Bioresource Technology 128 (2013) 118–124

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Effect of various stress-regulatory factors on biomass and lipid production in microalga *Haematococcus pluvialis*

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HIGHLIGHTS

- Specific growth conditions for biomass yield in *Haematococcus pluvialis.*
- Specific growth conditions for biodiesel feedstock lipids.
- Specific growth conditions for UFA-rich lipids for multiple use.
- Refined biomass proposed for biofuel feedstock.

ARTICLE INFO

Article history:
Received 5 September 2012
Received in revised form 10 October 2012
Accepted 12 October 2012
Available online 23 October 2012

Keywords:
Haematococcus pluvialis
Stress conditions
Biomass
Feedstock lipids
UFA-rich lipids

ABSTRACT

To maximize the biomass and lipid production for applications in food or biofuel feedstock, nine stress conditions were tested considering N and/or P limitations, light intensity & quality, for *Haematococcus pluvialis* SCCAP K-0084 cultivation. Photosynthetically active radiation (PAR), warm white light emitting diode (WWLED), and white light emitting diode (WLED) at illumination of 240 μmol photons m^{-2} sec^{-1} were the best stress-regulatory factors. PAR without P & low N conditions yielded high biomass with 33% lipids containing increased C16:0 and C18:0 saturated fatty acids, and reduced unsaturated fatty acids (UFAs) (oleic, linoleic, and α/γ-linolenic). WWLED and WLED without P conditions also yielded high biomass, but 25% lipids with increased amounts of UFAs. Red light emitting diode (RLED) without P & low N conditions yielded 46% lipids with lowest biomass. PAR and WWLED & WLED illuminated conditions were found suitable respectively for biodiesel feedstock lipids and UFA-rich lipids for multiple applications.

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1. Introduction

Haematococcus pluvialis, a freshwater green microalga has been proven to be a significant natural source of astaxanthin, a carotenoid with high antioxidant capacity and has long been used as food coloring agent in aquaculture and poultry (Lorenz and Cysewski, 2000). The microalga grows as motile bi-flagellated green cells under favorable growth conditions, but during extreme environmental conditions, such as nutrient limitation, increased light intensity or high salt concentrations, these cells undergo morphological and biochemical changes as their survival strategies. They form red cysts by losing their motility, increasing cell size, thickening cell walls, increasing lipids biosynthesis and carotenogenesis, particularly accumulation of orange-red pigment astaxanthin esters that may constitute up to 95% of the total carotenoids (Lee and Zhang, 1999). Generally, during most of the stress conditions microalgae accumulate neutral lipids, and particularly in *H. pluvialis*, the accumulation of neutral lipids and astaxanthin esters are simultaneous processes (Boussiba, 2000; Thompson, 1996). The increased neutral lipids under unfavorable conditions were hypothesized to serve as a matrix for solubilizing astaxanthin esters (Boussiba, 2000). This hypothesis was further supported by increased production of oleic acid (C18:1n-9 *cis*) enriched lipids that is essential for astaxanthin accumulation (Zhekisheva et al., 2002). It was reported that the neutral lipids of either nitrogen starved or high light stressed cells possess enhanced palmitic (C16:0) and linoleic (C18:2n-6 *cis*) acids content (Zhekisheva et al., 2002). Therefore,

Abbreviations: BLED, blue LED; DMSO, dimethyl sulfoxide; DW, dry weight; FAs, fatty acids; FFAs, free fatty acids; GLED, green LED; LED, light emitting diode; PAR, photosynthetically active radiation; PUFAs, polyunsaturated fatty acids; RLED, red LED; SFAs, saturated fatty acids; UFAs, unsaturated fatty acids; WLED, White LED; WWLED, Warm white LED.

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0960-8524/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biortech.2012.10.049

microalgae producing enhanced amounts of lipids with essential fatty acids and the antioxidant astaxanthin would have added-value as an ingredient for various functional food, cosmetics and nutraceutical products. Additionally, microalgal lipids can serve as biodiesel feedstock because of negligible unsaturated fatty acids (UFAs) content, and the significant amount of saturated fatty acids (SFAs) mainly contributed by C16 and C18 lengths (Chisti, 2007). The lipids containing astaxanthin of *H. pluvialis* in a biodiesel blend can significantly increase the oxidative stability (Oliveira et al., 2006), which is one of the important properties of biodiesel. Further, the high levels of saturated and mono-unsaturated fatty acids of microalgal lipids make them ideal for biodiesel that remains as liquid at low temperatures with a high energy content. Microalgal biomass after refining of value-added molecules (e.g., astaxanthin in *H. pluvialis*) can also be used as feedstock for bioethanol or biomethane production (John et al., 2011; Mussgnug et al., 2010; Nigam and Singh, 2011).

The aims of the present study were to identify suitable stress conditions for maximum production of biomass and lipids in a standard microalga *H. pluvialis* SCCAP K-0084 that is readily available for any users, and recommend the optimum stress conditions to produce lipids applicable as biodiesel feedstock or as ingredient for functional foods or health products.

