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Research Article

Nutrient Stimulation of Indigenous Microorganisms for Oil-in-Water Emulsion in a Medium Temperature Petroleum Reservoir with Ca^{2+}-Rich Brine

Guoxin Shi,[1] Yuanyuan Jia,[2] Lei Bai,[1] Guoqiang Li,[2] Xiaoli Liu,[1] Meichen Liu,[2] Xuecheng Dai,[1] Xuefeng Tian,[2] Hongbo Wang,[1] and Ting Ma[2]

[1]*Experimental Inspection Research Institute, Xinjiang Oilfield Co. Ltd., Karamay 834000, China*
[2]*Key Laboratory of Molecular Microbiology and Technology, Ministry of Education, College of Life Sciences, Nankai University, Tianjin 300071, China*

Correspondence should be addressed to Ting Ma; tingma@nankai.edu.cn

Guoxin Shi and Yuanyuan Jia contributed equally to this work.

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One of the challenges indigenous microbial enhanced oil recovery (MEOR) is facing is the high percentage of divalent ions, which obstruct the growth and metabolism of microorganisms and destabilize the oil-in-water (o/w) emulsion. Six formulas were selected for the stimulation of indigenous microbes and to compare their performances on the oil emulsification and oil spreading in the Luliang oilfield containing Ca^{2+}-rich brine. Illumina MiSeq sequencing of 16S rRNA genes was applied to investigate the structural response of microbial communities to various formulas. The results showed that the addition of proper organic phosphorus and the optimal P/N ratio (0.01) can facilitate production of biosurfactant and create stable o/w emulsion with specific reservoir condition containing Ca^{2+}-rich brine, through direct stimulation of certain functional microbes. This study provides a promising path for direct enrichment of biosurfactant-producing and oil-degrading *Dietzia* genus and a potential instructional approach of indigenous MEOR in Luliang oilfield.

1. Introduction

Great efforts have been made in indigenous microbial enhanced oil recovery (IMEOR) technique as an environmentally friendly tertiary recovery method to release trapped residual oil from a porous medium. In this technology, in situ reconstruction of microbial community has been significantly focused along with the increasingly global concerns on environmental risks. Compared with other conventional oil recovery techniques, IMEOR is also able to save oil production costs with a decline in oil prices in recent years [1, 2].

IMEOR is a multidiscipline process that increases oil recovery through stimulation of microorganisms in a reservoir with an injection of specific nutrients for artificially directing metabolic activities to exert functions such as the degradation of heavy friction of crude oil, the formation of stable oil-water emulsions, low interfacial tension, increment of inner pressure, change of wettability, and redirection of injection fluids through clogging high permeable zones [3, 4]. Among these beneficial effects, the formation of stable oil-in-water (o/w) emulsion by biosurfactant has been well acknowledged with extensive evidences from worldwide laboratories and oilfield trials [5, 6]. Therefore, numerous literatures have reported the biosurfactant and its related producer from ex situ and indigenous environment [7–9]. Furthermore, these biosurfactant producers generally have inherent ability to improve oil production, such as the reduction of the content of C20-C40 alkane derivatives, biodegradation of higher molecular weight frictions of the crude oil, and biotransformation sulfur-containing organic compounds.

Although emulsification has successfully demonstrated great practicability in improving the oil production, the

Table 1: Stimulation formulas.

Formulas	Corn steep powder (%)	Molasses (%)	$NaNO_3$ (%)	NH_4Cl (%)	Crude oil (%)	CH_3COONa (%)	Soybean powder (%)
OR	—	—	—	—	—	—	—
OC	—	—	0.6	0.25	2	0.25	—
OCN	—	0.25	0.6	0.25	2	—	—
ONP1	0.15	—	0.6	0.25	2	—	—
ONP2	—	—	0.6	0.25	2	—	0.25
FN1	0.15	—	0.6	/	2	0.25	—
FN2	0.15	—	0.6	0.25	2	0.25	—

OR used as the control, referred to the original oil production water without nutrient addition.

Table 2: Chemical characteristics in the six formulas.

