

Study on a Strain of *Lysinibacillus* sp. with the Potential to Improve the Quality of Oil Sands

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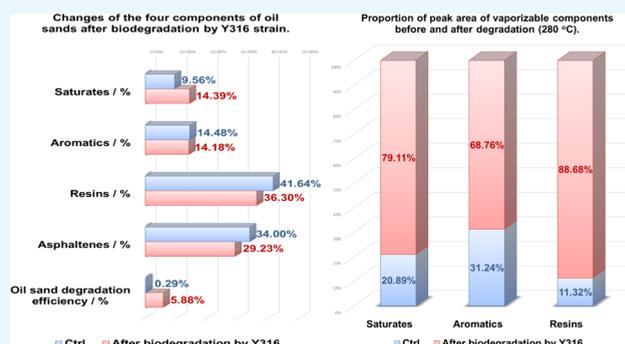


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ABSTRACT: A strain of *Lysinibacillus* sp., named as Y316, can degrade heavy fractions such as resins and asphaltenes in oil sand. We used Y316 to degrade oil sand samples for 35 days. After bacterial degradation, the oil sand degradation efficiency was 5.88%, while the degradation efficiency of the control group was only 0.29% under the same conditions. Compared with the control group, the saturated content of oil sand in the degradation group increased from 9.56 to 14.39%. After degradation, the resin and asphaltene fractions decreased by 5.34 and 4.77%, respectively. The results of the vaporizable fraction analysis also confirmed the degradation of heavy fractions and the formation of light fractions. After 35 days of degradation, the vaporizable fractions of saturates increased by 3.76 times. The results indicate that Y316 has great significance for improving the quality of oil sands and assisting in oil sand exploitation.



1. INTRODUCTION

Microorganisms capable of degrading crude oil are everywhere,¹ and these microorganisms vary in their degradation mechanism, degradation rate, and degradation ranges. For example, *Bacillus subtilis* can efficiently degrade the concentration of total petroleum hydrocarbons in contaminated soil from 84 to 39 g/kg in a simulated polluted environment.² With the help of an auxiliary carbon source, a fungus of *Pestalotiopsis* degraded 92% of the samples in the medium within 30 days (the initial concentration of crude oil was 1000 mg/L).³ At present, researchers mainly focus on the environmental pollution caused by the petroleum industry. Zhang et al. found a strain of *Pseudomonas aeruginosa* DQ-8 that can effectively degrade *n*-alkanes and polycyclic aromatic hydrocarbons in petroleum pollutants.⁴ Microbial treatment can be used as a supplementary method after secondary oil recovery to extend the life of oil fields.⁵ Zhang et al. isolated three strains of *Bacillus* sp. The viscosity decreased by an average of 20–30% after the crude oil samples were degraded by three microorganisms.⁶

There are many studies on microbial degradation of petroleum hydrocarbons but few on the biodegradation of oil sands. Oil sand mining requires a lot of energy and causes serious environmental pollution. It is mainly because oil sands contain many heavy components, such as resins and asphaltenes. Some studies have shown that light fractions are conducive to the degradation of crude oil by microorganisms because these fractions can provide the necessary carbon source for the initial stage of microbial growth and increase the

solubility of heavy fractions.^{7,8} The low content of light fractions in the oil sand sample makes it difficult to biodegrade.

As an unconventional oil resource, oil sands have become an essential part of fossil energy globally. There are about 5.6 trillion barrels of asphalt and heavy oil resources in the world, mainly distributed in Alberta, Canada, the Orinoco heavy oil belt in Venezuela, and California.⁹ Canada has the third largest total oil volume in the world, behind Venezuela and Saudi Arabia.¹⁰ However, Canada's oil sand resources account for 85% of the world's total resources, about 1867 billion barrels.¹¹ Canada has been mining oil sands for decades. The current mining methods are mainly open-pit mining and in situ mining technology.¹² Open-pit mining will cause damage to the surface vegetation and produce a large amount of production of sewage.^{10,13} In situ mining requires a large amount of energy to heat the ore bed in advance.¹⁴

Increasing the content of light fractions through biodegradation can improve the quality of oil sands and effectively reduce the energy cost during the oil sand mining and processing. Existing studies have pointed out that microorganisms utilize petroleum hydrocarbons to produce methane under anaerobic conditions.¹⁵ Jones et al. observed significant

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amounts of associated methane in heavy oil and oil sand reservoirs.¹⁶ The above research studies show that under anaerobic conditions, the light fractions in crude oil are degraded by microorganisms, leaving heavy components. Aerobic degradation is a more feasible degradation scheme. We used a strain of *Bacillus* for the preliminary study. Similar to *P. aeruginosa*, *Bacillus* can degrade heavy fractions in crude oil, which is conducive to the initial development of this research.^{5,17,18} Kshirsagar et al. conducted a study of the biodegradation of different petroleum hydrocarbons in the experiments with mixed cultures. In the study, a strain of *Lysinibacillus* was isolated and tested for the biodegradation of a crude oil fraction rich in saturated hydrocarbons, one rich in aromatic hydrocarbons, deasphalted oil, and pure asphaltene. The biodegradation efficiencies were 17, 13, 10, and 17%, respectively. Among the six selected bacteria, *Lysinibacillus* showed higher asphaltene degradation efficiency.¹⁹ So far, there are no reports on the direct degradation of oil sands by *Bacillus* sp. bacteria or the changes in oil sand fractions in the process of biodegradation.

