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Hydrocarbons degradation by a newly isolated thermophilic *Anoxybacillus sp.* with bioemulsifier production and new alkB genes

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Abstract: Interests in biodegradation of petroleum derived pollutants by thermophilic bacteria have been steadily increasing in recent decade. In this work, a newly isolated thermophilic bacterial strain was isolated from the deep petroleum reservoir and identified as *Anoxybacillus. sp* WJ-4 based on the analysis of the physiological characteristics, 16S rRNA sequencing, GC content and cellular fatty acids. It is the first report that strain WJ-4 can degrade a wide range of hydrocarbons (C8-C22) at 67^{o}C. The production of oligosaccharide-lipid-peptide bioemulsifier was detected. It exhibited the excellent emulsification activity with various oil phases (EI_{24}>60%), and the ability of increasing the cell surface lipophilicity during the degradation, but has no significant impact on the surface tension with reducing from 72.22 mN/m to 52.45 mN/m. Four alkane monooxygenase genes showed high phylogenetic relationship (>95%) with alkB genes from *Geobacillus*. These results indicated that this newly isolated bacterial strain and its bioemulsifier have great potentials in the environmental remediation and petroleum recovery under thermophilic condition.

Key words: Biodegradation; Bioemulsifier; *Anoxybacillus*; Thermophilic; Cell surface Lipophilicity.

1. Introduction

Petroleum is the predominant energy resource and chemically industrial raw materials in modern society. Spills, leaks, and other releases of petroleum, however, often result in soil and groundwater contamination.[1] Main sources of petroleum contamination include petrochemical industry processes, oil field installations, petroleum plants, liquid fuel distribution and storage devices, transportation equipment for petroleum products.[2] Pollution from such sources has significant environmental impact and presents human health hazardous. Petroleum is a complex mixture of hydrocarbons and related compounds. It is generally classified into four fractions: saturated alkanes, aromatics, resins, and asphaltenes, the latter two of which consist of polar molecules containing N, S, and O_2. Alkanes can

constitute 50% to 95% of crude oil, depending on source. As the main component of fuels and oils, its relative inertness poses ecological problems upon release to the environment. Due to their lack of functional groups as well as poor water solubility, alkanes exhibit both low chemical reactivity and low bioavailability for microorganisms.[3,4] Various linear, branched and cyclo-alkanes are known to cause respiratory, renal, or central nervous system disorders. As a result, considerable attention has been paid to the treatment of these pollutants.

Microbial bioremediation has been evaluated as one option in various polluted environments and is claimed to represent an efficient, economical, and versatile alternative to physicochemical treatments.[5,6] Hydrocarbon-degrading bacteria are widely distributed in nature. They include members of at least 60 genera of aerobic and five genera of anaerobic bacteria,[3,7-9] particularly *Acinetobacter*, *Rhodococcus*, *Alcanivorax*, *Bacillus*, *Mycobacterium*, *Pseudomonas* and *Dietzia*, which are among the most well known and studied.[10-13] However these bacteria generally exhibit good performance only under mesophilic conditions. Few thermophilic hydrocarbon-degrading species have been reported, although many thermophiles have been described.

Degradation of petroleum hydrocarbons by thermophilic bacteria has advantages over that by mesophilic or psychrophilic organisms, especially when they are incorporated into biotechnological applications.[14,15] An increasing attention has been paid directly to this field in recent decade.[16,17] Thus it is necessary to isolate more thermophilic strains with the capability to degrade these water-insoluble molecules. The majority of thermophilic strains are obtained from hot springs and oil reservoirs. High temperature petroleum reservoirs with temperatures exceeding 50 ºC are one type of biotope attracting great interest as sites for the collection and screening of new thermophilic hydrocarbon-degrading bacteria.[4,14,17,18] Genera collected from these sites have been limited to *Bacillus*, *Geobacillus*,

Thermoactinomyces, and *Brevibacillus*.[19,20]. Only four studies on hydrocarbon degradation by *Anoxybacillus* have also been reported,[21-24] where the degraded compounds were aromatic. As new thermophilic genus, *Anoxybacillus* could be utilized in a large number of applications as previous description,[25] however these applications are currently limited to the hydrolysis of the starch and lignocellulosic biomasses, not the degradation of alkanes.

