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The Potential Application of Microorganisms for Sustainable Petroleum Recovery from Heavy Oil Reservoirs

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Abstract: A microbial enhanced oil recovery (MEOR) technique was tested at low-temperature heavy oil reservoirs (Russia). The bioaugmentation approach used is based on the introduction of hydrocarbon-oxidizing bacteria into the oilfield in combination with an injection of oxygen as a H_2O_2 solution in order to initiate the first stage of hydrocarbon oxidation and of $(NH_4)_2HPO_4$ as a source of biogenic elements. Before the pilot trials, the microorganisms of petroleum reservoirs were investigated by high-throughput sequencing, as well as by culture-base and radioisotope techniques. Molecular studies revealed the differences in microbial composition of the carbonate and terrigenous oil reservoirs and the communities of injection and formation water. Aerobic bacteria *Rhodococcus erythropolis* HO-KS22 and *Gordonia amicalis* 6-1 isolated from oilfields oxidized oil and produced biosurfactants. Fermentative enrichment and pure cultures produced considerable amounts of low fatty acids and alcohols from sacchariferous substrates. In core-flooding tests, 43.0–53.5% of additional heavy oil was displaced by aerobic bacteria, producing biosurfactants, and 13.4–45.5% of oil was displaced by fermentative bacteria, producing low fatty acids, alcohols, and gas. A total of 1250 t additional oil was recovered as a result of the application of an MEOR technique at the Cheremukhovskoe heavy oil reservoir and Vostochno-Anzirskoe reservoir with light conventional oil.

Keywords: microbial enhanced oil recovery (MEOR); heavy oil reservoirs; microbial community; high-throughput sequencing; hydrocarbon-oxidizing bacteria; fermentative bacteria; surface and interfacial tension; core flooding; MEOR pilot trials

1. Introduction

The estimated reserves of heavy oil and bitumen in Russia are 6–7 billion tons, exceeding the remaining reserves of conventional oil, and may be considered the main basis for the development of oil production in the next few years. The most promising methods for heavy oil recovery are thermal methods, which decrease the oil viscosity (fire flooding, steam flooding, and cyclic or joint injection of steam, solvents, and surfactants). However, the application of these methods requires a large energy

consumption for steam generation, a large amount of fresh water and expensive equipment for its preparation, and the use of expensive chemicals (surfactants, solvents), which significantly increases the cost of oil and adds environmental risks such as contact between groundwater and the reactants.

Microbially enhanced oil recovery (MEOR) technologies were developed and successfully implemented in reservoirs with light conventional oil in Russia, USA, and China in 1980–2010 [1,2]. Oilfield microorganisms were shown to have a significant effect on oil displacement. Microbially produced gases, acids, solvents, polymers, and surfactants exhibit oil-displacement properties similar to those of their synthetic analogs. All microbial metabolites probably have a synergistic effect on oil displacement. The method of microbial stimulation compares favorably with chemicals injection into the reservoir, since bacteria may thrive in the oil reservoir, producing metabolites in the reservoir pores where they have the greatest effect on oil displacement. Furthermore, the nutrient source for bacteria is either the oil itself or waste food production, such as molasses, which significantly reduces the cost of the additionally recovered oil in comparison with other methods.

Relatively few works have dealt with field MEOR experiments at heavy oil reservoirs. The microbial ecology of heavy oil reservoirs and factors controlling in-reservoir petroleum biodegradation were the main study topics [3]. The ability of sulfidogenic and methanogenic communities to carry out the biodegradation of heavy oil components was shown both under laboratory conditions and in reservoirs [4–9]. The microorganisms inhabiting heavy oil deposits were investigated [10–14]. A number of aerobic, biosurfactant-producing bacteria were isolated from oilfields, and heavy oil displacement from model oilfields by the isolates was demonstrated [15–17].

Although field experiments at the heavy oil reservoirs have been few, it was shown that heavy oil recovery became more sustainable when MEOR techniques were applied. The patterns of activity of a thermophilic microbial community were investigated at the Dagang high-temperature (56.9–59.4 °C) heavy oil reservoir (China), and three field trials were performed (at the North block and block No. 1 of the Kongdian bed and at the Gangxi bed) [18–20]. At the Kongdian bed, characterized by high temperature, complex geological conditions, and heavy oil with a density 0.955–0.969 g/cm^3, the biotechnology involved injecting oxygen as an air–water mixture or H_2O_2 together with an aqueous solution of nitrogen and phosphorus mineral salts through injection wells in order to activate the oilfield microbial community [18]. The microbial oxidation of heavy oil resulted in the formation of lower alcohols and volatile fatty acids, which were subsequently utilized by anaerobic microorganisms producing gases (CH_4 and CO_2). The production of biosurfactants, resulting in decreased surface tension of formation water, was observed both in cultures and in oil reservoirs. Oil viscosity in the zone of production wells located at the North block of the Kongdian bed decreased by 11% (from 501.2 to 458.9 mPa s), wax content in oil decreased from 6.64% to 4.72% [12,20]. Under conditions of a high-temperature oil reservoir, methane was found to be produced from acetate in the course of the syntrophic growth of acetate-oxidizing bacteria and H_2-utilizing methanogens. Pure cultures of acetate-oxidizing *Thermoanaerobacter ethanolicus* strain 1017-7b and H_2-utilizing methanogen *Methanothermobacter thermautotrophicus* strain KZ3 were isolated from an association producing methane from acetate. While none of these strains were able to grow on acetate in monoculture, methane formation from acetate was observed in the combination of these cultures [21]. A total of 46,152 t additional oil was recovered at three experimental sites of the Dagang oilfield, which is an indication of the high efficiency of the technology for activation of the oilfield microorganisms for the purpose of heavy oil displacement from high-temperature oilfields [20].

MEOR field trials were also performed at low-temperature heavy oil reservoirs. In the experiment at the Xinjiang No. 6 oilfield (China), air, molasses, and mineral salts were injected into the reservoir to initiate an indigenous process of microbial enhanced oil recovery (MEOR), which resulted in an increment of 1872 t heavy oil [22]. Oil oxidation by *Pseudomonas* and the metabolic products, including biosurfactants, were proposed to be the primary factors responsible for improving heavy oil recovery.

The aim of this work was to study the microbial community composition and the presence of microorganisms able to produce oil-releasing compounds in production waters of low-temperature

heavy oil reservoirs (Russia), to estimate heavy oil displacement from the cores by aerobic organotrophic and fermentative bacteria, and to perform a pilot trial of the MEOR technique at the oilfield.

2. Materials and Methods

2.1. Characteristics of the Studied Heavy oil Reservoirs and Sampling Procedures

The microorganisms of injection and production water from petroleum reservoirs in Volgograd oblast and Tatarstan (Russia) were studied. Oil strata were located at the depth of 830–1650 m and had a temperature of 20–40 °C (Table A1). According to analyses of surface samples, oil density varied from 0.813 g/cm^3 to 0.924 g/cm^3 (at 20 °C). This made it possible to classify the oil from two out of four oilfields as heavy oil. The studied areas of the Arkhangelskoe and Romashkinskoe oilfields (Tatneft, Tatarstan) and Cheremukhovskoe and Vostochno-Anzirskoe oilfields (Ritek, Volgograd oblast) are exploited with water-flooding [23].

Water samples were collected directly at the head of injection wells and production wells into sterile bottles, hermetically sealed without air bubbles, and used for radioisotope and microbiological analyses on the day of sampling. Within 4–6 h after sampling, inoculations were carried out in order to determine cell numbers of cultivated microorganisms. To estimate the rates of sulfate reduction and methanogenesis, aliquots of the water samples were supplemented with labeled compounds ($Na_2{}^{35}SO_4$, $NaH^{14}CO_3$, and $^{14}CH_3COONa$) and analyzed as described previously [24]. The samples for chemical analyses were stored at 6 °C. For molecular biological research, water samples (1 L each) were fixed with ethanol (1:1 vol/vol) at the day of sampling, filtered through 0.22 μm membranes (Millipore, United States), and stored at −20 °C prior to analysis.

2.2. Analysis of Microbial Diversity in Heavy oil Reservoirs

The composition of microbial communities from injection and formation water was determined by high-throughput sequencing of the 16S rRNA genes on the Illumina platform. Cell biomass obtained by the filtration of ethanol-fixed water samples was washed off with the lysing solution containing 0.15 M NaCl and 0.1 M Na_2EDTA (pH 8.0) and used for DNA isolation. Total DNA was isolated using the PowerSoil DNA Isolation Kit (MoBio, USA) according to the manufacturer's recommendations and was stored at −20 °C. The purified DNA preparation was used as a template for polymerase chain reaction (PCR) with a pair of universal primers to the V3–V4 regions of the 16S rRNA gene: 319F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3). The primers were supplemented with oligonucleotide identifiers for sequencing on MiSeq (Illumina, USA). Sample preparation and sequencing were carried out according to the manufacturer's recommendations. Quality control of obtained reads was carried out using UPARSE [25]. After qualification, the reads were grouped into operational taxonomic units (OTUs) with 97% similarity using USEARCH (https://www.drive5.com/usearch/) [26]. The taxonomic position of the representative sequence for each OTU was determined using the RDP (https://rdp.cme.msu.edu/tutorials/classifier/classifer_cover_page.html) classifier [27]. Analysis of community composition using heatmaps was carried out using ClustVis [28]. Statistical analysis was carried out using Microsoft Excel, Rstudio (the vegan package) [29].