We plan to screen a microorganism that mainly degrades heavy petroleum fractions. The purpose of this study was to assess the improvement effect of strain Y316 on the quality of oil sands. After optimizing the culture temperature, the effect of bacterial degradation on the quality of oil sands was analyzed by comparing the changes in the proportion of light fractions and heavy fractions between the degradation group and the control group. At the same time, the genome of the strain was amplified and identified, and the performance of the biosurfactant secreted by the strain was evaluated. This study provides evidence for the feasibility of using microbial degradation methods to improve the quality of oil sands.

2. RESULTS AND DISCUSSION

2.1. Isolation and Identification of Strain Y316. In this research, a strain of *Lysinibacillus* sp. Y316 was used to degrade oil sands. The strain from the Lenghu oil field exhibited good growth in the medium and improved the proportion of light fractions of oil sand.

The cell morphology of this strain is shown in Figure 1. The colonies appeared to be white with wet edges and convex, and the surface was dry with approximately circular edges. The cells were 3.0–4.0 μm long and 0.5 μm wide.

The results of 16S rDNA sequence analysis showed that the sequence similarity between this strain and *Lysinibacillus* sp. is 100%. The phylogenetic tree was constructed based on the 16S rDNA sequences of Y316 (Figure 2). According to the results of morphological observation and 16S rDNA sequence

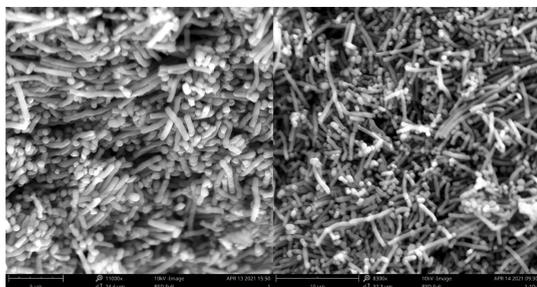


Figure 1. Scanning electron microscopy image of *Lysinibacillus* sp. Y316.

analysis, Y316 was identified as *Lysinibacillus* sp. and registered with the sequence number MZ604311 in the NCBI.²⁰

2.2. Optimization of Incubation Temperature. After adjusting the culture temperature, the optimum condition of Y316 was determined to be 40 °C. The growth curves of microorganisms at different temperatures are shown in Figure 3.

Between 30 and 40 °C, the growth rate of the strain was positively correlated with the increase of temperature. When the culture temperature reached 45 °C, Y316 could maintain growth and metabolism, but the growth rate decreased.

Overall, in the subsequent biosurfactant separation test and oil sand degradation test, the culture temperature was set to 40 °C. The appropriate culture temperature can promote the growth rate of the strain, and the biodegradation efficiency is directly proportional to the growth and development of bacterial cells.²¹ At the same time, we tested the initial pH of the medium at 40 °C, which was the most suitable for strain growth. The results showed that the strain grew better in neutral, weak acid, and weak alkaline environments, and strong acidity would inhibit the growth of the strain (Figure S1).

2.3. Isolation and Identification of the Biosurfactant. The extract from *Lysinibacillus* sp. Y316 appeared as a sticky residue with a yellowish-brown color. The biosurfactant was soluble in aqueous solutions and organic solvents such as methanol and chloroform. Its physical and chemical properties were similar to those of glycolipid biosurfactants.²² The thin-layer chromatography (TLC) experimental results confirmed this view (Figure 4).

The Fourier transform infrared spectrum of the biosurfactant showed strong absorption bands at 3278.88 cm^{-1} resulting from the hydroxyl group (Figure 5). The C–H stretching vibrations at 2871.97–2976.11 cm^{-1} and 1233.74–1455.51 cm^{-1} indicate the presence of aliphatic chains. The C=O bond vibration occurs at 1711.03 cm^{-1} , and the C–O–C bond stretching vibration occurs at 1074.16 cm^{-1} .^{23,24} These results strongly indicated that the biosurfactant contains a glycolipid structure.²⁵

2.4. Surface Tensions and Critical Micelle Concentration of the Biosurfactant. The surface-active properties of the biosurfactants mainly depend on their ability to lower the surface tension and critical micelle concentration (CMC) values. The ability to reduce the surface tension depends on the CMC, which is defined as the minimum concentration of a surfactant required for maximum surface tension reduction of water and initiate micelle formation. High-efficiency surfactants have a meager CMC value. In other words, only a little surfactant is required to reduce the surface tension.²⁶

The surface tensions versus the biosurfactant concentrations were plotted (Figure 6). With the increase of biosurfactant concentration, the surface tension of the solution decreased from 73 to 50 mN/m. At this time, the concentration of the biosurfactant was 16540 mg/L. This value is significantly different from the CMC value range of 1–2000 mg/L for general biosurfactants,²⁷ such as the CMC value (120 mg/L) of biosurfactants produced by *P. aeruginosa* LB1.²⁸ The difference may have resulted from differences in the purity and composition of the biosurfactants.²²

Glycolipids secreted by strains are often a series of homologues with different structures and different proportions.²⁹ Ma et al., in the study on *P. aeruginosa* DN1, obtained a mixture of six rhamnolipid homologues.³⁰ The yield and proportion of these homologues depend on factors such as