Ingredient (%)	OC	OCN	ONP1	ONP2	FN1	FN2
Ammonium nitrogen	0.2500	0.2513	0.2590	0.2520	0.0090	0.2590
Inorganic phosphorus	0	0	0.0011	0.0003	0.0011	0.0011
Total phosphorus	0	0.0001	0.0036	0.0018	0.0036	0.0036
Organic phosphorus	0	0.0001	0.0025	0.0015	0.0025	0.0025
OP/N ratio	0	0.0004	0.01	0.006	0.28	0.01

OP/N ratio refers to organic phosphorus divide by ammonium nitrogen.

similar challenge IMEOR facing is to emulsify crude oil under in situ reservoir condition of high salinity, especially of high bivalent cation, when compared with conventional surfactant oil recovery [10–12]. Furthermore, the higher percentage of divalent cations and salinity in the reservoir brine may impede the growth of indigenous microorganisms through the higher osmotic pressure, the inactivation of functional enzymes, or the deficiency of nutrients [13, 14]. Thus, how to successfully stimulate indigenous microorganisms and emulsify crude oil under the condition of high divalent ions and salinity require more efforts.

Herein, we targeted the oil formation water of Lu-9 oil well located in Luliang block (Xinjiang oilfield, China) where the high content of Ca^{2+} and salinity was detected in formation brine. The characteristics of the selected oil reservoir have been elaborated in the previous studies [15]. 11.48 million tons of the recoverable reserve attracted the innumerable investigations of oil recovery techniques, including chemical EOR, CO_2 flooding, and thermal recovery. However, few of the trials were successfully achieved due to the high percentage of divalent ions (Ca^{2+}) [16]. Compared with conventional technologies for oil recovery in this oilfield, IMEOR is a suitable and practical technique for a number of reservoirs [17]. Although the potential implementation of IMEOR has been investigated, the efforts were mostly made on the connections between emulsification and oil degradation. However, the understanding of microbial community structure and dominating functional bacteria after stimulation is still limited. Moreover, it is necessary to explore the interactions between microbial structure and community function when indigenous microorganisms stimulate oil reservoirs, especially those exposed under Ca^{2+}-rich brine.

In this study, we compared six optimized formulas for indigenous stimulation to create stable o/w emulsion of the targeted reservoir and tentatively elaborated the relationships among the emulsification, microbial diversity, and functional bacteria via Illumina MiSeq sequencing of 16S rRNA genes. The real-time PCR was employed to quantify the key gene for the well-known glycolipid biosurfactant production. The approach for direct stimulation of biosurfactant-producing bacteria, such as *Dietzia* genus, was further expounded. This study provided a practical way for applying IMEOR in the targeted oil reservoir and especially a technical strategy of oil recovery in the reservoir with a high percentage of divalent ions.

2. Materials and Methods

2.1. Sample Collection and Stimulation with Various Formulas for Emulsification. The produced water, containing indigenous microorganisms of the petroleum reservoir, is separated into crude oil and formation water after static stratification. The crude oil was collected from the Lu-9 oil well located in Xinjiang oilfield, and the formation water was collected from nine adjacent oil wells located at the Luliang block. The crude oil and liquid samples were placed in 25-liter sterile plastic buckets, sealed, and transferred to a cool and dry place in the laboratory for further analysis. After characterization, the physical and chemical properties and the formation conditions of Lu-9 are suitable for MEOR. More details about the targeted oilfield are tabulated in Supplementary Table S1.

Based on the previous reports [15, 18–20], six formulas (Table 1) were optimized for the stimulation of indigenous microorganisms with calcium-rich formation brine to evaluate the emulsifying ability of various nutrients. Each formula was composed of various organic or inorganic substrates, and the elemental compositions of each formula were analyzed. As shown in Table 2, the formulas varied in crucial

components. The six formulas were prepared in the produced water by 100 mL and injected into conical bottles of 250 mL in total volume. The bottles were incubated at 37°C while in shaking at 180 rpm for 7 days. The emulsification condition of the crude oil was graded through a visual determination method, which was demonstrated in detail (Table S2) [18]. The biosurfactant production capability was determined by oil spreading test. The procedure was to add hot water and sterile n-dodecane dyed by Sudan III to the culture dish, then add samples to the surface of the plate, and measure the diameter of the oil drain ring with a scale [21–24].

