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EXPERIMENTAL ARTICLES

Hydrocarbons and Fatty Acid Methyl Esters in Bacterial Biomass before and after Physicochemical Treatment

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Abstract—Hydrocarbons and fatty acid methyl esters were identified by chromatography–mass spectrometry in extracts of the native biomass of bacteria: chemoorganoheterotrophic *Arthrobacter* sp. and *Pseudomonas aeruginosa* and chemolithoautotrophic *Carboxydothermus* sp. Ultrasound treatment of bacterial biomass and mild thermolysis were shown to promote formation of a broad spectrum of hydrocarbons from bacterial biomass. The biomarker stigmastane belonging to the sterane group was found in *P. aeruginosa* biomass after thermolysis at 110°C in an open vial. Alkane composition in *P. aeruginosa* biomass before and after thermolysis at 300°C in a sealed container remained unchanged, indicating the possibility of preservation of hydrocarbons of bacterial origin in sealed layers under high temperature and elevated pressure.

Keywords: hydrocarbons and fatty acid methyl esters, bacteria, genesis of petroleum, ultrasound and heat treatment

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Hydrocarbons (HC) are widespread in both animate nature and abiocoen. In the geosphere, the major HC resources are concentrated in oil- and gas fields, gas hydrates, oil shales, and coal beds. In the biosphere, HC are produced by both eukaryotes and prokaryotes: alkanes, alkenes, alkadienes, and polymeric isoprenoids are found in eukaryotes [1–7]; methane is produced by methanogenic prokaryotes; arenes (alkylbenzenes) were revealed in the *Thermoplasma* sp. and *Sulfolobus* sp. archaea [8]. Bacteria are capable of producing alkanes, cyclic alkanes, alkenes, arenes, and alkadienes, as well as various polymeric isoprenoides [2, 3, 9–14].

The HC sources in the biosphere and geosphere are undoubtedly interrelated. Two theories for genesis of petroleum HC were proposed and experimentally substantiated: abiogenic (inorganic, mineral) and biogenic (organic, sedimentary-migrational) [15]. It had been suggested that oil was formed in the Earth's mantle as a result of inorganic synthesis in the presence of minerals acting as catalysts under thermobaric conditions; this process was associated with the Earth degassing and occurred most probably prior to archebiosis. In the course of degassing, H_2, CH_4, NH_3, H_2O, CO, H_2S, C_2H_6, CO_2, N_2 were released; the presence of great reserves of gas hydrates indicates immense scale of this process. Earth degassing was among the primary factors of evolution which formed the hydrosphere, atmosphere, and, eventually, biosphere. At archebiosis, organic naphtidogenesis appeared to prevail. It is customarily assumed that the biomass of phyto- and zooplankton scattered throughout the bottom sediments of seas and other reservoirs was the basic material for oil and gas formation. In the course of biomass transformation into the sedimentary rocks, highly condensed macromolecules (kerogen) were formed from organic matter (OM); this process was accompanied by gradual segregation of the HC components (micro oil). The energy required for OM transformation into HC originated from different sources: enhanced thermal flow, biochemical and chemical energy, radioactive materials of enclosing rocks, etc. The thermocatalytic conversion of OM from the oil- and gas-containing matrix at the catagenetic stage of lithogenesis (the main phase of oil formation) occurred within a temperature range from 60 to 170–180°C [16].

At present, organic theory is the best to explain the essence and scale of the oil field formation. The biogenic conception of oil origin conventionally suggested an important role of prokaryotes in initial transformation of eukaryotic biomass, as well as in the terminal stages of oil migration into the country rock. However, by the time eukaryotes developed, the Earth's crust had been restructured due to prokaryotic biochemical activity, which is continuing today [17]. Therefore, initial supply of OM for naphtidogenesis was probably provided by the deep biosphere prokaryotes; their estimated biomass was half of the surface mass [18–20]. The deep biosphere contains both archaea and bacteria and is characterized by great biodiversity; methanogens, acetogens, sulfate-,

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sulfur-, iron-, and manganese-reducing bacteria, as well as anaerobic organotrophs, have been isolated from the oil strata [21]. According to the current hypotheses, the concentrated OM of bacterial mats and colonies of chemolithoautotrophic anaerobic bacteria formed the maternal oil substance [18, 19]. Taking into account the calculated amount of deep biomass and conditions for oil formation, it may be suggested that the processes of oil generation from prokaryotic residues are going on today.

