenogenesis. As shown in Fig. 3, the cell number decreased significantly with increasing concentration of nicotine and diphenylamine. The optimal inhibitory concentration was determined at the point when the cell number decreased to 50%, so 0.20 μmol l−1 nicotine and 80 μmol l−1 diphenylamine was used to evaluate the carotenogenesis inhibition for *H. pluvialis*.

3.3. Chemical mappings of single *Haematococcus pluvialis* cell

Fig. 4 shows the photomicrographs and the chemical images of the *H. pluvialis* cells in presence of different carotenogenesis inhibitors under high light irradiation condition for 1, 5 and 10 days. In addition, the chemical FTIR spectra of pure triacylglycerols, bovine albumin and cellulose representing the components of lipid, protein and polysaccharide were included in Fig. 4b. We observed the significant increase of signals at 3010 and 2925 cm−1 in the non-treated *H. pluvialis* cells, suggesting the accumulation of carotenoids during the algal cell encysting (Fig. 4a). The increase of the absorbance at 1740 cm−1 assigned to lipids indicates that the accumulation of astaxanthin in the algal cells from green vegetative cells to red cysts is associated with increased levels of lipids, which is involved in the transport of β-carotene across the chloroplast membrane into cytoplasm and the storage of astaxanthin in lipid vesicles [20,21].

Compared to the cells without treatment, the signals of astaxanthin (3010 cm−1), the C=O band of lipids (1740 cm−1), and the Amide I of proteins (1650 cm−1) all decreased significantly in the
cells treated by nicotine and diphenylamine, suggesting that lipids, proteins and astaxanthin biosynthesis were blocked by nicotine and diphenylamine (Fig. 4b and c). As the alga transforms from a green vegetative cell to a red cyst accompanied by extensive accumulation of astaxanthin and expansion of cell volume, the cell wall undergoes significant structural and functional modifications from an outer primary wall to a secondary wall eventually to a thick tertiary cell wall [22]. Our FTIR chemical mappings showed an increase of signal at 1035 cm\(^{-1}\) with light irradiation time in non-treated cells (Fig. 3a), resulted from the vibration of polysaccharides formed in the cell wall. Similar increasing in polysaccharides' signal was also observed in the cells that were treated by nicotine and diphenylamine, indicating that the carotenogenesis inhibitors didn’t block the cell wall formation during the algal cell encysting.

4. Conclusions

In summary, in this study we have demonstrated that FTIR microspectroscopy imaging is a powerful and useful tool to reveal the detailed spatial information in a single algal cell exposed to carotenogenesis inhibitors of nicotine and diphenylamine. It can also be used as a promising approach to visualize the spatial locations and relative concentrations of lipids, proteins and polysaccharides during the accumulation of astaxanthin in \textit{H. pluvialis}. The application of FTIR microspectroscopy imaging, therefore, may
serve as a novel method to investigate astaxanthin accumulation mechanisms in the algal cell.

Conflicts of interest

The authors declare that they have no conflict of interest.

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