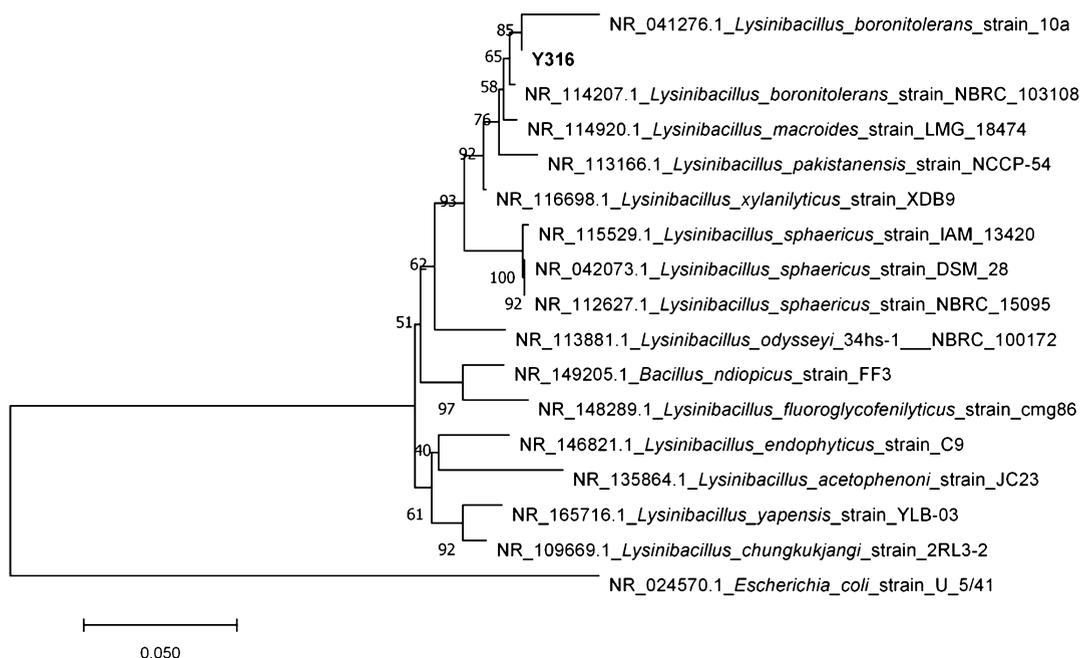


Figure 2. Neighbor-joining tree is based on partial 16S rDNA gene sequences (1424 bp) of the cultured and related species found by BLAST search. The bootstrap analysis was performed with 1000 repetitions. Bar = 0.050 nucleotide substitution per site.

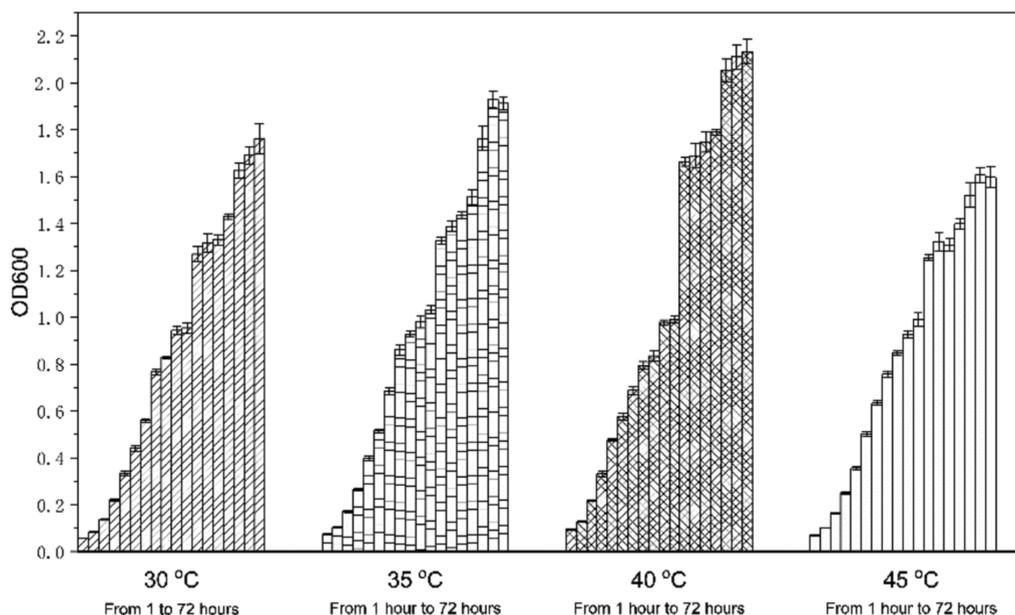


Figure 3. Influence of different culture temperatures on the growth of the Y316 strain (cultivation at 120 rpm).

medium composition, culture temperature, pH, substrate, microbial species, and so forth.^{31–33} Furthermore, the surface properties of glycolipid homologues also differ. Nitschke et al. reported that the CMC value of di-rhamnolipids was only 5 mg/L compared to mono-rhamnolipids (CMC = 40 mg/L).³⁴ It can be seen that the purified biosurfactant has better surface properties. In this study, we did not further isolate and purify the obtained biosurfactant, which is one reason for the high CMC value of the biosurfactant produced by Y316.