Recognized bacterial mechanisms for enhancing hydrocarbon substrate availability and utilization for simplicity generally divide into (a) those related to the uptake of soluble fractions and (b) those related to the production of surfactants for physical modification of substrates and cell surface lipophilicity;[26-28] the mechanisms however overlap. Extremely limited water solubility is the most serious hurdle for the uptake alkane as a metabolic substrate, although higher temperatures can contribute to improved mass transfer rates. The uptake of hydrophobic components therefore commonly and frequently involves the production of microbial surfactant molecules as emulsifying agents or cell surface lipophilicity (CSL) altering agents to facilitate ultimate biodegradation.

Produced by a wide range of hydrocarbon-degrading microorganisms, microbial surfactants can be grossly classified into two major categories based on molecular weight (MW).[29,30] Examples of low-MW biosurfactants include glycolipids and lipopeptides, which dominant function is the lowering of the surface/interfacial tension. While examples of high-MW biosurfactants include emulsan, alasan, biodispersan, and extracellular or cell membrane-bound bioemulsifiers (such as exopolysaccharide and lipopolysaccharides), which main function is emulsion stabilization.[30,31]. For many hydrocarbon-degrading microorganisms, extensive changes of the cell surface lipophilicity were detected during growth on hydrocarbons.[32] Several reports in the literature find correlation among CSL, microbial surfactant production, and hydrocarbon degrading capability.[26,30-32]

In the present report, a novel thermophilic hydrocarbon-degrading strain WJ-4 was isolated and investigated. This is the first report that this *Anoxybacillus* strain can utilize alkanes (C8 - C22) as the sole source of carbon for growth. With a goal of designing future applications in remediation of crude oil contaminated environments, the alkane-degrading kinetics of strain WJ-4 was characterized. A bioemulsifiers was detected during biodegradation and its structure was analyzed. Emulsification stability was evaluated as well. A tentative comparison of the *alkB* gene from this novel thermophilic strain was also carried out. This paper highlights an important potential use of a novel thermophilic strain for the cleanup of alkane or petroleum polluted environments.

2. Experimental

2.1. Isolation of microorganisms

The brine and oil samples were collected from the reservoir formation located at depths of 1300 to 1600 meters subterranean with the temperature of 58-68°C in Longhupao block (latitude 46.798383, longitude 124.350554) of Daqing oilfield. Fifty milliliter of the brines and five grams of crude oil were transferred to the 250 ml flask filled with 50.0 ml of the autoclaved minimal medium supplemented with trace-element solution (0.1%, v/v), and then incubated at 65°C with 180 rpm for 20 days. The minimal medium contains (g/L): NH_4NO_3 3.4, K_2HPO_4 1.5, NaH_2PO_4, 1.5, $MgSO_4$ 0.3, yeast extract 0.3, Glucose 0.3, pH 6.8-7.2; and the composition of the trace-element solution contains (g/L): $MnCl_2{\cdot}4\ H_2O$ 0.1, $CoCl_2{\cdot}6H_2O$ 0.17, $CaCl_2{\cdot}2H_2O$ 0.02, $FeCl_2$ 0.4, H_2BO_3 0.019, $ZnCl_2$ 0.1, $NaMoO_4{\cdot}2H_2O$ 0.1, vitamin B_{12} 0.01, Vitamin C 0.01, $NiCl_2{\cdot}6H_2O$ 0.05. After 20-days incubation, fifty milliliter of the cultured sample was transferred to 50 ml of the above fresh sterilized medium again for the second 20-days incubation at the same condition. Then 100 ul of the final cultured sample was spread onto LB agar plates and incubated at 65°C for 48 h. Pure cultures of each morphologically

distinct colony were selected by repetitive streaking onto solid LB agar medium. In order to isolate the strains with ability of degrading hydrocarbon and biosurfactant production, the selected pure strains were cultured for 10 days in the above sterilized minimal medium with crude oil as carbon source, then the cell-oil-free supernatant of each cultured sample was conducted the oil spreading agar plate and crude oil emulsification test following the described methods.[33]

2.2. Characterization of strain

The final selected bacterial strain was characterized according to the standard biochemical tests (morphology and biochemistry) following the Bergey's manual of systematic bacteriology. Growth was determined by measuring the OD_{600} value at different temperature (20-80°C), pH (4-11) and NaCl concentrations (0-25%, g/L). The ability to utilize various carbon sources was examined in minimal medium. Carbon sources were added at final concentration of 0.05% (w/v) with: fructose, glucose, L-rhamnose, sucrose, lactose, molessess, starch, xanthan, n-dodecane and n-hexadecane.

G+C content were determined according to Mesbah[34] by using HPLC. Nonmethylated Lambda-DNA (Sigma) with GC-content 49.858 mol% was served as an external standard.

