Algal Research 12 (2015) 289–294

Contents lists available at ScienceDirect

Algal Research

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

Characterization of newly isolated oleaginous microalga *Monoraphidium* sp. for lipid production under different conditions

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ARTICLE INFO

Article history:
Received 21 May 2015
Received in revised form 21 August 2015
Accepted 22 August 2015
Available online xxxx

Keywords:
Biodiesel
CO_2 capture
Lipid production
Monoraphidium
Oleaginous microalgae

ABSTRACT

Green microalga *Monoraphidium* sp. was isolated during the mass cultivation study of *Ettlia* sp., when it was found that the culture was dominated by smaller unidentified cells with high lipid content. The strain was identified and named as *Monoraphidium* sp. based on its morphological characterization under light and confocal microscopy followed by genomic characterization via 18 s sequencing. The lipid content, fatty acid profiles, biomass and lipid productivity of microalga were examined under autotrophic cultivation condition using various CO_2 concentrations as well as different nitrogen sources. Isolated *Monoraphidium* sp. was found to have high lipid content of 28.92% determined via gas chromatography and sulfo-phospho-vanillin method (SPV). Lipid profile of the algal cells exhibited a suitable distribution of fatty acids for biodiesel production, with cultivation using urea producing particularly high quality of biodiesel. Therefore, the results suggest that *Monoraphidium* sp. is a promising feedstock for biodiesel production.

1. Introduction

Social and economic development of any country is always accompanied by an increase in energy consumption per capita. Most of this energy demand is being met by fossil fuels, which is a non-renewable source of energy that will be inevitably exhausted. The combustion of petroleum based fuels is a major source of greenhouse gases which contributes to atmospheric pollution and global warming [1,2]. Therefore, intensive research has been carried out in the last decades for the production of alternative sources of energy. In particular, biofuels production from sustainable feedstocks is getting immense importance worldwide [1,3].

All of the world's biodiesel is currently being produced from energy crop oils or animal fats. As such immense studies have been conducted on biodiesel production using crops as feed stocks, namely soybean, rapeseed, sunflower and safflower based oils [4]. However, fuel production using such feedstocks competes directly with human food production, which can destabilize food security in poor countries as well as resulting in spikes in food prices. Therefore, alternative feedstock is required for biodiesel production [5,6].

Among the several existing renewable feedstocks, microalgae have raised a great interest because of several advantages it offers, which includes high rates of atmospheric carbon dioxide sequestration [7], higher biomass production, higher growth rates as compared to other energy crops [8] and higher photosynthetic efficiency. The yield per area of oil from algae can be over 200 times greater than that of the best-performing plant/vegetable oils, primarily due to the fact that unlike land plants, algae is not restricted to annual or biannual growth and harvest cycles due to their unicellular nature [9]. Moreover, the cultivation of microalgae can be done on non-arable land which can help reduce the competition with other food crops. Microalgae have the ability to accumulate more than 50% of cell dry weight in lipids, mostly in the form of triacylglycerides (TAGs) as storage lipids. However, under optimized growth conditions, TAGs are only produced in small quantities; cells only accumulate large quantities of TAGs under growth limiting stress conditions like nutrient limitation (nitrate and phosphate), temperature [10], pH [11], or light stress [12,13]. Lipid accumulation under nutrient limitation is a well-known phenomenon. Among the various mentioned stress conditions, lipid accumulation under nitrogen deficiency is the most widely studied [14].

The identification of species with desirable attributes, such as high lipid content and suitable fatty acid profiles is a significant challenge associated with the algal biofuel research [15]. Even though many lipid rich microalgal strains have already been isolated, there are large and undetermined number of unknown microalgal strains present in different environments that can have even greater potential for the

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http://dx.doi.org/10.1016/j.algal.2015.08.015
2211-9264/

production of biodiesel [16]. Thus, the isolation and characterization of algae from different environments should be always performed.

