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Indigenous microalga *Parachlorella* sp. JD-076 as a potential source for lutein production: Optimization of lutein productivity via regulation of light intensity and carbon source

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ABSTRACT

Lutein has attracted attention because of its beneficial roles, including alleviating cardiovascular diseases. We compared lutein production of diverse indigenous algal candidates, and selected *Parachlorella* sp. JD-076 as a lutein producer. The strain was cultivated under different types of photo-bioreactors (PBRs). Maximal growth was obtained from the tubular type with 2.7-fold higher dry cell weight (DCW) than in the cylindrical type. To optimize lutein production, *Parachlorella* sp. was exposed to various levels of illumination (100–1000 μmol m^{-2} s^{-1}) and combinations of glucose and CO_2 in the tubular-type PBR. High-light intensity efficiently increased DCW and lutein productivity in an intensity-dependent manner, and 5% CO_2 resulted in the maximum biomass and lutein productivity compared to that of other conditions, with 2.1 g L^{-1} day^{-1} and 25 mg L^{-1} day^{-1}, respectively. We speculated that the strain *Parachlorella* sp. JD-076 will provide a cost-effective lutein bioprocess via regulation of illumination and CO_2 supply.

1. Introduction

Over the last few decades, studies regarding the enhancement of microalgal biomass production have been performed to produce value-added compounds, including polyunsaturated fatty acids, proteins, vitamins, and carotenoids, for commercial use [1,2]. Among these products, carotenoids have attracted much attention from investigators because of their outstanding biological functions, as well as high economic values and the growing worldwide carotenoid market [3]. The global carotenoid market value reached approximately $1.24 billion in 2016, and it is anticipated to reach nearly $1.53 billion by 2021 [4]. Carotenoids are yellow, red, or orange-colored natural pigments, and play essential roles in photoautotrophic organisms because of their photoprotection activity; that is, they reduce photo-oxidative damage by immoderate illumination [5]. To date, over 600 different natural carotenoids have been found, and many studies regarding the role of carotenoids in human health, such as antioxidant activity, protective effects against eye disease, and anti-cancer activity, have been widely reported [6]. However, the commercially produced carotenoids from microalgae are limited to β-carotene and astaxanthin, and they are generally used as food colorants, or supplements in human and animal food [7–9]. Because many carotenoids exhibit different bioactivities, it is necessary to develop bioprocesses for the production of various algal carotenoids, such as those with lutein and fucoxanthin.

Lutein is a yellow pigment of the xanthophyll class that is prevalent in vegetables and terrestrial plants, such as carrots, spinach, and kale, and it has a substantial potential to reduce the chances of age-related macular degeneration (AMD) [10]. Moreover, previous studies have also reported that lutein content efficiently prevents cardiovascular disease and cancers in humans [11,12]. Therefore, the attention paid to lutein and its demand has increased over recent years, with a 3.6% annual growth rate in the worldwide market [13]. Although commercial lutein production is now achieved from marigold flowers, including *Tagetes erecta* and *Tagetes patula*, microalgae also have great potential compared to that of conventional resources. Most microalgae have a 5–10 times higher growth rate than higher plant resources, and their cultivation does not require wide arable lands. Furthermore, most species are easily cultivated using seawater or brackish water, and a daily production of algal biomass can be achieved via control of a mass cultivation system [13]. However, for commercial use of microalgae in

Abbreviations: DCW, dry cell weight; ETR, electron transfer rate; HPLC, high performance liquid chromatography; ANOVA, one-way analysis of variance; PBR, Photobioreactor; PCR, Polymerase chain reaction; SEM, scanning electron microscope

