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Spectral characterization of β, ε-carotene-3, 3′-diol (lutein) from marine microalgae *Chlorella salina*

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ABSTRACT

This study aimed on modern analytical techniques for the isolation, separation and structural identification of the essential bioactive carotenoid Lutein, from green microalga, *Chlorella salina*. Identification was done by comparing their absorption and mass spectral data with those of reference standard values reported. The extract is separated by selective C_{18} columns and the data were then combined with spectroscopic information. Structural assignment of the separated compound is done by HR-MS. The results of the spectral investigation showed that the isolated pigment showed absorbance peak at 445 nm. Total luminescence spectra were recorded by measuring the emission spectra in the range 350 −720 nm at an excitation wavelength of 455 nm. The excitation-emission matrices were recorded and two basic fluorescence regions have been obtained. The compound was resolved within 4.36 min by using a C_{18} column with a flow rate at 1 ml/min and detection at 450 nm. The compound was detected by a High Resolution Orbitrap-MS with regard to specificity and sensitivity (with limits of detection ranging from 1.0 to 3.8 pg μL^{-1}).

1. Introduction

There has been an increasing interest on the possibility of using photosynthetic micro organisms such as microalgae for converting light and carbon-di-oxide into valuable biochemical products with a wide range of applications including food, feed, biofuels, cosmetics and pharmaceuticals. They contain many substances like fatty acids, tocopherols, vitamins, and pigments [1]. Among the products extracted from algae with potential for commercial exploration, the natural bioactive compounds are highlighted. The carotenoids derived from microalgae have significant antioxidant and anti-inflammatory effects, which allow them to provide health benefits. These could be alternative nutraceuticals one of the main important advantages of microalgae is their rapid growth over traditional plants [2].

Carotenoids are lipophilic substances that are chemically classified in two groups: Carotenes and Xanthophylls [3]. Carotenoids are indeed potent biological antioxidants that are able to absorb the excited energy of singlet oxygen radicals into their complex ringed chain, thus leading to degradation thereof; this will concomitantly prevent tissues from becoming damaged by these radicals. Hence natural carotenoids are in high demand by the world market, owing to a number of obvious applications in the pharmaceutical, food and cosmetic industries. Carotenoids are produced either via chemical synthesis or they can be separated from naturally occurring plants or algae. Algae production has gained a lot of interest during recent years due to its high growth rate and tunability of the algae composition. In addition to fatty acids there are also other compounds in algae that are of interest as potential feedstock for synthesis of valuable compounds, such as carotenoids, chlorophylls and proteins. Emphasis is placed on Lutein, a xanthophyll with extraordinary potential for protecting against a wide range of diseases.

Lutein, one of the primary xanthophyll plays an important role in preventing or ameliorating the effects of a number of degenerative human diseases, such as chronic diseases, age related macular degeneration. Due its wide spread use the global lutein market has grown significantly in the recent years with US sales of over $150 million per year [4]. Marigolds are the commercial source of lutein, but relatively high operating cost and low lutein content (0.03%) tends to find alternative source. However, several microalgae

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species including *Muriellopsis* sp. [5], *Chlorella sorokiniana* [6] and *Scenedesmus obliqus* [7] are known to produce lutein at higher content and thus considered as a viable alternative source. They are potential lutein producers because they are capable of accumulating a much higher lutein content (0.5%–1.2% dry weight) than marigold petals, and their growth is independent of season or weather, producing a homogeneous biomass which is ready for processing without labor-intensive separation required. *Chlorella* strains are regarded as competent candidates due to their highest chlorophyll content, fast growth rate and ease of cultivation. They are considered as a rich biochemical reservoir comprising of 45% protein, 20% fats and carbohydrates, 5% fiber, carotenoids, minerals, vitamins, phenolics, antioxidants and immunostimulators that find potential applications in health, food, pharmaceutical and cosmetic industries. With this approach, the present work was carried out on a fast growing, non-fastidious strain of *Chlorella salina*, of marine origin. The present study aims to identify and determine the concentration of lutein produced by *Chlorella salina* based on simple models that allow prediction of microbial development and eventual enhancement of growth. The identification of the carotenoid in *Chlorella salina* was done using chromatographic/spectroscopic techniques. Their quantification was achieved by high performance liquid chromatography (HPLC).

