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# Structural Diversity of the Lipopeptide Biosurfactant Produced by a Newly Isolated Strain, *Geobacillus thermodenitrifcans* ME63

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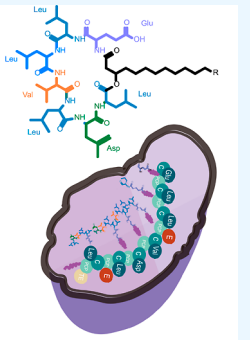
**ABSTRACT:** The genus *Geobacillus* is active in degradation of hydrocarbons in thermophilic and facultative environments since it was first reported in 1920. Here, we report a new strain, *Geobacillus thermodenitrificans* ME63, isolated from an oilfield with the ability of producing the biosurfactant. The composition, chemical structure, and surface activity of the biosurfactant produced by *G. thermodenitrificans* ME63 were investigated by using a combination of the high-performance liquid chromatography, time-of-flight ion mass spectrometry, and surface tensiometer. The biosurfactant produced by strain ME63 was identified as surfactin with six variants, which is one of the representative family of lipopeptide biosurfactants. The amino acid residue sequence in the peptide of this surfactin is N-Glu → Leu → Leu → Val → Leu → Asp → Leu-C. The critical micelle concentration (CMC) of the surfactin is 55 mg $L^{-1}$, and the surface tension at CMC is 35.9 mN $m^{-1}$, which is promising in bioremediation and oil recovery industries. The surface activity and emulsification properties of biosurfactants produced by *G. thermodenitrificans* ME63 showed excellent resistance to temperature changes, salinity changes, and pH changes.




## INTRODUCTION

Thermophilic, Gram-positive, spore-forming, chemo-organotrophic, and aerobic or facultatively anaerobic *Geobacillus* species attract industry attention as metabolic factories for the production of thermophilic enzymes, biomass energy, and antibiotics.[1] The genus *Geobacillus* were once classified as the genus *Bacillus*, and the earliest report of *Geobacillus* was the isolation of a strain named *Geobacillus stearothermophilus*.[2] In 2001, taxonomists reclassified *Bacillus kaustophilus*, *Bacillus stearothermophilus*, *Bacillus thermocatenulatus*, *Bacillus thermodenitrificans*, *Bacillus thermoglucosidasius*, and *Bacillus thermoleovorans* into a new genus *Geobacillus*.[3] The genus *Geobacillus* contains 20 species, of which 21 strains have been reported.[4] The reduction of nitrate to nitrogen gas by *Geobacillus thermodenitrificans* was originally named "Denitrophilum" by Ambroz[5] and was once officially recognized as *G. stearothermophilus*.[6] Research on *G. thermodenitrificans* has focused on three areas: hydrocarbon degradation, thermophilic enzyme production, and antibiotic biosynthesis.[7−9] A variety of thermophilic enzymes with diverse functions were isolated from *G. thermodenitrificans*, including esterase, amylase, glucosidase, arginase, and xylan-degrading enzyme.[9,10] Furthermore, *G. thermodenitrificans* NG80-2 with hydrocarbon degradation ability focuses academia and industry spotlight.[11] Whole-genome sequencing analysis of *G. thermodenitrificans* NG80-2 expounds the metabolic pathway of alkane degradation. Alkanes entering the cell are primary oxidized in vitro by alkane oxygenase released by *G. thermodenitrificans* NG80-2 into primary or secondary alcohols, then converted into aldehydes by dehydrogenase, and finally oxidized into fatty acids. Fatty acids pass through the cell membrane and enter the cell through the classic $\beta$-oxidation pathway and tricarboxylic acid cycle to decompose into carbon dioxide and water to release energy.[12,13] It is well known that the hydrocarbons collected and decomposed by *Geobacillus* species are often inseparable from biosurfactants. Emulsification, micellization, adhesion, and deadhesion of microorganisms to hydrocarbons and desorption of hydrophobic compounds play a key role in enhancing the bioavailability of hydrocarbons by biosurfactants.[14]

Biosurfactants are structurally diverse amphiphilic compounds usually composed of glycolipids, phospholipids, lipopeptides, fatty acids, polysaccharides−protein complexes, and natural lipids.[15] Microbial lipopeptides with excellent surface activity and broad-spectrum antibacterial properties have been widely used in the pharmaceutical industry since surfactin was first discovered.[16] Microbial lipopeptides with rheology and resistance to extreme environments have been widely used in the petroleum industry.[17] Considering that the