The goal of the present work was comparative studies of HC from native biomass and from the samples exposed to either sonication or elevated temperature. The objects of this study were an aerobic bacterium *Arthrobacter* sp. RV, *Pseudomonas aeruginosa* RM, a bacterium capable of anaerobic growth due to denitrification, and an obligately anaerobic thermophilic chemolithoautotrophic bacterium *Carboxydothermus* sp. SET-IS9. The latter bacterium is of special interest due to its ability to utilize CO as the sole source of carbon and energy; this property is important for the initial geological stages of OM accumulation and oil formation in deep biosphere.

MATERIALS AND METHODS

Subjects of the study. Strain *Carboxydothermus* sp. SET-IS9 was isolated from thermal springs (Iceland) in the Laboratory of Hyperthermophilic Microbial Communities (Institute of Microbiology, Russian Academy of Sciences) headed by E.A. Bonch-Osmolovskaya. Cultivation of this freshwater hydrogenogenic CO-oxidizing prokaryotic strain was carried out at 65°C for 48 h under 100% CO in anaerobically prepared liquid medium containing the following (g/L distilled water): NH_4Cl, 1.50; K_2HPO_4, 0.50; $MgSO_4 \cdot 7H_2O$, 0.20; KH_2PO_4, 0.10; $CaCl_2 \cdot 2H_2O$, 0.02; NaCl, 0.50; pH 6.0. The medium was supplemented with vitamin and trace element solutions (1 mL/L). Reducing agent (sodium dithionite) was added dropwise until the Eh level became below 300 mV; resazurin was used as an indicator [22, 23].

Hydrocarbon-oxidizing strains *Arthrobacter* sp. RV and *Pseudomonas aeruginosa* RM were isolated from the Volga and Moskva rivers, respectively. The genus level identification of the strains was performed according to the Bergey's Manual; the species level identification of *P. aeruginosa* RM was carried out by molecular biological methods [24]. Both submerged and surface methods of cultivation were used. *P. aeruginosa* RM was grown in mineral–organic medium containing the following (g/L distilled water): $NaNO_3$, 2.0; KH_2PO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.25; $CaCl_2 \cdot 2H_2O$, 0.01; yeast extract, 2.0; glucose, 20.0; pH 7.0 [25]. *Arthrobacter* sp. RV was grown in "rich" liquid medium containing the following (g/L tap water): peptone, 2.0; yeast extract, 1.0; casein hydrolyzate, 1.0; glucose, 1.0; chalk, 2.0; glycerol, 10 mL/L; pH 6.7–7.2 [24]. Submerged cultivation of *P. aeruginosa* RM and *Arthrobacter* sp. RV was carried out in flasks on a rotary shaker (280 rpm) for 24 and 96 h, respectively. Surface cultivation of the strains was performed on petri dishes with the medium containing agar (17.0 g/L) at 28°C; after 24–48 h, the biomass was scraped off. Submerged-grown biomass of *Arthrobacter* sp. and *P. aeruginosa* was collected on an OPN-8UKhL4.2 centrifuge (Russia) at 7000 *g* for 20 min; the cells of *Carboxydothermus* sp. SET-IS9 were filtered through a NPF nylon membrane filters (Biokhrom, Russia) with a pore size of 0.2 μm.

Biomass thermolysis. Mild thermolysis of the biomass in open vials was performed at 110 and 150°C, which corresponded to the temperature of the main phase of oil formation according to Vassoevich [16]. Biomass samples (0.5–1 g) were heated gradually and incubated at 110°C for 2–3 h or at 150°C for 1 h.

Additionally, thermolysis of the biomass was carried out in hermetically sealed vials. The biomass sample was dried at 80°C to the constant weight; then it was transferred into a quartz ampoule, sealed, and heated in a muffle furnace at 300°C for 20 h.

Biomass sonication. The cell suspension in phosphate buffer (pH 7.0) was sonicated on an UZDN-2T ultrasonic disintegrator (Russia) (22 kHz, 20–25°C) for 3 min (0.5-min sonication with 1-min intervals).

Extraction and analysis of hydrocarbons. The fraction of hydrophobic compounds was isolated from native biomass and from the samples subjected to thermolysis or sonication by extraction with chloroform at 25°C in an ultrasonic bath at 20°C for 15 min. After solvent removal, the residue was dried over $CaCl_2$; HC were extracted from the residue with *n*-hexane in an ultrasonic bath at 40°C (three times for 5 min).

