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Isolation of prospective microalgal strains with high saturated fatty acid content for biofuel production

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ABSTRACT

Isolation of new microalgal strains with unique and valuable properties from diverse environmental conditions is an important starting point for the establishment of high quality feedstock for biofuel production. In this study, we have isolated twelve strains of microalgae, belonging to the genera *Chlorella*, *Botryococcus*, *Scenedesmus*, *Nannochloris* and *Bracteacoccus* from samples obtained from various natural sources by flow cytometry. Five of them were selected for further evaluation, as lipid producers on the basis of Nile Red fluorescent microscopy screening. Analysis of the biomass in the exponential and stationary phases of growth showed that the strain *Scenedesmus abundans* A1175 has the highest specific growth rate ($0.20 \pm 0.01\ d^{-1}$), biomass productivity ($73.82 \pm 4.53\ mg\ L^{-1}\ d^{-1}$) and lipid content ($44.4 \pm 2.7\%$) in nitrogen depleted conditions. Strains *Chlorella vulgaris* A1123 and *S. abundans* A1175 have a high total content of saturated (SFA) and monounsaturated (MFA) fatty acids (67.0% and 72.8% respectively). *S. abundans* A1175 also had low polyunsaturated fatty acid (PUFA) content that would allow for its use as a source of high quality biofuels.

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1. Introduction

Microalgae are widespread in nature, including soil, freshwater and marine environments [1]. Microalgae biomass has been considered as a promising source for production of motor fuels in comparison with other sources of renewable biomass [2]. It was shown that oil productivity of microalgae is 100 times higher than that of some agricultural crops, such as canola, soy and oil palm [3,4]. Currently, microalgal biomass is used to produce third generation biofuels, biogas and bioethanol, but is also considered as a source of other valuable components: protein, polyunsaturated fatty acids, pigments, sugars and antibiotics [5].

High oil content of some microalgal strains makes them suitable for biodiesel production. Microalgae belonging to such genera as *Chlorella*, *Dunaliella*, *Nannochloris*, *Nannochloropsis*, *Neochloris*, *Porphyridium*, and *Scenedesmus* contain 20–50% of lipids by weight, making them suitable for biodiesel production [4]. Nevertheless, it was shown that strains with high lipid content have a low biomass productivity that significantly reduces their effectiveness as lipid producers [6]. In addition to high lipid content, prospective microalgal strains need to have high growth rates and a specific composition of produced lipids. An important criterion for production of high quality biodiesel that meets biodiesel standards EN 14214:2003 and ASTM D6751 is a low content of polyunsaturated fatty acids in raw oils used a feedstock. Biofuels that contain components with more saturated bonds are more resistant to oxidation, and at combustion, glycerides remaining in the fuel are not polymerized, which increases the reliability of diesel engines [7]. Thus, to find a prospective microalgae strain for the production of high-quality biofuel, it is necessary to screen for natural habitat strains with a high content of SFA and MFA with no additional requirements for expensive supplements for fast growth (e.g. vitamins). A number of studies have shown that the total content of SFA and MFA can reach high values (60–68% in some strains like in *Chlorella vulgaris* ESP-31 [8]). However, features of such strains remain poorly understood in terms of the physiology of the accumulation and composition of lipids, and their applicability to the production of valuable by-products, mainly protein and carbohydrates.

In this study, we have isolated a number of microalgal strains from various natural sources. The biomass accumulation, productivity, lipid content and composition of the isolated strains were studied during the exponential and stationary phases of autotrophic growth. Using flow cytometry, a number of microalgal strains were isolated and identified. Five of them were selected through screening with Nile Red microscopy as lipid producers. Analysis of their productivity for biomass and lipids, lipid accumulation and biochemical composition revealed that two strains, *S. abundans* A1175 and *C. vulgaris* A-1123, have optimal characteristics for production of high quality oil with high total content of MSA and SFA.

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2. Materials and methods

2.1. Isolation of microalgae by cell sorting with flow cytometry

Samples of soil and water were inoculated in 50 cm^3 Bold's Basal Medium (BBM) [9] and cultivated for two weeks at a temperature of 28 °C, under a 16 h-light/8 h-dark cycle, and illumination of 60 μmol m^{-2} s^{-1}. Microalgae cells were separated from the contaminating microorganisms by flow cytometry. Cell sorting was performed on BD FACSAria flow cytometer, and a sapphire laser with a wavelength of 488 nm was used for excitation of chlorophyll fluorescence. The fluorescence intensity was determined by photodetector with a dichroic mirror 655LP and a narrowband optical filter 695/40 nm. FACSFlow™ was used as a compressing fluid in the sorting process. Before sorting, suspension was purified from large aggregates, using cell strainers (35 μm, BD Falcon). Microalgae cell fraction had at least 6×10^4 cells that were plated onto BBM agar and cultured as described until visible colonies were observed. The purity of microalgal cultures was verified by microscopy and by the absence of bacterial and fungal growth on LB and PDA agar layered with diluted microalgae cultures from BBM agar plates.

2.2. Identification of the isolated strains of microalgae and phylogenetic analysis

Genomic DNA from microalgal cultures was isolated using the Wizard SV Genomic DNA Purification System (Promega, USA) according to the manufacturer's instructions. The partial sequence of the 18S rRNA gene was amplified from the 50 ng genomic DNA by PCR by Taq polymerase using universal primers 5′-ACCTGGTTGATCCTGCCAGT-3′ (forward) and 5′-TCAGCCTTGCGACCATAC-3′ (reverse) [10], PCR was performed under the following conditions: 1 cycle 95 °C — 3 min, 40 cycles (95 °C — 30 s, 54 °C — 20 s, and 72 °C —1 min 30 s), 1 cycle — 72 °C for 10 min and products were analyzed by electrophoresis. DNA sequencing was performed with BigDye® Terminator v3.1 Kit (Applied Biosystems) and the nucleotide sequence was determined on ABI 3730XL sequencer (Applied Biosystems). The obtained sequences were aligned with BLAST to sequences of microalgae 18 s rRNA from GenBank database [11]. Multiple sequence alignment and phylogenetic tree construction were performed using MEGA 5 [12]. The phylogenetic tree was built using the Neighbor-Joining algorithm [13], and the statistical reliability of its topology was determined by the Bootstrap test [14].

