

Research Article

Effects of Air Injection on the Metabolic Activity of Emulsifier-Producing Bacteria from Oil Reservoirs

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Oil emulsification is one of the major mechanisms for microbially enhanced oil recovery (MEOR). Although air injection is generally recommended for field trials of MEOR in China, its influence on the microbial community structure in oil reservoirs remains poorly understood, especially activation of emulsifier-producing bacteria. Herein, the effects of air injection on oil emulsification, nutrient consumption, oil properties, and microbial community structures were compared for activated cultures under four different oxygen content conditions: anaerobic, facultative anaerobic, intermittent aeration, and aerobic. The results showed that crude oil in aerobic and intermittent aeration cultures was emulsified effectively when nutrients were thoroughly depleted. The particle diameter of emulsified droplets was 4.74–10.02 μm . High-throughput sequencing results showed that *Bacillus* and *Aeribacillus* were effectively activated under aerobic and intermittent aeration conditions, while *Tepidimicrobium* and *Coprothermobacter* were activated under facultative anaerobic and anaerobic conditions. Real-time quantitative PCR results showed that the initial emulsifying effect was positively correlated with the abundance of *Aeribacillus pallidus*.

1. Introduction

Crude oil is a nonrenewable fossil fuel buried in the lithosphere and a basic energy source for industrialisation and economic development [1]. However, due to technical constraints, the average recovery ratio for conventional reservoirs in China is currently less than 35%; according to existing geological reserves and mining speeds, the country will face significant energy security risks in the near future. For this reason, the “National Medium- and Long-Term Science and Technology Development Plan (2006–2020)” includes “exploration and utilisation of complex geological oil and gas resources” as a key area and priority theme for future research and development [2]. At present, the main problem for Chinese oil exploitation is that most of oil fields have entered the late stages of production with the characteristic of high water cut. Traditional processing technologies have struggled to meet the needs of postwater flooding and chem-

ical flooding processes. Therefore, exploring and researching sustainable and economically effective oil recovery technologies are of great strategic significance for maintaining the sustainable development of the Chinese petroleum industry and the national economy [3].

Microbially enhanced oil recovery (MEOR) is a new technology based on injecting a large amount of microbial liquid (exogenous MEOR) into an oil reservoir, or using nutrients to stimulate the metabolic activities of indigenous microorganisms (indigenous MEOR) underground, followed by interaction with underground oil and oil reservoirs to enhance oil recovery. Between 40 and 45% of the world's oil reservoirs have great potential for the application of microbial flooding approaches [4]. A large number of laboratory experiments and field trials related to MEOR indicated that reduction of water cut can be reduced and oil recovery can be enhanced during the exploitation process. For example, biosurfactant-producing *Pseudomonas aeruginosa*

was injected into 60 production wells in Daqing Oilfield in China, and enhanced oil recovery was detected in 80% of experimental wells after shutting these wells for a month [5]. From 2001 to 2005, two field trials were carried out in Dagang Oilfield; 722 m³ of exogenous bacteria (*Arthrobacter* sp., *Pseudomonas* sp., and *Bacillus* sp.) and 8400 m³ of nutrients were injected into the oil reservoir through injection wells in the Guan 69 block, and 8700 t of incremental oil was exploited during this process [6]. In Kongdian and Gangxi blocks, 18,217 t of incremental oil was acquired by injecting nutrients, air, or H₂O₂ to activate indigenous microorganisms [7, 8]. From 2010 to 2011, Xinjiang Oilfield in China carried out a field trial of MEOR in the Liuzhong District, in which 15,600 m³ of nutrients and 62,400 m³ of air were injected into the oil reservoir and 1872 t of incremental oil was obtained [9]. In Bebee Oil Field in the United States, biosurfactant-producing bacteria were similarly injected into oil reservoirs through production wells in 2005 and 2011, and water cut was reduced in concert with enhanced oil recovery [10, 11]. A common feature of these successful field trials is that biosurfactant production or emulsification of crude oil occurred during the experimental process.

Among oil displacement mechanisms mediating MEOR, biosurfactant production and crude oil emulsification are currently considered to be one of the key factors driving enhanced oil recovery [12, 13]. Therefore, research has focused on functional microorganisms in microbial flooding technology, especially those capable of producing surfactants to maintain their high metabolic activity. Existing biosurfactants mainly include glycolipids (rhamnolipids, sophorolipids, and trehalolipids), lipopeptides (surfactins, lichenysins), some proteins, and bioemulsifiers [14, 15]. Small-molecule surfactants can not only reduce oil/water interfacial tension but also emulsify crude oil, while macromolecular emulsifiers have better emulsifying ability and emulsion stability [16]. Biosurfactant-producing microbes mainly include *Acinetobacter*, *Alcaligenes*, *Bacillus*, *Enterobacter*, *Pseudomonas*, and *Rhodococcus*, together with some strains in the genus *Serratia*, most of which are strictly aerobic bacteria [16, 17]. Therefore, the injection of air during the microbial flooding process is essential for activating surfactant-producing bacteria. Herein, a series of laboratory stimulation experiments were performed to determine the optimal air injection regime, probe the influence of air injection on the activation of emulsifier-producing bacteria, and explore shifts in the microbial community structure and the properties of crude oil during air-coupled MEOR. Although these experiments do not completely simulate the pressure of actual oil reservoirs or the migration behaviour of microorganisms and crude oil in porous media, the results provide important reference values for field trials of MEOR.