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oil reservoir is a special ecosystem with high-temperature, high-salt, and high-pressure conditions.[18] The stable performance of lipopeptide to high temperature and high salinity is critical in the process of crude oil recovery. Keharia et al. demonstrated that the biosurfactant from *Bacillus licheniformis* DS1 maintained good stable emulsifying activity at pH 4−10, elevated temperature up to 120 °C, and NaCl concentration up to 10% (w/v).[19] Although research studies on *Geobacillus* species in the oil recovery process have been carried out in detail, there is limited information on biosurfactants synthesized by *Geobacillus* species. *Geobacillus* sp H2OVP3 was reported to produce anionic biosurfactants from crude oil in 2013.[20] The ability of *Lysinibacillus sphaericus* and *Geobacillus* sp to produce biosurfactants has been determined using the drop method by Manchola and Dussán.[20] The biosurfactant produced by *Geobacillus thermoleovorans* JQ912239 showed a maximum emulsification index of 87% and a lower surface tension reduction of 43 mN $m^{-1}$.[21] Bioemulsifiers produced by the facultative anaerobic bacterium *G. thermodenitrificans* NG80-2 proved to be polysaccharide−protein complexes.[22] Detailed mechanisms of oxidation and degradation of hydrocarbons by *Geobacillus* sp have been reported. However, there are only four reports that *Geobacillus* sp can produce biosurfactants, and the structural information of biosurfactants is scarce.

In this study, the biosurfactants produced by a new strain *G. thermodenitrificans* ME63 were identified as surfactin with six variants. The amino acid residue sequence in the peptide of this surfactin is N-Glu → Leu → Leu → Val → Leu → Asp → Leu-C. The yield of the surfactin by strain ME63 was 0.81 g $L^{-1}$, as determined by high-performance liquid chromatography (HPLC). The critical micelle concentration (CMC) was estimated to be about 55 mg $L^{-1}$, corresponding to a surface tension of 35.9 mN $m^{-1}$. Surfactin produced by *G. thermodenitrificans* ME63 exhibits excellent resistance to changes in temperature, salinity, and pH, making it a promising candidate for the oil recovery application.

## MATERIALS AND METHODS

**Materials.** All the chemicals and biochemicals used, unless otherwise stated, were obtained from Shanghai Meryer Chemical Technology Co., Ltd, Shanghai, China. Acetonitrile and methanol (HPLC-grade) were obtained from Shanghai Boer Chemical reagents Co., Ltd, Shanghai. Acetonitrile and methanol (LC−MS grade) were obtained from Shanghai Macklin Biochemical Co., Ltd, Shanghai, China. The genomic DNA miniprep kit was obtained from Axygen Bioscience Co., Ltd, Union City, CA, USA. Produced water was collected from the Daqing oilfield in China for microorganism isolation.

**Screening for Biosurfactant-Producing Microorganisms.** *Medium.* Luria−Bertani medium with 10.0 g of peptone, 5.0 g of yeast extract, and 10.0 g of NaCl per liter was used. Mineral salt medium (MSM)[23] containing 15 g of $NaNO_3$, 1.1 g of KCl, 1.1 g of NaCl, 0.00028 g of $FeSO_4{\cdot}7\ H_2O$, 3.4 g of $KH_2PO_4$, 4.4 g of $K_2HPO_4$, 0.5 g of $MgSO_4{\cdot}7\ H_2O$, and 0.5 g of yeast extract per liter was used, and an additional 20 g of sucrose per liter was added for strain cultivation. All media were adjusted from their original pH to 7.2 using 1.0 M NaOH or 1.0 M HCl. The medium was sterilized at 115 °C for 30 min.

*Screening Method.* The mineral salt medium was boiled under the protection of $N_2$−$CO_2$ gas to remove dissolved oxygen, then it was put it into serum bottles and sterilized at 115 °C for 30 min, and the resazurin reagent as the indicator of dissolved oxygen (red in the aerobic state, colorless in the anaerobic state) was added. The mixture was inoculated with 1% (v/v) oilfield-produced water and cultured for 15 days. The isolated bacteria were obtained by aerobic separation of the samples of anaerobic enrichment culture. After the isolated bacteria were cultivated, the production potential of the biosurfactant was detected by the surface tension test.

*Surface Tension Test.* The microorganisms were incubated for 72 h at 37 °C (180 rpm/min). Then, the cells were detached by centrifugation at 8000*g* for 10 min. The supernatant was collected and used for surface tension. The surface tension was measured using a fully automatic tensiometer JK99B (Shanghai Wang Xu Electric Co., Ltd., China) by the du Nouy ring method.[24−26] The measurement temperature is set at 25 °C, the pH is adjusted to 7.2, and all test data were averaged three times in parallel.