2.3. Media Composition and Cultures Used in the Study

Cell numbers of the microorganisms of the main metabolic groups were determined by inoculation of ten-fold dilutions of the water samples into liquid media in triplicate, as described earlier [23]. Salinity of the studied water samples was taken into consideration when preparing the media. Samples of injection water were inoculated into the media containing 10 g NaCl per liter. Samples of high-salinity formation water were inoculated into the media with 45 g NaCl per liter. Aerobic organotrophs were enumerated in trypton-extract-glucose (TEG) medium containing the following (g·L^{-1}): Bacto tryptone, 5.0; yeast extract, 2.5; glucose, 1.0; and NaCl, as required; pH 7.0–7.2. The numbers of hydrocarbon-oxidizing bacteria were determined using a mineral medium supplemented with a

mixture of C_{10}–C_{22} *n*-alkanes (2% vol/vol). Anaerobic fermentative bacteria were enumerated in the medium supplemented with peptone (4 g·L^{-1}) and glucose (10 g·L^{-1}). The numbers of sulfate-reducing bacteria were determined by an increase in sulfide concentration in dilution series of formation water in Postgate B medium with sodium lactate (3.5 g·L^{-1}), supplemented with microelements and reduced with $Na_2S{\cdot}9H_2O$ (200 mg·L^{-1}). The numbers of methanogens were assayed by methane production in dilution series in the Zeikus medium with acetate (2 g·L^{-1}), methanol (1 mL·L^{-1}), and H_2/CO_2 (4:1), supplemented with microelements and yeast extract (1 g·L^{-1}). The composition of the media used is cited in the publication by Bonch-Osmolovskaya et al. [24]. Inoculated media were incubated for 30 days at 22–24 °C and then examined using an Olympus microscope with a phase contrast device.

Aerobic heavy oil degrading bacteria *Gordonia amicalis* strain 6-1 (=VKM Ac-2795D) and *Rhodococcus erythropolis* strain HO-KS22 (=VKM Ac-2807D) were isolated earlier from the Cheremukhovskoe and Vostochno-Anzirskoe oilfields (Russia), respectively [23]. Fermentative enrichment 7SA was obtained by inoculation of the medium with peptone and glucose with water injected into well 1010 of the Vostochno-Anzirskoe oilfield. Pure culture of a fermentative bacterium *Oscillibacter ruminantium* strain AIK was obtained from the sludge of a biogas reactor processing oil-containing and municipal waste [30]. For core flooding experiments, *O. ruminantium* strain AIK was grown in the medium for fermentative bacteria (FM) containing the following (g·L^{-1} distilled water): KH_2PO_4, 0.2; NH_4Cl, 0.25; NaCl, 1.0; $MgCl_2{\cdot}6H_2O$, 0.4; KCl, 0.5; $CaCl_2{\cdot}6H_2O$, 0.1; peptone, 1.0; glucose, 2.0; sucrose, 2.0; yeast extract, 1.0; $Na_2S{\cdot}9H_2O$, 0.5; and 1 mL·L^{-1} trace elements [31]. Argon was used as the gas phase. Fermentative enrichment 7SA was grown in liquid mineral FM medium supplemented with glucose (2.0 g·L^{-1}) and sucrose (2.0 g·L^{-1}) as substrates, with 10.0 g·L^{-1} NaCl; without addition of the reducing agent. Strain AIK and fermentative enrichment 7SA were grown for 20 days under stationary conditions at 25 °C. Grown liquid cultures with microbial biomass and metabolites were used in core flooding experiments on the enhancement of oil recovery.

Oil biodegradation by aerobic hydrocarbon-oxidizing bacteria was studied in the medium of the following composition (g·L^{-1} distilled water): $CaCl_2{\cdot}6H_2O$, 0.01; $MnSO_4{\cdot}5H_2O$, 0.02; $FeSO_4{\cdot}7H_2O$, 0.01; $Na_2HPO_4{\cdot}12H_2O$, 1.5; KH_2PO_4, 1.0; $MgSO_4{\cdot}7H_2O$, 0.2; NH_4NO_3, 2.0; NaCl, 5.0; pH 6.8–7.2; oil 1.0% (vol/vol). The experiments were carried out using crude oil of the Cheremukhovskoe oilfield, which contained 23.5% saturated hydrocarbons, 42.3% aromatic hydrocarbons, 30.1% resins, and 4.1% asphaltenes. The cultures and uninoculated medium with oil (control) were incubated for seven days on a shaker (60 rpm) at 28 °C. After incubation, oil fractions were separated as described previously [32]. Residual oil was extracted with chloroform; the extract was dried with Na_2SO_4, filtered, and the solvent was removed on a rotary evaporator. Oil was then dispensed into vials, dried for 24 h under draught, and analyzed by gas-liquid chromatography (GLC).

2.4. Analytical Methods

The contents of methane, hydrogen, and carbon dioxide accumulated in the gas phase of the primary enrichment cultures were determined by gas chromatography. Sulfide was determined by the colorimetric method of Pachmayr with dimethyl-*p*-phenylenediamine [33]. The chemical composition of formation water was determined as described by Bonch-Osmolovskaya et al. [24]. Volatile fatty acids and alcohols were analyzed with a Shimadzu GC 2010 Plus chromatograph (Japan) in a column (30 m × 0.32 mm) with a Zebron ZB-FFAP phase thickness of 0.25 µm, as described previously [34]. The surface tension was measured at the interface between formation water and air. The interfacial tension was determined at the interface between the studied liquid and the *n*-hexadecane by the ring-tearing-off method using a Kruss K10 ST tensiometer (Kruss GmbH, Germany) at 22 °C.

The aliphatic fraction of oil was analyzed on a Crystal 5000.1 gas–liquid chromatograph (Chromatec, Russia) equipped with a flame ionization detector and a 15 m ZB-FFAP capillary column, with helium as the carrier gas. The column temperature changed from 100 °C to 320 °C in the course of analysis at the rate of 5 °C/min.

2.5. Core Flooding Experiments

The oilfield models were constructed using several cores of carbonate rocks with intact structure; each core was ~30 mm in diameter and ~40 mm long. The cores were cleaned from hydrocarbons by using the Soxhlet extraction method, using benzene and methanol in the proportion of 75:25 vol/vol. After cleaning, the cores were dried at 100–110 °C for 24 h, and their absolute permeability was determined. The columns packed with two cores each were 8.0 cm in length and 3.02 cm in diameter and were made of stainless steel. The cores were saturated with filtered formation brine with 195 $g \cdot L^{-1}$ salinity using vacuum desiccators for 24 h, and pore volume (PV) was determined using the dry and wet weights of each core, as described previously [16]. The cores were then flooded with heavy crude oil with a density of 0.93 g/cm^3 and viscosity of 190 mPa·sec at 1 cm^3/min until no more water was removed. This filtration rate was chosen since microbial processes in the near-bottom zone of injection wells were simulated, which is characterized by a high rate of injected water flow. The oil initially in place (OIIP) was determined, which was indicated by the volume of water displaced. The core was subjected to water flooding at 1 cm^3/min until no further oil was removed. The residual oil was calculated by measuring the amount of oil recovered by water-flooding. After preliminary operations, the model was incubated for one to seven days at the experimental values of temperature and pressure. Then, 10 PV of the grown liquid culture was injected as a tertiary recovery stage and extra oil recovery was determined. All core floods were conducted at 7–8 MPa and 22–24 °C to mimic the pressure and temperature of the oilfield, respectively.

2.6. Nucleotide Sequence Accession Numbers

The 16S rRNA gene sequences of strains *Gordonia amicalis* 6-1 (= VKM Ac-2795D), *Rhodococcus erythropolis* HO-KS22 (= VKM Ac-2807D), and *Oscillibacter ruminantium* AIK were deposited in the NCBI database under accession numbers MN101280, MN622878, and MN623416, respectively. The libraries of the 16S rRNA gene fragments, obtained as a result of high-throughput sequencing, were deposited in the NCBI Sequence Read Archive (SRA) under accession number PRJNA589080.

3. Results and Discussion

3.1. Physicochemical and Microbiological Characteristics of Injection and Production Water of the Studies Oilfields

The petroleum reservoirs studied are exploited with water-flooding with fresh water from the surface or with its mixture with formation water, separated from oil. The injection of fresh water results in decreased salinity in the area of water mixing. Formation water from the Arkhangelskoe heavy-oil reservoir with carbonate collectors had salinity from 36 $g \cdot L^{-1}$ to 244 $g \cdot L^{-1}$, belonged to the chlorine-calcium type, and contained high levels of bicarbonate (0.12 $g \cdot L^{-1}$ to 0.70 $g \cdot L^{-1}$) and sulfates (0.44 $g \cdot L^{-1}$ to 1.58 $g \cdot L^{-1}$) (Table 1). In the terrigenous Cheremukhovskoe heavy oil reservoir, production water with salinity from 12 $g \cdot L^{-1}$ to 186.9 $g \cdot L^{-1}$ belonged to the chlorine-calcium type; the content of bicarbonate in studied water samples varied within a narrow range from 0.29 $g \cdot L^{-1}$ to 0.32 $g \cdot L^{-1}$, and that of sulfate from 0.08 $g \cdot L^{-1}$ to 0.22 $g \cdot L^{-1}$. At the terrigenous Romashkinskoe oilfield (Almetievskaya bed) with light conditioned oil, which was used for comparison, the salinity of production water samples varied within a wide range exceeding 190 $g \cdot L^{-1}$. Fresh river water injected into the Almetievskaya bed through injection wells 20990* and 10055* had a density of ~1.000 kg dm^{-3}.

Table 1. Physicochemical characteristics of injection and formation water at the studied oilfields.