2.2. DNA Extraction and Miseq Sequencing of 16S rRNA Genes. Microbial cells of the collected samples were obtained by centrifugation at 4°C and 12,000 g for 20 min (Beckman, CA 94304, USA). Total genomic DNA was extracted following the previous description with minor modifications [25]. The pretreatment with the homogenizer (Bioprep-24, Allsheng, China) and 0.1 mm glass beads was carried out for 1 min at room temperature, following with one hour of enzymolysis. DNA was then extracted using AxyPrep™ Bacterial Genomic DNA Mini-prep Kit (Axygen Biosciences, Tewksbury, MA 01876, USA) according to the manufacturer's instructions, and the concentration of DNA was determined by a μLite spectrophotometer (BioDrop, BD1274, Cambridge). The DNA was then stored at −30°C for high-throughput sequencing [26].

The PCR products of V4 conserved region of bacterial 16S rRNA genes were sequenced by Illumina MiSeq sequencing platform. The total genomic DNA of each sample was amplified using bacterial primer sets 515F (5′-GTGCCAGC MGCCGCGGTAA-3′) and 806R (5′-GGACTACNNG GGTATCTAAT-3′). High-throughput sequencing library was constructed by GENEWIZ (Suzhou, China) for OTU analysis [27]. Sequence clustering was performed using VSEARCH (1.9.6) (sequence similarity set to 97%), and the representative sequence of each OTU was compared using NCBI BLASTN. Based on the RDP (Ribosomal Database Program), Bayesian algorithm was applied to taxonomic analysis, and the community composition of each sample was obtained at genera levels.

2.3. RT-PCR of the Key Gene for Glycolipid Production. Real-time PCR was conducted to estimate the glycolipid producing microbes. Primers were designed using Oligo 6 [28], and the primer sequences were named *rhl*F (5′ -GCTSAGCGACGAACTGACCT-3′) and *rhl*R (5′ -GRCTGCCCTGMTCGAGRAAG-3′) [29]. The *rhl* was used as marker gene for rhamnolipid producing bacteria [30]. The reactions are performed in a real-time PCR system (Bio-Rad, iCyclerTM Thermal Cycler, USA), following a protocol described previously [18].

2.4. Microbial Community Analysis. Based on OTU database, the random sampling method was used to calculate the α diversity index including Ace and Chao1 [31, 32]. The Bray-Curtis intersample distance matrix was used for PCoA analysis to demonstrate β diversity [32]. The unweighted-unifrac analysis was used to compare the differences between the samples. Redundancy analysis (RDA) reflected the sample distribution and environmental factors on a two-dimensional ranking diagram, and CANOCO 4.5 was used for detrended correspondence analysis (DCA) as follows [33, 34].

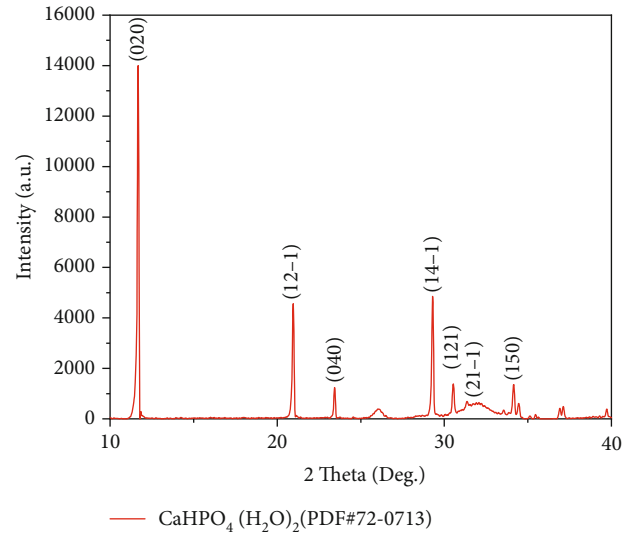


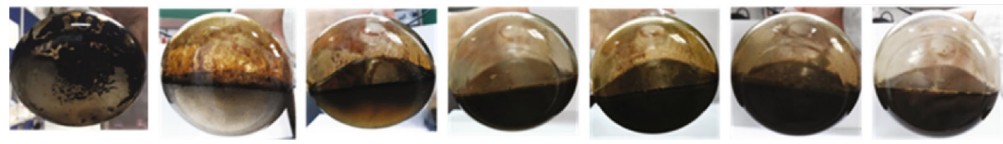
Figure 1: XRD analysis of the precipitation by adding inorganic phosphorus salt to Ca^{2+}-rich brine.