2. Materials and Methods

2.1. Experimental Samples. Baolige Oilfield is located in the Nanxun trough of the Bayindulan depression in the Erlian Basin. Experimental samples were collected from the Ba 19

and 38 fault blocks located in the Ba-II structural belt [18]. For sample collection, the sampling valve of the production well was opened to release the dead volume, and a sterile plastic bucket (25 L) was filled with production water and sealed to prevent air entering and affecting the microbial community composition. Samples were delivered to the laboratory within 24 h and stored at 4°C.

2.2. Nutrients. Optimised nutrients were selected according to the nutritional requirements of microorganisms and the ionic composition of production water. The specific composition was 0.4% glucose, 0.2% corn steep powder, 0.27% NaH₂PO₄, and 0.38% NH₄Cl. Nutrients (except glucose) and glucose were separately mixed with 50× mother liquor using distilled water and sterilised at 121°C for 30 min.

2.3. Simulation Experiments. A 94 mL sample of production water, 2 g crude oil, and 2 mL 50× sterilised mother liquor containing nutrients (except glucose) and glucose were added to a 250 mL anaerobic bottle, and cells were cultivated at 58°C with shaking at 200 rpm in the presence of different amounts of oxygen. For aerobic cultivation, bottles were sealed with gauze. For anaerobic cultivation, bottles were sealed with a rubber stopper and air was completely replaced with nitrogen. For facultative anaerobic cultivation, bottles were sealed with a rubber stopper and the ratio of air and liquid in the bottle was 1.5:1. For intermittent aeration cultivation, gas in the bottle for facultative anaerobic cultivation was replaced with sterile air every day.

2.4. Evaluation of the Oil Emulsification Effect. The size of oil droplets in the emulsified culture was measured to evaluate the crude oil emulsification effect. Firstly, the emulsified culture was appropriately diluted with sterilised water to ensure the desired number of dispersed oil droplets in the field of the microscope. A drop of a diluted sample was then placed on a glass slide for image acquisition using an optical microscope. Following acquisition, the image was binarised using Micro-Color Image Analysis System v5.2 (Zhongchen, Shanghai, China). The binary filtering function of the software was used to filter out pixels with a very small area, images with voids in the centre of binarisation were filled using an image processing tool, and particles on the boundary or bonded together were removed or divided by the image processing tool to generate separate particles. Next, the particle distribution calculation function of the software was used to calculate particle distribution parameters. These parameters included the perimeter, area, equivalent diameter, maximum diameter, and minimum diameter of each particle. The calculated values were saved in the system database, imported into a text document, and statistically analysed by SPSS (IBM, New York, USA).

2.5. Surface Tension Measurement. The surface tension of the culture was measured by a POWEREACH JK99B automatic interfacial tension meter (Zhongchen, Shanghai, China) according to the instruction manual.

2.6. Determination of Residual Sugar. The remaining glucose in the culture was measured using a SBA-40C biosensor (Yanke, Shandong, China) according to the instruction manual.

2.7. Determination of Crude Oil Viscosity. The emulsification viscosity and dehydration viscosity of crude oil were measured using a Brookfield CAP2000+ (Brookfield, New York, USA) cone and plate viscometer and measured with a 6# rotor at 58°C and 400 rpm. When the emulsified viscosity of the crude oil was measured, 1 mL of emulsified crude oil was directly measured. Before determining the dehydration viscosity of crude oil, the sample was dehydrated [19], and 1 mL of dehydrated crude oil was taken for viscosity measurement.

2.8. Analysis of the Alkane Composition of Crude Oil. Cultures in anaerobic bottles were extracted three times with an equal volume of n-hexane, the extracted organic phases were combined, and squalene was added to a final concentration of 0.008% (*w/v*) as an internal standard. An Agilent 6820 gas chromatography system (Agilent, California, USA) equipped with an on-column injection, FID detector, and HP-5MS capillary column (30 m × 0.53 mm i.d., 1.5 μm thickness) was employed for gas chromatography. Nitrogen was used as a carrier and set at a constant flow rate of 35 mL/min. Chromatography involved an inlet temperature of 320°C, an initial column temperature of 80°C for 5 min, a 5°C/min gradient to 290°C for 35 min, and a detector (FID) temperature of 320°C.