**Identification of Biosurfactant-Producing Microorganisms.** The AxyPrepTM bacterial genomic DNA miniprep kit (Axygen Bioscience, Union City, CA, USA) was chosen to extract total bacterial DNA. Universal bacterial primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) were applied as PCR primers for the strain DNA template.[26] The National Center for Biotechnology Information (NCBI) serves as a database for nucleic acid sequence comparisons. Phylogenetic tree construction was carried out using MEGA7.0[27] unweighted pairwise method of set arithmetic mean (UPGMA) and bootstrap.[28]

**Biosurfactant Extraction.** *Biosurfactant Extraction.* The cell-free medium was obtained by the centrifugation of the culture at 5000*g* for 25 min. 6.0 M HCl was added to the obtained cell-free culture supernatant to adjust to a final pH of 2.0, then left overnight in the refrigerator, followed by centrifugation at 5000*g* for 20 min to obtain a precipitate.[29] The obtained precipitate was extracted three times with ethyl acetate, and the organic phases were combined. Finally, the organic phase is removed by rotary evaporation to obtain a purified biosurfactant.[30]

*Determination of Molecular Masses.* The purified biosurfactant was dissolved in methanol and analyzed by ESI−MS (Micromass Co., UK). The electrospray source was operated at an ionization source temperature of 80 °C, an electrolyte voltage of 100 V, and a spray inlet temperature of 120 °C. The spectrum was acquired in the negative- and positive-ion mode.

*Reversed-Phase HPLC.* The purified biosurfactant mixture was separated into individual biosurfactants by reverse-phase HPLC using an HPLC system (LC 100) equipped with a HiQ sil C18 W column (KYA TECH, Japan; 5 μm, 250 × 21.2 mm). The HPLC system was set at 30 °C where the mobile phase was an 80 to 100% linear gradient of 0.1% (v/v) formic acid in acetonitrile and 0.1% (v/v) formic acid in water. The mobile phase was eluted at a flow rate of 1 mL·$min^{-1}$ and detected by an UV monitor at 205 nm. All test data were averaged three times in parallel.[31]

*Tandem Mass Spectrometry of Biosurfactants.* 5 mg of the purified biosurfactant was dissolved in acetonitrile and analyzed by LC−MS/MS (Thermo Q-Exactive plus). The collision energy of the mass spectrometry system was set at 30 V. The first-stage quadrupole mass spectrometer was used to select the target hydrogen-ionized molecule, and then, time-of-flight secondary ion mass spectrometry was used to analyze


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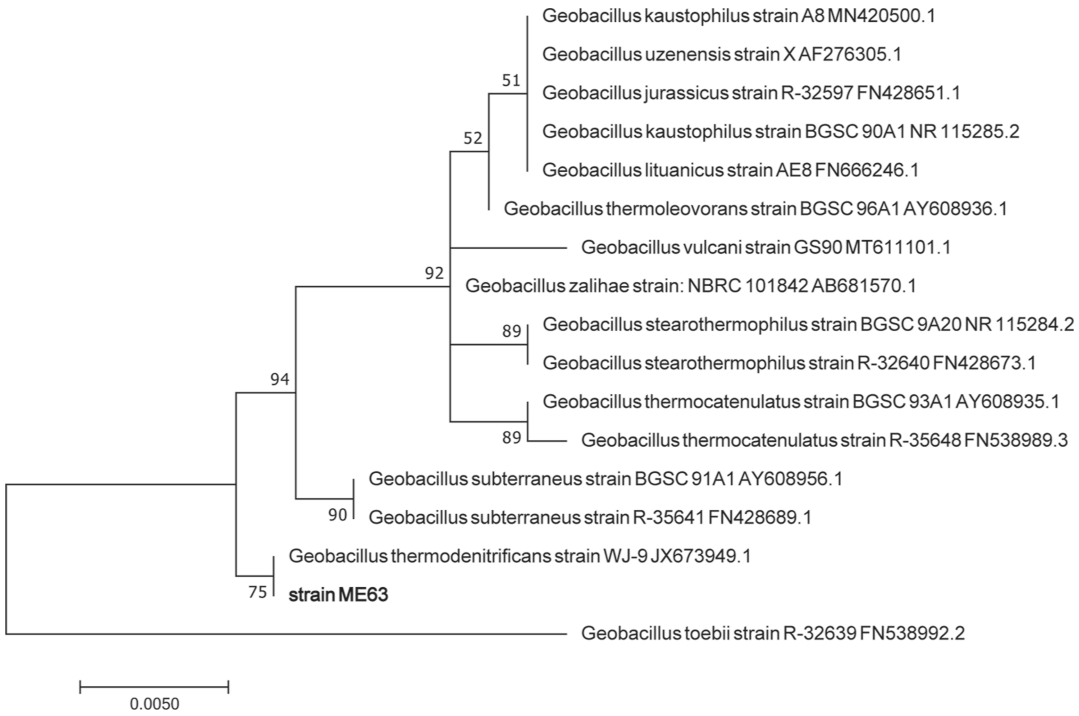


**Figure 1.** Phylogenetic tree based on 16S rRNA gene sequences showing the relationship between strain ME63 and different *Geobacillus* species-type strains. The Genbank accession number of each species is shown in the end.