Nonetheless, the biosurfactant secreted by Y316 still promotes the degradation of oil sands. In the process of degrading organic compounds, most strains secrete biosurfactants and extracellular enzymes to assist the degradation

process,³⁵ such as alkane hydroxylase, phenol oxidase, lipase, alcohol dehydrogenase, and so forth.^{4,36} Through enzymatic reactions and the action of biosurfactants, organic compounds are gradually emulsified, and the bioavailability of hydrophobic components is enhanced.³⁷ With the gradual emulsification of hydrophobic components, microorganisms also obtained the carbon source needed for growth. In conclusion, the role of biosurfactants is very important in the biodegradation of oil sands. In this study, the high CMC value of biosurfactants limited the biodegradation effect of oil sands. However, this also reflects that the degradation effect of Y316 on oil sands still has room for improvement. Adding additional biosurfactants should further enhance the degradation perform-

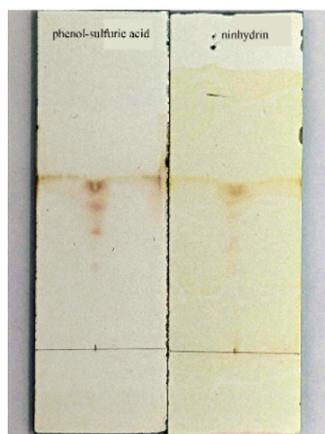


Figure 4. Color development result of TLC (left: the color development result of phenol-sulfuric acid reagent; right: the color development result of ninhydrin reagent).

ance of Y316. However, the amount of addition, the type of surfactant, and so forth must be carefully controlled because in some cases, the addition of biosurfactants may inhibit the growth of microorganisms and reduce the degradation efficiency.³⁸

2.5. Biodegradation of Oil Sand. In this test, the glucose and yeast powder supports the initial rapid proliferation of bacterial cells and thus facilitates oil sand degradation. It can be seen in Table 1 that after biodegradation with Y316, the oil sand degradation efficiency was about 6%. Compared with the control groups, the values were more than about 20 times higher. The saturated content of oil sands in the degraded groups was 4.83% higher than that in the control groups. In contrast, the contents of aromatics, resins, and asphaltenes were reduced by 0.3, 5.34, and 4.77%, respectively.

It can be seen in the degradation results that the proportion of resin and asphaltene components decreased, and the proportion of saturated hydrocarbon components increased. Such a phenomenon also occurred in the study by Etoumi et

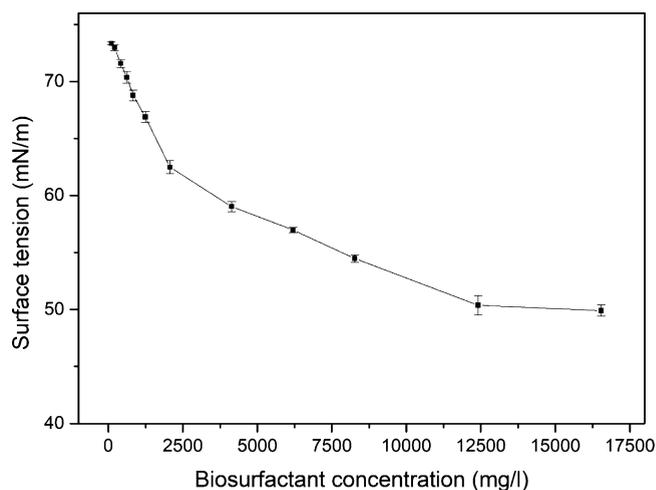


Figure 6. Surface tension of aqueous solutions of biosurfactants at different concentrations.

al., where *P. aeruginosa* degraded asphaltenes and resins in the crude oil, and the results showed that the proportion of saturated hydrocarbons increased by 10% with the decrease of asphaltenes and aromatic hydrocarbon components.³⁹ A research in 2001 showed that *P. aeruginosa* degraded 60% of asphalt in 120 days with asphalt as the carbon source. The test of degradation products showed that asphalt degradation produced saturated and aromatic components.⁴⁰ Gao et al. used two strains of *Pseudomonas aeruginosa* (Gx and Fx) to degrade asphaltene fractions in pure bitumen and heavy crude oil. The results showed that the content of saturates and aromatics increased with the degradation of asphaltenes.⁵

Tables 2–4 show that bacterial degradation has a significant effect on the relative amount (i.e., peak area) of the vaporized fractions (280 °C) in oil sands. After biodegradation, compared with the control groups, 67 new components appeared, three components disappeared, four components increased, and three components decreased in the saturated