The hexane extracts were analyzed by gas chromatography–mass spectrometry on a TRACE DSQII mass spectrometer (Thermo Scientific, United States) equipped with a Thermo TR-5ms capillary column (30 m length, 0.25 mm inner diameter, and 0.25-μm-thick immobile phase) packed with 5% phenyl polysilphenylene-siloxane. The spectra were recorded in the scanning regime (*m/z* from 45 to 500) at the total ion current (TIC); programmed temperature increase (70–280°C) was linear with the rate of 10°C/min; the temperature of ionization chamber was 200°C; the carrier gas was helium. The products were identified with the use of the standard *n*-alkane mixture (C_8–C_{20}) (Supelco) and by comparison of their mass spectra with those given in the NIST08 database. Some biomass samples after thermolysis were analyzed on an Agilent 6890N device equipped with a 5975B mass-selective detector by using an HP-5ms capillary column (30 m × 0.25 mm × 0.5 μm). All analyses were carried out in triplicate.

Table 1. Composition of HC and their derivatives in native biomass of *P. aeruginosa* RM, *Arthrobacter* sp. RV, and *Carboxydothermus* sp. SET-IS9

HC and their derivatives	*P. aeruginosa* RM	*Arthrobacter* sp. RV	*Carboxydothermus* sp. SET-IS9
Pentadecane, $C_{15}H_{32}$	+	+	–
Hexadecane, $C_{16}H_{34}$	+	+	+
Octadecane, $C_{18}H_{38}$	–	–	+
Nonadecene, $C_{19}H_{38}$	–	–	+
Eicosene, $C_{20}H_{40}$	–	–	+
Docosene, $C_{22}H_{44}$	–	–	+
2-Butyl-4-dimethyl benzyl phenol, $C_{19}H_{24}O$	–	–	+
Methyl ester of hexadecanoic acid, $C_{17}H_{34}O_2$	+	+	+
Methyl ester of octadecanoic acid, $C_{19}H_{38}O_2$	+	+	+
Methyl ester of octadecenoic acid, $C_{19}H_{36}O_2$	+	–	–

RESULTS AND DISCUSSION

Hexane extracts of the *Arthrobacter* sp. RV and *P. aeruginosa* RM biomass showed similar spectra of HC and their derivatives (Table 1). In submerged cultures of the studied strains, *n*-alkanes (pentadecane and hexadecane) and methyl esters of fatty acids: palmitic (hexadecanoic) and stearic (octadecanoic) were revealed. Moreover, methyl ester of oleic (octadecenoic) acid was found in *P. aeruginosa* RM biomass. There is information that *n*-alkanes were revealed in strains of *P. fluorescens* (C_{21}–C_{33}) and *Arthrobacter* (C_{15}, C_{16}, C_{21}, and C_{22}) [10, 13]. The difference between our results and the literature data may be explained by the differences in the procedures of sample preparation and analysis. In our experiments, pentadecane and hexadecane content of *P. aeruginosa* RM was 0.19 and 0.47 mg/g dry biomass, respectively; according to the literature data, the amount of HC in various bacteria ranged from 0.035 to 26.9 mg/g dry biomass [13].

The widest range of HC was revealed in the chemolithoautotrophic bacterium *Carboxydothermus* sp. SET-IS9; it included alkanes (hexadecane and octadecane), alkenes (nonadecene, docosene, and eicosene), 2-butyl-4-dimethylbenzyl phenol, and methyl esters of hexa- and octadecanoic acids (Table 1). These results are of special interest since obligately anaerobic thermophylic chemolithoautotrophic bacteria are inhabitants of the ecosystems resembling oil-bearing strata. Bacteria of the genus *Carboxydothermus* utilize CO as the sole source of carbon and energy and produce H_2 according to the equation $CO + H_2O = CO_2 + H_2$ [22]. Apart from methane synthesis by archaea, HC biosynthesis by chemolithoautotrophic prokaryotes remains practically unstudied. Of interest is the fact that the synthesis of HC during autotrophic growth was found in sulfate-reducing bacteria and clostridia [12, 13]. Detection of HC and their derivatives in *Carboxydothermus* cells indicates the possibility of HC synthesis by prokaryotes in the Earth's deep biosphere from the products of mantle degassing. This assumption virtually integrates both the mineral and organic theories. The contribution of hyperthermophilic archaea capable of utilizing gaseous products (CO, H_2O, CO_2, nitrogen and iron oxides, and unsaturated HC) into the oil field formation has been discussed in literature [26].