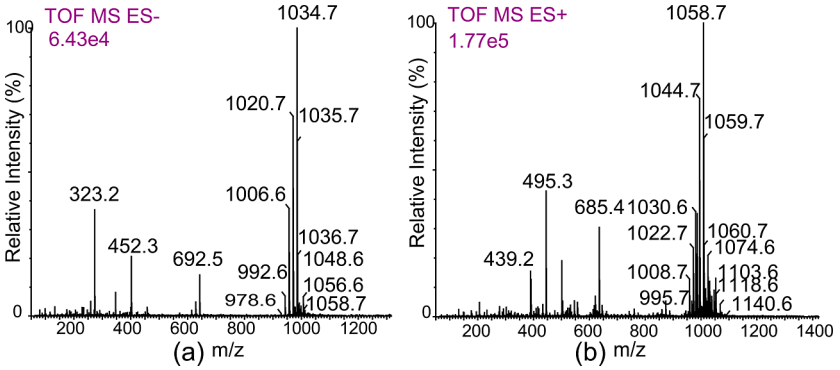


**Figure 2.** Mass spectrometry of biosurfactants produced by *G. thermodenitrificans* ME63. (a) Negative ionization mass spectrometry. (b) Positive ionization mass spectrometry.

and record the hydrogen ionization fragments produced by the fragmentation of the target hydrogen-ionized molecule.

**Stability Testing of Biosurfactants.** The bacteria were cultured in the fermentation medium at 37 °C and 180 rpm for 72 h. Then, the cells were separated from the fermentation solution by centrifugation at 8000 rpm for 10 min.

*Temperature Resistance Test.* The supernatant was incubated at different temperatures (20, 40, 60, 80, 100, and 120 °C) for 2 h to test the surface tension and emulsification index.

*Salt Resistance Test.* Surfactants are tested for surface tension and emulsification index after being kept for 2 h under different salinity conditions (0, 5, 10, 15, 20, 25, and 30%) (w/v).

*Acid and Alkali Resistance Test.* The pH of the supernatant was set to 2 to 12, and the surface tension and emulsification index were measured after keeping it stable for 2 h.[32]

## RESULTS AND DISCUSSION

**Biosurfactant-Producing Strain.** The biosurfactant-producing strain ME63 was isolated from production water of Daqing oilfield, China. Phylogenetic tree analysis based on 16S rRNA encoding gene sequence showed that this strain belonged to the *G. thermodenitrificans* (Figure 1). After 72 h of culture, the surface-active compound produced by *G. thermodenitrificans* ME63 reduced the surface tension of medium to 33.1 mN $m^{-1}$, and hemolysis assay and droplet collapse test showed positive results. The emulsification index $E_{24}$ (%) of the supernatant to liquid paraffin was 54.5%.

**Structural Characterization.** Biosurfactants produced by *G. thermodenitrificans* ME63 were determined by mass spectrometry with negative- and positive-ion modes (Figure 2). Negative ionization detected peaks with different *m/z* values of 978.6, 992.6, 1006.6, 1020.7, 1034.7, and 1048.6 (Figure 2a). The mass-to-charge ratio difference between each peak is 14 masses, corresponding to exactly one $CH_2$ residue. This indicates that the purified biosurfactant is a mixture of