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lutein production, high lutein-containing microalgae are required to reduce production costs. Compared to conventional lutein resources, such as marigold flowers *Tagetes erecta*, most lutein-containing microalgae are reported have lower lutein content (< 10 mg/g). According to Sun et al. [14], the maximum lutein content reported for several microalgal species are as follows: *Muriella aurantiaca* (2.6 mg/g), *Chlorella zofingiensis* (2.4–2.8 mg/g), *Tetracystis intermedium* (3.5 mg/g), *Coelastrum proboscideum* (3.4–5.0 mg/g), *Neospondiococcum gelatinosum* (4.4 mg/g), *Tetracystis tetrasporum* (4.4 mg/g), *Chlorella protothecoide* (4.6–5.4 mg/g), *Tetracysis aplanosporum* (5.9 mg/g), and *Dunaliella salina* (6.6 mg/g). The previous studies also revealed that the accumulation of carotenoid content significantly changed according to the regulation of culture conditions, including light intensity and nitrogen concentration [15,16]. The results of these studies showed that optimization of lutein production from microalgae is highly related to culture conditions. Thus, in the present study, we evaluated lutein accumulation from isolated algal strains to determine a potential lutein producer and investigated the effects of different light intensities and varying combinations of glucose and CO_2 on lutein productivity for the optimization of lutein production from algal biomass.

2. Materials and methods

2.1. Strains isolation and identification

For the screening test for lutein production of indigenous algal strains, freshwater samples were obtained for 3 years from throughout South Korea, including Seosan (36°49′36.8"N 126°19′31.7″E), Daejeon (36°22′01.9"N 127°22′09.9″E), Jeonju (35°49′45.7"N 127°07′42.6″E), Jinan (35°46′47.6"N 127°25′39.3″E), Jeongeup (35°32′32.5"N 126°50′25.6″E), Imsil (35°39′40.9"N 127°16′30.1″E), Namwon (35°27′08.4"N 127°26′04.5″E), Hampyeong (35°06′57.3"N 126°30′08.3″E), Naju (34°58′57.1"N 126°36′19.8″E), Damyang (35°17′09.6"N 127°03′11.0″E), Jangheung (34°44′36.0"N 126°50′41.7″E), Boseong (34°48′20.7"N 127°08′51.7″E), Suncheon (34°57′13.2"N 127°23′06.4″E), Goheung (34°36′43.9"N 127°28′59.9″E), Jinju (35°11′20.4"N 128°04′53.3″E), Busan (35°09′26.6"N 129°09′28.5″E), and Jeju (33°25′32.0"N 126°32′05.0″E) (Supplementary data 1). For the isolation of strains, 10 μL of the water sample was scrubbed into each agar plate (1.5% BG11), and generated algal colonies were streaked three-times into agar plates to remove contaminants. Axenic algal colonies were micro-picked and inoculated into a 250 mL Erlenmeyer flask with 100 mL of liquid BG11 medium [17]. Through the isolation process, a total of 100 different algal strains were axenically isolated. After the screening test for lutein accumulation, a 1 mL culture aliquot of algal strains that exhibited the highest lutein content was centrifuged at 4000 rpm for 5 min, and the pellet was used for DNA extraction using a DNA extraction kit (Intron, Korea). For the identification of each strain, 18S rDNA was amplified using two primers: 18S-ch165F (5’-CGACTTCTGGAAGGGACGTA-3′) and 18S-ch1200R (5’-GAGTCAAATTAAGCCGCAGG-3′) [18]. Polymerase chain reaction (PCR) was performed with 20 μL mixed solution containing 1 μL template DNA, 1 μL of each primer, 7 μL of distilled water, and 10 μL Go-taq (Promega, USA). The conditions for PCR amplification included an initial denaturation of 5 min at 95 °C, followed by 30 cycles of the denaturation step for 1 min at 95 °C, the annealing step for 30 s at 55 °C, and the elongation step for 1 min 30 s at 72 °C, and the final elongation step for 2 min at 72 °C. The amplified PCR products were identified by electrophoresis with 1% agarose gel, and sequence analysis was performed at Macrogen Inc., Korea.