Intracellular fatty acid methyl esters have not been previously found in bacteria. Extracellular methyl esters of palmitic, stearic, palmitoleic, and oleic acids have been earlier identified as growth factors and adaptogens in obligately methylotrophic bacteria *Methylophilus quaylei* methyl ester of 3-hydroxypalmitic acid, a communicative agent responsible for virulence, was found in the phytopathogenic bacterium *Ralstonia solanacearum* [27, 28].

To imitate the conditions of bacterial OM transformation in the Earth's deep biosphere and consequently of oil accumulation, bacterial biomass was subjected to sonication and thermolysis. No such data are available in the literature. The sonicated biomass of *P. aeruginosa* RM was characterized by a broader HC range than the native cells; moreover, methyl esters of methacrylic acid were revealed only after ultrasonic treatment (Table 2, Fig. 1). As distinct from the native biomass, the sonicated cells of *Arthrobacter* sp. RV contained additionally docosane, tricosane, and various esters of methacrylic acid, but did not contain pentadecane. Methyl esters of hexadecanoic and octadecanoic acids were revealed in sonicated biomass of both strains. The sonication of bacterial biomass appeared to induce HC formation from intracellular metabolites; in deep Earth, this process can be evoked by acoustical waves of different etiology.

Temperature is considered the main factor responsible for transformation of OM into the oil HC. It is known that the temperature enhancing with depth occurs in a disproportionate manner depending on the

Table 2. Composition of HC and their derivatives from the biomass of *P. aeruginosa* RM before and after physicochemical treatment

HC and their derivatives	Native biomass	Sonication	Thermolysis	
			110°C	300°C
Pentadecane, $C_{15}H_{32}$	+	–	–	+
Hexadecane, $C_{16}H_{34}$	+	+	+	+
Heptadecane, $C_{17}H_{36}$	–	–	+	–
Octadecane, $C_{18}H_{38}$	–	–	+	–
Nonadecane, $C_{19}H_{40}$	–	–	+	–
Eicosane, $C_{20}H_{42}$	–	–	+	–
2,6,11,15-Tetramethyl hexadecane, *iso*-$C_{20}H_{42}$	–	–	+	–
Heneicosane, $C_{21}H_{44}$	–	–	+	–
Docosane, $C_{22}H_{46}$	–	+	–	–
Tricosane, $C_{23}H_{48}$	–	+	–	–
9-Hexylheptadecane*, *iso*-$C_{23}H_{48}$	–	–	+	–
Tetracosane, $C_{24}H_{50}$	–	+	–	–
Pentacosane, $C_{25}H_{52}$	–	–	+	–
7-Hexyleicosane, *iso*-$C_{26}H_{34}$	–	+	–	–
Heptacosane, $C_{27}H_{56}$	–	+	+	–
Octacosane, $C_{28}H_{58}$	–	+	–	–
Nonacosane, $C_{29}H_{60}$	–	–	+	–
Hentriacontane, $C_{31}H_{64}$	–	–	+	–
Hexatriacontane**, $C_{36}H_{74}$	–	–	+	–
Pentadecyl cyclohexane, $C_{21}H_{42}$	–	–	+	–
Sesquiterpene	–	–	+	–
1,5,9-trimethyl cyclododecatriene, $C_{15}H_{24}$				
Stigmastane, $C_{29}H_{52}$	–	–	+	–
Ditretbutyl benzoquinone, $C_{14}H_{20}O_2$	–	–	+	–
Esters of 2-methyl propenic (methacrylic) acid	–	+	–	–
Methyl ester of hexadecanoic acid, $C_{17}H_{34}O_2$	+	+	–	+
Methyl ester of octadecanoic acid, $C_{19}H_{38}O_2$	+	+	–	–
Methyl ester of octadecenoic acid, $C_{19}H_{36}O_2$	+	–	–	+

* Retention time 18.36 min.