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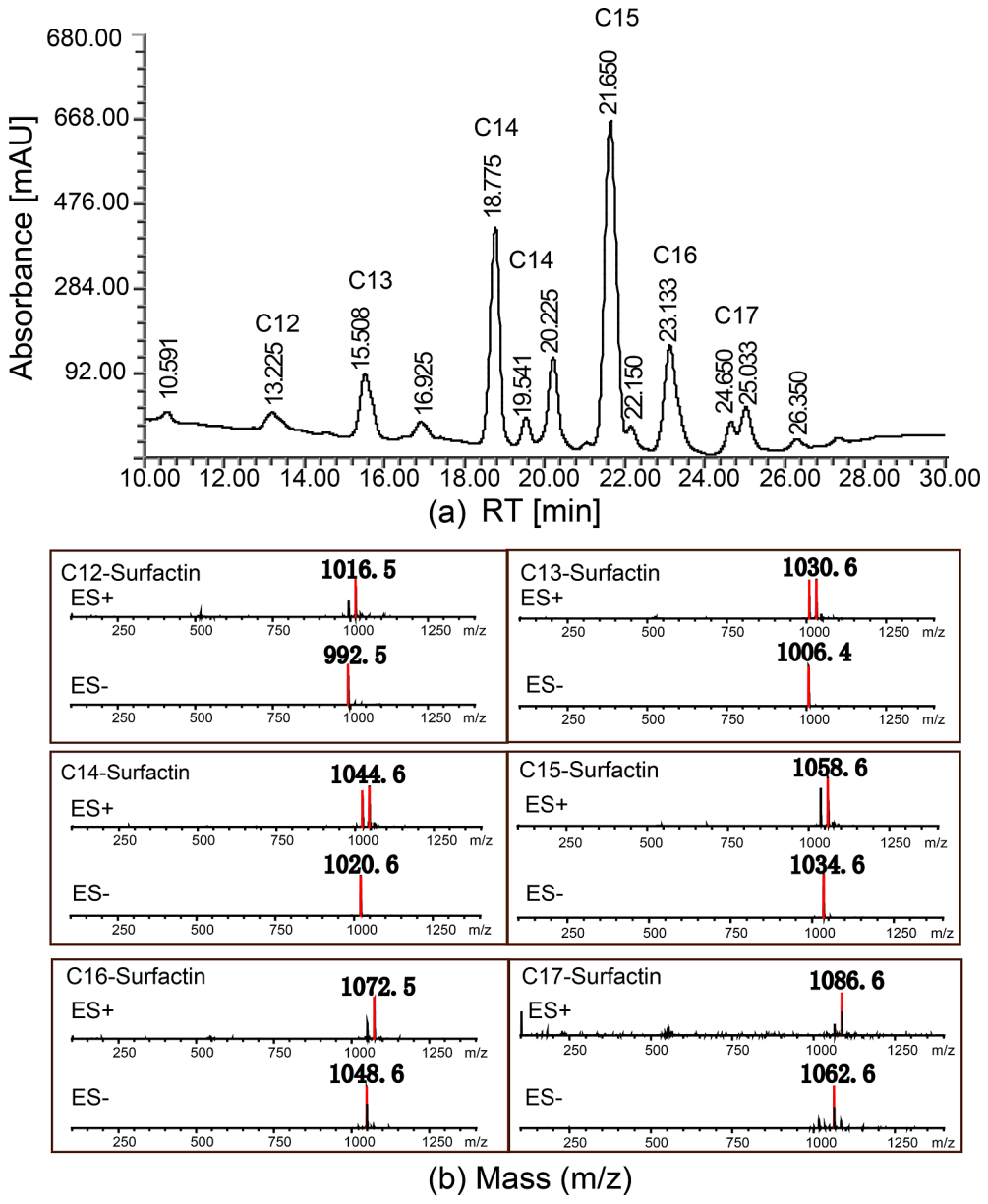


**Figure 3.** Composition of the biosurfactants produced by *G. thermodenitrificans* ME63. (a) HPLC chromatogram. (b) Mass-to-charge ratios with positive- and negative-ion mass spectra.

surfactin with a series of different fatty acid chain lengths. Positive ionization detected peaks (*m/z* 1008.7, 1022.7, 1030.6, 1044.7, 1058.7, 1074.6, 1103.6, and 1118.6), corresponding to the mass-to-charge ratios of negative ionized mass spectrum peaks (Figure 2b). Therefore, the biosurfactant produced by *S. luteola* ME44 was determined as surfactin homologues.

As shown in Figure 3, 13 peaks were separated with retention times ranging from 10 to 30 min by HPLC. The highest peak with retention time of 21.550 min represents C15-surfactin (corresponding to mass-to-charge ratios of 1034.6 and 1058.6), followed by C16-surfactin with a peak retention time of 23.133 min, corresponding to mass-to-charge ratios of 1048.6 and 1072.5. An incompletely resolved doublet represents C17-surfactin with retention times of 24.650 and 25.033 min. The three peaks with retention times of 18.775, 19.541, and 20.225 min were shown as hydrogen-ionized C14-surfactin with a mass-to-charge ratio of 1020.6 and sodium-ionized C14-surfactin with a mass-to-charge ratio of 1044.6. The peak of C13-surfactin appeared at 15.508 min (corresponding to mass-to-charge ratios of 1006.4 and 1030.6), and the peak of C12-surfactin appeared at 13.225 min with mass-to-charge ratios of 992.5 and 1016.5. The biosurfactants produced by *G. thermodenitrificans* ME63 contain six surfactin variants. The yield of this strain ME63 was 0.81 g $L^{-1}$, as determined by HPLC. The proportions of the surfactin variants C12-surfactin, C13-surfactin, C14-surfactin, C15-surfactin, C16-surfactin, and C17-surfactin were 2.6, 8.2, 32.4, 38.6, 13.8, and 4.4%, respectively.

As shown in Figure 4, the upper and lower images are the mass spectra of components C13 and C14 respectively, and the difference between any two peaks is the mass of the missing fragment. The peptide ring of surfactin is composed of 1 Val, 1 Asp, and 4 Leu.[33] Both mass spectra contain the same characteristic peaks of hydrogen ion amino acid residues 227.4 and 685.3 by comparing the spectra of component C13 and