2.9. DNA Extraction. A 10 mL sample of culture was centrifuged at 12,000 rpm for 10 min to collect cells. The pellet was resuspended in 1 mL lysis buffer (0.05 mol/L TRIS, 0.04 mol/L EDTA, and 0.1 mol/L NaCl (pH 8.0)), the suspension was added to a grinding tube containing 0.3 g of 0.1 mm glass beads, shaken at 4800 rpm for 1 min, and allowed to stand for 1 min, and this was repeated three times. A 100 μL sample of lysozyme (100 mg/mL) was added to the grinding tube, mixed, and incubated at 37°C for 1 h. A 100 μL sample of 20% sodium dodecyl sulphate (SDS) was then added, mixed, and incubated at 65°C for 30 min. DNA was finally extracted using a bacterial genomic DNA mini-prep kit (Axygen, New York, USA) and stored at -80°C.

2.10. Analysis of the Microbial Community Structure. Extracted genomic DNA was subjected to quality testing and sent to Majorbio (Shanghai, China). The V4 region of the 16S gene was amplified using primers of 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGAC TACHVGGTWTCTAAT-3') and the extracted genomic DNA before high-throughput sequencing. The amplified product was sequenced on an Illumina HiSeq2500 platform. All sequencing data were submitted to the Majorbio cloud (<https://cloud.majorbio.com/>) for microbial community structure analysis online.

2.11. Real-Time Quantitative PCR Analysis of Functional Bacteria. Abundance of functional bacteria was analysed by real-time quantitative PCR. The reaction system comprised

TABLE 1: Real-time quantitative PCR analysis primers.

Gene name	Primers
16S	27F: 5'-AGAGTTTGATCCTGGCTCAG-3'
	1429R: 5'-GGTTACCTTGTTACGACTT-3'
<i>Aeribacillus pallidus</i>	q16SF: 5'-ACCACGAGAGTTTGTAACACCC-3'
	qgap1R: 5'-CGTACCCTATTTCAATGTAT ATATC-3'
Lipase	qlipF: 5'-CCTGGCAACTCCGCATAAT-3'
	qlipR: 5'-CTTGAGCCCCAAAGGTAGTTAA-3'
alkB	For fusion primer sets, see Li et al. [23]

FastStart Universal SYBR Green Master Mix (10 μL), 6 pmol of each of the quantitative primers (Table 1), 1 μL of template DNA, and 20 μL of ddH₂O. PCR amplification was performed at 95°C for 10 min, followed by 40 cycles at 95°C for 20 s and 60°C for 1 min to measure the fluorescence signal, with an increase of 0.5°C per cycle from 60°C to 95°C for 10 s per cycle to obtain a dissolution curve. Three replicates were included for each reaction system, and the DNA copy number was calculated from the standard curve.

3. Results and Discussion

3.1. Emulsification of Crude Oil under Different Redox Potential Conditions. In the long-term water flooding process of reservoirs, although injected water brings a small amount of nutrients and dissolved oxygen into the reservoir environment, it cannot completely alter the environmental characteristics of the reservoir and oxygen, nitrogen, and phosphorus remain lacking. Therefore, in order to maximise microbial metabolism in reservoirs, improve the fluidity of crude oil, and increase the swept volume of injected water, it is common to inject nutrients (including carbon, nitrogen, and phosphorus sources) and air into the reservoir to enhance the metabolic activity of microorganisms, and this technology is known as MEOR [20]. The introduction of oxygen can stimulate the metabolic activity of microorganisms in reservoirs, thereby improving the emulsification and recovery of crude oil [7–9, 21]. However, determining the optimal amount of air injection in the microbial flooding process remains challenging.

In order to determine the effect of air injection on the emulsification of crude oil during the microbial flooding process, a series of laboratory simulation experiments were carried out in anaerobic bottles. The amount of air injected was manipulated by controlling the gas-liquid ratio in anaerobic bottles during the experiment. There was no air introduced in the anaerobic culture, unlimited air was introduced in the aerobic culture, the air-liquid ratio of the facultative anaerobic culture was 1.5:1, and the air-liquid ratio upon intermittent ventilation was 1.5:1-7.5:1. The experimental results showed that crude oil in the aerobic culture began to be emulsified on the second day and achieved a maximum emulsification effect on the third day,