2. Methods

2.1. Microalgal strain and culture conditions

The microalga *H. pluvialis* SCCAP K-0084 (hereafter *H. pluvialis*) was cultured in 250 ml Erlenmeyer flasks containing 100 ml of modified Bold's basal (MBB) medium enriched with $NaNO_3$ (0.875 g/L) for actively growing green-phase cells. Briefly, 500 μl of actively growing cells were inoculated per 100 ml culture medium to obtain an initial cell density (*in vivo* absorbance) of 0.05–0.06 at 680 nm. The cultures were incubated in environmental growth chamber (M-series walk-in, http://www.egc.com/prod_m_-series.php) at 20 °C, under the PAR (photosynthetically active radiation, 400–700 nm) illumination 80 μmol photons m^{-2} sec^{-1} for 16/8 h light/dark cycle with shaking at 150 rpm. On the 10th day of growth, these cultures were harvested and either retained as control cells for certain analysis or used as inoculum for red-phase cultivation for two weeks. To identify a suitable stress regulatory factor for lipids biosynthesis, a total of nine stress conditions were tested for green- and red-phase culturing. The stress experiments were set-up using the ten day old green-phase cells, which were carefully and quickly harvested by centrifugation at 3000*g* at 20 °C for 3–4 min, and the pellets were washed twice with sterile water before final re-suspension in respective stress medium (see details below). For certain stress conditions, the green phase growth of cells were under particular LED (Light Emitting Diode) illumination of 80 or 100 μmol photons m^{-2} sec^{-1}. We have designed a total of nine best possible stress conditions taking into account of the various favorable factors available in literature. The specific growth and corresponding stress conditions tested are outlined in Table 1.

2.2. Microalgal pigments and biomass determination

Both green- and red-phase culturing was monitored by measuring the content of chlorophyll *a* and total carotenoids of cells at indicated time points. Chlorophyll *a* was indicative of green-phase culturing, while carotenoids were indicative of red-phase culturing. For spectrophotometric measurement of these pigments, 2 ml of cells from a specific time-point of cultivation were centrifuged at 4000*g* for 5 min and the pellets were extracted overnight with dimethyl sulfoxide (DMSO) at room temperature (~20 °C) in the dark. The optical density of the supernatant was read at 665 and 480 nm, in 96-well plate reader (BioTek Synergy 4) to estimate respectively the content of chlorophyll *a* and total carotenoids by using the equations of Wellburn (1994). The cells harvested after two weeks of red-phase culturing were freeze-dried and the end-point biomass as growth determinant was estimated gravimetrically.

2.3. Lipids extraction and estimation

Extraction of lipids was done following the method of Folch et al. (1957). A known amount of biomass (~50 mg dry weight (DW)) was soaked in 5 ml extraction solvent (2:1 chloroform:methanol) for at least four hours at 4 °C. Then, the biomass was ground in a mortar and pestle by adding pulverized glass powder (~0.5 mm, Sigma). To ensure complete extraction, further grinding was done by adding 10 ml of extraction solvent. All extracts were pooled and made-up to 15 ml with extraction solvent in a tube, where 5 ml of Milli-Q water was added and vortexed mildly to remove water-soluble impurities. Then the tubes were centrifuged at 4600*g* for 8 min for the separation of two layers and the lower lipid layer was transferred carefully. The moisture content of lipids was eliminated by adding sodium sulfate crystals, and the clear supernatants of lipids were dried in a fume hood. These dried lipids were then measured gravimetrically.

2.4. Lipids fractionation and LC/MS analysis of fatty acids

The dried lipids were re-constituted in chloroform for fractionation into neutral, glycolipids, and phospholipids by using Qiagen DNA spin column packed with 500 μl bed volume of silica gel (pore size 60 Å, 230–400 mesh; Sigma). The re-constituted lipids (600 μl) were loaded onto the column, and the flow-through was re-loaded. The column was eluted with 600 μl of chloroform: acetic acid (9:1, v/v) and considered as neutral lipids. The second flow-through obtained was pooled with neutral lipids fraction. Then, glycolipids were eluted by 600 μl of acetone: methanol (9:1, v/v), and finally phospholipids were eluted by 600 μl of HPLC grade methanol. All fractions were collected in amber GC-vials by gravity and dried with nitrogen flush.

The above lipid fractions were dissolved in 1 ml of methanol: dichloromethane: acetonitrile: water (2:1:1.5:1.5, v/v) and 10 μl of each fraction was used to obtain complete free fatty acids profile using HPLC (Agilent 1260 series) equipped with Q-TOF mass spectrometer (Agilent 6520). Analytes were resolved by an Agilent C-18 Poroshell 120 column (2.7 μm, 3.0 × 150 mm) with gradient elution. Mobile phase A consisted of 2 mM ammonium acetate in water and mobile phase B consisted of 2 mM ammonium acetate in 95% acetonitrile. The mass spectrometer was operated in negative ionization mode, scanning from 50–1100 *m*/*z*. Drying gas flow rate, temperature and nebuliser pressure were at 5 L min^{-1}, 325 °C, and 30 psi, respectively. Fragmentor and skimmer voltages were kept at 175 V and 65 V, respectively. All MS/MS experiments were carried out at 15 V as collision energy (CE), while all other conditions kept the same.

2.5. Statistics and dendrogram construction

Results are averages of triplicates and the values in each graph are shown with 5% error bars. Quantitative data on dry weight biomass and corresponding lipids content were used as variables. The dendrogram was constructed using squared euclidean distance and average linkage between groups of hierarchical cluster analysis method (IBM SPSS 19 statistical software package).

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