In this study, a new microalgal strain *Monoraphidium* sp. was isolated during the mass cultivation of *Ettlia sp.* The taxonomic identity of the new isolate was confirmed using 18S rRNA gene sequence analysis. The algal growth rate, biomass productivity, lipid content, fatty acid profiling and lipid productivity of the newly isolated microalgal strain were also determined. Furthermore, *Monoraphidium* sp. was cultivated in various nitrogen sources to determine the most effective nitrogen sources for biomass and lipid productivity.

2. Material and methods

2.1. Algal strain, axenic isolation and cultivation conditions

Ettlia sp. was obtained from Korea Research Institute of Biology and Biosciences (KRIBB), Daejeon in South Korea and was mass cultivated in the Bio-Energy Engineering Research Lab of KAIST. The algae were cultured in BG11 medium with the following compositions: 1500 mg/L $NaNO_3$, 40 mg/L $K_2HPO_4 \cdot 3H_2O$, 75 mg/L $MgSO_4 \cdot 7H_2O$, 20 mg/L Na_2CO_3, 27 mg/L $CaCl_2$, 6 mg/L citric acid monohydrate, 6 mg/L ammonium ferric citrate, and 1 mg/L Na_2EDTA, with 1 mL trace metal solution (2.86 mg/L H_3BO_3, 1.81 mg/L $MnCl_2 \cdot 4H_2O$, 0.222 mg/L $ZnSO_4 \cdot 7H_2O$, 0.079 mg/L $CuSO_4 \cdot 5H_2O$, 0.050 mg/L $CoCl_2 \cdot 6H_2O$, 0.39 mg/L $Na_2MoO_4 \cdot 2H_2O$). All culture media used for this study were made using tap water supplied in KAIST. The cells were photoautotrophically cultivated in 20 L plastic bottle plugged with plastic cap with a flow rate of 100 mL/min. A silicon tube for supplying 2% CO_2 was connected through the cap and extended close to the bottom of the container, and the gases were sterilized by a 0.20 μm PTFE gas filter diaphragm Midisart-2000 (SRP65, Sartorius, Germany). Culture was subjected under continuous illumination, and the temperature of the room was maintained at 25 ± 2 °C. Cell growth was monitored by measuring the $O.D._{680}$. After the 6th month of serial batch cultivation, the culture was contaminated and dominated by a new microalgal species. The unknown algal sample was collected from a 20 L bottle of *Ettlia* mass culture. These samples were centrifuged at 3000 *g* for 10 min at room temperature to concentrate cells after the serial dilution of 10^{-5} and spread onto 1.5% agar plates of BG-11 to grow single colonies of microalgae. These agar plates were illuminated under cool continuous light of 80 μmol/m^2 s at 25 °C.

2.2. A morphological study by light microscopy, fluorescence microscopy using BODIPY staining

The isolated algal culture was grown in BG-11 media. For microscopic analysis, a small amount of this culture was taken and analyzed via inverted light optical microscopy (Leica, DM2500, Germany) equipped with a Leica microscope camera (DFC425C) with 100× magnification. The lipid staining dye, BODIPY 505/515 (4, 4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacen), was obtained from Sigma Aldrich. A 5 mM BODIPY 505/515 stock was prepared by dissolving it in anhydrous DMSO and stored in −20 °C in the dark. For efficient staining of the intracellular lipid molecules of *Monoraphidium* sp., an aliquot of 20 μl BODIPY (10 μl BODIPY stock + 4990 μl dH_2O with 0.2% DMSO (*v*/*v*) was added to the 180 μl microalgal culture suspension. The resulting suspension of dye and alga was agitated for 1 min on a vortex mixer. The suspension was then incubated in the dark for 10 min at room temperature. After the incubation period, the samples were mounted on the glass slide and observed under the confocal microscope [17].