Oilfield, Well No.	Total Salinity, g L^{-1}	pH	Content, g L^{-1}					
			$Na^+ + K^+$	Ca^{2+}	Mg^{2+}	Cl^-	HCO_3^-	SO_4^{2-}
Arkhangelskoe oilfield								
7528*	0.966	7.6	0.195	0.134	0.037	0.110	0.354	0.136
4610	36.291	7.2	9.932	3.206	0.608	21.272	0.592	0.681
7492	51.712	6.8	16.289	3.138	0.737	29.261	0.708	1.579
4634	73.850	6.5	22.302	4.584	1.668	43.219	0.573	1.504
7806	104.628	6.9	32.134	6.528	2.117	62.423	0.622	0.804
4308	244.092	5.9	81.615	11.538	3.683	146.695	0.122	0.439
Romashkinskoe oilfield								
20880	11.819	7.5	3.222	1.052	0.213	7.115	0.207	0.013
20963	49.869	6.6	13.9	4.259	0.912	30.636	0.159	0.0029
47B	81.813	6.4	22.762	7.014	1.52	50.101	0.098	0.0177
2356D	165.14	5.7	46.123	14.279	2.888	101.79	0.049	0.0108
21058	190.78	5.4	53.230	16.533	3.344	117.6	0.049	0.0177
Cheremukhovskoe oilfield **								
5600*	0.621	7.86	0.114	0.060	0.015	0.114	0.244	0.074
5454	12.098	7.70	4.125	0.516	0.138	6.946	0.293	0.080
5463	72.274	6.69	24.324	3.373	0.993	43.124	0.317	0.143
5464	186.890	6.30	64.849	8.531	1.564	111.435	0.293	0.218

* Injection well, other wells are production wells. ** Data from Nazina and co-authors [23].

A tendency to higher abundance of culturable microorganisms in the desalinated zone compared to the highly mineralized formation water was observed. In the Arkhangelskoe heavy oil reservoir with carbonate collectors, the highest microbial numbers were found in injection fresh water (well 7528) and in the production water sample with salinity up 73 g·L^{-1} (well 4634) (Figure A1). In production water with higher salinity, the numbers of microorganisms of the major physiological groups did not exceed tens of cells per mL. Sulfate reduction rate was registered by the radioisotope method in water samples with salinity below 105 g·L^{-1}, but methanogenesis rate was detected up to 244 g·L^{-1} (Table A2).

In the terrigenous Cheremukhovskoe heavy oil reservoir, the numbers of microorganisms were considerably lower in production water samples with salinity exceeding 72 g·L^{-1}, although fermentative microorganisms occurred at up to 186.9 g·L^{-1} (well 5464). In spite of the broad salinity range of formation water at the terrigenous Romashkinskoe oilfield (Almetievskaya bed) with light oil, the numbers of aerobic organotrophs, as well as of fermentative and methanogenic prokaryotes, were higher than in the studied heavy oil reservoirs. While sulfide formation was detected in fermentative enrichments after long-term incubation, cultured sulfate-reducing bacteria were not retrieved when medium with lactate and 45 g·L^{-1} NaCl was inoculated with formation water of the Romashkinskoe oilfield.

3.2. Phylogenetic Diversity of Archaea and Bacteria in Injection and Production Water

High-throughput sequencing of the V3–V4 region of the 16S rRNA genes was used to determine the composition of microbial communities in the samples of injection and formation water from the Arkhangelskoe and Romashkinskoe petroleum reservoirs. A total of 107,846 reads were obtained and operational taxonomic units (OTUs) were defined based on 97% identity. The 16S rRNA gene

fragments of prokaryotes of the Bacteria domain were predominant, with *Proteobacteria*, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes* being the most abundant groups (Figure 1)

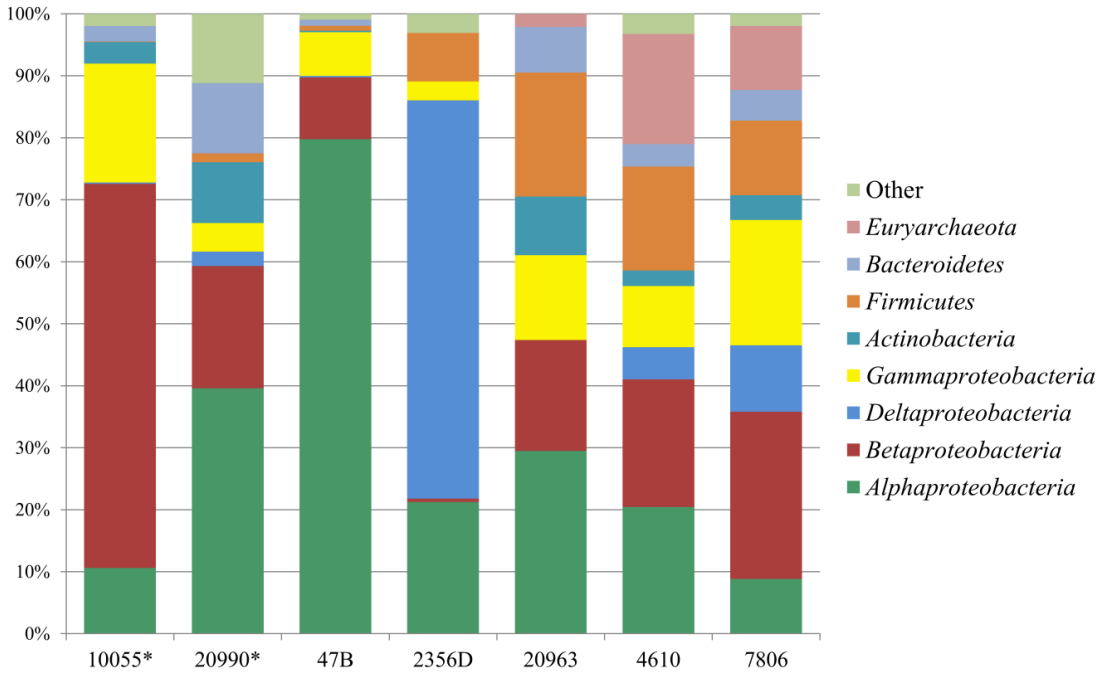

Figure 1. Taxonomic classification of archaeal and bacterial 16S rRNA gene fragments in the libraries from injection and production water samples at the phylum level (at the class level for *Proteobacteria*) using the RDP classifier. Injection wells are marked with *, the rest are production wells.

The sequences of *Archaea* were revealed in the libraries of production water from wells 4610 and 7806 of the Arkhangelskoe heavy oil reservoir, as well as in library 20963 from the Romashkinskoe oilfield, where they constituted 18.0%, 10.1%, and 2.1% of the total number of genes in the library, respectively. Microbial communities of these reservoirs with high salinity of formation water are characterized by a noticeable occurrence of methylotrophic prokaryotes (aerobic methylotrophic bacteria and anaerobic methylotrophic methanogenic archaea).

The libraries of production water from wells 4610 and 7806 of the Arkhangelskoe heavy oil reservoir revealed the sequences of aerobic organotrophic bacteria of the genera *Bacillus*, *Pseudomonas*, *Methylobacterium*, of anaerobic fermentative *Halanaerobium*, and of syntrophic bacteria *Smithella* and *Syntrophus*, typical for water-flooded petroleum reservoirs. Sequences of less frequently occurring bacteria of the genera *Herminiimonas*, *Acidovorax*, and *Sphingomonas* were also detected (Figure 2).

The minor sequences belonged to fermentative bacteria of the genera *Geotoga* and *Mesotoga* and to sulfate-reducing bacteria of the genera *Desulfocurvus*, *Desulfotignum*, *Desulfonatronovibrio*, *Desulfovibrio*, *Desulfosalsimonas*, *Desulfovermiculus*, and *Desulfoglaeba* (PRJNA589080). *Euryarchaeota* sequences belonged mainly to organotrophic halophilic archaea of the genus *Halomicrobium* (class *Halobacteria*), which are capable of both aerobic and anaerobic (by nitrate reduction) growth, and to halophilic methanogenic archaea of the genus *Methanohalophilus* (class *Methanomicrobia*). The coccoid methylotrophic methanogen *Methanococcoides euhalobius*, capable of growth in the presence of 14% NaCl (wt/vol), was originally isolated from the Bodyuzhskoe oilfield of Tatarstan [35,36].