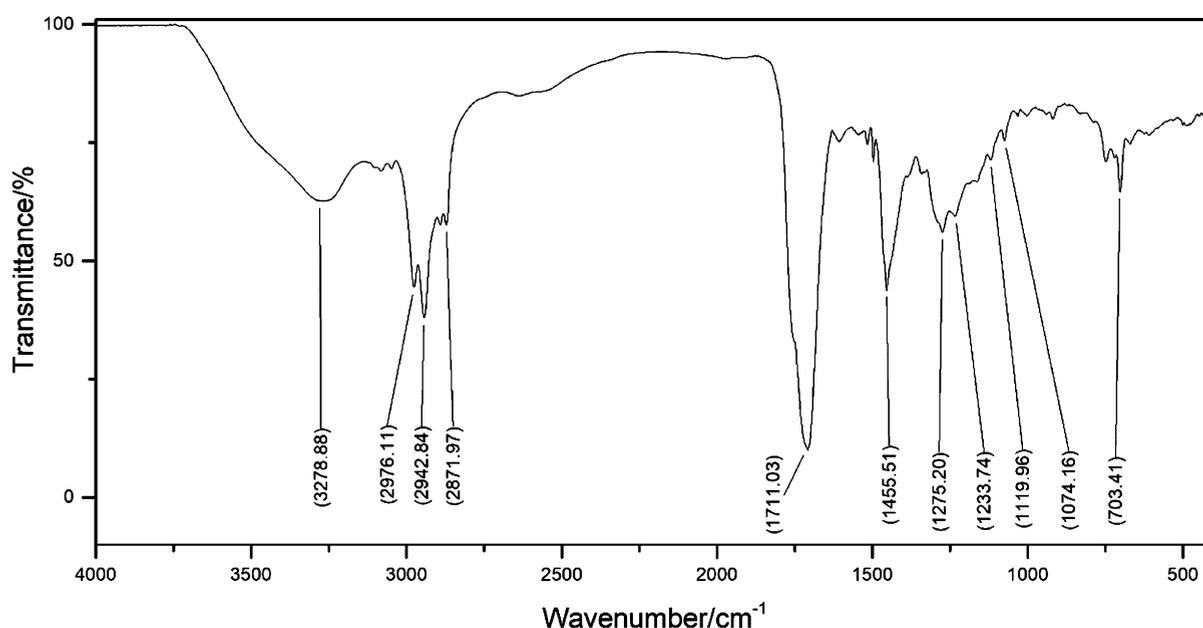


Figure 5. Infrared spectrum of the biosurfactant produced by the strain Y316.

Table 1. Changes in the Relative Content of the Four Components of Oil Sand Oil after Biodegradation by the Y316 Strain

	saturates/%	aromatics/%	resins/%	asphaltenes/%	oil sand degradation efficiency/%
ctrl	9.56 ± 0.07	14.48 ± 0.08	41.64 ± 0.65	34.00 ± 0.55	0.29 ± 0.06
Y316	14.39 ± 1.05	14.18 ± 0.53	36.30 ± 0.89	29.23 ± 0.32	5.88 ± 1.25

Table 2. Relative Quantity (Chromatographic Peak Area) of the Vaporizable Fractions (280 °C) in Oil Sands (Saturates) after Degradation by the *Lysinibacillus* sp. Strain Y316

saturates RT (s)	ctrl peak area	Y316 peak area	ΔR %
408.723–862.944	0	11 new components appear	
865.464	6,198,691	0	−100.00
1096.88	9,872,218	7,539,395	−23.63
1208.1	0	1,186,587	
1222.42	0	822,123	
1539.14	1,156,799	0	−100.00
1542	2,365,819	0	−100.00
1792.66	4,247,559	3,529,585	−16.90
1918.99–2425.72	0	eight new components appear	
2427.9	386,031	1657063	329.26
2439.62–2572.23	0	12 new components appear	
2594.52	7,055,6084	61,393,177	−12.99
2628.62–2707.12	0	eight new components appear	
2710.3	14,339,565	30,118,854	110.04
2715.06~2985.86	0	22 new components appear	
3029.3	18,201,563	111,515,826	512.67
3135.4	65,906,140	284,078,892	331.04
3139.51	0	2,129,815	
3189.49	0	22,555,304	
3314.96	0	329,683	
3319.7	0	455,649	
vaporizable components (280 °C)			
	saturates	ctrl	Y316
total number of vaporizable components		10	74
number of different components		3	67
number of new components			67
number of disappearing components			3
number of increased components			4
average increase rate (%)			320.8
number of reduced components			3
average increase rate (%)			−17.8

components (280 °C), with an average change rate of 320.8 and −17.8%, respectively (Table 2). In the aromatic components (280 °C), a new component appeared, five components disappeared, four components increased, and five components decreased, with an average change rate of 199.5 and −20.1%, respectively (Table 3). As for the resin components (280 °C), 25 new components appeared, six components disappeared, two components increased, and seven components decreased, with an average change rate of 35.9 and −39.8%, respectively (Table 4).

The total peak areas of the vaporizable components (280 °C) in the three components of the degradation group and the control group were calculated (Figure 7). The results showed that the total peak area of vaporizable components in the degradation group was 3.76 times that of the control group in

Table 3. Relative Quantity (Chromatographic Peak Area) of the Vaporizable Fractions (280 °C) in Oil Sands (Aromatics) after Degradation by the *Lysinibacillus* sp. Strain Y316

aromatics RT (s)	ctrl peak area	Y316 peak area	ΔR %
675.54	3,858,933	804,095	−79.16
791.628	1,559,569	0	−100.00
865.464	7,458,714	7,373,513	−1.14
1044.64	3,592,277	0	−100.00
1096.8	13,398,942	11,603,048	−13.40
1370.24	3,310,499	0	−100.00
1518.73	3,431,601	3,347,291	−2.46
1539.25	753,061	675,311	−10.32
1542.11	2,286,581	2,935,837	28.39
1659.26	3,698,347	0	−100.00
1792.57	4,395,338	3,776,865	−14.07
2428.13	447,414	1,131,915	152.99
2594.18	20,784,302	147,556,828	609.94
2628.24	0	488,262	
2710.19	18,257,924	19,489,161	6.74
3594.88	3,276,847	0	−100.00
vaporizable components (280 °C)			
	aromatics	ctrl	Y316
total number of vaporizable components		15	11
number of different components		4	1
number of new components			1
number of disappearing components			5
number of increased components			4
average increase rate (%)			199.5
number of reduced components			5
average increase rate (%)			−20.1

the saturated hydrocarbon component, 2.22 times in the aromatic hydrocarbon component, and 8.09 times in the resin component. The increase in the peak area of the vaporizable components of saturates further proves the degradation of heavy fractions and the production of light fractions during the degradation process.⁵