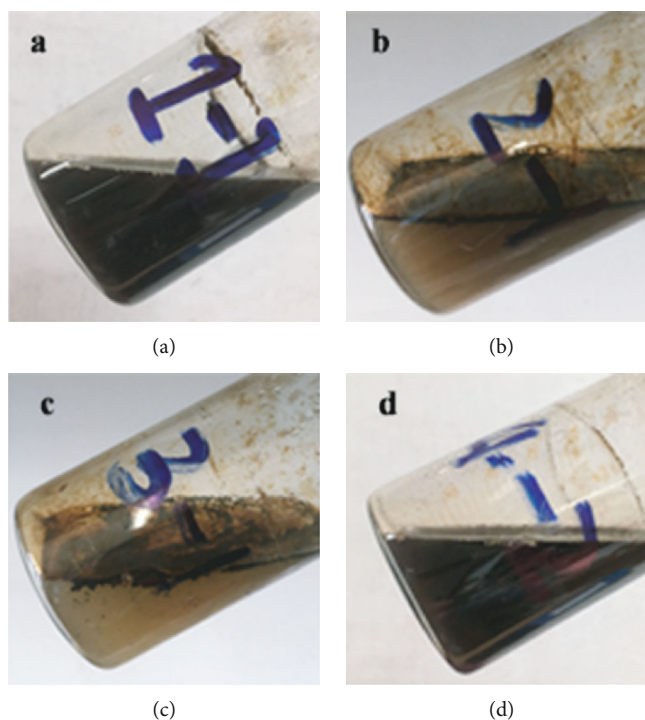


FIGURE 1: Emulsification of crude oil in aerobic culture (a), facultative anaerobic culture (b), anaerobic culture (c), and intermittent aeration culture (d).

TABLE 2: Physicochemical properties of different cultures.

Oxygen condition	Oil droplet size (μm)	Surface tension (mN/m)	Residual sugar (mg/100 mL)	Crude oil viscosity (cP)	Emulsifying viscosity (cP)	Dehydration viscosity (cP)
Aerobic	4.74	52.40	124	184	67	274
Intermittent aeration	10.02	53.44	135		71	263
Facultative anaerobic	—	46.24	2		150	187
Anaerobic	—	50.21	2		166	182

and this was maintained until day six. Crude oil in the intermittent aeration culture began to be emulsified on the third day, the emulsification effect was greatest on the fourth day, and this was also maintained until the sixth day. Crude oil in the facultative anaerobic culture began to be emulsified on the fourth day, but the emulsification effect was not optimal until day six, after which the cultivation process was stopped. Crude oil in the anaerobic culture was not emulsified until the sixth day (Figure 1). These results indicate that air is very important for activating emulsifier-producing bacteria in oil reservoirs. To ensure effective activation of emulsifier-producing bacteria and to maintain high metabolic activity, the volume of injected air should be 4.5-fold higher than that of injected water. If the injected air-liquid ratio is less than 1.5, emulsifier-producing bacteria in the reservoir are not effectively activated, which may be the main reason for the poor emulsification of crude oil in the facultative anaerobic and anaerobic cultures.

3.2. Physicochemical Properties of Cultures. Physicochemical properties of different cultures were analysed to further

determine the causes of the different emulsification effects in different cultures, including oil droplet size, surface tension, residual sugar, crude oil viscosity, and alkane degradation (Table 2 and Figure 2). Glucose was nearly exhausted in all cultures after 6 days of cultivation, indicating that differences in the emulsification effects among cultures could not be explained by inadequate activation of the metabolic activity of microorganisms.

The results of surface tension measurements indicate that the emulsification of crude oil in cultures may be at least partially dependent on the production of emulsifiers because the surface tension of cultures will be lower than 40 mN/m if small-molecule biosurfactants contribute to the emulsification of crude oil [16]. Although the emulsification of crude oil in the aerobic culture and the intermittent aeration culture appeared to be similar, the droplet size and uniformity of emulsified oil in the aerobic culture were clearly superior to those in the intermittent aeration culture (Figure 3). These results also indicate that oxygen can promote the production of emulsifiers during the activation of microorganisms in reservoirs. The emulsifying viscosity of