** Retention time 25.91 min.

rock constitution, tectonic fracturing, etc. [29]. Thermolysis of the biomass from liquid culture of *P. aeruginosa* RM was performed either in open vials (at 110 and 150°C) or in sealed vials (at 300°C). The yield of the extract from the biomass subjected to thermolysis in open vials at 110°C was 2.5% of dry weight; this value was threefold higher than that from the biomass heated in open vials at 150°C and tenfold higher than that from the native biomass. The extract from the biomass heated in open vials at 110°C was characterized by a wider HC range than those from the native cells and sonicated biomass (Table 2, Fig. 2). For the first time, the sterane stigmastane $C_{29}H_{52.}$ was revealed in the extract from the biomass heated at 110°C. Steranes are important compounds; their presence confirms organic origin of oil. It is known that 4-methylsteranes are used as biomarkers of bacterial origin of oil [30]. No pentadecane was found either in the biomass heated at 110°C or in the sonicated sample, possibly because of its evaporation. No methyl esters of fatty acids were determined in the sample heated at 110°C.

Squalen, an acyclic unsaturated isoprenoid $C_{30}H_{50}$ was revealed in the solid medium-grown biomass of *P. aeruginosa* RM subjected to thermolysis at 110°C. Squalen is known as an oil biomarker and as an intermediate of steroid and hopanoid biosynthesis; it was found in various groups of bacteria including evolutionary ancient archaea and purple bacteria

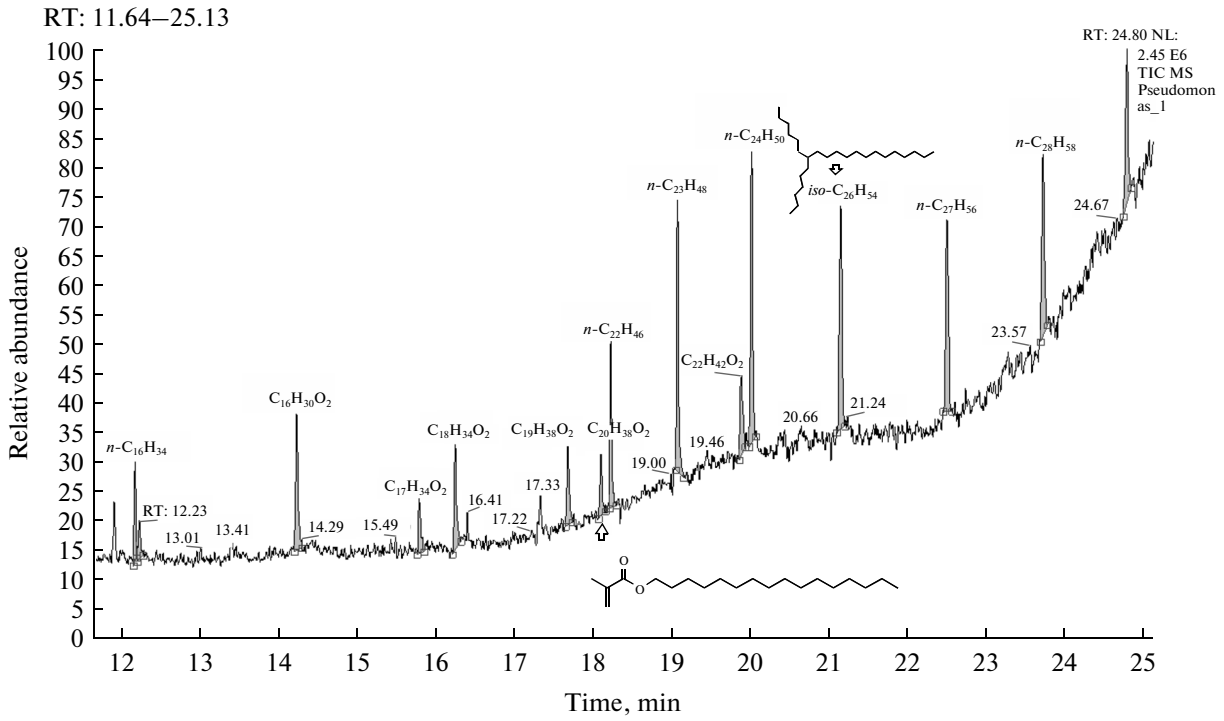

Fig. 1. Chromatogram of the hexane extract of sonicated *P. aeruginosa* RM biomass.