3. Results and Discussion

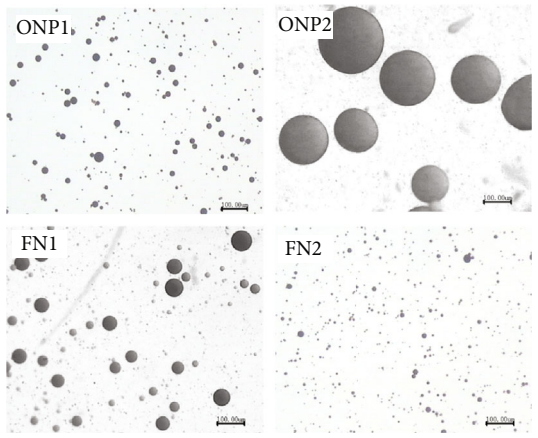
All raw sequence data presented in this article are available in the repository of the NCBI under Bioproject number PRJNA682271.

3.1. Stimulation of Oil Recovery Functional Microorganisms. The stimulation of indigenous microorganisms to create functional metabolites for crude oil emulsification is an important strategy of IMEOR technology. Crude oil was generally employed as the main carbon source. Nitrogen source, phosphorus source, and the supplementary organic carbon source are required to stimulate indigenous microbes under original oligotrophic environments.

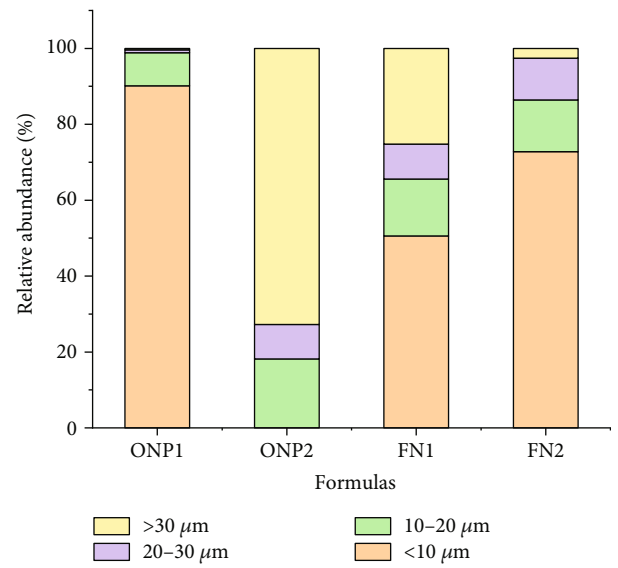
When adding nutrients, the compatibility of the formation of water with the phosphorus source is an essential issue that cannot be ignored. The addition of inorganic phosphorus ($H_2PO_4^-$, HPO_4^{2-}, or PO_4^{3-}) to the Ca^{2+}-rich brine could cause precipitation, which was identified as calcium hydrogen phosphate using XRD analysis (JCPDS: 72-0713) (Figure 1). In the XRD profiles, the peak positions (2θ) around 11.65°, 20.95°, 29.30°, and 30.54° correspond to the (020), (12-1), (14-1), (121) reflections, respectively. In the quantitative experiment of precipitation, the addition of 0.2% ammonium dihydrogen phosphate generated 0.21 g calcium hydrogen phosphate in divalent cation enrichment solution and adding 0.2% of diammonium hydrogen phosphate produced 0.1824 g of calcium hydrogen phosphate. Inorganic phosphorus precipitated with divalent cations, resulting in the deficiency of effective phosphorus source for microbial metabolism. Therefore, corn steep powder,

Formula	OR	OC	OCN	ONP1	ONP2	FN1	FN2
Stimulation effect							
Emulsify grade	–	+	++	++++	+++	+++	++++

(a)



(b)



(c)

Figure 2: Emulsification effects of the stimulated formation water with crude oil: (a) emulsify grade determination of six formulas and the original formation water sample; (b) microscopic images of emulsified crude oil; (c) droplet size distribution of crude oil emulsion.

molasses, and soybean powder were primarily considered as phosphorus candidates with additional advantages of easy accessibility and low cost. As shown in Figure 2(a), six formulas exhibited distinct effects on emulsification ability, and the formulas with sufficient phosphorus source (ONP1/ONP2/FN1/FN2) had a better performance than others.