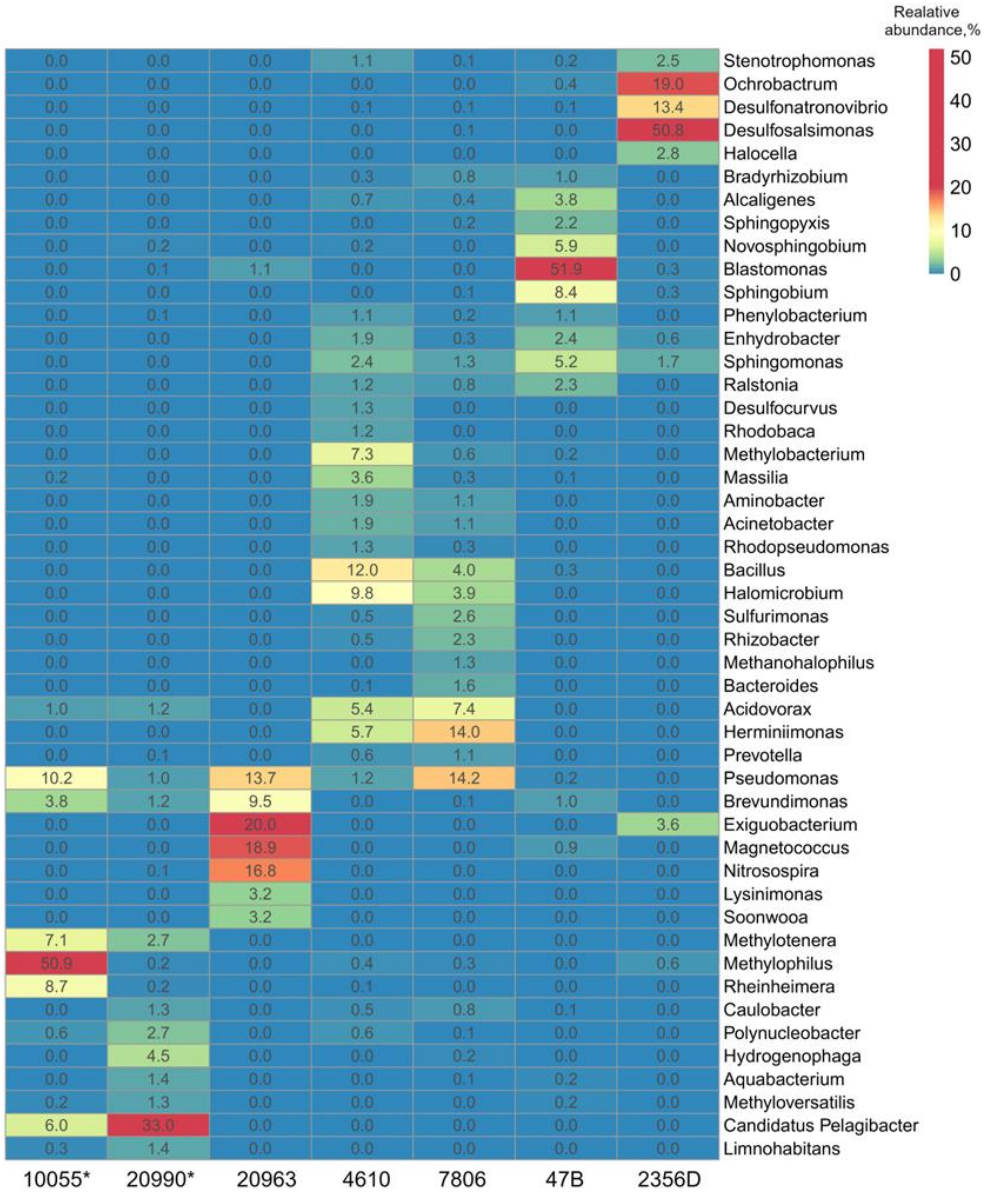

Figure 2. Heatmap analysis of the distribution of the dominant genera of Archaea and Bacteria in injection and production water samples from the Arkhangelskoe and Romashkinskoe oilfields. The relative values for microbial genera are marked by colors from blue to red, designating the least abundant to most abundant. Abundance is expressed as the ratio of the number of targeted sequences to the total number of sequences from each sample. Injection wells are marked with *, the rest are production wells.

Among bacteria revealed in the libraries of production water 20963, 47B, and 2356D from the Romashkinskoe oilfield were halophilic sulfate-reducing bacteria of the genera *Desulfosalsimonas* and *Desulfonatronovibrio*, aerobic organotrophic bacteria of the genera *Ochrobactrum*, *Pseudomonas*, and *Brevundimonas*, as well as members of the genera *Exiguobacterium*, *Blastomonas*, *Nitrosospira*, which seldom occur in oilfields, and magnetotactic bacteria of the genus *Magnetococcus*. Members of the genus *Exiguobacterium* are facultatively anaerobic organotrophs [37]. *Magnetococcus* can grow chemolithoautotrophically with thiosulfate or sulfide as electron donors, and chemoorganoheterotrophically on acetate; thus, the conditions in the water-flooded oilfield are in agreement with their growth requirements [38].

In injection water 10055*, methylotrophic bacteria of the genera *Methylophilus* (50.9% of the total number of genes in the library) and *Methylotenera* (7.1%) predominated, as well as members of the genera *Pseudomonas* (10.2%), *Rheinheimera* (8.7%), and '*Candidatus* Pelagibacter' (6%). In injection water 20990*, bacteria of the genera '*Candidatus* Pelagibacter' (33%), *Hydrogenophaga* (4.5%), *Methylotenera* (2.7%), and *Polynucleobacter* (2.7%) were found. Aerobic chemoorganotrophic bacteria of the genus *Polynucleobacter*, which utilize a narrow range of substrates (acetate, pyruvate, malate, etc.) under aerobic conditions or grow as facultative anaerobes, are known to be common in freshwater environments [39]. The chemoheterotrophic marine bacteria of the SAR11 clade *Candidatus* Pelagibacter, which were revealed in both samples of injection water, are "the Earth's most abundant organisms" [40]. The reason for the occurrence of these bacteria far from marine habitats remains unclear.

Application of the principle component analysis (PCA) method revealed considerable similarity in the composition of microbial communities in production water samples from the same petroleum reservoirs (Figure 3). The 4610 and 7806 communities from Arkhangelskoe oilfield, where formation water contained high concentrations of sulfate, a diverse archaeal population, and sulfate-reducing bacteria as minor phylotypes, formed a separate group. Another separate group was represented by communities of production water from the Romashkinskoe oilfield (47B, 2356D, and 20963), where sulfate concentration in the water was low and various halotolerant aerobic organotrophic bacteria and halophilic sulfate reducers were present. Microorganisms of injection water communities formed two groups, which were different from formation water communities.

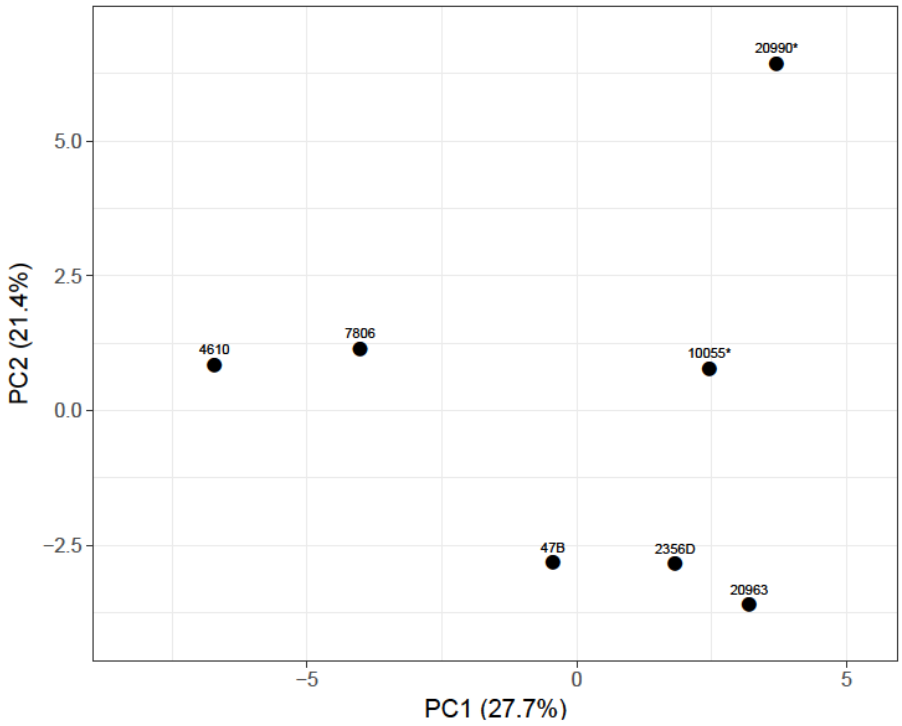

Figure 3. Principal component analysis (PCA) based on relative abundance of 16S rRNA gene operational taxonomic units (OTUs) from injection and production water samples of the Arkhangelskoe and Romashkinskoe oilfields.

Earlier analysis of the 16S rRNA genes of the microbial community from injection water 5600* of the Cheremukhovskoe oilfield revealed bacteria of the genera *Dechloromonas* (15%), *Phenylobacterium* (12%), hydrocarbon-oxidizing bacteria of the genera *Acinetobacter* (5%) and *Rhodococcus* (1.2%), and of sulfate- and sulfur-reducing bacteria of the genera *Desulfovibrio*, *Desulfomicrobium*, and *Desulfuromonas* [23]. Denaturing gradient gel electrophoresis (DGGE) analysis of the *mcrA* genes in injection water 5600* revealed methanogens of the genera *Methanospirillum*, *Methanobacterium*, and *Methanoregula*; production

water from sample 5464 revealed methanogens of the genera *Methanosarcina*, *Methanoculleus*, and unidentified *Thermoplasmata*. These results indicate that both microorganisms from the surface freshwater basins and the reservoir microbiota adapted to oilfield conditions are delivered into the oil reservoir with injection water (a mixture of surface fresh water and formation water after oil removal).

3.3. Production of Oil-Releasing Compounds by Bacteria from Heavy Oil Reservoirs

3.3.1. Aerobic Bacteria Producing Biosurfactants

A number of aerobic organotrophic bacteria from the genera *Rhodococcus, Gordonia, Nocardia, Pseudomonas, Bacillus, Salinicola, Chromohalobacter*, and *Cellulomonas* were previously isolated from Russian heavy oil reservoirs [23]. These bacteria produced biosurfactants in the media with a variety of organic substrates (acetate, glucose, sucrose, ethanol, peptone, and glycerol), as well as in the medium with crude oil.

