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Nitrogen-doped carbon dots prepared from bovine serum albumin to enhance algal astaxanthin production

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ABSTRACT

The aim of this work was to use green and simply synthesized nitrogen-doped carbon dots (N@CDs) based on carbonized bovine serum albumin (BSA) to boost the accumulation of astaxanthin in microalgae. The BSA carbon dots revealed a high blue emission having a quantum yield of up to 44%, which is superior to all previously reported carbon dots. By directly adding the N@CDs at low concentrations (1 mg L^{-1}) to the algal culture of *Haematococcus pluvialis*, astaxanthin production increased more than two-fold (66 mg L^{-1}), compared to the control (29 mg L^{-1}), in a shorter time of 1 week instead of 2 weeks at the reddening stage. After 1 month of continuous irradiation, the HRTEM images and fluorescence spectra of N@CDs looked similar to as-prepared N@CDs, suggesting their photostability and reusability. Our novel N@CDs could be a promising tool for the future industry of natural astaxanthin and other value-added products from microalgae.

1. Introduction

The ketocarotenoid pigment astaxanthin has important applications in industries such as cosmetics, feed as a coloring agent, and it is used as a powerful antioxidant for several health conditions [23]. This red pigment is accumulated in the green microalga *Haematococcus lacustris* (synonym: *H. pluvialis*) [20], which is the richest natural source of astaxanthin [4] during the development of the palmelloids (green cells) to aplanospores (red cysts) under stress conditions, mainly under high light. However, this process of producing astaxanthin by *H. pluvialis* is relatively long (7 days for biomass accumulation "green stage" and up to 14 days for astaxanthin accumulation "red stage") [17], for a final amount of only 1–5% of total biomass [6], which makes its production very costly [21].

It is well-know that there is a direct relationship between pigment accumulation by photosynthetic organisms and the illumination conditions [3]. It was shown that blue light efficiently increases *H. pluvialis* productivity of astaxanthin [14,16,22,29]. However, the energy used to operate blue light emitting diodes (LEDs) increases the cost of astaxanthin production. Although some studies have focused on removing the unwanted regions of light spectrum, to improve algal growth and thus eventually the yield of the target product, by using monochromatic or polychromatic filters, the actual application of these techniques in large scale is poorly feasible due to complexity, high cost of construction, maintenance and control.

In recent years, carbon light-emitting (fluorescent) nanoparticles, namely, carbon dots (CDs), have attracted much attention due to their unique optical and electronic properties such as their excellent biocompatibility, aqueous solubility, low cytotoxicity, easy functionality and environmental friendliness [9]. Fluorescence quantum yield of CDs can be improved by nitrogen (nitrogen-doped carbon dots, N@CDs), and several reports have shown that the N@CDs are non-toxic for living organisms with no significant changes in their vitalities even when used at different concentrations, and environmentally safe, if prepared from clean sources [18].

The synthesis of nitrogen-doped carbon material such as nanotubes or nanoparticles is well established in the literature, and the methods to synthesize these nitrogen-doped nano objects are diverse [28]. However, the quantum yield of the N@CDs previously reported and their photostability features are still relatively low for long biological processes, with the challenge of keeping their ability to function without aggregation or precipitation.

In this study, low-cost, non-toxic, and one-pot hydrothermally-synthesized N@CDs were engineered, with blue emission of a high quantum yield (QY) up to 44% to boost astaxanthin production by *H. pluvialis*. The concept of excitation of algal cells by these novel non-

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metal nanoparticles is that they convert some of the UV and visible spectra of the solar energy into blue light. The blue light has higher energy than the other longer wavelengths of the visible light. This energy creates a glare that is integrated with the high sunlight, which together can significantly increase the algal productivity of astaxanthin. The novel N@CDs are easily used by directly adding them at low concentrations (1 mg L^{-1}) into the algal culture before the reddening stage (high light stress) without any toxicity. This technology is unique since the other literature reports have shown that specific nanometals can improve the growth of algae indirectly by coupling to localized surface plasmon resonances placed outside of closed photobioreactors [11,26].

2. Materials and methods

2.1. Synthesis of N@CDs

The synthesis of N@CDs was achieved via a hydrothermal method using aqueous BSA solution (Sigma-Aldrich). In detail, 0.5 g BSA (66.5 kD) was taken in 100 mL of ultrapure water and stirred for 15 min at room temperature to prepare a homogeneous BSA aqueous solution. This solution was then transferred into 100 mL stainless steel autoclave with teflon lining inside and heated at 195 °C for 6 h in a hot air oven to promote carbonization of BSA. The carbide slag was removed from the product solution via centrifugation at 10,000 rpm for 15 min. The obtained pale yellow brown solution containing the N@CDs was afterwards analyzed for its fluorescence properties at different excitation wavelengths (λ_{ex} = 330, 350, 370, 390, 410, 430, 450, and 470 nm). The fluorescence emitted from the N@CDs in all the emission spectra manifested narrow bands with the emission maxima centered between 455 and 480 nm. The maximum emission fluorescence intensity was observed at the excitation wavelength of 390 nm, revealing the most intense emission at 460 nm with a corresponding narrow full-width at half-maximum value of 92 nm, with a relatively small size distribution of the particles of an average size of ~4 nm.