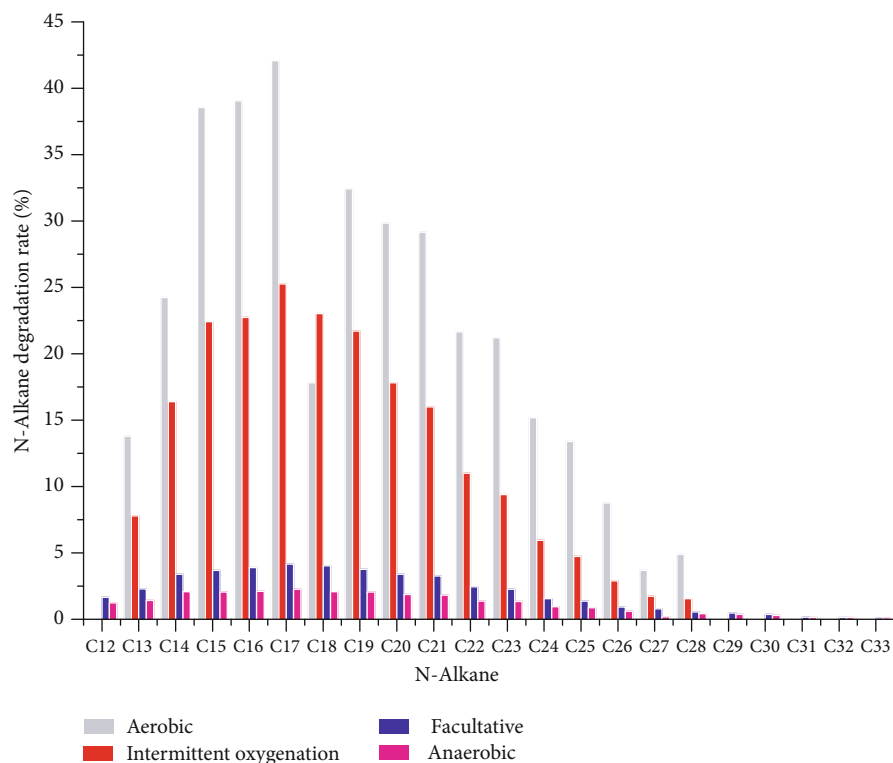
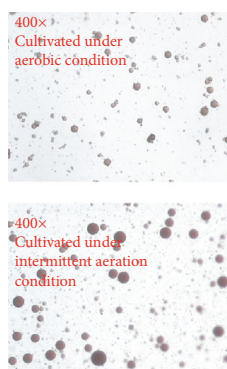


FIGURE 2: Degradation of alkanes of different chain lengths in different cultures.



Parameters	Aerobic	Intermittent oxygenation
Valid samples	366	159
Mean	4.74	10.02
Std. deviation	2.25	5.58
Range	19.23	31.95
Minimum	1.87	4.17
Maximum	21.10	36.12
Percentiles		
50	4.36	11.47
97	9.60	23.18

FIGURE 3: Morphology of emulsified oil droplets in cultures.

crude oil in the aerobic culture and intermittent aeration culture decreased by 63.6% and 61.4%, respectively (Table 2). These results indicate that activation of emulsifier-producing bacteria has the potential for improving the mobility of crude oil in reservoirs.

However, unlike the emulsifying viscosity of crude oil, the dehydration viscosity of crude oil in the aerobic culture and intermittent aeration culture increased by 48.9% and 42.9%, respectively (Table 2), which was negatively correlated with the emulsification effect. In order to further determine the cause of this increase in dehydration viscosity of crude oil, the degradation of alkanes in each culture was analysed. The results showed that the degree of degradation of alkanes in crude oil increased with increasing oxygen concentration and alkanes of medium chain length (C14-C23)

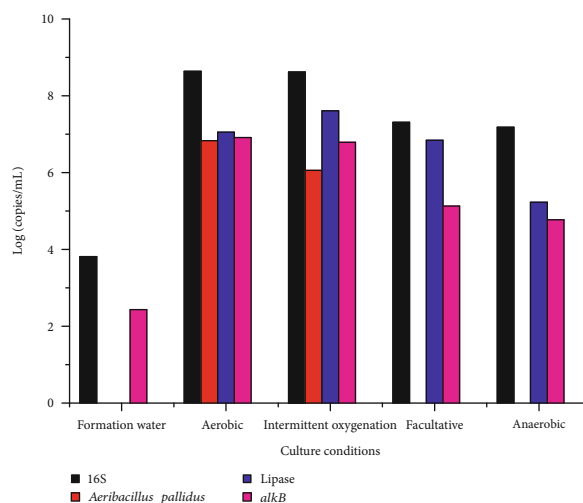


FIGURE 4: Abundance of emulsifying bacteria in different cultures.

were degraded faster than those with longer chains ($\geq C29$; Figure 2). Degradation of alkanes of medium chain length is the main reason for the increase in dehydration viscosity of crude oil. Quantitative analysis indicated that abundance of the *alkB* gene was 8.13×10^6 copies/mL in aerobic culture, 6.17×10^6 copies/mL in intermittent aeration culture, 1.35×10^5 copies/mL in facultative anaerobic culture, and 5.89×10^4 copies/mL in anaerobic culture (Figure 4). These results were positively correlated with the biodegradation degree of alkanes in cultures (Figure 2). The *alkB* gene encodes alkane hydroxylase, an enzyme responsible for the

initiation of alkane degradation in different culture systems. AlkB is widely distributed in nature, and medium-chain alkanes are its optimum substrates [22].