The strains *Gordonia amicalis* 6-1 and *Rhodococcus erythropolis* HO-KS22, which exhibited the most pronounced decrease in the surface tension, were studied in more detail. *R. erythropolis* HO-KS22 grew aerobically within broad ranges of salinity (0 g·L^{-1} to 80 g·L^{-1} NaCl, optimum at 10 g·L^{-1}), pH (5.5–9.5, optimum at pH 7.0–7.5), and temperature (5–37 °C, optimum at 28 °C). *G. amicalis* 6-1 grew aerobically at 0 g·L^{-1} to 100 g·L^{-1} NaCl (optimum at 15 g·L^{-1}), pH from 6.5 to 8.2 (optimum at 7.4), and at 5 °C to 45 °C (optimum at 28 °C). Biodegradation of heavy oil by *G. amicalis* 6-1 resulted in the utilization of C_{11}, C_{12}, C_{13}, $C_{15–C21}$, and C_{23} *n*-alkanes (Figure A2a,b). After seven days of incubation, *R. erythropolis* HO-KS22 consumed most of the *n*-alkanes from heavy oil (Figure A2c). In the medium with crude oil, the strains *G. amicalis* 6-1 and *R. erythro*polis HO-KS22 decreased the interfacial tension from 21 mN/m to 0.2–1.0 mN/m. In the medium with glucose, *G. amicalis* 6-1 synthesized biosurfactants during the highest biomass accumulation (late logarithmic to early stationary growth phases), as was evident from the decrease in surface tension from 72 mN/m to 20 mN/m and of the interfacial tension against *n*-hexadecane from 47 mN/m to 1–2.1 mN/m (Figure A3a–c). The most pronounced decrease in surface tension was observed at the glucose/nitrate ratio of 5/0.5 (g/g per 1 L); the highest decrease in interfacial tension occurred at the 20/1.0 ratio (g/g). Salinity and temperature optima of *G. amicalis* 6-1 and *R. erythropolis* HO-KS22 and the ability to consume crude oil indicate their adaptation to the conditions of the near bottom zone of injection wells, where dissolved oxygen is supplied by injected water; these physiological characteristics may be used in the application of strains 6-1 and HO-KS22 for injection into oilfields in order to increase oil recovery or to remove asphaltene–resin–paraffin deposits.

The microorganisms capable of decreasing surface tension by more than 10 mN/m are considered potential biosurfactant producers [41]. The isolation of biosurfactants from the media and investigation of their structure were beyond the scope of the present work. The surface tension decrease of the media caused by the isolates *G. amicalis* 6-1 and *R. erythropolis* HO-KS22 was compared to that caused by sulfonol, an anionic synthetic surfactant used in the oil industry for oil recovery enhancement. The biosurfactants present in liquid cultures of the strains decreased the surface tension to the same degree as a sulfonol solution at 1.3 g L^{-1}. Thus, *G. amicalis* 6-1 and *R. erythropolis* HO-KS22 are promising for application in the biotechnologies for enhanced oil recovery and for oil removal from contaminated environments. *G. amicalis* 6-1, which is also able to use dibenzothiophene as a sulfur source in the presence of other organic substrates, was patented [42].

3.3.2. Fermentative Bacteria Producing Low Fatty Acids and Alcohols

While the population of anaerobic fermentative bacteria was the most numerous one in formation water samples (Figure A1), their growth on heavy oil was very poor (data not shown). The growth of fermentative enrichments in media with peptone and glucose did not result in surface tension changes. The studied fermentative enrichment 7SA and pure culture *Oscillibacter ruminantium* strain AIK fermented organic substrates with the production of low fatty acids, alcohols, and gases (H_2 and CO_2). The 7SA fermentative enrichment produced low fatty acids (acetic, 50.0 mg·L^{-1}; propionic, 190.0 mg·L^{-1}; and *n*-butyric, 3590.0 mg·L^{-1}), the pH of the medium decreased from 7.0 to 5.5. *O.*

ruminantium AIK produced low fatty acids (LFA) (acetic, 1895.0 mg·L^{-1}; propionic, 390.0 mg·L^{-1}; isobutyric, 370.0 mg·L^{-1}; *n*-butyric, 3830.0 mg·L^{-1}; and other LFA, 790.0 g·L^{-1}), isopropanol, 110.0 g·L^{-1}; and gases (H_2 and CO_2); pH decreased to 4.5. Volatile fatty acids are known to promote the dissolution of carbonate oil-bearing rocks and to increase their porosity, permeability, and oil recovery.

Methanogens were detected in a number of samples by molecular, cultural, and radiotracer techniques. It is, however, hardly probable that fermentative bacteria may provide enough substrates for methanogens for methane to have a significant effect on oil displacement, even in the presence of sugar-containing substrates. The cultures of aerobic bacteria and anaerobic fermentative bacteria were therefore used in core flooding experiments.

3.4. Core Flooding for Heavy Oil Recovery

The cultures of biosurfactant-producing aerobic bacteria and of fermentative bacteria-producing volatile acids and alcohols were grown in liquid media with sugar-containing substrates and then injected into the models. In these experiments only the effect of introduced liquid cultures of aerobic and anaerobic bacteria on heavy oil recovery was estimated. The distribution of bacteria in the pore space of the models and bacterial growth in time were not analyzed.

The oilfield models were prepared using the natural carbonate cores from oilfields and were saturated with heavy oil (density 0.93 g/cm^3). The cores were then flooded with NaCl solution (30 g·L^{-1}), which was similar to formation water salinity, to 100% water content in the outflowing liquid. After primary oil displacement with the NaCl solution, the residual oil saturation of the cores varied from 67.3% to 80%, indicating that most of the water present in the column was replaced by oil. The results obtained with the carbonate cores are shown in Table 2.

Secondary core flooding with 10 PV of the liquid cultures of aerobic and anaerobic bacteria resulted in decreased residual oil saturation in the cores and additional oil displacement after MEOR. Injection of the liquid culture *O. ruminantium* AIK, which contained low fatty acids (7.27 g·L^{-1}), isopropanol, and the biomass, was accompanied by additional displacement of 30.5% and 60.4% of oil (45.5% on average) from cores #1 and #2. Additional oil recovery from cores #3 and #4 flooded with fermentative enrichment 7SA was 11.6% and 15.3% (13.4% on average), which was probably the result of lower concentrations of the fermentation products in the culture 7SA (3.83 g LFA L^{-1}).

The flooding of cores #5 and #6 with the aerobic culture *R. erythropolis* HO-KS22 resulted in the highest additional oil displacement of 60.6% and 46.4% (53.5% on average) (Figure 4).

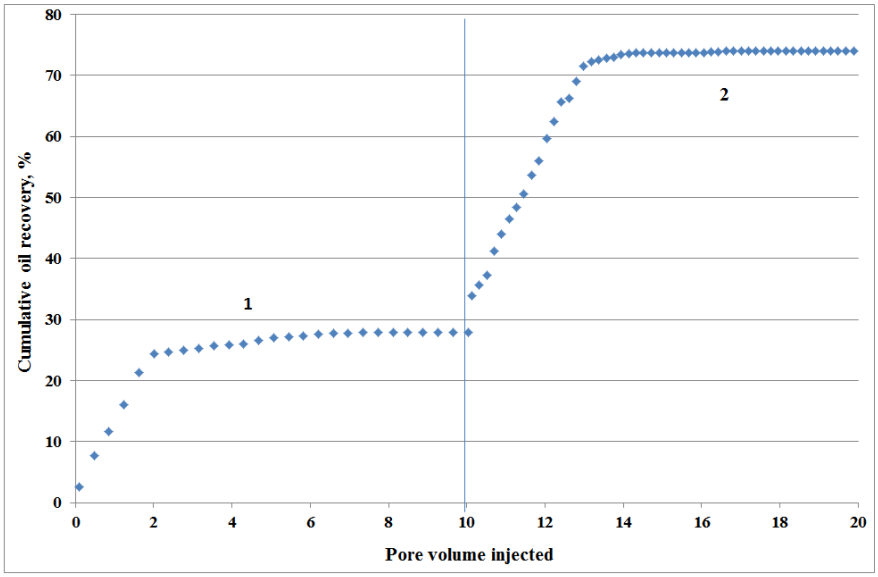

Figure 4. Cumulative oil recovered from the core by successive injection of water with a density of 1.04 g·cm^{-3} (1) and liquid culture of R. erythropolis HO-KS22 (2). Rate of injection was 1 mL·min^{-1}.

Table 2. Core properties and heavy oil recovery as a result of flooding of aerobic and anaerobic cultures.

