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RESEARCH PAPERS

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

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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 λ = 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-

79). The linear rate of the carrier gas in the column was 15 cm/s. A flame ionization detector was used. Detector chamber temperature was 230°C. Hydrogen consumption in the detector chamber was 30 mL/min [9].

In the further work, joint capillary GLC and mass spectrometry (MS) of FAME were performed on an Agilent 7890A GC device (Agilent Technologies, Inc., United States), where a 60-meter DB-23 capillary column (grafted stationary phase 50%- cyanopropylmethylpolysiloxane) with an inner diameter of 0.25 μM and a thickness of stationary phase layer of 0.25 micron was used for the separation. The terms of FAME separation were: carrier gas (helium) pressure in the injector was 245 kPa; gas consumption was 1 mL/min; the volume of a sample was 1 μl; flow divider was 1 : 5; the evaporator temperature was 260°C. Column temperature gradient program was from 130°C to 170°C at 6.5°C/min, from 170 to 215°C at 2.75°C/min, holding at 215°C for 25 minutes, from 215 to 240°C at a rate of 40°C/min, and holding at 240°C for 50 min. Working temperature of MS detector was 240°C, the ionization energy was 70 eV. Determination of the amount of total lipids in cultures, calculated for esterified FA, was conducted via Excel software package (Microsoft Windows).

Isolation of MA methyl ester product. We mixed 17 g of *P. hollandica* raw biomass with 17 mL of *iso*-propanol and 1.33 g of NaOH and heated at reflux for 1 hour. The alcohol was distilled off. The pasty residue was repeatedly washed with hexane until the disappearance of an intense orange color of hydrocarbon layer, and we periodically separated and discarded the nonpolar phase. By adding hydrochloric acid, the pH of the aqueous phase was adjusted to 2. The resulting free FA were quantitatively extracted with hexane. The solvent was distilled off completely from this extract. We dissolved 0.25 g of the residue on a warm water bath in a homogeneous solution of 1.5 g of urea in 4 mL of methanol. The solution was first cooled slowly to room temperature and then to 4°C during 24 h. The supernatant was separated from the precipitate by decantation. The precipitate, containing predominantly myristic acid and less than 10% of MA from the sum of all FA, was washed with hexane three times and then discarded. Wash hydrocarbon solutions were combined with uterine alcohol and mixed with 100 mL of diluted (1 : 20) aqueous solution of hydrochloric acid. Free FA were reextracted from the aqueous layer with hexane until the disappearance of yellow color of the upper hydrophobic phase. The lower aqueous alcohol phase was discarded. The solvent was distilled off from the hydrocarbon extract. The residue (0.16 g), containing approximately 40% of MA from the sum of all FA, was quantitatively transferred with methanol to a thermostatic (30°C) chromatography column packed with 6 g of chemically grafted lipophilic particles C_{18} Hi-Flosil (200/325 mesh, Applied Science Laboratories Inc., United States). Lipids were eluted from the column with 50% aqueous methanol, which included 0.5% acetic acid. The volatile solvents from the first fraction of eluate, which contained approximately 60% of MA, were distilled off. The aqueous residue was washed with benzene until the disappearance of yellow color of the layer. The solvent was distilled off from the hexane extract; 0.06 g of the residue was dissolved in 10 mL of acetyl chloride and methanol mixture (9 : 1 by volume) and incubated for 1 hour at reflux temperature. Volatile solvents were evaporated. Preparation, comprising mainly MA methyl ester, was separated from the residue by washing with benzene (until the disappearance of the yellow color of the hydrocarbon layer). The benzene solutions were combined. The solvent was distilled off. Methyl myristoleate was separated from the other lipophilic components of the drug by preparative GLC, described previously [10], and 30 mg of chromatographically pure MA methyl ester were obtained. The benzene solution of the drug was stored in sealed glass ampules under an inert atmosphere in the frozen state [10].