DNA was extracted from the isolated strain following the instruction of the extraction Kit (MoBio, USA) for phylogenetic analysis. The methods for 16S rRNA gene amplification and sequencing have been reported previously.[23] The phylogenetic analysis was examined by BLAST of the National Center for Biotechnology Information.[35] Multiple sequence alignments were carried out using ClustalX 1.8 and the neighbor-joining tree was constructed using MEGA Software Version 6.0.

Late exponential phase cells were harvested by centrifugation (10000 rpm for 10 min) and washed triple with distilled water for the analysis of the cellular fatty acids. Lipid extraction and cellular fatty acid analyses were performed according to the method of Siristova.[36] The relative percentages of the

148 fatty acids were determined from the peak areas of the methyl esters by gas chromatograph (Agilent
149 5890) equipped with a flame-ionization detector. The running method was as follows: injector
150 temperature, 240°C; detector temperature, 240°C; carrier gas (He) flow rate, 10 ml/min. The oven
151 temperature was programmed from 50 to 300 °C at 8°C/min. The results are means of two independent
152 experiments.

153 *2.3. Kinetics of Alkanes Degradation*

154 Cell harvested from 50 ml LB medium of the selected strain was washed with distilled water triply
155 and transferred to a 250 ml flask with 2 g of ultrafilter-sterile alkane mixture (n-alkane, C8-C22,
156 purity>99%, Sigma, USA) and 90 ml of minimal medium, then incubated at 70℃ with 180 rpm for 40
157 days with using the cell-free sterile medium as control. 4 parallel samples were prepared as interval
158 samples for analyzing the biodegradation kinetics, including cell growth, residual alkane, cell surface
159 lipophilicity, surface tension and emulsification.

160 Cell growth was monitored by measuring the dry cell weight. Cell was collected by centrifugation
161 (10000 × *g*, 10 min) of a 100 ml culture broth and washed with distilled water twice, and then dried by
162 heating at 40^{o}C until constant weight was attained. Surface tension of cell-free samples was measured
163 by the digital tensiometer (KRUESS klot, Germany) using the ring detachment method. Emulsification
164 activity was determined by addition of 5 ml alkanes mixture to 5 ml of the cell-alkanes-free supernatant
165 in a 15 ml graduated tube according to the previous description.[37] Adherence of bacteria to
166 hydrocarbons was used as a measurement of cell-surface lipophilicity according to the reported
167 method.[32]

168 Alkanes were extracted with dichloromethane in triplicates (>99%, Sigma, USA), collected the
169 organic phase and removed the solvent in rotary evaporator at 30^{o}C, and then weight the residual

alkanes. In order to profile the degradation characterization, gas chromatograph equipped with FID detector (Agilent 5890, USA) was applied to detect the changing of each fraction in the alkanes mixture. The GC running program was as follow: injector temperature, 240°C; detector temperature, 240°C; carrier gas (He) flow rate, 10 ml/min. The oven temperature was programmed from 50 to 300°C at 8°C/min. The relative percentages of the n-alkanes were determined from the peak areas; and the weight of each alkane was calculated by the relative percentage and the total weight of residual alkanes mixture. The results are means of two independent experiments.

2.4. Analysis of bioemulsifier

Purification of the bioemulsifier was performed by solvent extraction and alcohol precipitation.[37, 38] The measurement of surface tension and emulsification were applied to testify the surface/interface activities of the obtained materials, and then the chemical characteristics of these materials were further analyzed with or without the hydrolysis. The carbohydrate moiety was detected by phenol sulfuric acid method using glucose as standard.[39] Protein moiety was detected by Lowry method using bovine serum albumin (BSA) as calibration standard.[40] The lipid moiety was detected by dichloromethane-methanol method.[41] The number-average molecular weight (*Mn*), weight-average molecular weight (*Mw*) and polydispersity index (PDI) of the biosurfactant were measured by gel permeation chromatography (GPC) using pullulan standards as described previously.[42,43]

Monosaccharide composition was determined according to the method reported by Carrion.[38] The hydrolysates were used to identify and quantify the constituent monosaccharides by high-perfomance liquid chromatography (HPLC) using Aminex HPLC Carbohydrate Analysis Columns HPX-87P (300 × 7.8 mm) and HPX-87C (300 × 7.8 mm) (BioRad) with the commercial sugars as standard for monosaccharide identification. Amino acids were analyzed following the methods from Xia[37] with an

automatic amino acid analyzer (1100 series, Hewlett Packard, USA). Fatty acid compositions were extracted from the hydrolyzates with ether in triplicates and esterified with methanol at 100°C for 1 h, then subjected to gas chromatograph mass spectrometer analysis.[44]

Effect of the environmental factors (salinity, temperature, pH, and metallic ions) on the performance of the emulsification against the crude oil was determined. The concentration of the purified active bioemulsifier tested in emulsification tests was 2000 mg/L.