Parameters	Experiments											
	# 1	**# 2**	**Average**	**# 3**	**# 4**	**Average**	**# 5**	**# 6**	**Average**	**#7**	**#8**	**Average**
Pore volume, cm^3	8.14	8.53	8.34	9.0	9.5	9.25	10.46	10.12	10.29	8.13	13.3	10.7
Porosity, %	15.4	15.5	15.5	16.2	17.3	16.8	19.0	18.7	18.9	14.9	24.0	19.5
Absolute permeability, $10^{-3} \times \mu m^2$	254	372	313	331	220	276	333	324	328.5	379	289	334
Connate water saturation, fraction	0.251	0.235	0.243	0.28	0.27	0.27	0.204	0.200	0.202	0.26	0.23	0.25
Primary oil displacement with the NaCl solution (30 g L^{-1})												
Pore volume injected, PV	10	10	10	10	10	10	10	10	10	10	10	10
Residual oil saturation before MEOR, %	67.3	77.6	72.4	79.5	68.6	74.0	74.6	72.1	73.3	80.0	69.3	74.6
Additional oil displacement by grown culture in the liquid medium												
Culture	*O. ruminantium* AIK			Fermentative enrichment 7SA			*R. erythropolis* HO-KS22			*R. erythropolis* HO-KS22 + *G. amicalis* 6-1		
Pore volume injected, PV	10	10	10	10	10	10	10	10	10	10	10	10
Residual oil saturation after MEOR, %	36.6	17.2	26.9	67.9	53.3	60.6	14.0	25.7	19.8	25.8	37.5	31.6
Heavy oil recovery enhancement as a result of MEOR, %	30.7	60.4	45.5	11.6	15.3	13.4	60.6	46.4	53.5	54.2	31.8	43.0

The flooding of cores #7 and #8 with liquid cultures of strains HO-KS22 and 6-1 in the 1:1 ratio (vol/vol) caused additional oil replacement of 54.2% and 31.8% (43.0% on average). The increase in oil recovery observed in core-flooding experiments #5–#8 was probably due to the ability of the aerobic *Rhodococcus* and *Gordonia* isolates to produce biosurfactants, both associated with their cell walls and released into the medium. Biosurfactants are known to be bipolar molecules concentrating at the oil–water interface and causing oil emulsification and a decrease in the interfacial tension between oil and water.

Additional oil displacement in experiments with fermentative cultures was probably caused by the dissolution of carbonate rocks and by their porosity increasing due to the action of the fermentation products (primarily acetic and butyric acids and CO_2). Short-chain fatty acids interacted with the carbonate matrix according to the following equations:

$$\begin{gathered} 2CH_3COOH + CaCO_3 \rightarrow (CH_3COO)_2Ca + H_2CO_3; \\ 2CH_3CH_2CH_2COOH + CaCO_3 \rightarrow (CH_3CH_2CH_2COO)_2Ca + H_2CO_3. \end{gathered} \quad (1)$$

As a result, soluble salts were produced and rock permeability in the contact zone increased, while calcium and magnesium salts of fatty acids with over seven atoms in their carbon chain are insoluble, so their accumulation resulted in decreased permeability. In the collectors consisting of aragonite and calcite, increased carbonate concentration in the medium may initiate the process of dolomitization, with magnesium incorporating into the structure of carbonates. The dolomite thus formed has lower volume than calcite or aragonite, and this process should in theory increase the rock permeability [43]. Inflow of the biomass of aerobic and anaerobic bacteria was accompanied by sealing of the highly permeable interlayers, which resulted in the changed phase permeability of the models (data not shown). The interaction of microbial metabolites with the rock, oil, and water resulted in more widespread water flooding and increased oil recovery. The results of model experiments were confirmed in the course of pilot trials at heavy oil reservoirs.

3.5. Pilot Trials of MEOR Biotechnology at Petroleum Reservoirs

As a substrate for microbial growth, heavy oil, being by its nature a product of biodegradation, is therefore a poor source of the easily utilized fractions most available to microorganisms. It may be expected, however, that in the presence of oxidants, the oilfield microbial community will oxidize oil with the production of a spectrum of oil-displacing metabolites. A field pilot experiment for enhancing oil recovery using the microbiological method was carried out at the Cheremukhovskoe oilfield, in the zone of the injection well 5600. The Cheremukhovskoe oilfield, containing heavy oil with a density of 0.932 kg/m^3, has been exploited since 1997. Water-flooding with fresh river water mixed with production water separated from oil was initiated in 2001. The initial recoverable reserves were 802×10^3 t. The average oil saturation was 0.810 U. The average water cut of oil production is presently 51%. The same experiment was carried out in the zone of the injection well 1010 at the Vostochno-Anzirskoe oilfield with oil with a lower density of 0.856 kg/m^3. Since 2003, the Vostochno-Anzirskoe oilfield has been exploited with focal water injection in order to preserve the seam pressure. The initial recoverable reserves were 582×10^3 t. The average oil saturation was 0.713 U.

The biotechnological treatment at the Cheremukhovskoe and Vostochno-Anzirskoe oilfields included the injection of oil-oxidizing bacteria adapted to oilfield conditions into the reservoir through injection wells. For the sustainable growth of oil-oxidizing bacteria, oxygen (as a H_2O_2 solution) and $(NH_4)_2HPO_4$ solution as a source of biogenic elements were injected in order to initiate the first stage of the oxidation of oil hydrocarbons. After the treatment of injection wells 5600 and 1010, the cultured microorganisms and the rates of microbial processes in the zone of production wells were studied and the effect of microorganisms on oil recovery was assessed. Unfortunately, we had no possibility of monitoring the changes in the microbial community during the pilot trials by molecular techniques.

The water in wells 5454 and 5463 of the Cheremukhovskoe oilfield showed the highest oil displacement, the highest numbers of aerobic organotrophic bacteria (including hydrocarbon oxidizers), and anaerobic fermentative bacteria were found to increase. The numbers of hydrogenotrophic and acetate-utilizing methanogens did not change in the course of the biotechnological treatment, although radiotracer analysis revealed a 1.5–4.5-fold increase in methanogenesis rates in a number of production wells (Table 3). In sulfate-containing formation water, sulfate reduction is unavoidable, unless special measures for its suppression are taken. Sulfate reduction rate in the zone of responding wells increased from 4–98 ng $S^{2-} \cdot L^{-1} \cdot day^{-1}$ (during the baseline period) to 13–213 ng $S^{2-} \cdot L^{-1} \cdot day^{-1}$ in the course of the trial. Heavy oil biodegradation was accompanied by increased concentrations of acetate (from 1.3–3.0 mg·L^{-1} to 20–30 mg·L^{-1}) and bicarbonate (from 244–305 mg·L^{-1} to 476–671 mg·L^{-1}) in the formation water of responding wells. In the course of the experiment, microbial metabolites spread from the near bottom zone of the injection well and affected oil displacement 5–7 months after the introduction of bacteria, the oxidant, and the nitrogen and phosphorus salt in the reservoir (Figure 5). As a result of the pilot trial at the Cheremukhovskoe oilfield, 1141 t of oil was additionally recovered in the course of a year after the onset of the treatment. Although the abundance of hydrocarbon-oxidizing and fermentative bacteria, as well as bicarbonate concentration, increased the zone of production well 5464, this did not result in enhanced oil recovery, probably due to the high salinity of formation water in this area (over 186 g·L^{-1}), which prevented microbial growth and the formation of oil-releasing metabolites.

At the Vostochno-Anzirskoe oilfield, the biotechnological treatment resulted in a very low increase in oil recovery. A total of 1250 t of oil was obtained in the course of two pilot trials. The efficiency of the biotechnological treatment was confirmed by the water content in the production of the responding wells decreasing by 18–35% (Figure 5).

In the MEOR pilot trials at the heavy oil reservoirs, both at the high-temperature Dagang oilfield (China) [18–20] and at the low-temperature Cheremukhovskoe and Romashkinskoe oilfields [44,45] (Russia), oil recovery became more sustainable after the biotechnological treatment, which had a positive effect of total oil recovery. The Cheremukhovskoe oilfield is characterized by a low reservoir temperature (20.2–21.3°C). The efficiency of carbonate dissolution by acetate at a low temperature was estimated in laboratory experiments [46]. It was shown that the dissolution of calcium carbonate in aqueous solutions of acetic acid occurs even at 10 °C. At the low-temperature (17–23 °C) bed 302 with carbonate collector and heavy oil at the Romashkinskoe oilfield, an MEOR pilot trial was performed based on the introduction of *Clostridium tyrobutyricum* and molasses into the oil stratum [44,45]. Monitoring the concentrations of fatty acids, calcium, and carbonates dissolved in the formation water of bed 302 provided direct evidence of carbonate dissolution by acidic products of microbial metabolism. According to the van't Hoff equation, the rate of this reaction would have certainly been higher at higher temperatures. The dissolution rate of carbonate rock is probably less important than the fact of formation of low fatty acids directly in the pore water, where their effect on the oil-bearing rocks is maximal.

Due to the multistage and interrelated nature of microbial processes occurring in oilfields, it is difficult to attribute enhanced oil recovery to a single mechanism. Field experiments at heavy oil reservoirs indicated that all groups of microbial metabolites produced during molasses fermentation [44,45] and oil oxidation [18–20] affected oil displacement. Volatile fatty acids dissolved the carbonate rocks, increasing their porosity and permeability. The gases produced in the course of transformation of the fermentation products in the microbial trophic chain (CO_2, hydrogen, and methane) caused local restoration of the seam pressure, increased gas factor, oil swelling, and decreased oil viscosity, as well as increased collector permeability due to carbonate dissolution by CO_2. The surfactants caused a decrease in the interfacial tension at the oil–water and oil–rock boundaries and to oil emulsification. Both microbial exometabolites and the biomass itself affected the motility of formation fluids and the direction of hydrodynamic flows in the oilfield, also increasing the waterflooding coverage.

Table 3. Microbial numbers, acetate, HCO_3^-, and SO_4^{2-} concentrations and rates of sulfate reduction and methanogenesis in production water before biotechnological treatment (1) and 10 months after the treatment (2).