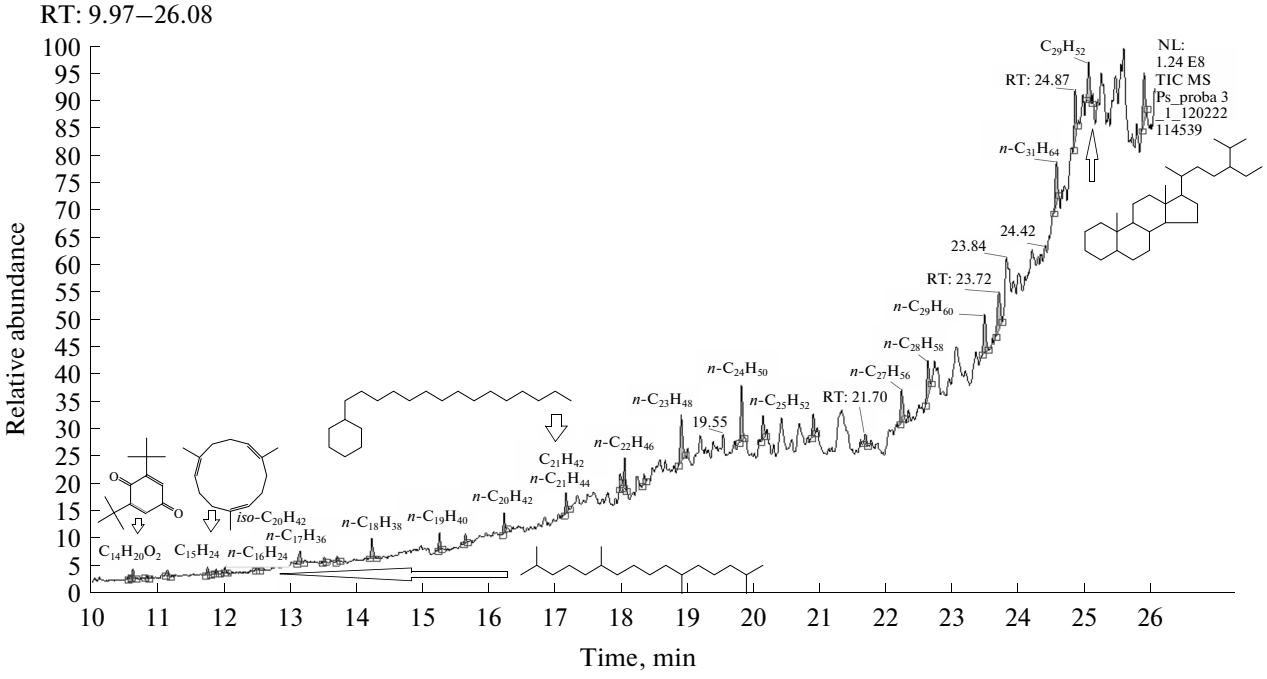

Fig. 2. Chromatogram of the hexane extract of *P. aeruginosa* RM biomass after thermolysis in open flask at 110°C.

[9, 13, 30]. Hopanoids, the most important oil biomarkers, were revealed in many bacteria, e.g., methanotrophs, sulfate-reducing bacteria and cyanobacteria [14, 30]. Thermolysis of the *P. aeruginosa* RM biomass at 150°C promoted formation of considerable amounts of pitchy substances that complicated identification of HC peaks on chromatograms.

No degradation of *n*-alkanes occurred during thermolysis of the *P. aeruginosa* RM biomass in sealed vials at 300°C (Table 2); it indicates the possibility of

preservation of bacterial HC in sealed oilfields at a rather high temperature. However, proportions of HC can be changed during thermolysis; in particular, a ratio of pentadecane to hexadecane was 3.5 and 2.5 in the heated and native biomass samples, respectively. Comparison of two methods of biomass thermolysis (in open vials at 110°C and in sealed vials at 300°C) showed that in the former case, formation of a wide range of new HC was accompanied by disappearance of the fatty acid methyl esters, whereas in the last case, the fatty acid methyl esters were retained.

Thus, the finding of HC, especially oil biomarkers, among metabolites and products formed during sonication and thermal treatment of the biomass can be considered experimental evidence of the prokaryotic origin of oil HC and of the possibility of modern oil formation from bacterial OM. In practice, OM directly accumulated in the operating oil-producing strata (especially, at high temperatures) can be used in the technology of microbial enhanced oil recovery (MEOR) based on activation of the microbiota activity.

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