3.3. Microbial Community Structure in Different Cultures. Reservoirs are extreme ecological environments featuring high pressure, hypoxia, and oligotrophy. Except for hydrocarbons, nitrogen, phosphorus, and organic carbon sources for microorganism growth and metabolism are extremely scarce in oil reservoirs [1]. However, even in such an extreme environment, there are still a number of indigenous microorganisms (typically less than 10^5 CFU/mL) that convert hydrocarbons into methane and carbon dioxide at extremely slow rates under anaerobic conditions [23]. Microorganisms involved in this conversion of hydrocarbons form stable microbial communities in the reservoir ecosystem through metabolite transport and long-term competition. During the water flooding and MEOR, injected water and air introduce oxygen into the reservoir, and this disrupts the relative balance in the ecological environment of the reservoir, shifting the original microbial community to gradually form a new microbial community [23].

In order to clarify the effect of air injection on the microbial community structure of the reservoir, high-throughput sequencing was used to analyse changes in the microbial community structure under different cultivation conditions (Figure 5). At the phylum level, *Proteobacteria* was the dominant group in the production water, accounting for 95.1% of total bacteria, while *Firmicutes* dominated in aerobic, intermittent aeration, facultative anaerobic, and anaerobic cultures, accounting for 99.9%, 99.3%, 96.0%, and 81.1%, respectively. At the class level, aerobic and intermittent aeration cultures shared a similar community structure, in which the dominant group was *Bacilli*, accounting for 99.9% and 85.8%, respectively. Facultative anaerobic and anaerobic cultures also shared a similar community structure, with *Clostridia* and *Bacillus* dominating; *Clostridium* accounted for 64.0% and 56.9%, and *Bacillus* accounted for 32.0% and 24.2%, respectively. *Clostridium* were also relatively abundant (13.5%) in the intermittent aeration culture, and *Coprothermobacteria* was present in the anaerobic culture (11.5%). At the order and family levels, the distribution of dominant groups in each culture was similar to that at the class level. The distribution of dominant groups in different cultures was strictly restricted by the oxygen content; *Coprothermobacteria* are strictly anaerobic and only occur in anaerobic cultures [24], while *Bacillus* can grow under aerobic and facultative anaerobic conditions [25]. Thus, *Bacillus* can grow well in both aerobic and intermittent aeration cultures, but not in facultative anaerobic or anaerobic cultures.

3.4. Abundance of Emulsifying Bacteria in Different Cultures. In cultures with a superior emulsification effect on crude oil, *Bacillus thermolactis* and *Bacillus thermoamylovorans* were detected in large quantities, accounting for 15.3% and 5.0% in the aerobic culture and 8.4% and 25.2% in the intermittent aeration culture, respectively. In addition, another dominant bacterium, *Aeribacillus pallidus*, was detected in the aerobic culture (Figure 6). *A. pallidus*, a strictly aerobic

bacterium, is commonly found in production fluids of high-temperature reservoirs, hot springs, and oil-contaminated soils. Most strains belonging to this species have the ability to degrade hydrocarbons under aerobic conditions and can also produce biosurfactants to emulsify crude oil during the degradation process [26]. Herein, a strain belonging to *A. pallidus* was also isolated from the aerobic culture and named *A. pallidus* HB-1. Strain HB-1 could emulsify crude oil effectively but could not reduce the surface tension of the culture. Therefore, biosurfactants produced by HB-1 are likely to be emulsifiers.

Bacillus thermophilus is a facultative anaerobic bacterium, and most strains belonging to this species can produce extracellular lipases that possess multiple catalytic capabilities, including hydrolysis, alcoholysis, esterification, transesterification, and reverse synthesis of triacylglycerides and other water-insoluble esters [27]. The catalytic ability of lipases depends on the characteristics of the reaction system; ester hydrolysis is promoted at the oil/water interface, and enzymatic synthesis and transesterification occur in the organic phase. The affinity of lipases at the oil/water interface can stabilise the oil/water emulsion to some extent [27]. *Bacillus thermoamylovorans* is a newly reported species sharing a close genetic relationship with *Bacillus thermolactis* [28].

In order to further determine the correlation between the emulsification of crude oil and emulsifying bacteria in different cultures, specific primers for *A. pallidus* and lipase-encoding genes were used to measure the abundance of emulsifying bacteria in different cultures by real-time fluorescence quantitative PCR (Figure 4). Similar to the results of high-throughput sequencing, *A. pallidus* was not detected in the anaerobic or facultative anaerobic cultures, and the emulsification of crude oil and abundance of lipase-encoding genes in these cultures were positively correlated. Although the concentration of lipase-encoding genes in these cultures reached 6.92×10^6 copies/mL and 1.70×10^5 copies/mL, respectively, crude oil was not sufficiently emulsified, indicating that the lipase has weak emulsifying activity for crude oil. In aerobic and intermittent aeration cultures, *A. pallidus* was present at 6.76×10^6 copies/mL and 1.15×10^6 copies/mL, respectively, and the lipase-encoding gene was present at 1.12×10^7 copies/mL and 4.07×10^7 copies/mL, respectively. The emulsification of crude oil in aerobic and intermittent aeration cultures was strongly correlated with the abundance of *A. pallidus*, while the abundance of the lipase-encoding gene contributed less to the emulsification of crude oil.