Oilfield, Well No.	Time of Analysis	Microbial Numbers, cells·mL^{-1}			Concentration, mg·L^{-1}			Methanogenesis Rate, nl CH_4·L^{-1}·day^{-1} from			% from Acetate	Sulfate Reduction Rate, ng S^{2-}·L^{-1} ·day^{-1}	Additional Oil, t
		Aerobes	Fermenters	Sulfate Reducers	HCO_3^-	Acetate	SO_4^{2-}	HCO_3^-	Acetate	Total			
Cheremukhovskoe oilfield													
5600*	1	10^6	10^6	Nd	244	Nd	74						
5452	1	10^2	10^6	10^3	6	2.1	96	4	44	48	92	4	
	2	10^3	10^3	10^4	641	20.6	96	86	127	213	60	13	72
5454	1	10	10^5	10^2	305	2.6	80	26	24	50	47	91	
	2	10^4	10^5	≤10	671	30.4	80	31	45	76	59	164	599
5463	1	0	10^2	0	305	3.0	143	78	17	95	18	98	
	2	≤10	10^4	10	165	26.0	143	82	311	393	79	213	507
5464	1	0	10^2	≤10	244	1.3	218	70	47	117	40	108	
	2	≤10	10^5	0	476	1.4	218	65	12	77	16	96	−37
Vostochno-Anzirskoe oilfield													
1010*	1	10^6	10^4	Nd	293	Nd	732						
245D	1	10^2	10^3	10^2	171	1.2	147	90	0	90	0	198	
	2	0	10^6	10^4	342	2.3	147	31	26	57	46	65	−4
1012	2	Nd	Nd	Nd	756	9.8	147	28	53	81	65	137	42
1009	2												71

* Injection well, other wells are production wells. Nd means no data.

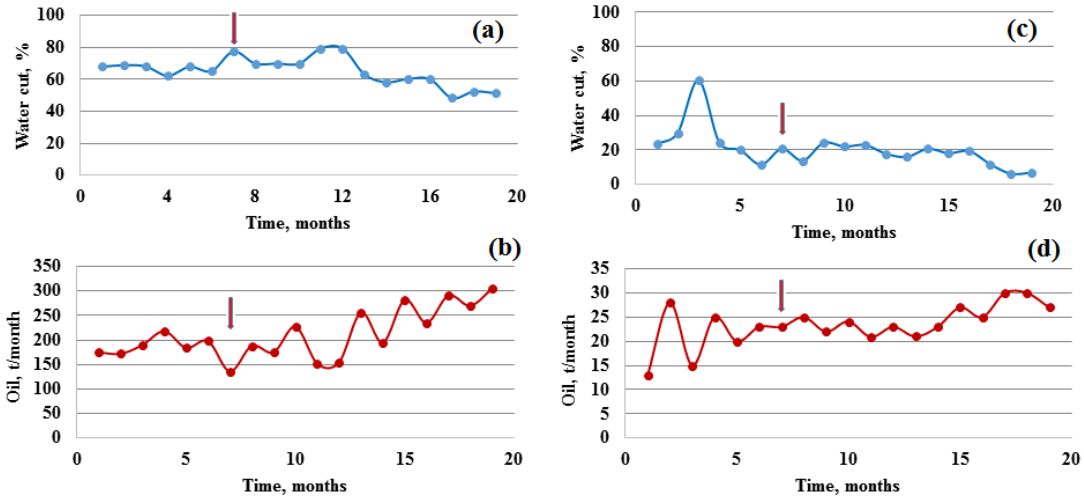

Figure 5. Water cut (%, **a**,**c**) and oil recovery (t/month, **b**,**d**) from production wells 5463 (**a**,**b**) and 1009 (**c**,**d**) during the baseline period and during the biotechnology trial at the Cheremukhovskoe and the Vostochno-Anzirskoe oilfields, respectively. The arrow designates the beginning of the biotechnological treatment.

4. Conclusions

Our results indicate that the studied low-temperature water-flooded heavy oil reservoirs (Russia) contain microbial populations capable of producing oil-displacing metabolites.

Aerobic bacteria *Rhodococcus erythropolis* HO-KS22 and *Gordonia amicalis* 6-1 isolated from oil reservoirs were capable of oxidizing heavy crude oil, and produce biosurfactants which significantly decrease the surface and interfacial tension of the media. *G. amicalis* 6-1 and *R. erythropolis* HO-KS22 have potential for applications in paraffin control, microbial enhanced oil recovery (MEOR), and the bioremediation of hydrocarbon-polluted environments.

The aerobic microbial community of heavy oil reservoirs was poor. Thus, its enrichment (bioaugmentation) with hydrocarbon-oxidizing bacteria adapted to oilfield conditions is a prerequisite for application of the MEOR technologies based on oil oxidation.

Fermentative bacteria from heavy oil reservoirs did not grow on oil; however, they produced volatile fatty acids, alcohols, and gases (H_2 and CO_2) when growing on sugar-containing substrates.

In core-flooding tests, liquid cultures of aerobic bacteria-producing biosurfactants were more effective in heavy oil displacement than fermentative bacteria-producing low fatty acids, alcohols, and gas, which resulted in a recovery of 43.0–53.5% and 13.4–45.5% of additional oil.

The pilot trial at the Cheremukhovskoe oilfield confirmed the efficiency of the MEOR technology based on the introduction of hydrocarbon-oxidizing bacteria together with injection of the oxidant as an H_2O_2 solution and of $(NH_4)_2HPO_4$ as a nitrogen and phosphorous source for low-temperature heavy oil reservoirs.

5. Patents

Borzenkov, I.A.; Sokolova D.S.; Nazina, T.N.; Babich, T.L.; Semenova, E.M.; Ershov, A.P.; Khisametdinov, M.R. *Gordonia amicalis* strain with ability of generation directly in oil reservoir of oil-displacing agent—bioPAV and decreasing content of organosulfur compounds of oil. Patent RU No. 2,673,747 C1. Published: 29.11.2018. (In Russian). http://www1.fips.ru/fips_servl/fips_servlet?DB=RUPAT&DocNumber=2673747&TypeFile=html

Author Contributions: Conceptualization, T.N. and I.B.; Data curation, D.S. (Dmitriy Serdukov) and M.K.; Formal analysis, D.S. (Diyana Sokolova), D.G. and M.K.; Investigation, D.S. (Diyana Sokolova), D.G., E.S., T.B., S.B., D.S. (Dmitriy Serdukov), A.E., M.K. and I.B.; Methodology, M.K.; Project administration, T.N., D.V., K.B. and I.B.; Resources, D.V. and K.B.; Supervision, T.N.; Writing—original draft, T.N., D.S. (Diyana Sokolova), D.G., E.S., M.K. and I.B.; Writing—review and editing, T.N. All of the authors read and approved the submitted manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. Characteristics of the studied petroleum reservoirs.

Parameters	Cheremukhovskoe Oilfield	Arkhangelskoe Oilfield	Romashkinskoe Oilfield, Almetievskaya Bed	Vostochno-Anzirskoe Oilfield
Horizon/sedimentation	Bobrikovsky	Bashkirian deposits	Devonian	Kynovsky
Lithology	Terrigenous, sandstone	Organogenic limestones with sandstone and dolomites	Terrigenous, sandstone, siltstone	Terrigenous, sandstone
Average depth below sea level, m	1225	830	1640–1650	1586
Average porosity, %	24.0	12.8	18.0	20.5
Average permeability, μm^2	0.479	0.278	0.400	0.176
Effective oil-saturated reservoir capacity, m	2.0	3.2–12.8	3.0	2.4
The formation temperature, °C	20.2–21.3	20	40	23.3
Oil density in surface condition, kg/m^3 (20 °C)	0.932	0.924	0.813	0.856
Oil viscosity in reservoir condition, mm^2/sec	72.6	43.9–161.9	3.5–6.5	19–86.2
Water cut (average), %	51	68	89	33

Table A2. Rates of sulfate reduction and methanogenesis in production water of the Arkhangelskoe oilfield.

Well No.	Concentration, mg·l^{-1}			Methanogenesis Rate, nl CH_4·L^{-1}·day^{-1} from		Sulfate Reduction Rate, ng S^{2-} L^{-1}·day^{-1}
	HCO_3^-	Acetate	SO_4^{2-}	HCO_3^-	Acetate	
4308	122	1.8	439	0	67.2	0
4634	573	38	1504	0	1722	2610
4647A	305	1.2	960	0	0	0
7492	708	6.1	1579	0	0	0
7806	622	1.8	804	0	8.4	750

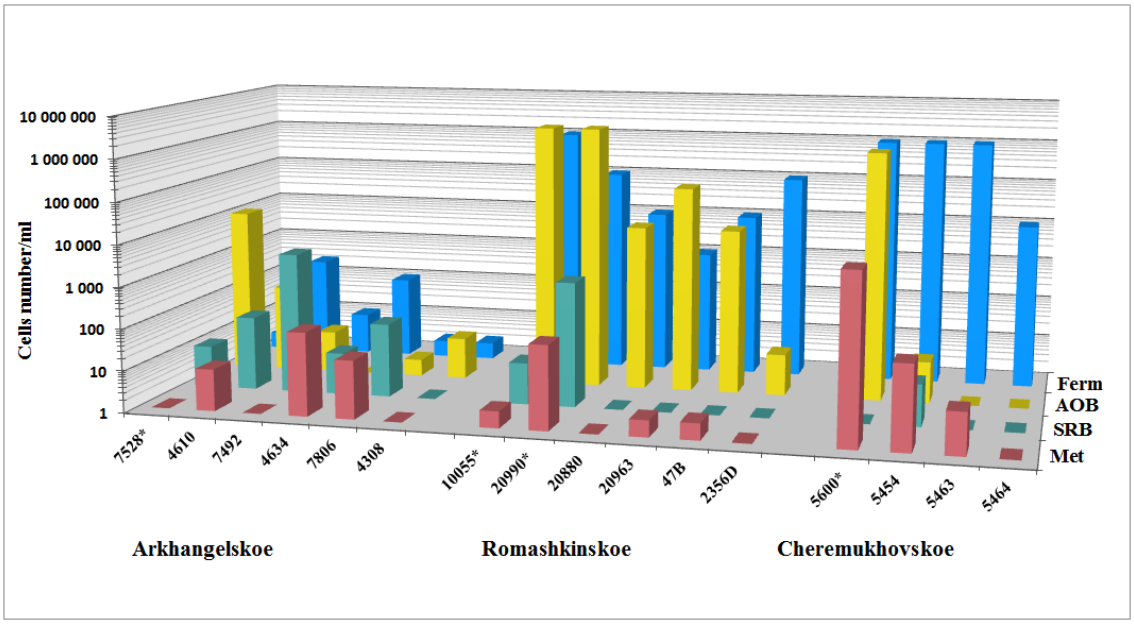

Figure A1. Numbers of aerobic and anaerobic microorganisms in injection and production water samples from the Arkhangelskoe, Romashkinskoe and Cheremukhovskoe oilfields. Designations: Ferm—fermentative bacteria, AOB—aerobic organotrophic bacteria, SRB—sulfate-reducing bacteria, Met—methanogenic archaea. Injection well marked with *, the rest are production wells.