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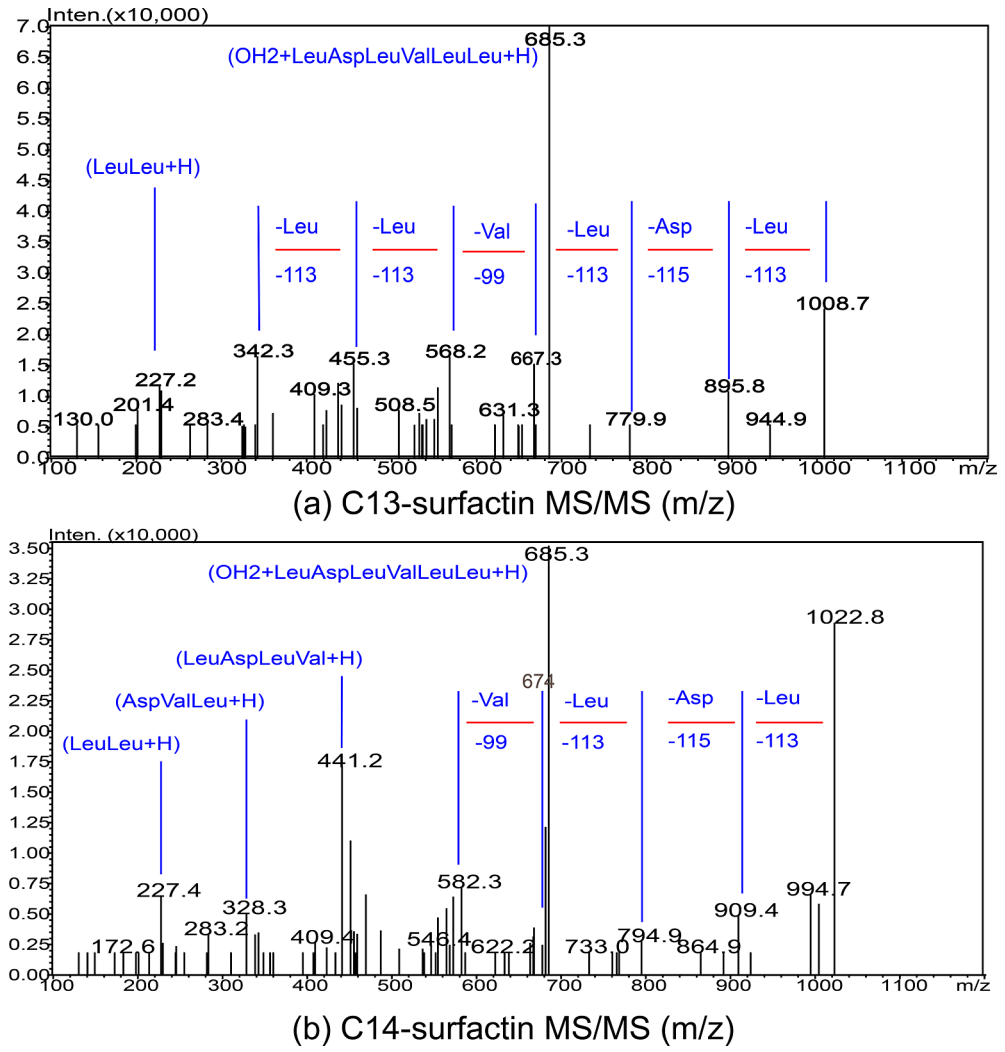


**Figure 4.** Mass spectrum of C13-surfactin and C14-surfactin hydrogen ion lipopeptide components and their hydrogen ion fragments. (a) Mass spectrum of C13-surfactin hydrogen ion fragment. (b) Mass spectrum of C14-surfactin hydrogen ion fragment.

component C14. Besides, each peak in the mass spectrum of C14 (582, 674, 794, 909, and 1022) has a mass-to-charge ratio of 14 more than the corresponding peak of C13 (568, 666, 779, 895, and 1008), showing that the aliphatic part of C14 has one more $CH_2$ residue than that of C13. Mass differences in mass spectral peaks can be used to determine the linking peptide chains of amino acid residues. Therefore, the mass difference between peak 1022 → 909 → 794 → 674 → 582 of C14 and peak 1008 → 895 → 779 → 666 → 568 of C13 indicates that the amino acid residues are connected in the form of Leu-Asp-Leu-Val. In addition, looking at the peaks of the C13 and C14 groups from low to high 227 → 328 → 441 → 685 indicates that the sequence is Leu-Leu-Val-Leu-Asp-Leu. The last residue Glu in the peptide can only be supported by the Glu-Leu-Leu-Val-N fragment in the MS spectrum as the N-terminus after N-Leu. According to the amino acid analysis of the peptide chain, the amino acid residue sequence was N-Glu → Leu → Leu → Val → Leu → Asp → Leu-C in the peptide. The amino acid sequence of surfactin produced by *G. thermodenitrificans* ME63 is slightly different from the normal amino acid sequence of surfactin.[34]

**Surface Activity.** Determination of CMC of biosurfactants produced by *G. thermodenitrificans* ME63 via surface tension changes. The surface tension curve first decreased and then remained constant with the increase of biosurfactant concentrations. According to the above results in Figure 5, the turning point of the surface tension curve corresponds to the biosurfactant concentration of 55 mg $L^{-1}$, and the surface tension remained at 35.9 mN $m^{-1}$.