4. Conclusions

- (1) Injection of air can dramatically alter the microbial community structure of oil reservoirs. The abundance of aerobic bacteria increases with increasing introduction of air, but anaerobic bacteria dominate the microbial community of oil reservoirs
- (2) Most biosurfactant-producing bacteria are aerobic or facultative anaerobic microorganisms. Injection of air

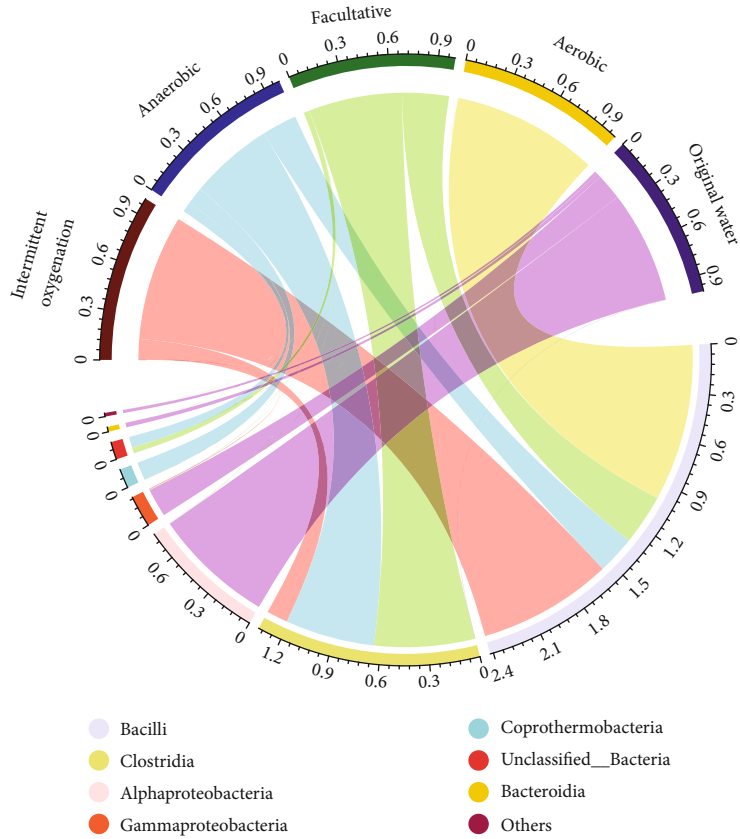
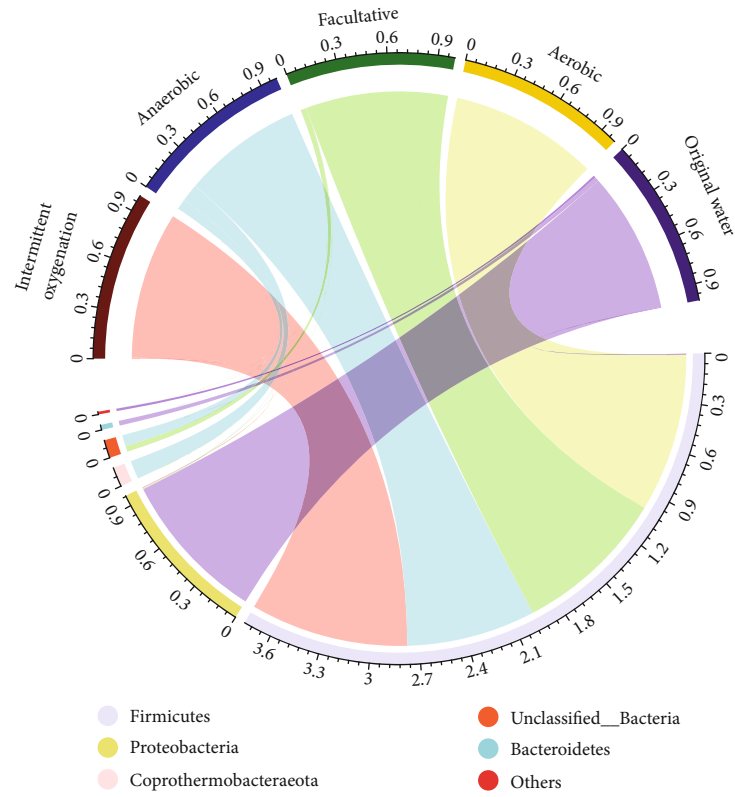


FIGURE 5: Composition of microorganisms at the phylum and class levels in different cultures.

