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Enhanced astaxanthin extraction efficiency from *Haematococcus pluvialis* via the cyst germination in outdoor culture systems

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ABSTRACT

Haematococcus pluvialis is the richest source of natural astaxanthin (3S, 3′S), but the rigid cell wall of mature red cyst (aplanospore) complicates the efficient extraction of astaxanthin from the strain. Herein, the cyst germination method was developed by using nitrate and light for practical application for an efficient astaxanthin extraction from *Haematococcus* cells cultured in outdoor condition where flue gas, solar radiation and photobioreactor were used. Notably, autotrophic germination rate was easily regulated by light intensity. Under conditions of low germination rate, total astaxanthin concentration was highly maintained and astaxanthin extraction efficiency was rapidly enhanced during autotrophic germination. As a result, under homogenization (30 s), the extracted astaxanthin concentration in the cells treated with 1 mM KNO_3–150 μE/m^2/s was highly increased by 58% compared to the cells treated without germination. Our technical solution will definitely improve an astaxanthin extraction titer with the practical application in outdoor *Haematococcus* culture system.

1. Introduction

Astaxanthin (3,3′-dihydroxy-β-carotene-4,4′-dione), which is one of the most powerful antioxidants among carotenoids, is extensively used as a pigment source in aquaculture and also gained broad application in pharmaceutical and nutraceutical industries due to the strong antioxidant property [1]; astaxanthin possesses 500-fold and 38-fold stronger free radical antioxidant activity of vitamin E and β-carotene, respectively [2–4]. *Haematococcus pluvialis* accumulates high levels of the red ketocarotenoid pigment, natural astaxanthin up to 4% of its dry mass [5]. The market price of natural astaxanthin from *H. pluvialis* is approximately $7000 per kg because it is preferred for human consumption [6].

H. pluvialis has various phases in lifecycle of differentiating morphology [7]. Under low stress conditions, *H. pluvialis* exists as a flagellate (green motile cell) and a palmelloid (green nonmotile cell) during vegetative growth. Under high stress conditions such as high irradiance, nutrition deficiency, high salinity and drought, *Haematococcus* cells show morphological and physiological transformation [8–11] in which green flagellates are turned into red cysts (aplanospore) to accumulate astaxanthin. However, under favorable conditions, germination is occurred to form flagellated zoospores in order to start a new vegetative growth [7].

Various methods of cell wall disruption such as bead-beating, sonication, microwaves, acid- and alkali-treatment, enzyme lysis and supercritical CO_2 have been investigated for efficient astaxanthin extraction from astaxanthin-enriched aplanospore in *H. pluvialis* [12]. However, compared to a flagellate, an aplanospore has a rigid cell wall which is made up of sporopollenin-like material, algaenan, thereby hindering solvent extraction of astaxanthin [13–15]. To enhance the extraction efficiency of astaxanthin from *H. pluvialis*, cell wall-deficient mutant was isolated by strain improvement of upstream process [16].

Herein, under outdoor autotrophic conditions in which flue gas and solar radiation were used, astaxanthin extraction efficiency from *H. pluvialis* was successfully improved via germination by controlling nitrogen supplement and light intensity. Notably, in the conditions of high cell density of aplanospore, one of the most crucial factors for an increase in germination rate was a light intensity. Consequently, under conditions of 1 mM KNO_3 and 150 μE/m^2/s solar irradiance, maximal astaxanthin extraction was obtained after 3 days of autotrophic germination. Above all, high amount of dividing cysts in the cells cultured with 1 mM KNO_3 and 150 μE/m^2/s solar irradiance were easily harvested without addi-

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tional costs and energy prior to release of zoospores from mature red cyst (aplanospore). In large-scale outdoor *Haematococcus* culture system, the practical application of the germination strategy will surely reduce energy costs for astaxanthin extraction from *H. pluvialis* by efficiently weakening the rigid cell wall of aplanospore.

2. Materials and methods

2.1. Algal strain and culture conditions

H. pluvialis NIES-144 (wild-type) was purchased from the National Institute for Environmental Studies in Tsukuba, Japan. The strain was cultured photoautotrophically in NIES-C and NIES-N media during the green and red stages, respectively [17–18]. NIES-C medium (pH 7.5) of green stage is comprised of 0.15 g/L $Ca(NO_3)_2$, 0.10 g/L KNO_3, 0.05 g/L β-glycerophosphoric acid disodium salt pentahydrate, 0.04 g/L $MgSO_4 \cdot 7H_2O$, 0.50 g/L Tris-aminomethane, 0.01 mg/L thiamine, 0.10 μg/L biotin, 0.10 μg/L vitamin B12, and 3.00 mL/L PIV metal solution, which consisted of 1.0 g/L Na_2EDTA, 0.196 g/L $FeCl_3 \cdot 6H_2O$ and (in mg/L) 36.0 $MnCl_2 \cdot 4H_2O$, 22.0 $ZnSO_4 \cdot 7H_2O$, 4.0$CoCl_2 \cdot 6H_2O$, and 2.5 $Na_2MoO_4 \cdot 2H_2O$. The NIES-N medium (pH 7.5) of red stage was prepared by excluding an N source from the NIES-C medium, by substituting (in gram per liter) $CaCl_2 \cdot 2H_2O$ 0.13 and KCl 0.07 for $Ca(NO_3)_2$ and KNO_3, respectively, for supplement of calcium and potassium ions.