The microscopic images of crude oil dispersed in different formulas (ONP1/ONP2/FN1/FN2), and the droplet size distributions of the crude oil in the culture broth were presented in Figures 2(b) and 2(c). The smaller the droplet size, the more uniform distribution, and the better emulsification performance. As shown in Figure 2(b), under the action of microorganisms and their products, the crude oil of ONP1 and FN2 was dispersed in very fine droplets, while the crude oil of ONP2 and FN1 was dispersed as large droplets and medium-sized oil droplets in the culture medium. This showed that among the four formulas, ONP1 and FN2 had relatively better emulsifying properties, while FN2 had the second-best emulsifying ability, and OPN2 had the relatively poor emulsifying ability. The droplet size distribution showed that 90.15% of the oil droplets in OPN1 were smaller than 10 μm, and 1.17% of the oil droplets were larger than 20 μm. In FN2, 72.83% of the oil droplets were below 10 μm in size, and 13.58% were larger than 20 μm (Figure 2(c)). In comparison, OPN1 had better emulsification performance.

As shown in Table 2, the concentration of organic phosphorus in corn steep powder was 1.7-fold of soybean powder and 25-fold of molasses. Its corresponding emulsification activity of the formulas (ONP1/FN1/FN2) with corn steep powder is higher, especially the formulas (ONP1/FN2) with specific N/OP ratio (~100 : 1), indicating that organic phosphate plays a crucial role in the functional indigenous microbial activation of this type of reservoir.

Emulsification generally has a positive correlation with biosurfactant production [35]. To further validate the emulsify condition, oil spreading test was applied to estimate biosurfactant production (Figure 3). Similarly, the formulas with organic phosphorus source (ONP1/ONP2/FN1/FN2) exhibited better surface activity than others, especially ONP1/FN2 with a specific P/N ratio. As a negative control, the diameters of oil spreading by OR was 0 cm, while following by 4.0 cm of ONP1, 3.5 cm of FN2, 2.5 cm of ONP2, 3.0 cm of FN1, 0 cm of OC, and 0.2 cm of OCN, respectively.

Although the quantity and structure analysis of biosurfactants in all samples were not carried out so far due to the presence of crude oil obstructed the effective extraction of

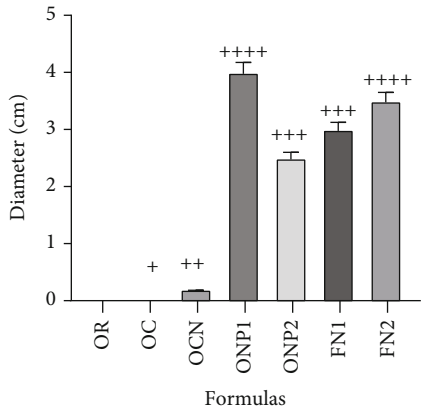


Figure 3: Oil spreading test represents the surfactant production ability by indigenous microbial consortia; "+" indicated the emulsify level of crude oil; OR used as the control.

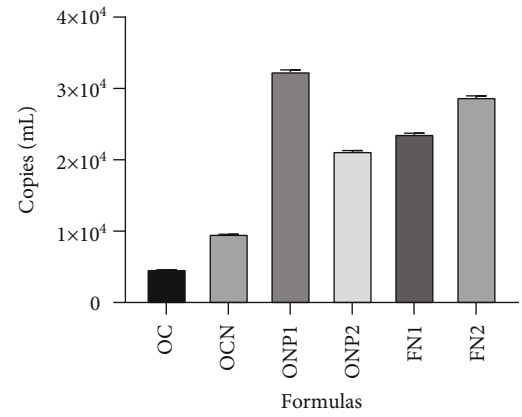


Figure 4: Real time-PCR results for *rhl* gene of the six stimulated formulas.

surfactants. The related biosurfactant-producing gene was determined by the quantification of *rhl* through RT-PCR, which is a key gene for one kind of glycolipid production that is inherently produced by lots of microorganisms [28]. As a similar trend, results showed in Figure 4 that formulas with organic phosphorus source (ONP1/ONP2/FN1/FN2) can enrich more *rhl* copies, indicating these samples have a promising potential in producing more glycolipid than others.