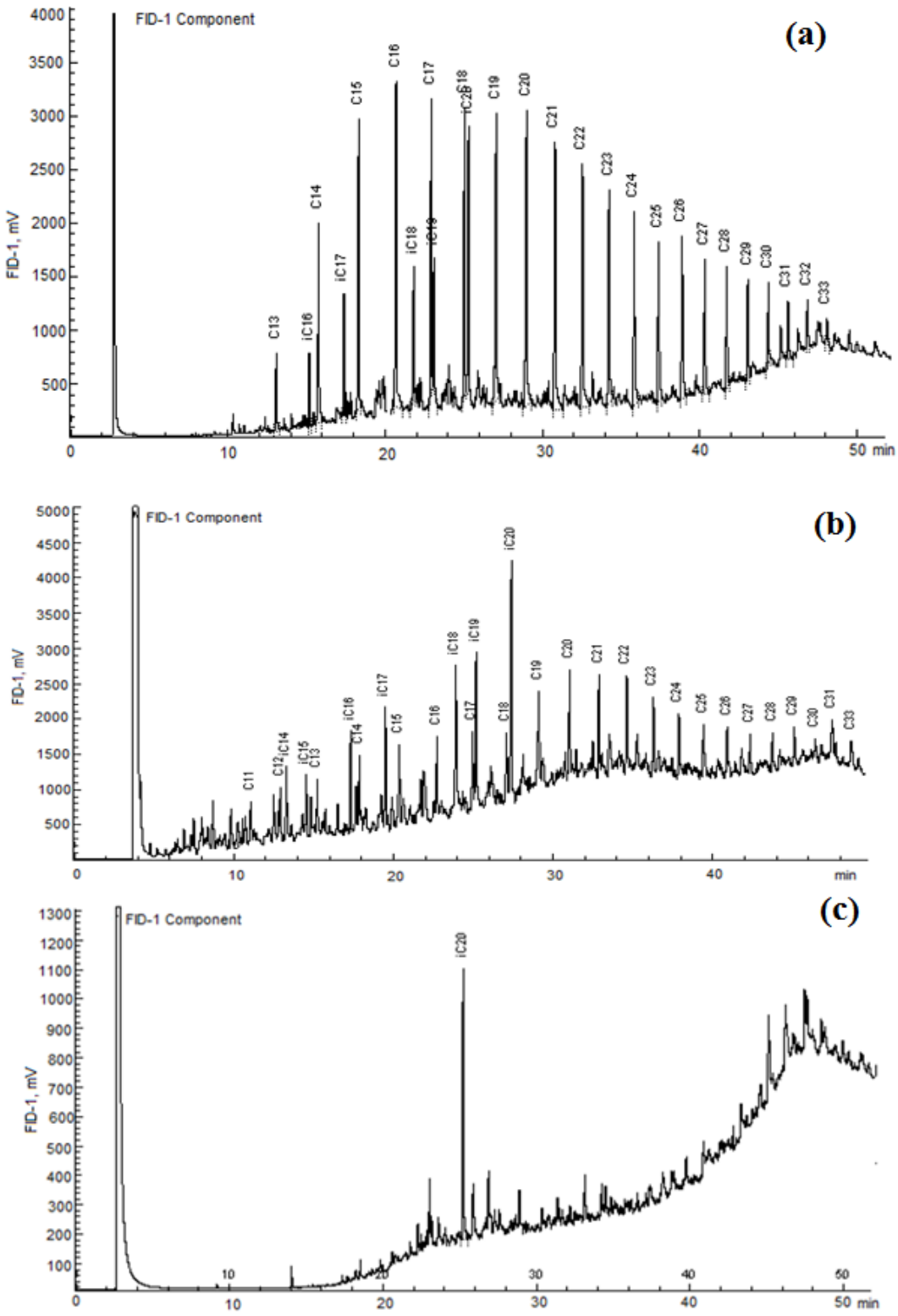

Figure A2. Chromatograms of *n*-alkanes of heavy oil from well 5452 (control, **a**), oil degraded by *Gordonia amicalis* strain 6-1 (**b**) and by *Rhodococcus erythropolis* HO-KS22 (**c**). Strains were incubated in the presence of 10 g oil L^{-1}. Strain 6-1 was incubated for 20 days at 23 °C, strain HO-KS22 for 7 days at 28 °C.

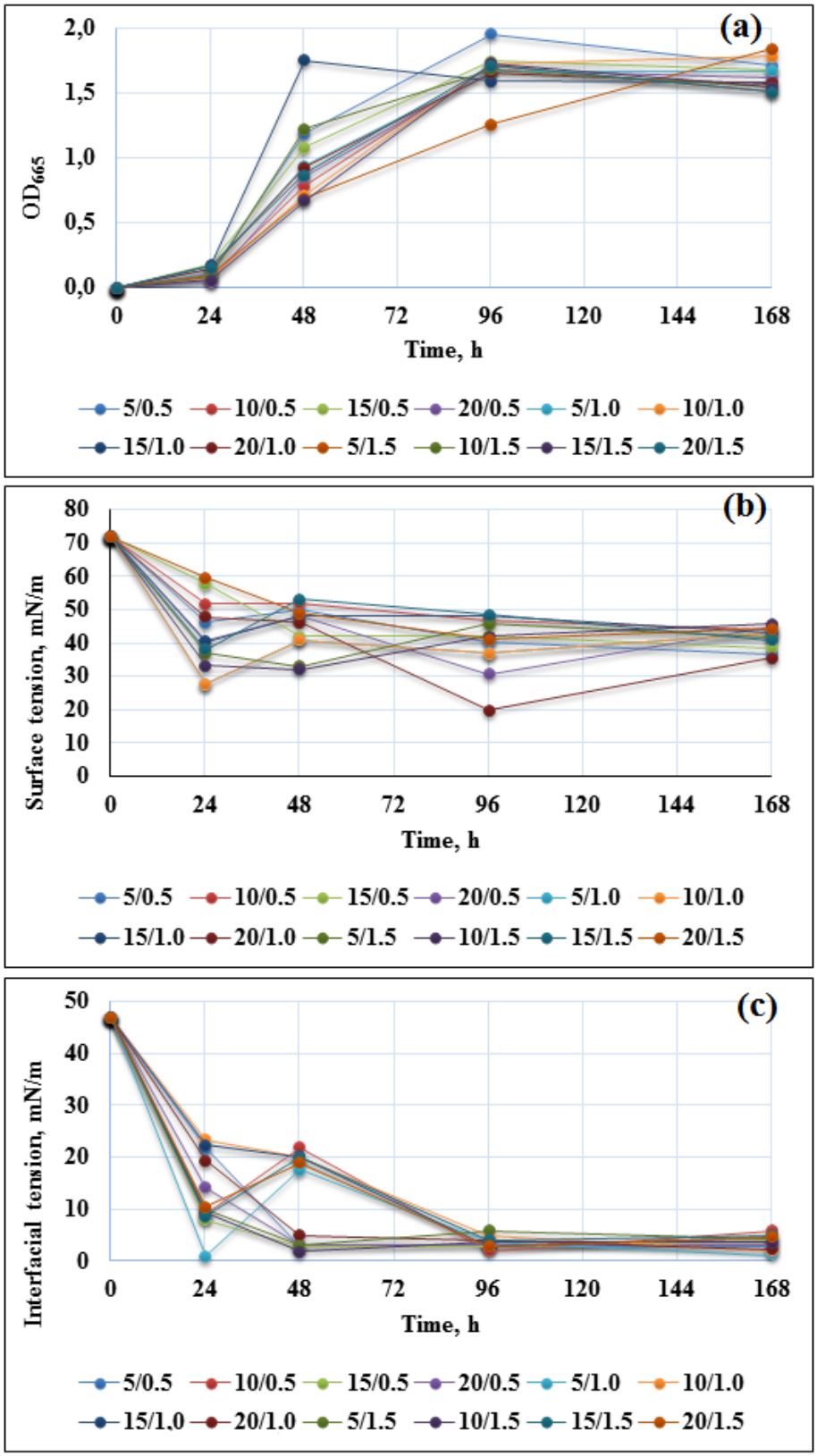

Figure A3. Accumulation of biomass (OD_{665}, **a**) by *G. amicalis* 6-1 strain in media with different glucose–nitrate ratios (g/g in L^{-1}) and change of surface tension (**b**) and interfacial tension of media against hexadecane (**c**) in dynamic.

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