The proportion of aromatic components did not change significantly between the control group and the degradation group, but the peak area increased in the detection of vaporizable components. It may be because that during the degradation process, the part of aromatics produced by the degradation of resin and asphaltene components and the part of biodegraded aromatics offset each other. PAHs are degraded by microorganisms under the action of lignin-degrading enzymes through a series of intermediate products such as 2, 2-diphenic acid, phthalic acid, protocatechuric acid, and pyruvic acid.⁴¹ Yu et al. also described the degradation mechanism of PAHs. The metabolites secreted by microorganisms assisted the ring-opening degradation of PAHs and turned them into chain-like structures.⁴² Ma et al. reported that *Bacillus* sp. could secrete enzymes to disrupt chemical bonds

Table 4. Relative Quantity (Chromatographic Peak Area) of the Vaporizable Fractions (280 °C) in Oil Sands (Resins) after Degradation by the *Lysinibacillus* sp. Strain Y316

resins RT (s)	ctrl peak area	Y316 peak area	ΔR %
674.364	3,863,109	0	-100.00
791.628	1,751,199	0	-100.00
865.296	8,811,340	6,045,962	-31.38
1044.72	0	2,964,711	
1096.8	15,249,541	9,144,060	-40.04
1277.23–1367.6	0	five new components appear	
1370.15	3,784,043	0	-100.00
1371.32–1516.88	0	10 new components appear	
1518.82	4,223,688	2,709,900	-35.84
1525.51	0	12,634,297	
1528.91	0	4,425,200	
1538.98	2,627,504	492,702	-81.25
1542.08	0	2,947,563	
1612.14	0	6,439,119	
1659.18	5,686,767	3,140,974	-44.77
1792.66	5,337,456	4,628,668	-13.28
2006.6	0	2,604,671	
2116.81	2,762,297	0	-100.00
2117.57	0	465,152,771	
2349.82	0	486,719	
2381.33	6,281,142	0	-100.00
2427.95	1,365,753	1,090,797	-20.13
2533.28	0	1,928,040	
2569.99	17,605,555	24,020,330	36.44
2594.6	0	77,143,336	
2628.42	713,198	369,603	-48.18
2710.27	60,130,502	34,184,228	-43.15
2881.46	26,912,082	0	-100.00
2907.72	1,266,393	1,713,123	35.28
vaporizable components (280 °C)			
resins	ctrl	Y316	
total number of vaporizable components	18	36	
number of different components	6	25	
number of new components		25	
number of disappearing components		6	
number of increased components		2	
average increase rate (%)		35.9	
number of reduced components		9	
average increase rate (%)		-39.8	

between aromatic and naphthenic rings in fused-ring compounds.¹⁸

This indicates that the resin and asphaltene components also contain aromatics. In fact, resin and asphaltene are complex mixtures with high molecular weight. They are mainly composed of aromatic rings, nonaromatic rings, side chains, and heteroatoms. The difference is that the molecular weight of asphaltenes is higher than that of resins, and the composition is more complex.⁴³ So far, there is no precise definition for the chemical structure of resin and asphaltene components. Although there have been many studies discussing their structure,^{44,45} no consensus has been reached.

At present, the four fractions are mainly divided according to the different solubility. For example, asphaltenes can be dissolved in toluene but insoluble in *n*-alkanes; resins can be

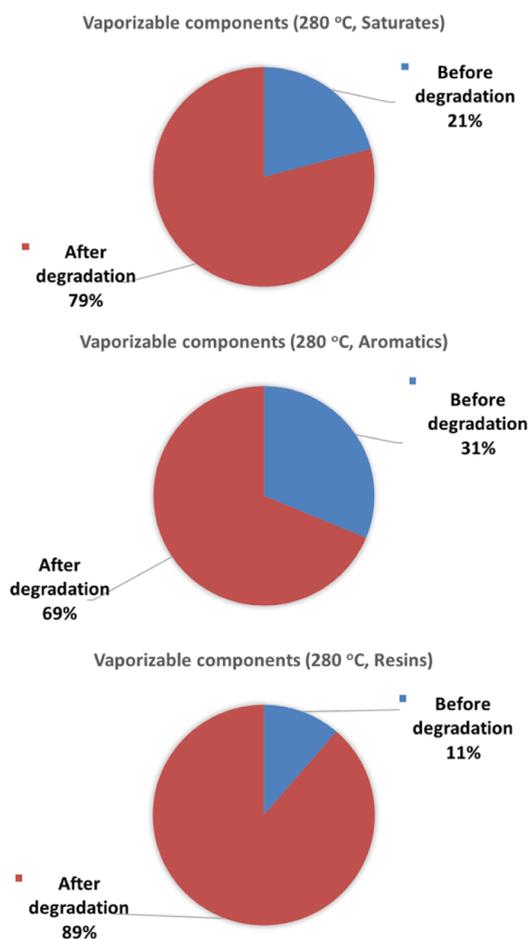


Figure 7. Proportion of the peak area of vaporizable components before and after degradation (280 °C).

dissolved in *n*-hexane and *n*-heptane. As the molecular weight increases, the structure becomes more complex, and the polarity of the resin and asphaltene increases continuously.⁴⁵ This explains why in this experiment, the proportion of resins in the degradation group decreased, but the total vaporizable peak area increased. Under aerobic conditions, the four fractions of oil sands were gradually degraded under the action of oxygenase, and intermediate products such as aldehydes, ketones, and carboxylic acids were generated in the process.⁴³ The polarity of these intermediates is close to that of the resin, causing the intermediates and resins to elute together in the silica gel/alumina column. Therefore, the total peak area of the resins (degradation group) increased abnormally.