2.3. Preliminary screening for lipid production in microalgae

Equal amounts of cells from each microalgae isolates obtained during the isolation were cultured in 24 well plates in 1.5 cm^3 BBM-3 N medium supplemented with 0.75 g L^{-1} $NaNO_3$ under a 16 h-light/8 h-dark cycle illumination at 60 μmol m^{-2} s^{-1} in 2% CO_2 atmosphere for 7 days and were agitated in an orbital shaker at 150 rpm. After incubation, cells were harvested by centrifugation at 1000 g for 5 min, transferred to the BBM medium without $NaNO_3$, and similarly cultured in 24 well plates for up to 10 days to trigger lipid accumulation in cells. During cultivation in the BBM medium, the pattern of accumulation of lipids was observed by fluorescent microscopy with Nile Red cell staining.

2.4. Cultivation of microalgae

A single colony of each strain cultivated on BBM agar was picked up and inoculated into 50 cm^3 Erlenmeyer flacks in 25 cm^3 BBM media and cultivated at 80 rpm agitation on an orbital shaker at 28 °C (without CO_2 supplementation), at an illumination of 60 μmol m^{-2} s^{-1} under a 16 h-light/8 h-dark cycle until the mid-exponential phase was reached. Culture growth was monitored by measuring the optical density (OD) at 680 nm using a UV–VIS spectrophotometer (Epoch, Biotek). Cells were inoculated in 250 cm^3 conical flasks, containing 100 cm^3 of BBM at OD 0.08, and cultured at 150 rpm in an orbital shaker at 28 °C as described until the stationary phase of growth was reached.

2.5. Determination of dry cell weight

The relationship between OD and dry cell weight (DW) was determined for each strain. During the exponential phase of growth, the OD was measured at 680 nm, and 10 cm^3 of liquid was withdrawn from culture (not less than 5 times at different time points in triplicate) and transferred on a glass fiber filter, dried at 105 ºC overnight and weighed. The relationship between OD and DW was determined through linear regression and converted to g L^{-1} with $R^2 > 0.98$.

2.6. Evaluation of growth rates and productivity parameters

Biomass productivity (BP) was calculated through the equation BP (mg L^{-1} d^{-1}) $= (X_2 - X_1) \cdot (t_2 - t_1)^{-1}$ where X_1 and X_2 were the biomass dry weight concentrations (mg L^{-1}) on days t_1 (start of the exponential phase) and t_2 (end of the exponential phase), respectively.

Specific biomass growth rate (μ) was calculated in the exponential growth phase by $\mu\,(d^{-1}) = (\ln X_2 - \ln X_1) \cdot (t_2 - t_1)^{-1}$ where X_1 and X_2 are the lipid dry weight concentrations (mg L^{-1}) on days t_1 (start of exponential growth) and t_2 (end of exponential growth), respectively.

Doubling time (DT) is defined as $DT = \ln 2 \cdot (\mu)^{-1}$.

All experiments were carried out in triplicate, and data are expressed as mean ± SD.

2.7. Fluorescent microscopy of cells after Nile Red staining

Fluorescent staining of 1 cm^3 of microalgal cell suspension (10^5–10^6 cells) was performed using 0.05 cm^3 of the Nile Red solution in acetone (0.1 mg cm^{-3}). The mixture was incubated for 10 min at 37 °C in darkness. The fluorescence of the cells of stained and control samples of microalgae were observed on Axioskop 2 Plus (Carl Zeiss) in the Interinstitutional Shared Center for Microscopic Analysis of Biological Objects SB RAS. Zeiss filter sets FS10 (excitation bandpass, 450 to 490 nm; emission bandpass, 515 to 565 nm) and FS20 (excitation bandpass, 546/12 nm; emission bandpass, 575 to 640 nm) were used for fluorescence detection. Images were taken with Axiovision imaging v4.6. Acquisition and processing of data was done using the Axiovision v4.6 software.

2.8. Determination of Nile Red fluorescence

Fluorescent staining of neutral lipids of microalgae was performed using the Nile Red dye. For this purpose, 0.025 cm^3 of Nile Red in acetone (0.1 mg cm^{-3}) was added to 0.5 cm^3 of the cell suspension containing 20% DMSO, with the concentration of cells normalized at OD 0.2 (680 nm). The mixture was incubated for 10 min at 37 °C in darkness. Analysis was performed with Perkin-Elmer EnVision2103 Multilabel Reader using 525/579 excitation/emission filters. The relative fluorescence intensities were obtained by subtracting of both the autofluorescence of unstained algal cells and fluorescence intensity of Nile Red of blank medium containing DMSO and Nile Red Dye from the fluorescence intensities measured in the stained cells.

2.9. Analysis of nitrate concentration in medium

Nitrate concentration during the cultivation of microalgal strains in medium was measured as described in [15]. Samples of cultural liquid were centrifuged at 3000 g for 5 min. The supernatant was collected and the absorbance was measured at 220 nm. Sodium nitrate solution with concentrations of up to 500 μM were used as a standard in all measurements.

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