2.5. Analysis of alkane hydroxylase genes

DNA isolated from the Anoxybacillus strain was used as a template for PCR. Amplification of alkB fragments of the themophilic bacteria was carried out with various degenerate oligonucleotide primers as designed in literatures.[45,46] The purified PCR products of alkB genes were cloned using a pGEM-T vector system (Promega). Clones were sequenced with the universal M13 primers in an ABI 3700XL genetic analyzer (Applied Biosystems). The preliminary analysis of the new sequences was done with the BLAST program of NCBI. The nucleotide sequences were aligned with homologous sequences retrieved from GenBank with ClustalX 1.8, and a neighbor-joining tree was constructed using MEGA Software Version 6.0.

3. Results and discussion

3.1. Characterization of bacteria

The well-performed strain WJ-4 is a facultative aerobic, gram-positive, motile, spore-forming, rod-shaped bacterium with a length of 3.9 to 5.6 μm and a width of 0.6 to 0.9 μm (Table 1). Colonies were gray white, umbonate surface and undulate edge. The sporangium was not swollen, whereas the spores were oval and terminal positioned. Growth was observed both on carbohydrates (arabinose, fructose, glucose, L-rhamnose, mannose, sucrose, lactose, molessess, starch, xanthan) and

hydrocarbons (n-dodecan, n-hexadecane and xylene). Indol was not produced; nitrate was not reduced; Voges-Proskauer reaction and Methyl-red test were negative. Like the most thermophilic bacilli, catalase reaction was positive for this tested strain. It can survive at wide range temperature from 45^{o}C to 80^{o}C, and the maximum optical density was achieved at 68-72^{o}C. The tolerance to NaCl levels was weakened along with the increasing of the salt concentration, the growth was ceased almost when salinity reaching at 12%. The optimum pH for the growth was 6.0-8.0.

The phylogenetic tree (Fig. 1) showed that strain WJ-4 was almost positioned between *Anoxybacillus* genus from one side and *Geobacillus* genus from the other side. The closest (more than 98%) sequence relatives found by BLAST search was *Anoxybacillus* species. (KJ842629.1, EU710556.1, KF266689.1), *Geobacillus* species (FJ823100.2, EU087702.1). Although *Anoxybacillus* is phylogenetically close to *Geobacillus* species,[49,51] strain WJ-4 was related to genus *Anoxybacillus* on the basis of phylogenetic similarity with *Anoxybacillus* species combined with the morphological, physiological, and biochemical properties. The sequence of the 16S rRNA gene of the strain WJ-4 is deposited and available under the GenBank accession number JX673944.1.

The G+C content of the genomic DNA for the strain WJ-4 was 44.3 mol%, which was significantly lower than those for the genus *Geobacillus* (48.2-58 mol%),[51] but similar to the closest *Anoxybacillus* relatives (42-57 mol%, *Anoxybacillus amylolyticus* 43.5 mol%; *Anoxybacillus voinovskiensis* 43.9 mol%; *Anoxybacillus contaminans* 44.3 mol%). Although the G+C content of DNA was 43.2 mol% for *Geobacillus tepidamans*,[49] it currently has been reclassified as *Anoxybacillus tepidamans*. Therefore, based on the similar percentage range of G+C content between *Geobacillus* and *Anoxybacillus* genus, it is possible that the intimate phylogenetic relationship existed between them.

The cellular fatty acids (FAs) of the strain WJ-4 was largely composed of the branched saturated

aliphatic acid, and has similar percentage with other thermophilic bacilli containing anteiso-fatty acids as minor components (Table 2). Iso-branched Fatty acids constituted 83.22% of total WJ-4 fatty acids and greatly predominated over anteiso-branched members, and contains iso-branched saturated fatty acids (iso-C15:0 and iso-C17:0) as major fatty acids, expecially iso-C15:0 account for 48.79%. The presence of branched FAs is considered to be a means of maintaining membrane fluidity; iso-branched FAs generally have higher melting points, while anteiso-branched FAs typically have lower melting points. This is possible explanation for the theromophilic property of strain WJ-4. The percentage of the iso-C15:0 and iso-C17:0 for other genus representatives were: 48.30% for *Geobacillus Stearothermophilus* 5965, 68.59% for *Anoxybacillus contaminans* and 72.8% for *Anoxybacillus amylolyticus*, 86.36% for *Anoxybacillus rupiensis* R270.[50]