RESULTS AND DISCUSSION

Screening of Cyanobacterial Strains for the Presence of Myristoleic Acid

Strains from the collections of microalgae of IPPAS, belonging mainly to the Group 1 according to the classification of cyanobacteria that was based on FA composition, were selected for the screening of cyanobacteria for the presence of myristoleic acid [11–13]. Strain *Cyanobacterium* sp. IPPAS B-1200 with a high content of C_{14} FA that was recently described by us, belongs to the group [14]. In addition, we have included in the analysis the thermophilic strain *Phormidium laminosum*, belonging to the Group 3γ [10] and containing myristic acid [15], as well as a strain of *P. hollandica* from the Group 2, comprising myristic acid and MA [16, 17].

Among the studied strains, the highest relative amount of C_{14} FA was found in the strains of *Cyanobacterium* sp. B-1200 (of approximately 40% totally), *Synechococcus* sp. B-266 (of approximately 20% totally), and *P. hollandica* (of approximately 35% totally). However, the content of MA in *Synechococcus* sp. B-266 was characterized by the values of ~1%, that in *Cyanobacterium* sp. B-1200 of 10%, and that in *P. hollandica* of 17%. Based on these values, the strain of *P. hollandica* was selected for further work as the most promising MA producer (Table 1).

Growth Characteristics of Prochlorothrix hollandica Culture

It is known from the literature that *P. hollandica* cells are able to grow in standard cyanobacterial medium, BG-11 [18]. We compared growth rates of

Table 1. Relative content of fatty acids in the cells of different cyanobacterial strains under storage conditions in the collection of IPPAS: 18–20°C, 10–20 μM photons/(m^2 s)

Strain and its number in the collection of IPPAS	Fatty acids, M %					
	14:0	14:1Δ9	16:0	16:1Δ9	18:0	18:1*
Cyanobacterium sp. B-1200	30.2	10.5	18.8	37.5	1	2.0
Synechococcus sp. B-266	18.6	0.7	30.2	40.4	tr.	10.1
Synechococcus sp. B-434	0.5	0.4	75.7	10.0	2.4	11.0
Synechocystis sp. B-288	5.0	tr.	41.1	22.5	10.8	20.6
Thermosynechococcus vulcanus	tr.	0.5	66.0	17.4	2.1	14.0
Synechococcus elongatus B-267	1.5	1.6	50.7	21.2	2.0	23.0
Synechococcus sp. B-468	1.5	1.6	72.0	10.4	2.1	12.4
Phormidium laminosum B-407**	3.0	0.9	37.4	38.4	3.0	11.3
Microcystis firma B-260	0.5	0.5	86.0	10.0	1.0	2.0
Prochlorothrix hollandica	14.0	17.4	29.6	23.0	10.2	5.8

Screening of strains for the presence of MA was carried out according to the protocol [15]. tr. are traces, the amount is <0.5%. * Sum of 18:1Δ9 and 18:1Δ11 FA. ** In addition to FA, shown in the table, this strain contains 2% of 18:2Δ9,12 and 4% of 18:3Δ6,9,12. Mean values of three independent determinations are shown. Standard deviations are ±0.1–0.5.

P. hollandica culture cultivated in various media—Ph, BG-11, and Tamiya (Fig. 1)—at a constant temperature of 25°C and constant white light illumination with an intensity of 50 μM photons/(m^2 s). The results showed that the culture did not grow in Tamiya medium. The growth rates in Ph and BG-11 media were comparable during the first 1–3 days, but the culture further achieved higher density in Ph medium (Fig. 1). Based on these data, further culturing of *P. hollandica* was carried out in Ph medium.

It is known that the optimum temperature for the growth of *P. hollandica* culture ranges between 20–30°C [18, 19]. However, this is a relatively wide range of temperatures. To determine the optimum temperature, *P. hollandica* cells were grown at different temperatures: 20–22°C, 24–26°C, and 28–30°C (Fig. 2). The growth of *P. hollandica* at 20–22°C and 28–30°C was significantly slower than during culturing at 24–26°C. Thus, 24–26°C is the optimal temperature for the intensive culturing of *P. hollandica*.