3.2. Response of Emulsification Difference and Community Diversity to Different Nutrients. By sequencing the V4-V5 conserved region of bacterial 16S rRNA genes, a total of 936,462 bacterial 16S reads were obtained from the seven samples, including the original water sample and the six stimulated samples. After quality optimization, 468,231 effective reads were recovered and clustered into 109 OTUs. The rarefaction curves of the OTUs indicated that the depth of sequencing is sufficient for accurate and comprehensive analysis (Figure S1). The richness and alpha diversity (Figure S2) in samples (ONP1/ONP2/FN1/FN2) are higher than those without the addition of organic phosphorus, particularly the formulas (OPN1 and FN2) with specific N/OP ratio. The principal coordinates analysis (PCoA) also demonstrated that the similarity between ONP1 and FN2 was obviously high and held similar microbial community, and the same situation was found between FN1 and ONP2 (Figure 5).

Based on the results above, it was apparent that organic phosphorus and appropriate N/OP ratio made a significant difference in the microbial community among six samples. Numerous reports focused on the influence of exogenous carbon source on microbial community structure in the oil reservoir [36, 37], and the injection of the abundance of exogenous carbon source into reservoir formation became a conventional and indispensable step for the field tests of IMEOR [38]. However, it seemed that the supplementary of additional and water-soluble carbon source (CH_3COONa) had no obvious contribution to emulsify crude oil (Figure 2) and even a little bit of negative effect on the oil spreading with comparison of ONP1 and FN2 (Figure 3) and biosurfactant production (Figure 4), which has been well-documented as the main mechanism of oil recovery [39]. Exposure to

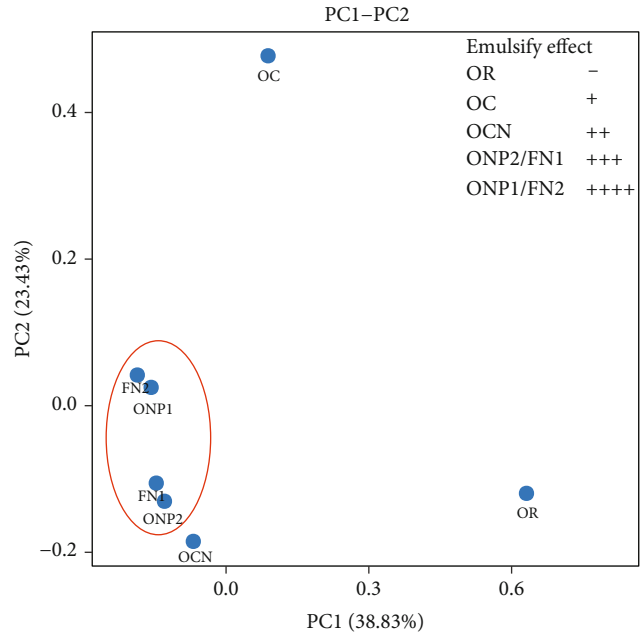


Figure 5: Principal coordinates analysis (PCoA) of each sample and the emulsifying effect.

water-insoluble carbon sources (like crude oil) can facilitate the production of biosurfactant for some specific microorganisms [40] and direct the structure of microbial community to generate more microorganisms which can utilize crude oil and produce biosurfactant [41]. However, some bacteria that do not contribute to emulsification will also use carbon sources to compete with functional bacteria for the limited nutrients and resources, which may explain the addition of carbon sources do not contribute to the emulsification in this reservoir. Therefore, organic phosphorus, N/OP ratio, and crude oil played important roles in the diversity and function of microbial communities.

3.3. Microbial Community Structure Shifts under Different Nutrition Stimulation. All clustered OTUs were assigned into nine phyla revealed that *Proteobacteria* (65.59~94.32%),

