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RESEARCH PAPERS

Optimization of *Prochlorothrix hollandica* Cyanobacteria Culturing for Obtaining Myristoleic Acid

S. S. Lapina[a], I. P. Maslova[a], V. P. Pchelkin[a], V. S. Bedbenov[a], K. S. Mironov[a], B. K. Zayadan[b], A. A. Userbaeva[b], M. A. Sinetova[a], and D. A. Los[a]

[a] *Institute of Plant Physiology, Russian Academy of Sciences (IPPAS), ul. Botanicheskaya 35, Moscow, 127276 Russia*
[b] *Faculty of Biology and Biotechnology, Al-Farabi Kazakh National University, pr. Al-Farabi 71, Almaty, Republic of Kazakhstan*
e-mail: losda@ippras.ru

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Abstract—Plankton filament cyanobacteria *Prochlorothrix hollandica* is characterized by a very high content of C_{14} and C_{16} fatty acids (FA) in the lipid membranes. Depending on culturing conditions of the cyanobacteria, total concentrations of myristic and myristoleic acids can reach 35% and those of palmitic and palmitoleic acids can reach 60% of all esterified FA cells. In *P. hollandica*, a variety of monounsaturated FA is represented by myristoleic and palmitic acids, and by hexadecenoic ($C_{16:1}$) acid with olefin bond of *cis*-configuration, located in the Δ4 position. The process of intensive culturing for *P. hollandica* cells to yield a maximal biomass in order to isolate the pure drug of myristoleic acid derivative has been optimized. The use of a three-stage purification gives 30 mg of chromatographically pure myristoleic acid methyl ester from 17 g of *P. hollandica* raw biomass (dry mass is 3 g), which is 1% of dry cell mass.

Keywords: Prochlorothrix hollandica, desaturase, fatty acids, lipids
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INTRODUCTION

Myristoleic acid (MA) is a monounsaturated fatty acid (FA), 14:1Δ9 (sometimes designated as 14:1ω5). MA induces apoptosis and necrosis in human prostate cancer cells (LNCaP) *in vivo* with a minimum inhibitory concentration of 9 μM. MA as a substrate is also involved in the acylation of regulatory proteins, such as tyrosine protein kinase Src is the main regulator in the formation of osteoclasts, destructing bone tissue [1]. Cetyl myristoleate, a drug used for the treatment of arthritis [2, 3] and fibromyalgia [4], is obtained from esterification of free myristoleic acid with cetyl alcohol.

Typically, MA is isolated from the fruit of aquatic grass staurogyne (*Staurogyne repens*) or from the seeds or Myristicaceae plants; nutmeg, which is rich in trimyristin—triglyceride with three saturated myristic acid residues—and the African nutmeg (*Pycnanthus angolensis* or *P. kombo*), from which oil (known as combo oil) MA are obtained, are the most famous [5]. *P. kombo* seeds contain 56–61.6% of oil, which includes 58–64% of myristic acid and 19–26% of MA. The average trading price of MA in sales of leading manufacturers of chemically pure reactants (Sigma–Aldrich, Cayman Chemical, MP Biochemicals, Protheragen, and Wako Pure Chemical Industries) is approximately US $1.000 per 1 g.

Photoautotrophic microalgae and cyanobacteria, having a plastic metabolism and ability to adapt to a variety of environmental conditions, are potential producers of many useful compounds, including fatty acids [6]. The objectives of this work were the screening of cyanobacteria strains from microalgae collection of IPPAS for the search of potential MA producer, the optimization of culturing conditions for promising strains and the obtaining of chromatographically pure MA methyl ester.

MATERIALS AND METHODS

The objects of the study were the strains of cyanobacteria from the collection of microalgae IPPAS (Moscow): *Cyanobacterium* sp. B-1200, *Synechococcus* sp. B-266, *Synechococcus* sp. B-434, *Synechocystis* sp. B-288, *Thermosynechococcus vulcanus*, *Synechococcus elongatus* B-267, *Synechococcus* sp. B-468, *Phormidium laminosum* B-407, *Microcystis firma* B-260; and *Prochlorothrix hollandica*, which was originally given by Prof. George S. Bullerjahn (Bowling Green State University, Bowling Green, OH, United States).

The culturing conditions for *Prochlorothrix hollandica* and other cyanobacteria. Periodic culturing of cyanobacterial strains was performed under sterile

Abbreviations: MA—myristoleic acid; FAME—fatty acid methyl esters

conditions similarly to the method of intensive culturing of unicellular algae [7]. Before the beginning of the experiment, *P. hollandica* cells were grown in flasks during 10–14 days in tubes with 100 mL of medium at 25° C and constant illumination ~20 μM of photons/(m^2 s). Unilateral room illumination (~50 μM of photons/(m^2)) was used during the first two days after inoculation. The vessels with a volume of 250 mL with glass bubblers and suspension layer thickness of 40 μM were used in the experiments, and the illumination of the culture was noctidial and unilateral or bilateral (~100 μM of photons/(m^2 s)); 50-watt fluorescent lamps of BS-80 type were used. Illumination was measured photometrically using LI-COR (model LI-189, Li-Cor Inc., United States) equipped with a Quantum sensor module (Q17227, Li-Cor Inc.). *P. hollandica* cells were grown in Ph media at 25°C at different intensities of illumination: 20–50, 50–100, 100–150, or 200 μM of photons/(m^2 s).