In addition, we attempted to determine the optimal illumination for the most efficient growth of *P. hollandica* biomass. Cells were grown in Ph media at 25°C at different illumination intensities: 20–50, 50–100, or 100–150 μM photons/(m^2 s) (Fig. 3a). Results showed that the strain preferred a relatively low illumination at 50–100 μM photons/(m^2 s). These indicators were typical for most species (mostly unicellular) of cyanobacteria. By reducing or increasing the illumination, the growth rate of culture decreased relatively to this level. Thus, *P. hollandica* relates to the strains of photoautotrophic microorganisms, preferring moderate illumination intensity.

The experiments also showed that, under the optimal culturing conditions and upon reaching a relatively high suspension density, illumination intensity can be greatly increased. This promotes further accumulation of biomass (Fig. 3b). These results are explained by the fact that, upon reaching the critical density at a given illumination intensity, surface layers of culture absorb and scatter incoming light quanta, which results in slowed growth rate of culture that

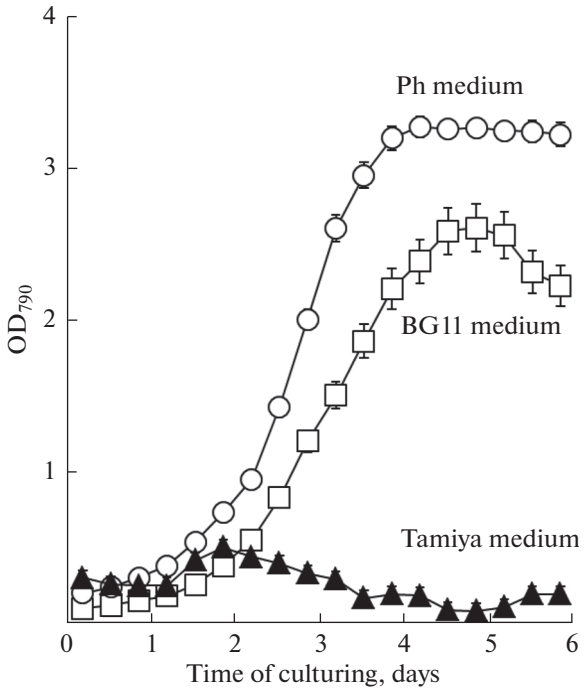

Fig. 1. Cultivation of *Prochlorothrix hollandica* cells during 6 days in different nutrient media (Ph, BG-11, and Tamiya).

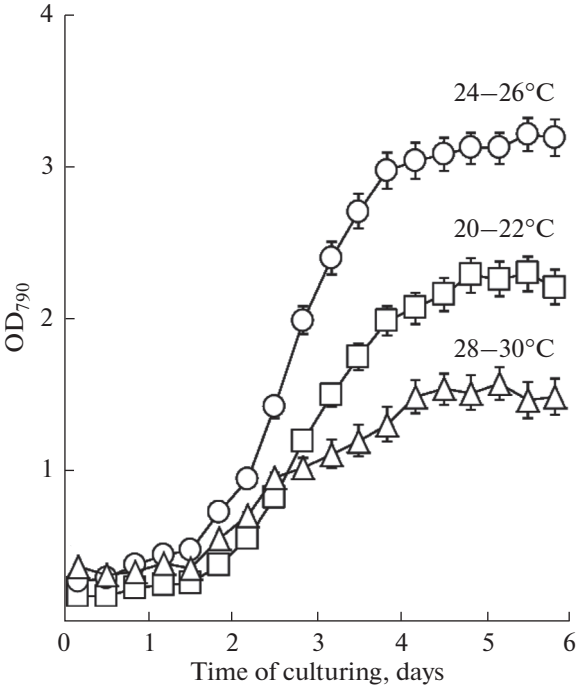

Fig. 2. Cultivation of *Prochlorothrix hollandica* cells during 6 days in Ph medium at different temperatures.

reaches a plateau. Increasing illumination intensity increases to a certain limit the penetration of photons into the suspension and, thus, supports the photosynthetic activity, which, in turn, provides continued culture growth and biomass accumulation. A further increase in the intensity of illumination can cause photoinhibition, disassembly of phycobilisomes (light-harvesting complexes of cyanobacteria), and, consequently, reduced photosynthetic activity and slowed growth. The conditions of superstrong illumination lead eventually only to the death of a culture (Fig. 3a).