In this study, a two-stage induction strategy was applied to improve astaxanthin production in *H. pluvialis* under outdoor photoautotrophic conditions. In the first ("green") stage, the cultures were fully grown in the N-replete medium (NIES-C, pH 7.5) under weak lights (~35 μE/m^2/s) for 21 days (green stage). In the second ("red") stage, the cells were transferred to N-deplete medium (NIES-N, pH 7.5) and cultured under strong lights (~300 μE/m^2/s) over 42 days (red stage) for highly efficient induction by facilitation of rapid nitrogen starvation and photo-inducibility. After 42 days of autotrophic induction, the concentration of biomass and astaxanthin was maximally reached to 3.82 g/L and 150.8 mg/L, respectively (Fig. S1).

After that, autotrophically induced red cyst cells (aplanospore) were germinated for efficient astaxanthin extraction from mature red cysts by weakening the rigid cell wall of aplanospore using nitrate and light. To demonstrate the effect of germination on the astaxanthin extraction efficiency, different levels of nitrate (KNO_3) (1.0–2.0 mM) and light intensity (150–300 μE/m^2/s) were used in outdoors for germinating the mature red cyst cell (aplanospore). Under the outdoor autotrophic conditions, *Haematococcus* cells experienced daily variations in the light intensity and temperature of solar radiation. Therefore, during 6 days of germination, cells were maximally exposed to light intensities of 150 μE/m^2/s for moderate light conditions and 300 μE/m^2/s for high light conditions (Fig. S2). Cultures were also roughly maintained in outdoors between 17.5 °C and 27.5 °C without temperature control (Fig. S2).

The outdoor *Haematococcus* culture system was developed for natural astaxanthin production using flue gas and natural solar radiation. The outdoor *Haematococcus* culture system for natural astaxanthin production was installed near the thermal power plant, Korea District Heating Corporation located at Baekhyeon-dong, Bundang-gu, Seongnam-si, Gyeonggi-do, in the Republic of Korea (latitude: 37°00'00"North, longitude: 127°30'00"East) [19].

In the system, our previously developed thin-film 25 L-photobioreactor (PBR) constructed from a polymer film (CPP; polypropylene-based cast polypropylene) was used for outdoor culture of *H. pluvialis* [20,21]. In the PBR, cells were cultured with flue gas, which was composed of 3.5 ± 0.5% (v/v) CO_2, 1.36 ± 4.12 ppm CO, 10.17 ± 0.73% O_2, and 21.63 ± 3.54 ppm NOx, at a flow rate of 0.2 vessel volumes per min (vvm). During the outdoor culture period, control of the photon flux density was applied by using shade.

2.2. Analytical methods

2.2.1. Measurement of dry cell weight and cell count

Cell biomass was determined by filtering aliquots of samples using GF/F microfiber filter paper (Whatman, Cambridge, UK). 10 ml of cell suspensions were filtered with pre-weighed filters and dried at 100 °C dry oven overnight to determine the biomass. Nitrates in the culture were analyzed by ion chromatography (DIONEX 500, Chelmsford, MA, USA).

The cells in the germination stage were classified into 4 types, namely mature cyst, dividing cyst with cell divisions, dividing cyst with differentiated zooids, releasing cyst with differentiated zooids, and were counted at 1 day intervals using an improved Neubauer counting chamber (C-Chip, DHC-N01, iNCYTO, Korea) [22].

2.2.2. Measurement for photosynthetic pigment (chlorophyll, carotenoid, and astaxanthin) analysis

To assay intracellular pigments, cell suspensions (10 mL) were collected by centrifuging the culture fluid at 5000 rpm for 5 min at 4 °C, discarding the supernatant, and rinsing the cell pellet with pre-chilled TE buffer (pH 7.5). The pellet was homogenized with Tissue Lyser II (Qiagen, Valencia, CA, USA) using pre-chilled Tissue Lyser adaptors in pre-chilled 100% methanol to extract pigments. The extraction procedure was repeated until the cell debris was colorless. The homogenized lysate was centrifuged at 15,000 rpm for 10 min at 4 °C to separate the supernatant and cell debris. The extracts (supernatants) were used to measure chlorophyll and astaxanthin. Total intracellular concentrations of chlorophyll were assayed using UV spectrophotometry [23].

Astaxanthin concentrations were quantified by a Shimadzu high performance liquid chromatography (HPLC) system equipped with two LC-10AD pumps and SPD-10A UV–vis detector (Shimadzu, Japan) [17,24–25]. The extracts were saponified with 0.01 M NaOH (in methanol) and separated using a 250 × 4.6 mm HS-303 hydrosphere C_{18} column (YMC, Japan). The mobile phase consisted of solvents A (dichloromethane: methanol: acetonitrile: water, 5.0: 85.0: 5.5: 4.5, v/v) and B (dichloromethane: methanol: acetonitrile: water, 22.0: 28.0: 45.4: 4.5, v/v). For the effective separation of free astaxanthin, the following linear gradient from 0 to 100% B for 12 min, and 100% B for 50 min. The flow rate was 1.0 ml/min and the peaks were measured at 480 nm [17,25].

2.2.3. Analysis of astaxanthin extraction efficiency

In our study, 120 min of homogenization treatment was sufficient for completely breaking the cell wall of astaxanthin-enriched *Haematococcus* cells in 10 ml of the fermented sample containing about 40 mg of mature red cysts (Fig. S3). Accordingly, we determined the astaxanthin concentration after 2 h of homogenization treatment is a total astaxanthin (100%) of the cell. Subsequently, 30 min of homogenization was highly efficient for astaxanthin extraction from 10 ml of the fermented sample containing high amounts of the dividing cyst with differentiated zooids (Fig. S3). Therefore, astaxanthin extraction efficiency was determined by comparing quantities of extracted astaxanthin after sufficient (120 min) and deficient (30 min) homogenization treatment; the percentage (%) of extraction efficiency was determined by the equation below.

$$\text{extraction efficiency}(\%) = \frac{\text{insufficient (0.5hour) homogenizing treatment}}{\text{sufficient (2hour) homogenizing treatment}} \times 100$$