The similar physiological characteristics, G+C content of DNA, fatty acid profile and phylogenetic similarity (98.0-99.0% to the closest relatives) with representatives of the genus *Anoxybacillus* allow us to place the strain WJ-4 in the genus *Anoxybacillus* as the type strain for the novel species. Particularly, it was possible that *Anoxybacillus* sp. WJ-4 has some similar characteristics with *Geobacillus* species.

3.2. Degradation kinetics

Due to lack of functional groups as well as low water solubility, alkanes exhibit low chemical reactivity and bioavailability for microorganisms. However, some microorganisms possess the metabolic capacity to use these compounds as carbon and energy sources for their growth. In this study, the isolated themophilic and halotolerant strain WJ-4 was evaluated to degrade the alkanes from C8 to C22. The results of the hydrocarbon degradation kinetic shown in Fig. 2A demonstrated the excellent ability of alkane degradation in this novel strain with maximum growth reaching at 20^{th} day. The

degradation rate became flatten after 20 days incubation but totally 58.75% of alkanes were decomposed after 40 days at 70°C under aerobic condition.

The thermophilic bacterial genera *Anoxybacillus* and *Geobacillus* were described nearly in the radiation from the Gram-positive genus *Bacillus*,[51,52] and *Bacillus* and *Geobacillus* has presented the ability of alkane degradation.[53] Compared with *Bacillus* and *Geobacillus*, *Anoxybacillus* is a relatively new genus that was proposed in the year 2000.[52] Few researches on the hydrocarbon degradation by *Anoxybacillus* genus have been reported so that the application of this new genus on the bioremediation and enhanced oil recovery was rare. *Anoxybacillus* strain was able to efficiently degrade the synthetic aromatic hydrocarbons.[21-24] Al-Jailawi et al. found that *Anoxybacillus* was the most predominant genus thermophilic bacteria with aromatic hydrocarbons degradation activities from hydrocarbon contaminated soil in Iraq.[22] However, none of literatures has been investigated to explore the possibility and characteristics of alkanes degradation by this relatively new genus, this study therefore was the first report about the alkane biogeradation by *Anoxybacillus* genus.

Gas chromatography was applied to present the changing of each component in alkane mixture during degradation, and the residual weight of each alkane was calculated as well. The results in Fig.3 showed that this strain can utilize the different chain alkane (C8-C22) with various efficiency, especially that C8 and C9 were almost depleted after the first 10 days degradation. The degradation kinetic of each alkane (in Fig.2B) demonstrated that the degradation rate of the alkanes can be classified into three types based on the rate calculation of the first 10-days degradation. The first type with the degradation rate (higher than 10 mg/d) is limited to C8-C10 with 14.49 mg/d, 14.33 mg/d and 11.92 mg/d respectively; for the second type, the degradation rate is between 5 mg/L to 9 mg/L which includes C11-C12 with 8.13 mg/d and 6.47 mg/d; for the third type, the degradation rate is less than 3

mg/d for the long chain alkane (C13-C22). Combined the results in Fig. 2A and 2B, it was obvious that the degradation of C13-C22 between 10^{th} and 30^{th} day was facilitated along with the cell surface lipophilicity increasing and reaching 65% of CSL at 30^{th} day, while the cell surface lipophilicity of the strain before 10^{th} day was relatively lower than 20%. Two possible mechanisms of alkane uptaking have been proposed and well proven: direct uptake of the small chain alkane, and the changing of physiochemical properties of cell to alkane.[26] In addition, suggested mechanisms for the uptake of hydrophobic contaminants by degrading bacteria include direct contact of substrates with microorganisms having a high CSH and biosurfactant-mediated uptake by microorganisms capable of producing biosurfactant (and bioemulsifier).[28] Therefore, the possible mechanism of degradation of *Anoxybacillus* WJ-4 when fed with different molecule alkanes could be explained by the above proposed mechanisms based on the results in Fig. 2 and 3. It hypothesized that this bacterial strain assimilated the small chain alkane (<C10) following the first mechanism (because of the depletion of C8 and C9 in Fig. 2B and 3); and the degradation of the long chain alkane (>C10) conformed to the second mechanism (because of the increasing of cell surface lipophilicity in Fig. 2A).