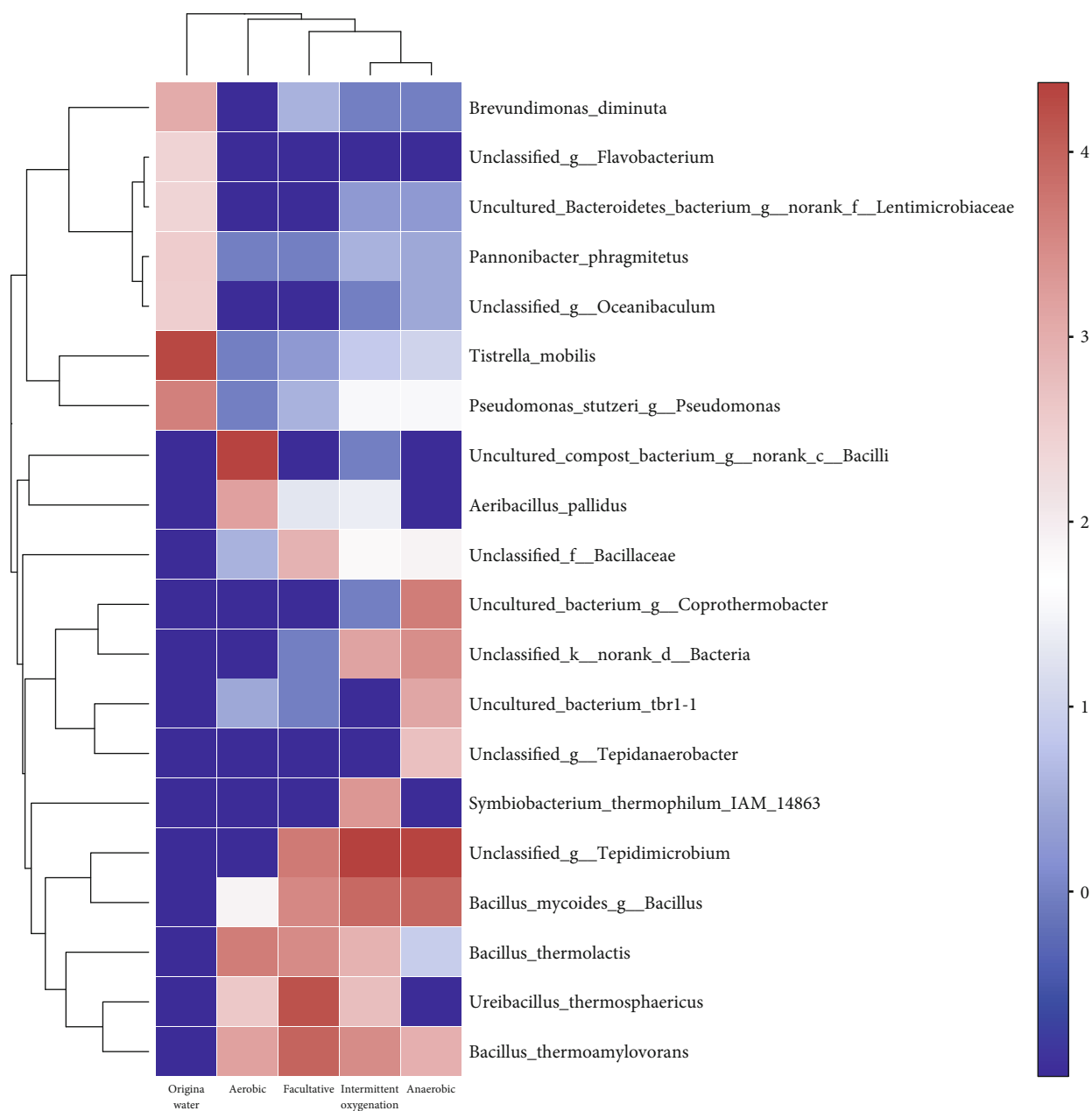


FIGURE 6: Species composition of microbial communities in different cultures.

during the MEOR process can increase the production of biosurfactants, which can emulsify crude oil and enhance oil recovery

- (3) According to the results of stimulation experiments, the abundance and metabolic activity of emulsifying bacteria can be improved by slug injection of air during MEOR, but the amount of injected air should be 4.5-fold higher than that of injected nutrients

Data Availability

The data used to support the findings of this study are available from the corresponding author.

Conflicts of Interest

The authors declare no conflict of interest.

Acknowledgments

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