P. hollandica was cultivated in BG-11, Ph, and Tamiya media

BG-11 medium [8] was prepared from concentrated solutions: 100 mL of solution 1 contained 0.3 g $C_6H_8O_7$, 0.3 g $(NH_4)_3Fe(C_6H_5O_7)_2$, 0.05 g EDTA-Na_2; 1 L of solution 2 contained 30 g $NaNO_3$, 0.78 g K_2HPO_4, 1.5 g $MgSO_4 \cdot 7H_2O$; 100 mL of solution 3 contained 1.9 g $CaCl_2 \cdot 2H_2O$; 100 mL of solution 4 contained 2 g Na_2CO_3; 1 L of solution 5 (a mixture of microelements) contained H_3PO_4, 1.81 g $MnCl_2 \cdot 4H_2O$, 0.222 g $ZnSO_4 \cdot 7H_2O$, 0.391 g $Na_2MoO_4 \cdot 2H_2O$, 0.079 g $CuSO_4 \cdot 5H_2O$, 0.0494 g $Co(NO_3)_2 \cdot 6H_2O$; 1 L of solution 6 contained 119.15 g Hepes, pH 7.5. To make 1 liter of medium, the solutions were mixed in the following proportions: solution 1—2 mL; solution 2—50 mL; solution 3—2 mL; solution 4—1 mL; solution 5—1 mL; solution 6—40 mL. The volume was adjusted with distilled water to 1 liter and autoclaved at a pressure of 1.0–1.5 atm for 30 minutes.

To make Ph medium, concentrated solutions were prepared as follows: 100 mL of solution 1 contained 15 g $NaNO_3$; 100 mL of solution 2 contained 3.6 g $CaCl_2 \cdot 2H_2O$; 100 mL of solution 3 contained 1.2 g $FeNH_4C_6H_8O_7$; 100 mL of solution 4 contained 0.1 g EDTA-Na_2; 100 mL of solution 5 contained 4.0 g K_2HPO_4; 100 mL of solution 6 contained 7.5 g $MgSO_4 \cdot 7H_2O$; 100 mL of solution 7 contained 2.0 100 g Na_2CO_3; 1 L of solution 8 (microelements) contained 2.8 g of H_3BO_3, 1.8 g $MnCl_2 \cdot 4H_2O$, 0.222 g $ZnSO_4 \cdot 7H_2O$, 0.39 g $Na_2MoO_4 \cdot 2H_2O$, 0.079 g $CuSO_4 \cdot 5H_2O$, 0.012 g $CoCl_2 \cdot 6H_2O$. To make 1 liter of medium, the solutions were mixed: 10 mL of solution 1 and 1 mL of each solution nos. 2–8. The volume was adjusted to 1 liter and autoclaved under the same conditions.

Tamiya medium [7] contained: 5.0 g/L KNO_3 2.5 g/L $MgSO_4 \cdot 7H_2O$, 1.25 g/L KH_2PO_4, 0.037 g/L EDTA-Na_2, 0.009 g/L $FeSO_4 \cdot 7H_2O$, 2.86 mg/L H_3BO_3, 1.81 mg/L $MnCl_2 \cdot 4H_2O$, 0.222 mg/L $ZnSO_4 \cdot 7H_2O$, 0.018 mg/L MoO_3, 0.023 mg/L NH_4VO_3; 1 L of Tamiya medium was autoclaved as described above.

Measurement of growth characteristics in cultures. The growth of cultures was monitored with a Genesis UV10 spectrophotometer (Thermo Scientific, United States) accordingly to changes in the optical density of the cell suspension at a wavelength $\lambda = 790$ nm (OD_{790}). We use distilled water for dilution of cell suspension at large densities of the culture that was needed for OD_{790} measuring. The density of the suspension was also compared to the density of distilled water. Quartz cuvettes with a width of 1 cm were used for the measurements.

Culture growth was also controlled by determination of dry mass. Cells were pelleted by centrifugation at 5000*g*. The culture was dried at 80°C for 3 days.

Determination of qualitative and quantitative composition of fatty acids in the cultures. To determine the absolute content of lipids in the cultures and their total FA content, samples with a volume of 15–50 mL (depending on the strain and culturing conditions) were fixed as follows. Cyanobacteria cells were pelleted by centrifugation for 3 minutes at 5000*g* at room temperature. Then, the supernatant was removed, pelleted cells were resuspended in 10 mL of distilled water, and they were quantitatively transferred into a 15 mL centrifuge tube and pelleted again under the same conditions. Supernatant was again removed and the precipitate was immediately resuspended in 5–7 mL of hot (60°C) isopropanol, containing 0.02% of ionol. The tubes were incubated for 10 minutes in a water bath at 65°C, then they were sealed and stored at –20°C. FA methyl esters (FAMEs) were prepared by transesterification of prefixed and dehydrated material, to which methanol and acetyl chloride (9 : 1 by volume) were added; the methanolic solution of lipids was heated at reflux for 1 h.

In the initial stage of our research, the resulting mixture of FAMEs was analyzed using capillary GLC on a Tracor gas chromatograph (540 GC, Tracor Instruments, United States) with data processing in ADC under the control of Ecochrom software (Institute of Organic Chemistry Russian Academy of Sciences, Moscow). The capillary column with a length of 50 m and the internal diameter of 0.25 μM included grafted cyanopropyl methyl silicone polar liquid phase (CPS-1; Quadrex, Japan); film thickness was 0.25 microns. The working program of the temperature gradient in the column of Tracor 540 GC provided temperature rise for 40 minutes; first, from 130 to 140°C for 2 minutes at a rate of 5°C/min, then from 140 to 170°C for 3 min at 10°C/min, then from 170 to 200°C for 30 minutes at 1°C/min, and finally with maintaining the temperature of 200°C for 5 min. The carrier gas was argon (99.993%, GOST 10157-