Because resins and asphaltenes have complex structures and strong hydrophobicity, they are difficult to biodegrade. In practical degradation, cometabolism is required to accelerate the degradation process. The cometabolism process generally contains two substrates (growth substrate and nongrowth substrate), among which the growth substrate is more easily degraded, which can provide the carbon source for microbial growth and then promote the degradation of the nongrowth substrate.⁴⁶ During the oil sand degradation process, the additionally added yeast powder and glucose, as well as the easily degradable saturates and aromatics fractions, could be regarded as growth substrates, and the difficult-to-degrade resins and asphaltenes could be regarded as the nongrowth

substrates. As mentioned in the **Introduction**, the light fractions of oil sands in the experiments serve as growth substrates to accelerate biodegradation, increase the solubility, and reduce the hydrophobicity of the heavy fractions in the medium. However, the use of some growth substrates may inhibit the secretion of nongrowth substrate-degrading enzymes by microorganisms, so the effect of different growth substrates on degradation should be fully evaluated before practical application.⁴⁶ Considering the facilitation of the degradation process by adding a suitable growth substrate, this direction still has an important research value.

Screening more strains that can effectively degrade resins and asphaltenes and studying the exact structure and degradation mechanism of resins and asphaltenes are very important topics in the field of heavy oil biodegradation. The degradation results and the vaporizable fractions analysis results confirmed that Y316 could degrade resin and asphaltene fractions in oil sands. As a new strain that can degrade heavy fractions, we believe that Y316 has the potential to improve the quality of oil sands. In the subsequent studies, we will focus on the identification of the strain's secreted enzymes, the effect of different growth substrates on the biodegradation, and the analysis and identification of degradation products and other issues.

3. CONCLUSIONS

This study found a *Lysinibacillus* sp. strain (Y316) with the ability to degrade resins and asphaltenes. The glycolipid biosurfactants secreted by the strain Y316 promoted biodegradation. In the degradation experiment, the heavy fractions in the oil sands were effectively degraded and the light fractions were generated. We consider that this *Lysinibacillus* strain has the potential to improve the quality of oil sands and to assist in oil sand development.

4. MATERIALS AND METHODS

4.1. Chemicals, Strains, and Oil Sand. All chemicals used in this study were of analytical purity and obtained from various commercial sources (Sinopharm Chemical Reagent Co., Ltd. and Beekman Biotechnology). *Bacillus* was isolated from the polluted soil of the Lenghu oil field, Qinghai Province, provided by the China Industrial Culture Collection Center. We named this strain Y316. For the degradation test, the oil sand was purchased from Budun Island, Indonesia. The sample contained about 25% oil and about 75% inorganic minerals.

4.2. Bacterial Isolation and Identification. **4.2.1. Bacterial Isolation.** 5.0 g of soil sample was weighed and added into a 250 mL flask containing 100 mL of Luria–Bertani (LB) medium and incubated at 30 °C and 100 rpm under aerobic conditions for 48 h. Then, the bacterial solution was inoculated on a mineral salt agar plate containing 1% (w/v) oil sands by gradient dilution and streak plate techniques and cultured at 30 °C under aerobic conditions for 72 h. The bacterial colonies with the fastest growth were transferred to a liquid mineral salt medium with oil sands as the carbon source. Finally, the isolated strain samples were freeze-dried and cryopreserved in ampoules.

4.2.2. Bacterial Seed Culture. The lyophilized bacterial powder was inoculated into a 250 mL flask containing 50 mL of LB medium (peptone, 10 g; yeast powder 5 g; NaCl 10 g; water 1000 mL; pH adjusted to 7.0; autoclaved at 121 °C for

30 min before use). The inoculated flask was incubated at 25 °C under aerobic conditions for 7 days. Bacterial growth was observed and recorded using an UV spectrophotometer at 600 nm (optical density).^{47,48}

4.2.3. Optimization of Incubation Temperature. Studies have shown that microbial biomass and activity play a decisive role in the degradation and utilization of oil sands.⁴⁹ Temperature is very important for bacterial growth. Five milliliters of the bacterial suspension (OD600 = 1.0) was transferred into a 250 mL flask containing 50 mL of the mineral salt medium (10 g of NaCl; 0.4 g of NH₄Cl; 0.3 g of KH₂PO₄; 0.3 g of K₂HPO₄; 0.33 g of MgCl₂; 0.05 g of CaCl₂; water, 1000 mL; oil sand 1.0 g; 1 mL of trace element solution (1.5 g of FeCl₂·4H₂O; 0.19 g of CoCl₂·6H₂O; 0.1 g of MnCl₂·6H₂O; 0.07 g of ZnCl₂; 0.006 g of H₃BO₃; 0.36 g of Na₂MoO₄·2H₂O; water 1000 mL); pH adjusted to 7.0; autoclaved at 121 °C for 30 min before use). Four identical inoculated flasks were incubated at 30, 35, 40, and 45 °C, respectively, with oscillation at 120 rpm under aerobic conditions for 3 days. After incubating, the OD600 value of the bacterial suspension was measured to determine the optimal growth temperature of the strain.

4.2.4. Bacterial Identification. The strain was inoculated into the LB medium (agar 15 g; peptone 10 g; yeast powder 5 g; NaCl 10 g; water 1000 mL; pH adjusted to 7.0; autoclaved at 121 °C for 30 min before use). The inoculated plates were incubated at 40 °C under aerobic conditions for 24 h. The colony morphology was observed with eyes, and cell morphology was examined by scanning electron microscopy.⁵⁰ 16S rDNA sequence analysis was conducted in previous research.⁵¹ The gene sequence was entered into the NCBI database for retrieval and deposited in GenBank under the accession number MZ604311. The phylogenetic tree was constructed by the neighbor-joining method in MEGA 7.0.