Thus, the optimal mode of culturing in the laboratory was selected for *P. hollandica* strain: Ph culturing medium, culturing temperature of 24–26°C, illumination intensity of 50–100 μM of photons/(m^2 s). Furthermore, this strain proved to be suitable for two-phase culturing, when the culture can grow to large values of optical density, then the biomass can be further increased by increasing the illumination intensity (Fig. 3b).

Composition and Amount of Fatty Acids in Prochlorothrix hollandica

Detailed FAME analysis by GLC and mass spectroscopy showed the presence of the following acids in the lipids of *P. hollandica*: 14:0, 14:1Δ9, 16:0, 16:1Δ4, 16:1Δ9, 18:0, 18:1Δ9, and 18:1Δ11 (Table 2). *Cis*-vac-

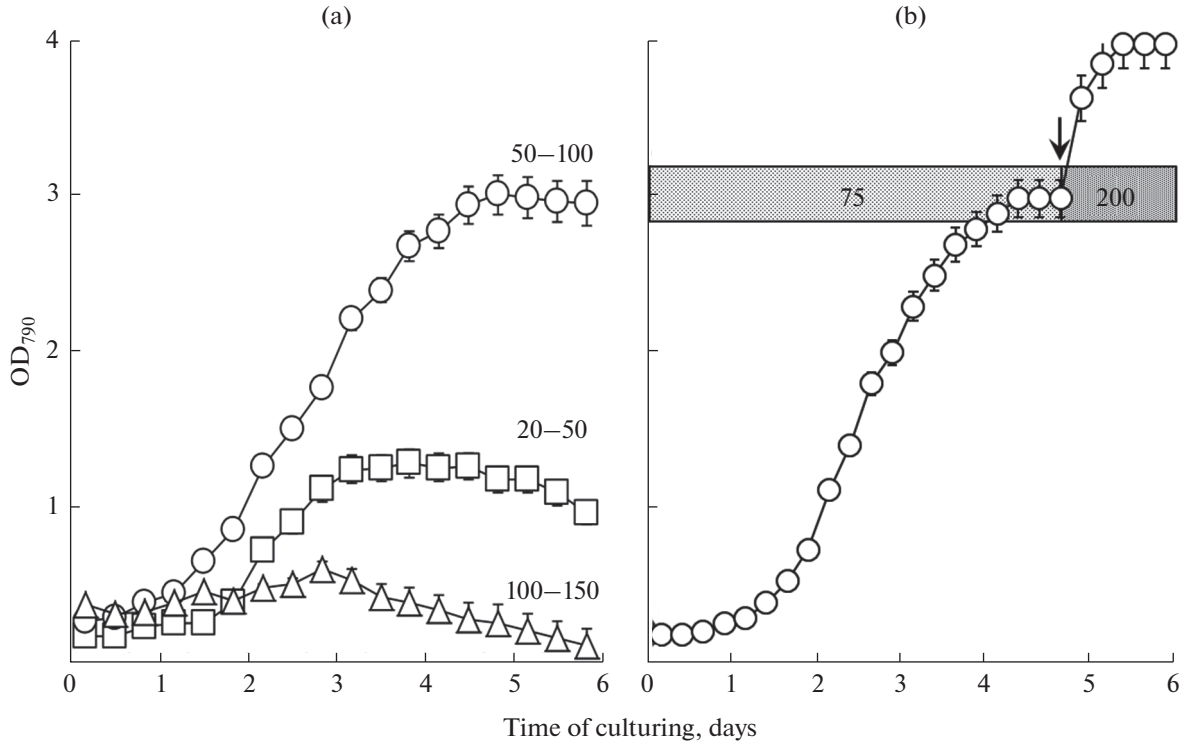

Fig. 3. Cultivation of *Prochlorothrix hollandica* cells. (a) 6 days in Ph medium at 26°C under varying illumination conditions (μM photons/(m^2 s)); (b) 5 days under the same conditions at illumination of 75 μM photons/(m^2 s) with subsequent increase in illumination intensity up to 200 μM photons/(m^2 s). Arrow shows the moment of change in the illumination intensity.