2.3. DNA isolation, 18S rRNA gene amplification, sequencing and identification of microalgae using phylogenetic analysis

1 ml of cultured cells was harvested by centrifugation at 12,000 RPM for 3 min at room temperature. The cell pellets obtained were resuspended in 600 μl of deionized water and mixed with equal volume of saturated phenol (pH 8.0). For DNA extraction, bead beating was performed to completely break the cells using a Mini bead beater (Biospec Corp., USA) with Zirconia/silica beads (0.5 g, 0.5 mm in diameter/0.4 g, 0.1 mm in diameter; Biospec, Oklahoma, USA) for 60 s at 3800 RPM. Then the sample was centrifuged at 12,000 RPM for 5 min and the supernatant was transferred to a fresh micro centrifuge tube. The transferred samples were mixed with the same volume of PCI (phenol: chloroform: isopropanol = 25:24:1, pH 8.0) and centrifuged. Thereafter, the aqueous layer was transferred to another fresh micro centrifuge tube and then was precipitated with isopropanol. Finally, the dried nucleic acid sample was suspended in sterilized distilled water and analyzed using a Colibri Microvolume Spectrophotometer (Berthold Corp., Germany).

Polymerase chain reaction (PCR) was performed in 50 μl reactions and each sample contained 2× PCR premix (EF) (Solgent Corp., Korea), and 50 ng of extracted DNA [18]. The 18S rRNA gene was amplified using universal primers [19], SR6 (5′-GTC AGA GGT GAA ATT CTT GG-3′) and SR9 (5′-AAC TAA GAA CGG CAT GCA C-3′), which resulted in an expected 395 bp PCR product. The PCR reaction was performed with a thermal program which consisted of — initial denaturation at 94 °C for 5 min and then 30 cycles of (denaturation 94 °C for 1 min, primer annealing at 50 °C for 1 min, and chain extension for 1 min at 72 °C), followed by a final extension at 72 °C for 10 min. The products were cloned into a T-Blunt vector (Solgent, Korea) according to the manufacturer's protocol to create artificial standard clones. Overnight grown *Escherichia coli* DH5α cultures were used to extract plasmid DNA using the QIA prep® Spin Miniprep kit (Qiagen, USA) and sequenced with M13F and M13R primer (T-Blunt Vector Systems manual). BioEdit sequence alignment editor ver 7.2.0 was used to assemble nearly full-length 18S rRNA gene sequences [20].

2.4. Effect of CO_2 on growth and lipid content

Two different levels of CO_2 in the bubbling aeration system were tested; low CO_2 (atmospheric, 0.035%) and high CO_2 (2%) and compared with one set in which no CO_2 was mixed. The normal air condition has small amount of CO_2, which can be increased by mixing of CO_2 with air during the cultivation. The culture was continuously aerated by gentle bubbling of air containing 2% CO_2 with a flow rate of 0.1 *v*/*v*/m, in addition to constant shaking of 120 RPM in an orbital shaker. The growth was measured at regular intervals and the biomass was harvested after 16 days of cultivation. Cell density was measured spectrophotometrically at 680 nm which can be translated to the dry weight by a standard curve. The lipid was also estimated by spectrophotometrically at 530 nm using SPV method which was correlated to the lipid quantity obtained by a previously generated standard curve [21].

2.5. Growth analysis of the new Monoraphidium strain under different nitrogen source

To investigate the optimum nitrogen source for the cell growth and lipid accumulation of *Monoraphidium* sp., different nitrogen sources, including sodium nitrate ($NaNO_3$) 17.6 mM, potassium nitrate (KNO_3) 17.6 mM, ammonium chloride (NH_4Cl) 17.6 mM and urea 8.8 mM were supplied with BG11 media in which no previous nitrogen source was added, The nitrogen sources were added on an equivalent weight basis of nitrogen presents in the nitrogen source. The cultures were cultivated with fluorescent light at 25 °C in a shaking incubator. The optical density (OD) measured at 680 nm was calibrated with biomass, and the lipid content was measured after every 48 h using Sulpho Phospho Vanillin (SPV) method [21]. The culture was harvested after it reached stationary phase and analyzed for biomass and lipid production. All experiments were conducted in duplicate and results were expressed as means of the replicates along with standard deviation.