2. Materials and methods

2.1. Micro algae culturing and harvesting

Chlorella salina was procured from CIBA, Chennai, Tamilnadu, India, and cultivated heterotrophically using sterile f/2 medium [8]. The microalga was cultivated in polythene bags of 200 L capacity, agitated bubbling air (1 v/m/ml-1 min-1) under 5000 lux illuminated with white fluorescent bulb for 12:12 h light and dark condition for 30 days, in order to guarantee high lutein accumulation during the stationary phase (15–20 days). Growth was monitored by measuring the optical density at 550 nm. When the culture reached stationary phase, the biomass was harvested by centrifugation at 8500 rpm for 10 min to get thick algal paste. Then the microalgal paste was rinsed with distilled water to remove residual salts and oven dried. The microalga has been biochemically characterized in order to know its total proteins, total lipids, chlorophyll, carotenoid content as well as its growth rate.

2.2. Measurement of algal growth and dry biomass

Algal cell growth was determined by absorbance at 550 nm. For the dry weight measurement, cells were collected by centrifugation at 3000 rpm, 10 min, washed with distilled water, and then dried by lyophilization before weighing.

2.3. Cell disruption

The overall procedures of extracting lutein from microalgae, composed mainly of cell disruption, saponification, solvent extraction steps, have been reported in the literature [9–11]. In this work, three different methods were used to break microalgal cells; namely, autoclaving, ultrasonication and acid lysis. In each test, 3 g of freeze dried algal sample was used. The performance of the cell disruption methods was monitored at 15 min interval for a period of 1hr. For, acid lysis 3 g of biomass added to the sterile seawater and the pH was reduced to 2.0 with HCl, and the solution was shaken for 1 h, 2 h, and 3 h using orbital shaker at 180 rpm [12]. The autoclave was operated at 121 °C with pressure of 15 lbs. The ultrasonicator was operated at a frequency and output power of 40 kHz and 400 W, respectively. A control experiment was also conducted under similar conditions, without any cell disruption procedure.

2.4. Saponification

The alkaline treatment was carried out using potassium hydroxide (KOH) to enhance the lutein extraction efficiency [13]. The microalgal biomass was mixed with KOH solution at a concentration of 0–80% (w/v). The mixture was placed in a water bath at 40 °C for 40 min. After that, Methanol: Hexane (2:1) was added to carry out organic solvent extraction.

2.5. Organic solvent extraction

Repeated extraction was carried out to recover lutein from the microalgal suspension. 19 ml methanol was subsequently added to mixture for the extraction of lutein. The mixture was then centrifuged at 3000 rpm for 15 min at 4 °C, and the supernatant was collected and kept at −12 °C for the subsequent determination of lutein stability [14]. The ratio of organic solvents to the sample mixture was 2:1 (v/v). The supernatant (organic phase) was then collected. The lutein content of the solvent extracts was measured by purging the organic solvent with a N_2 flow and then dissolving the precipitate in acetone for high performance liquid chromatography (HPLC) measurements.

2.6. Chromatography analysis and purification

The carotenoid extracts were then determined by high performance liquid chromatography (Waters- 2545, USA), as proposed by Ref. [15]. Separation was performed using a C_{18} column (5 μm, 150 mm × 46) at 30 °C. The mobile phase consisted of methanol/acetonitrile (90:10 V/V). The extracts were eluted at a flow rate of 1 ml/min and the lutein content was detected by measuring absorbance at the wavelength range of 220–750 nm. The maximal absorbance (450 nm) was chosen for quantification of lutein extracts.

2.7. Identification and quantification of the purified compound

High-resolution mass spectrometric analysis was performed on a single-stage orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, USA), equipped with an atmospheric pressure chemical ionization probe (APCI), operating in positive ionization mode. Ionization source working parameters were optimized and involved sheath gas pressure (35 au, arbitrary units), auxiliary gas pressure (0.01 au), sweep gas pressure (0.01 au), vaporizer temperature (275 °C), capillary temperature (275 °C), discharge current (5 μA), capillary voltage (57.50 V), tube lens voltage (90 V), and skimmer voltage (18 V). An m/z scan range of 100–650 was selected, and the resolution was set at 100,000 FWHM at 1 Hz (1 cycle per second). The automatic gain control (AGC) target was set at the high dynamic range ($3 \times e^6$ ions), and the maximum injection time was 50 ms. The option of "all ion fragmentation" using the high energy collision dissociation (HCD) cell was only used to investigate the confirmation potential of generated fragments and was turned off during the actual analysis. Instrument control and data processing were carried out by Xcalibur 2.1 software (Thermo Fisher Scientific, San Jose, USA). The tentative identification was based on UV–vis spectral characteristics and compared to standard data available in the literature. The concentration of lutein was determined by measuring the absorbance of the samples at 445 nm.