Table 2. Relative content of fatty acids (M %) in *Prochlorothrix hollandica* cells, grown under different conditions

FA	Storage conditions 20°C, 10–15 μM photons/(m^2 s)	Growing conditions	
		25°C, 50 μM photons/(m^2 s)	25°C, 100 μM photons/(m^2 s)
14:0	14.2 ± 0.2	11.1 ± 0.1	14.5 ± 0.2
14:1Δ9	16.3 ± 0.3	33.7 ± 0.5	21.0 ± 0.3
16:0	27.6 ± 0.5	23.2 ± 0.3	30.1 ± 0.3
16:1Δ9	23.6 ± 0.5	18.0 ± 0.2	22.8 ± 0.4
16:1Δ4	11.8 ± 0.2	9.0 ± 0.1	8.6 ± 0.1
18:0	0.9 ± 0.1	tr.	tr.
18:1Δ9	3.6 ± 0.1	tr.	tr.
18:1Δ11	2.0 ± 0.1	5.0 ± 0.1	3.0 ± 0.1

tr. are traces, the amount is < 0.5%.

cenic (18:1Δ11) acid is formed by FA synthetase by the path of prokaryotic synthesis of monounsaturated FA that does not involve desaturases. Thus, we can conclude that *P. hollandica* cells have two types of desaturases: Δ9- and Δ4-desaturases.

It should be noted that the overall FA composition in *P. hollandica* is not quite typical for cyanobacteria. The presence of desaturation products at Δ4 position is, perhaps, a unique feature of this organism. Furthermore, the strain is characterized by the presence of relatively large amounts of several C_{14} FA: saturated myristic acid (15%) and monounsaturated MA (34%). It was found that the qualitative and quantitative composition of FA varied considerably, depending on the culturing conditions. Thus, the largest number of MA was found in *P. hollandica,* growing at low illumination (50 μM of photons/(m^2)) at 25°C.

Equally high level of C_{14} FA was found in another representative of prochlorophytes, symbiotic *Prochloron* [20, 21], as well as in cyanobacteria *Cyanobacterium* sp. IPPAS B-1200, isolated from the salty waters of Lake Balkhash (Kazakhstan) [14]. C_{14} FA were not detected in such large numbers in other numerous representatives of the genus *Prochlorococcus* and in other cyanobacteria (both unicellular and filamentous) [13].

The data on the absolute content of individual FA (mg/g of dry mass) is shown in Table 3. Calculated per 1 g of dry mass, the largest proportion of these FA falls on palmitic (~12 mg) and palmitoleic (~14 mg) acids, while the absolute content of MA is approximately 10 mg per 1 g of dry mass of *P. hollandica* biomass (Table 3).

Isolation of Myristoleic Acid Methyl Ester

To isolate MA methyl ester, 17 g of *P. hollandica* biomass were used (approximately 3 g of dry mass). In the first purification step, a preparation of total free FA was obtained, as described in the Materials and Methods section. Initial preparation of total FA contained mostly 10% of myristic acid and 34% of MA from the sum of all FA. In the first purification step, FA preparation contained approximately 40% of MA (Fig. 4a, Table 4). After the second chromatographic purification step on a column filled with chemically grafted lipophilic particles C_{18} Hi-Flosil, enriched preparation of FA mixture from *P. hollandica* biomass contained approximately 65% of MA (Fig. 4b, Table 4).