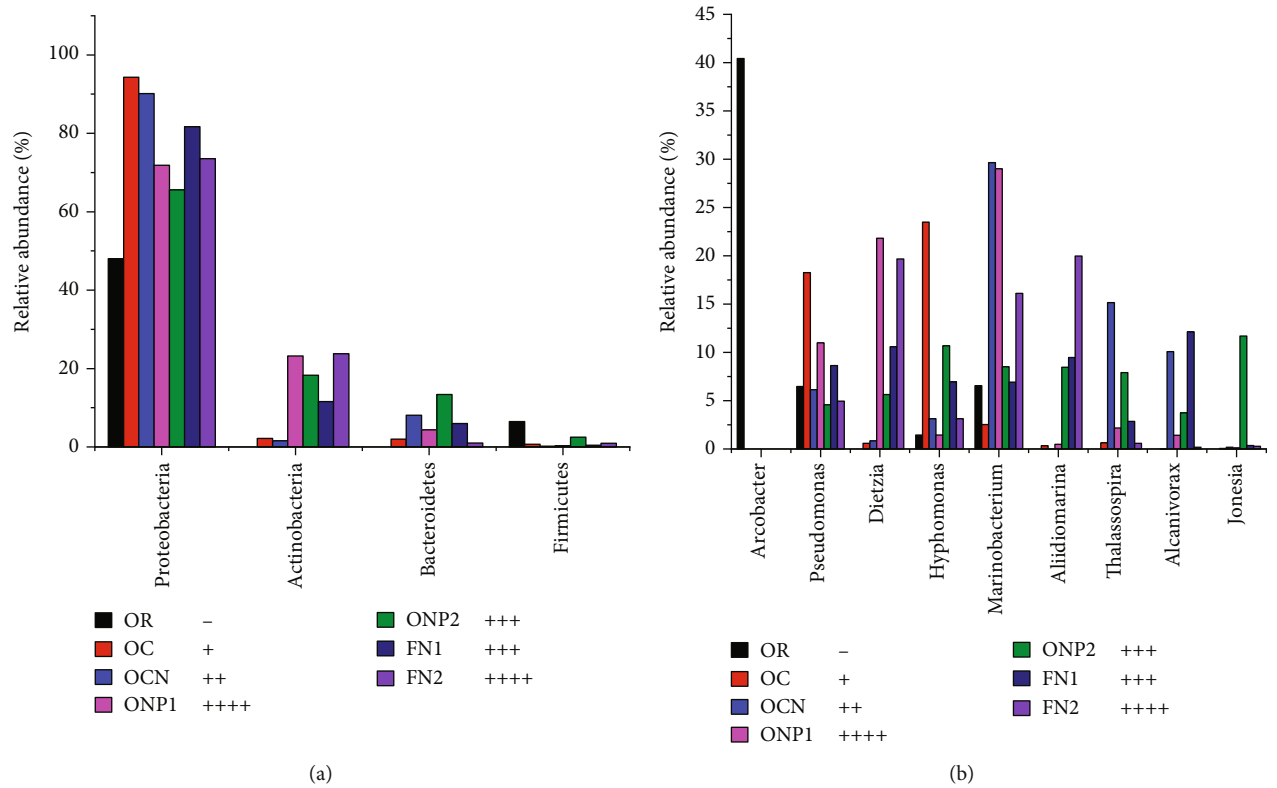


Figure 6: The relative abundances of microbial communities in the six stimulation formulas: (a) the four most abundant phyla; (b) the nine dominant genera.

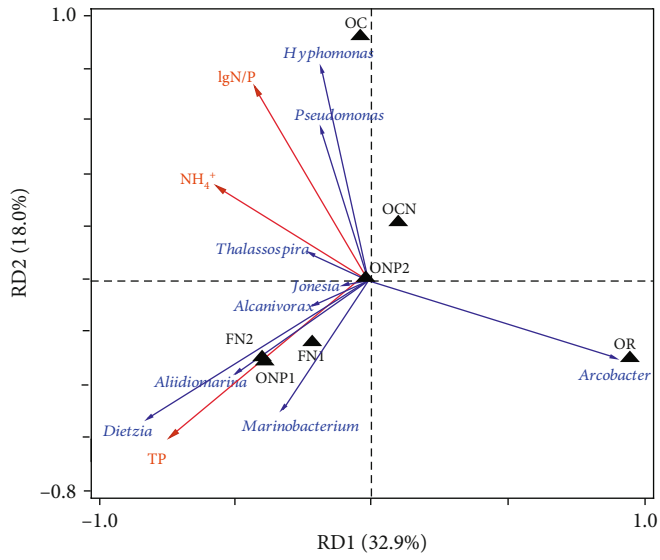


Figure 7: Redundancy analysis (RDA) of each sample; the right arrows indicated the contribution of each environmental factors; the dark blue arrows indicated genera of abundant relative abundance; the solid square indicated the seven samples.