**Properties of Biosurfactants.** Determination of surface activity and emulsification of biosurfactants produced by *G. thermodenitrificans* ME63 at different temperature conditions, different salinity conditions, and different pH (Figure 6) were analyzed. The surface tension of the biosurfactant produced by *G. thermodenitrificans* ME63 was 25.2 mN $m^{-1}$ at temperature of 20 °C, which slightly increased to 26.1 mN $m^{-1}$ as the temperature increased to 120 °C. The emulsification index ($E_{24}$) of the biosurfactant slightly fluctuated in the range of 54.3 to 56.6% as the temperature increased from 20 to 120 °C (Figure 6A). The surface tension of this biosurfactant shows a U-shaped change in the pH range of 2 to 12, the lowest surface tension of 24.2 mN $m^{-1}$ occurs at pH = 4, and the highest surface tension of 30.1 mN $m^{-1}$ occurs at pH 12. In addition, the change of the emulsification index presents an inverted U-shaped change, and the maximum emulsification index of 53.3% appears at pH = 6 (Figure 6B). High salinity is also a major feature of oil reservoirs. The surface tension of the biosurfactant increases slowly with the salinity, and its instant


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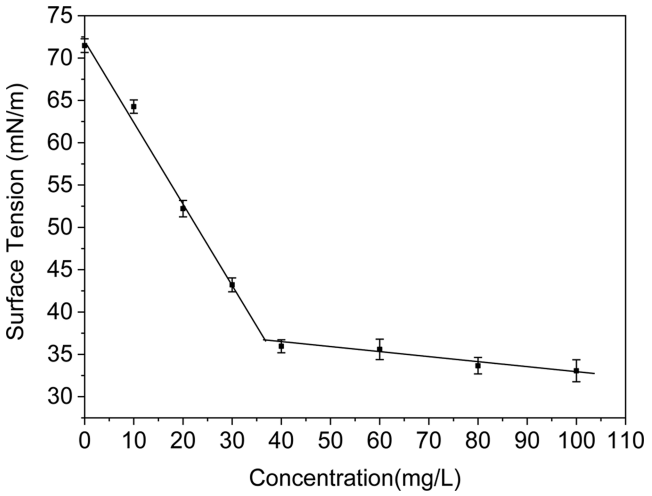


**Figure 5.** Surface tensions of the purified biosurfactant at different concentrations. The intersection of the two black lines represents the CMC, which is estimated to be approximately 55 mg $L^{-1}$.

surface tension is always lower than 30 mN $m^{-1}$ even under the condition of 30 (w/v) % salinity. As the salinity increases, the emulsifying performance ($E_{24}$ %) of the biosurfactant decreases significantly, it decreased from 56.5 to 42.0% (Figure 6C). The surface activity and emulsification of biosurfactants produced by *G. thermodenitrificans* ME63 were less affected by temperature changes, salinity changes, and pH, except for the effect of salinity on emulsification.

## DISCUSSION

*G. thermodenitrificans* has versatile metabolic pathways, flexible respiration systems, and robust transport systems for its survival under various natural condition. Furthermore, alkane oxygenase oxidation of alkanes has been reported as the primary step in the degradation of long-chain alkanes by *G. thermodenitrificans* NG80-2.[12] Biosurfactant-enhanced microbial degradation of hydrophobic hydrocarbons has been extensively studied.[35] One of the functions of lipopeptide is to increase the solubility and bioavailability of hydrocarbons, and another role for lipopeptide is to interact with phospholipid bilayers to change the permeability of cell membranes. Therefore, utilization of alkanes and surfactin biosynthesis by *G. thermodenitrificans* are expected to play a significant role in microbial enhanced oil recovery (MEOR) and oil pollution bioremediation. However, there is limited information on the structure of biosurfactants produced by *Geobacillus* species. Microbial lipopeptides were commonly produced by *Bacillus*,[36] *Acidobacterium*,[37] *Brevibacterium*,[38] *Paenibacillus*,[39] *Rhodococcus*,[40] *Streptomyces*,[41] *Pseudomonas*,[42] *Acinetobacter*,[43] and *Serratia*.[44] 34 families of lipopeptides containing 300 lipopeptide compounds have been reported.[45] To the best of our knowledge, this is first report about structure and composition of biosurfactants produced by *Geobacillus* species. The lipopeptides extracted from *G. thermodenitrificans* ME63 was determined as surfactin with six variants, including C12-surfactin (2.6%), C13-surfactin (8.2%), C14-surfactin (32.4%), C15-surfactin (38.6%), C16-surfactin (13.8%), and C17-surfactin (4.4%), respectively. Interestingly, the amino acid residue sequence N-Glu → Leu → Leu → Val → Leu → Asp → Leu-C of surfactin produced by *G. thermodenitrificans* ME63 is slightly different from the classical amino acid sequence of surfactin (N-Glu → Leu → Leu → Val → Asp → Leu → Leu-C).[46] The peptide ring of surfactin synthesis is performed by a cluster of non-ribosomal peptide synthetases.[47] Therefore, it is speculated that the amino acid sequence of the peptide ring of surfactin is related to the base sequence of the gene encoding NRPSs. Biosurfactants are secondary metabolites produced by microorganisms in response to environmental stimuli, especially oil reservoir. Composition and structure of biosurfactants produced by *G. thermodenitrificans* ME63 deepen our understanding of hydrocarbon degradation by *G. thermodenitrificans*. On the one hand, the biotoxicity of biosurfactants can reduce the competition between species. Second, biosurfactants contribute to biofilm formation because of their amphiphilic structure that alters the wettability of the environment. On the other hand, biosurfactants enhanced the degradation of hydrocarbons by *G. thermodenitrificans* by increasing the contact area between microorganisms and hydrocarbons.