Approximately 1000 bp of DNA was used for the DNA barcode study using a BLAST search with the help of the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast/cgi). The multiple DNA sequence alignment was carried out with the Clustal W program, and neighbor-joining phylogenetic tree constructed was conducted using MEGA6 software [19,20]. Morphology of strains was observed using an ECLIPSE 80i light microscope (Nikon, JPN) and scanning electron microscope (SEM) (Jeol, JPN). To obtain morphological images from the SEM, strains were prepared by a modified method of Pearl and Shimp [21] and Jung et al. [22]. In brief, for the fixation of the algal strain, 1 mL of 2% of glutaraldehyde (v/v) solution buffered with 0.1 mol/L sodium cacodylate was added to the cell pellet (1.5 mL centrifuge tube), and the mixture was incubated in a refrigerator for 1 h. Then, it was wash with distilled water, and freshly prepared 2% osmium tetraoxide was added to the cell pellet and incubated for 2 h in the dark. Subsequently, dehydration was performed using increasing concentrations of ethanol solution ranged from 10% to 100% (v/v), and hexamethyldisilazane (HMDS) was added to the cell pellet of the dry dehydrated sample. After platinum coating of the sample using an EMS150R Squtter coating system (Electron Microscopy Science, USA), an image was obtained by JSM-7900F Field Emission SEM (Jeol, JPN). Optimal temperature of the strains was determined using PhotoBiobox (SHINWHA SCIENCE, KOR) and the proposed method of [23].

2.2. Optimization of lutein productivity

The various types of photo-bioreactors (PBR) (tubular, cylindrical, flat panel, and intra-light) were tested to verify algal biomass productivity given equal energy input for optimization of lutein production (Supplementary data. 2A-2D). The working volume was 7 L (BG11 medium) and aeration was regulated with 0.5 vvm of 5% carbon dioxide. Cultivation was performed at 35 °C, and three 36 W cool-white fluorescent lights were used for each photobioreactor. The initial algal cell concentration was set at 1.0×10^6 cells mL^{-1}, and the dry cell weight (DCW) and algal cell concentrations were monitored daily for 5 days.

To optimize algal lutein productivity, various experimental conditions were tested using the tubular-type PBR. The light intensity regulated with cool-white fluorescence lights ranged from 100 μmol m^{-2} s^{-1} to 1000 μmol m^{-2} s^{-1}, and we also tested different combinations of 5% CO_2 supply and glucose supplementation in the medium: air, 5% CO_2, 5 g L^{-1} of glucose, and 5% CO_2 + glucose. The cultivation was performed at 35 °C, and the experiment testing 5% CO_2 and glucose combinations was performed at 500 μmol m^{-2} s^{-1} of light intensity.

2.3. Analytical methods

2.3.1. Determination of algal cell concentration and biomass productivity

Algal cell concentration was determined by the direct cell counting method using a hemocytometer (inCYTO, KOR) and an ECLIPSE 80i light microscope (Nikon, JPN). To determine algal biomass productivity, DCW was measured by the gravimetric method. Briefly, a 20 mL culture aliquot was filtered through dried and pre-weighed 25 mm glass microfiber filters (Whatman, UK), and incubated at 100 °C in a drying oven overnight. Subsequently, dried algal cells were precisely weighed using a microbalance (Mettler Toledo, USA). Biomass productivity was calculated using the proposed equation of Hempel et al. [24] based on DCW values.

2.3.2. Determination of photosynthetic pigments using high performance liquid chromatography (HPLC)

The analysis of photosynthetic pigments was performed using the modified method of Yun et al. [25]. After 96 h of cultivation time, microalgae were harvested by centrifugation at 6000 ×*g* for 5 min and freeze-dried for further analysis. For the extraction of lutein, the precisely weighed 10 mg of lyophilized samples were agitated and vortexed using 0.1 mm: 0.5 mm (1:3, v/v) zirconia beads (Biospec) with 1 mL ethanol (Merck) in 1.5 mL micro centrifuge tube. After being centrifuged at 10,000 ×*g* for 1 min, the supernatant phase was transferred into HPLC vials after filtering using 0.22 μm PTFE filters (Whatman, UK). The photosynthetic pigment analysis conducted was

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