Actinobacteria (1.55~23.77%), and *Bacteroidetes* (0.98~13.38%) were the predominant phylum and showed significant increments after stimulation with six formulas, while the abundances of *Firmicutes* and *Euryarchaeota* were obviously decreased (Figure 6(a)).

Further analysis of bacterial diversity in the genus level (Figure 6(b)) was carried out, and all clustered OTUs were classified into 66 genera; only 9 genera had the relative abundance greater than 10% in at least one sample, including *Pseudomonas* (4.56~18.25%), *Dietzia* (0~21.83%), *Hyphomonas* (1.44~23.49%), *Marinobacterium* (2.53~29.64%), *Alcanivorax* (0~12.13%), *Aliidiomarina* (0~19.97%), *Thalassospira* (0~15.15%), and *Jonesia* (0~11.68%). Among them, *Pseudomonas*, *Dietzia*, *Marinobacterium*, and

Alcanivorax were well-documented due to their ability of glycolipid biosurfactant production and hydrocarbon degradation [30, 41–45]. The total abundances of these four bacterial genera presented higher in OPN1 (63.24%) and FN2 (40.93%) than other samples, followed by FN1 (38.29%) and ONP2 (22.44%). Among these four bacteria genera, the ubiquitous genus *Dietzia* can efficiently degrade the medium-long-chain of saturated hydrocarbons which has a relatively higher percentage in Lu-9 crude oil [46] and simultaneously produce an amount of biosurfactant [42]. Among six samples, the relative abundance of *Dietzia* genus in ONP1 was the highest (21.83%) and then FN2, FN1, and OPN2. This genus was coincidently correspondent with the value of oil spreading, indicating that *Dietzia* may play a critical role in the emulsification of Lu-9 crude oil under the described condition.

RDA was also applied to evaluate the relationship between the stimulated genera and key nutrients. As shown in Figure 7, the distribution of the samples demonstrated a large consistency with the conclusion that formulas of ONP1 and FN2 with high abundances in *Dietzia* and *Marinobacterium* exhibited great correlations with NH_4^+ (N), organic phosphorus (OP), and ratio of N/OP, whereas formulas ONP2 and FN1 had less nitrogen and phosphorus content, which had no significant positive correlation with NH_4^+ or organic phosphorus. Formulas OCN and OC, as well as the original water sample (OR) with no obvious emulsification effect, had farther distance with those who had preferable effects.

These results demonstrated that the organic phosphorus and appropriate N/OP ratio were important for the emulsification stimulation of indigenous microorganisms from oil reservoir with high-content of medium-long-chain of saturated hydrocarbons and calcium-rich of brine. The thorough understanding of the difference in emulsification and microbial diversity after stimulation will be further explored coupling with the application of meta-transcriptomics techniques.

4. Conclusions

The corn steep powder is an excellent phosphorus and nitrogen source with outstanding compatibility in reservoir production water containing Ca^{2+}-rich brine. After stimulation, the community structure exhibited significant shifts under different nutritions. The dominant genus altered from *Arcobacter* (40.43%) to *Dietzia* (19.68~21.83%) in the commendable performance formulas (ONP1, FN2), indicating *Dietzia* as a biosurfactant producing bacteria plays a crucial role on the emulsification of crude oil. In addition, applying different nutrients have a striking impact on the community function which is followed by shifts on community structures. Conducting community-scale experiments, other than focusing on certain isolated single bacteria, is able to gain a more accurate and comprehensive understanding of the functional consortia. This study provides a perspective on the directed biostimulation of IMEOR and will assist the stimulation of $CaCl_2$ type formation water in reservoirs.

Data Availability

All raw sequence data presented in this article are available in the repository of the NCBI under Bioproject number PRJNA682271.

Conflicts of Interest

There are no conflicts to declare.

Authors' Contributions

Guoxin Shi and Yuanyuan Jia contribute equally to this work.

Acknowledgments

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Supplementary Materials

Table S1: physicochemical parameters of Luliang reservoir. Table S2: primers of rhl gene for RT-PCR. Figure S1: the rarefaction curve of the OTUs retrieved by Miseq sequencing in the seven samples. Figure S2: the alpha diversity of the seven samples represented by Ace and Chao1 indexes. *(Supplementary Materials)*

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