## CONCLUSIONS

A new lipopeptide-producing strain, *G. thermodenitrificans* ME63, was isolated from Daqing oilfield, China. The biosurfactant produced by *G. thermodenitrificans* ME63 was identified as the surfactin. The yield of the strain ME63 was 0.81 g $L^{-1}$, as determined by HPLC. The amino acid residue sequence in the peptide of surfactin produced from *G. thermodenitrificans* ME63 is N-Glu → Leu → Leu → Val → Leu → Asp → Leu-C. The biosurfactant contains six surfactin

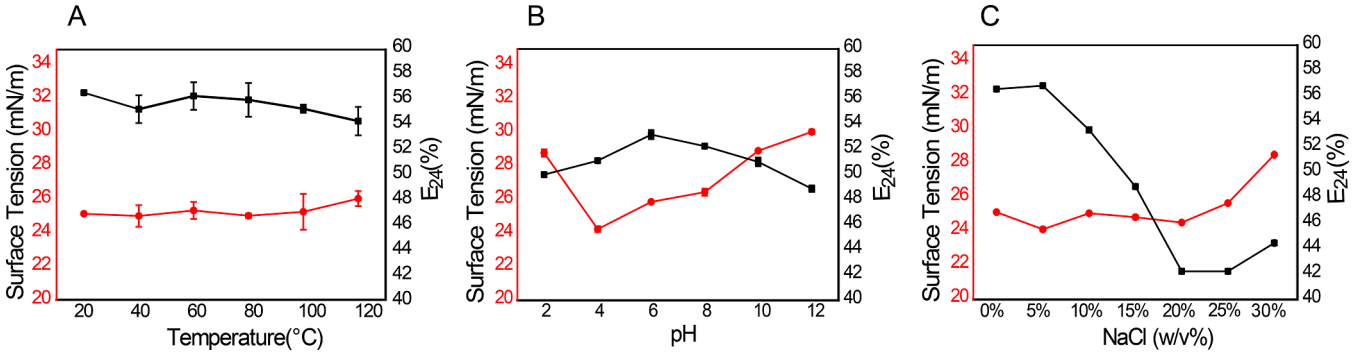


**Figure 6.** Temperature, salt, acid, and alkali resistance of the biosurfactant. The red line represents changes in surface tension, and black line represents changes in the emulsification index. (A) Temperature resistance test of the biosurfactant. (B) Acid and alkali resistance test. (C) Salt resistance test.



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variants, including C12-surfactin (2.6%), C13-surfactin (8.2%), C14-surfactin (32.4%), C15-surfactin (38.6%), C16-surfactin (13.8%), and C17-surfactin (4.4%), respectively. The CMC was estimated around 55 mg $L^{-1}$, with a corresponding surface tension of 35.9 mN $m^{-1}$. The surface activity and emulsification properties of biosurfactants produced by *G. thermodenitrificans* ME63 showed excellent resistance to temperature changes, salinity changes, and pH changes.

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### Author Contributions

Shi-Zhong Yang, and Bo-Zhong Mu conceptualized and supervised the overall work; Jia-Yi Li carried out the experiments and validated the data; Jia-Yi Li, Shi-Zhong Yang, and Bo-Zhong Mu—writing original draft preparation; and Yi-Fan Liu, Lei Zhou, Hong-Ze Gang, Jin-Feng Liu, Gang-Zheng Sun, and Wei-Dong Wang—writing, review, and editing. All authors have read and agreed to the published version of the manuscript.

### Notes

The authors declare the following competing financial interest(s): Author Jin-Feng Liu is employed by Daqing Huali Biotechnology Co., Ltd. Author Gang-Zheng Sun and Wei-Dong Wang are employed by Research Institute of Petroleum Engineering and Technology, Shengli Oilfield Company, Sinopec, Dongying, China. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

All data generated during this study are included in this article. DNA sequences: Genbank accession OP999715 (https://submit.ncbi.nlm.nih.gov/subs/rsearch=SUB12409787).

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