BSA microspheres (used as a negative control) were prepared by sonication [24,25] in the following manner: 30 mL of BSA aqueous solution (0.5% w/v) and 20 mL of pure dodecane were transferred into a reaction cell. The horn of an ultrasonic transducer (Sonic and Materials, Newtown, CT, VC-600) was inserted into the cell so that the tip was positioned at the interface between the aqueous and the organic phases. It was equipped with a Ti horn and operated at 20 kHz, 32% amplitude. The reaction cell was dipped in an ice-water bath during the sonication in order to maintain a low temperature, preventing denaturation of the BSA (which occurs at 65 °C). After sonication, the suspension was transferred into a separation funnel, which was kept at rest for 48 h at 4 °C. Three layers were formed: dodecane on top, an aqueous suspension of the BSA spheres in the middle and water at the bottom. After separation of the middle layer it was stored for longer periods at 4 °C.

2.2. Measurement of fluorescence quantum yield

The quantum yield (QY) of N@CDs was determined by the following equation:

$$QY = QY_{st}\frac{A_x}{A_s t}\times\frac{PL_{st}}{PL_x}\times\left(\frac{n_x}{n_{st}}\right)^2\times 100\%$$

whereas QY is the quantum yield, PL is the measured integrated emission intensity, 'n' is the refractive index of the solvent, and 'A' refers to the absorbance. The subscript 'st' and 'x' are refers to the standard sample QS and N@CDs, respectively. The QY was calculated to be ~44% using quinine sulfate as a reference.

2.3. Cultivation of Haematococcus pluvialis

Haematococcus pluvialis Flotow 1844 em. Wille K-0084 (Scandinavian Culture Collection of Algae and Protozoa, SCCAP) cultures were grown photoautotrophically on a complete RM medium agar [13]. A single colony was transferred to a 250 mL Erlenmeyer flask containing the same medium with a working volume of 100 mL. The vegetative cells were illuminated at 20–25 μmol m^{-2} s^{-1} by fluorescent lamps of a full sunlight spectrum, with continuous bubbling containing 2% CO_2, at 25 °C. Exponentially-growing cells from such cultures were used for all experiments.

2.4. Astaxanthin induction with BSA-prepared N@CDs

For the reddening stage, a cell density of ~1.5×10^6 mL^{-1} (ca. 1.0 g·L^{-1} of cell dry weight) was used [5,27]. Algal reddening stage experiments were conducted in clear glass tubes as mini bioreactors containing 10 mL of vegetative culture. Ten microliters of N@CDs, BSA microsphere suspension, BSA aqueous solution, or sterile distilled water (control) were added to the culture tubes to a final concentration of 1 mg L^{-1} (BSA equivalent, where applicable). The cultures were then maintained under high light of 450 μmol photons m^{-2} s^{-1} supplied by fluorescent lamps of a full sunlight spectrum with continuous shaking for 1 week in duplicates. The culture tubes were separated from each other by black barriers on the sides to prevent backscattered light from the adjacent tubes. Each experiment was performed twice.

2.5. Nile red staining

Nile red staining of the algal cells was carried out according to a standard protocol [7]. Briefly, microalgal cultures were sampled from the photobioreactors after 1 and 7 days of high light stress. Nile red dye (10 mg) was dissolved in 40 mL of acetone to produce a Nile red stock solution (0.25 mg mL^{-1}). Five microliters of Nile red stock solution was added to 500 μl of the collected microalgal cultures in 1.5 mL Eppendorf tubes and 0.2% dimethyl sulfoxide (DMSO) was added to facilitate the penetration of the stain into the cells. The tubes were inverted several times to ensure thorough mixing. The cells were washed with double distilled water and the fluorescence intensity of the stained cells was recorded under Zeiss Axio Imager Z2 microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany), after 30 min, with a fluorescence spectrophotometer excitation and emission wavelengths of 530 and 580 nm, respectively. Image acquisition was done using a Zeiss AxioCam MRm monochrome camera.

2.6. HPLC analysis of astaxanthin

To obtain a deeper insight into the response of the cultures to the different treatments, the pigment composition was analyzed by HPLC. For pigment extraction, 2 mL cell cultures were harvested (no significant differences in biomass between samples) by centrifugation (8000 rpm for 2 min) and supernatants were discarded. Cells were lysed by homogenization in 1 mL of 100% (w/w) acetone for 1 min in cold tubes. Samples were centrifuged at 10,000*g* for 2 min, and the supernatants were then filtered through a 0.2 μm Whatman glass microfiber filters. Extracts were analyzed by Elite LaChrom HPLC (Hitachi, Tokyo, Japan) equipped with an autosampler (L-2200) and a diode array detector (L-2455). Carotenoids and chlorophylls were separated on a Varian Microsorb-MV 100-S C18 (250 mm long, 4.6 mm i.d.) cartridge column (Waters, Milford, Massachusetts, USA) at 30 °C, as previously described in Abu-Ghosh et al. [1].

2.7. Reusability of N@CDs

After treatment of *H. pluvialis* cells with N@CDs, the cultures were allowed to settle for 1 h. Then the N@CDs were extracted from the