4.3. Separation and Detection of Biological Surfactants. **4.3.1. Biosurfactant Isolation.** Biosurfactants can improve the solubility of petroleum hydrocarbons and the efficiency of biodegradation.⁵² The biosurfactant (from Y316) was extracted from the mineral salt medium. First, the supernatant pH was adjusted to 2.0 with 6.0 M HCl after cell removal by centrifugation at 4900g for 30 min. Second, an equal volume of CHCl₃/CH₃OH (2:1) was added. After the phase separation, the organic phase was removed, and the extraction operation was repeated twice again. Third, the biosurfactant was concentrated from the organic phases at 40 °C. Finally, the yellowish product obtained was dissolved in methanol and concentrated again by evaporating the solvent at 40 °C.⁵³

4.3.2. Biosurfactant Characterization by TLC. A 0.1 mL surfactant sample was dissolved in chloroform and analyzed by TLC on silica gel plates. The chromatograms were developed using trichloromethane: methanol: water (65:15:2, v/v), and the detection method used is as follows: (1) glycolipids were detected with phenol sulfuric acid reagent and (2) lipopeptides were detected with 1% ninhydrin reagent.⁵⁴ The isolated biological surfactant was analyzed by infrared spectroscopy.

4.3.3. Surface Tension and CMC Determination. To determine the performance of the biosurfactant, the obtained surfactant was accurately weighed and dissolved in distilled water to prepare a series of solutions with the concentration gradient. Surface tension changes were determined by the pendant drop method using OCA-25 (optical contact angle measuring and contour analysis systems, Data Physics

Instruments, Germany) at room temperature. Each result was the average of nine determinations after stabilization. The value of CMC was obtained from the plot of surface tension against surfactant concentration. The CMC value was determined as the mg/L value of the biosurfactant.²⁵

4.4. Oil Sand Degradation Test. **4.4.1. Mineral Salt Medium.** The mineral salt medium was prepared as follows: 10 g of NaCl; 0.4 g of NH₄Cl; 0.3 g of KH₂PO₄; 0.3 g of K₂HPO₄; 0.33 g of MgCl₂; 0.05 g of CaCl₂; water, 1000 mL; 1 mL of trace element solution (1.5 g of FeCl₂·4H₂O; 0.19 g of CoCl₂·6H₂O; 0.1 g of MnCl₂·6H₂O; 0.07 g of ZnCl₂; 0.006 g of H₃BO₃; 0.36 g of Na₂MoO₄·2H₂O; water 1000 mL); 0.1 g of yeast powder and 1 g of glucose; pH adjusted to 7.0; autoclaved at 121 °C for 30 min before use.¹ The seed solution was prepared as follows: 5 mL of the bacterial suspension was transferred in a 250 mL flask containing 50 mL of LB medium; the culture was incubated at 40 °C with oscillation at 120 rpm under aerobic conditions for 12 h; the suspension optical density was 1.0 at 600 nm.

4.4.2. Biodegradation of Oil Sand. In the experiment, in order to prevent the influence of nonoil components on the experiment, the oil sand was washed three times with toluene at 60 °C. The organic phase was placed into flasks and weighed after the natural evaporation of toluene. The mineral salt medium (oil/medium, 1/100, w/v) was added to the flasks. The bacterial seed solution was inoculated into each oil-containing flask (seed solution/medium, 1/10, v/v) and incubated at 40 °C and 120 rpm under aerobic conditions for 35 days.

After incubation, the medium was extracted three times with 100 mL of toluene. The organic phase was dried in air and weighed. The residual oil was dissolved in excess *n*-heptane and centrifuged at 2000 rpm for 5 min, and the supernatant was collected. The above process was repeated until the supernatant was colorless. After drying, the remaining was weighed and regarded as asphaltene quality. The fractions were analyzed by silica gel/alumina column chromatography.^{55,56} In brief, the organic phase was loaded at the top of a column (600 × 20 mm, with a polytetrafluoroethylene stopcock at the bottom), and the column was successively eluted with 200 mL of *n*-heptane, 150 mL of *n*-heptane/toluene (v/v, 2:1), and 90 mL of toluene/dichloromethane/methanol (v/v, 1:1:1). The fractions eluted with these solvents were defined as saturates, aromatics, and resins, respectively. Different organic solutions were selected to elute various fractions of asphalt based on the composition of the target fractions. The alkane phase was a colorless transparent liquid, the aromatic phase was a yellow transparent liquid, and the resin phase was a dark-brown turbid liquid. After drying, the proportion of each component was calculated according to the weighing results. The degradation efficiency of the samples was calculated according to the following formula.

$$\frac{(\text{Initial mass of sample} - \text{mass of sample after biodegradation}) / \text{initial mass of sample} \times 100$$

4.4.3. Saturate, Aromatic, Resin, and Asphaltene Analysis of Oil Sand. The oil sample recovered from the degradation test was dissolved in 5 mL of *n*-hexane. The relative quantity of vaporizable fractions (280 °C) was estimated by measuring the peak area using gas chromatography (Agilent 7890). Gas chromatographic conditions are available in the report of Kim et al.⁵⁷ The effect of biodegradation on oil sand fractions could

be reflected by the difference in the chromatographic peak areas of specific fractions between the degraded group and control group at the same retention time.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.1c06451>.

OD600 value of Y316 at initial medium pH 5–9 (aerobic culturing conditions: 24 h at 40 °C with 120 rpm agitation) (PDF)

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Notes

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