Table 3. Absolute content and composition of individual fatty acids in *Prochlorothrix hollandica* cells

FA	fa, mg/g of dry mass
14:0	5.4 ± 0.2
14:1Δ9	9.3 ± 0.5
16:0	11.6 ± 0.5
16:1Δ9	13.6 ± 0.3
16:1Δ4	5.7 ± 0.2
18:0	0.8 ± 0.1
18:1*	2.8 ± 0.1
∑ FA, % of dry mass	5.0 ± 0.1

* Sum of 18:1Δ9 and 18:1Δ11 FA.

Table 4. Fatty acid composition of lipid preparations from the cells of *Prochlorothrix hollandica* at various stages of preparation of MA methyl ester

FA	Content of individual FA varieties, %			
	original preparation	I stage	II stage	III stage
14:0	10	7	15	2
14:1Δ9	34	39	65	98
16:0	23	12	tr.	no
16:1Δ9	18	18	13	no
16:1Δ4	10	11	7	no
18:0	tr.	4	no	no
18:1*	5	9	no	no

tr. are traces, the amount is < 0.5%. * sum of 18:1Δ9 and 18:1Δ11 FA.

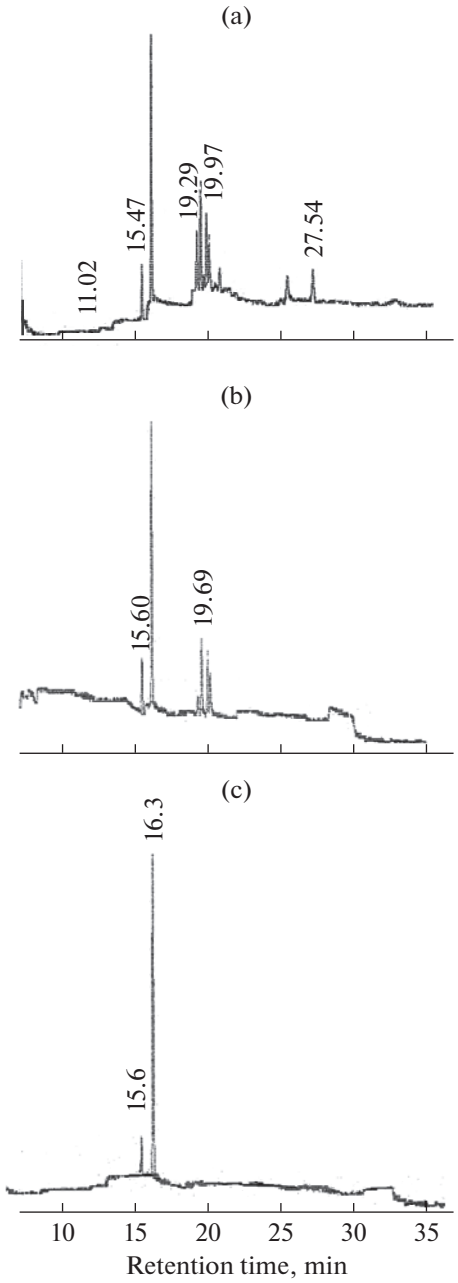

Fig. 4. Chromatograms of FA preparations obtained after three stages of MA methyl ester purification. (a) I stage, (b) II stage, (c) III stage of purification. See also the Materials and Methods section and Table 4.

The same preparation after the third purification step consisted mainly of MA methyl ester (Fig. 4c, Table 4). Subsequent application of preparative GLC made it possible to obtain approximately 30 mg of chromatographically pure MA methyl ester with its content of more than 98%.

Using this three-stage purification process, it was possible to obtain 30 mg of the desired product from 3 g of dry original material, which was, averagely, 1% of the dry cell mass. Thus, to obtain 1 g of MA methyl ester, it is necessary to use approximately 100 g of *P. hollandica* dry biomass, which is equivalent to 0.5 kg of raw material.

Rough economic calculation shows that the production of 1 g of MA methyl ester by the method that was described by us, excluding labor costs, energy costs, and depreciation costs, depending on the quality of reagents used, is several times cheaper than the production of its foreign counterparts.

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