Most of alkane-degrading bacteria secrete diverse surfactants that facilitate emulsification of the hydrocarbon. Zhao et al. elaborated a relationship between the cell surface lipophilicity and the level of biosurfactant production.[26] Hassanshahian and Emtiazi reported that a correlation between emulsification activity, cell adherence to hydrocarbon and growth rate of the crude oil degrading bacteria in crude oil media.[54] Kundu described the effect of biosurfactant production on the cell surface hodrophobicity and the degradation of hetero-aromatic hydrocarbon.[28] In this study, cell surface lipophilicity of *Anoxybacillus* cell and the emulsification index was increasing from 5.9% to 70.4% and from 0 to 72.2% respectively along with the decrease of the amount of the residual alkanes

(Fig. 2A). Therefore, it could be a conclusion that this novel thermophilic and halotolerant *Anoxybacillus* WJ-4 could synthesize the biosurfactant or bioemulsifier to facilitate the degradation of the alkanes.

Unlike biosurfactants (that can significantly decrease the surface/interfacial tension), bioemulsifiers are always high molecular biosurfactants that are able to form stable emulsions with hydrophobic materials (usually oil-in-water and less commonly water-in-oil), but hardly reduce the surface or interfacial tension. Fig. 2A presented the surface tension changing of the cell-alkane-free supernatant and indicated that the microbial surfactant produced by this novel strain WJ-4 has no significantly influence on the surface tension with decreasing from 72.22 mN/m to 52.45 mN/m. A 890 Da-biosurfactant from *Rhodococcus* could decrease the surface tension from 71 to 29 mN/m.[28] Haza found a 1044 Da-surfactin from *Bacillus* could reduce the surface tension from 69.07 mN/m to 30 mN/m.[55] Bao et al.[56] reported small-molecular lipopeptide biosurfactant has the lowest surface tension of 26.30 mN/m when using crude oil as carbon source. Bioemulsifiers are higher in molecular weight than biosurfactants as they are complex mixtures of heteropolysaccharides, lipopolysaccharides, lipoproteins and proteins.[30,57-59] They are also known as high molecular weight biopolymers or exopolysaccharides, which possess only emulsifying activity and not surface activity. Similar to biosurfactants, these molecules can efficiently emulsify two immiscible liquids such as hydrocarbons or other hydrophobic substrates even at low concentrations but in contrast are less effective at surface tension reduction.[30] In addition, the number-average molecular weight (*Mn*), weight-average molecular weight (*Mw*) and polydispersity index (PDI) of the bioemulsifier were measured by gel permeation chromatography (GPC) using pullulan standards. The results showed that the number-average molecular weights (M_n), and weight-average molecular weight (*Mw*) of the obtained bioemulsifier was

between 129,485±1,827 Da and 210,000±1,827Da with PDI average values of 1.212, respectively. Therefore, we can infer that the strain WJ-4 produces relatively high molecular bioemulsifier which was not able to reduce the surface tension obviously, but has ability of changing the cell surface lipophilicity and emulsifying the alkanes as evidence showed in Fig. 2A. Large amount of bacterial strains can produce high molecule bioemulsifier which can stabilize the various kinds of emulsion formed with the immiscible phases but cannot decrease surface tension significantly. The bioemulsifier of *Anoxybacillus* strain WJ-4 has similar characteristic with these reported bioemulsifiers.[31,42] Although numerous literatures on CSL, emulsification and biodegradation were limited to the small molecule biosurfactant (generally like glycolipids and lipopeptides), it was not ignorable and increasingly significant that bioemulsifier (high molecule weight biosurfactant) could be applied efficiently in many fields, especially the hydrocarbons bioremediation.

3.3. Analysis of biosurfactant

The chromogenic reaction was developed to analyze the composition of bioemulsifier from *Anoxybacillus* sp. WJ-4. Results showed in Fig. 3 that there were the blue-green spot, purplish-red spot and yellow brown spot on silica gel plates when separately using ninhydrin, anthrone and ammonium-perchlorate as color developing reagent, indicating that peptides, glycosides and lipids were constituted with approximate percentage of 6.25%, 53.12% and 40.63% respectively in this bioemulsifier. Thus the bioemulsifier produced by *Anoxybacillus* sp. WJ-4 was a high molecular weight oligosaccharide-lipid-peptide complex in which these moieties could be structurally associated involving either covalent or non-covalent bonds. The oligsaccharide composition of the hydrolysed bioemulsifier consisted of D-glucose, D-galactose, D-mannose and L-rhamnose in approximate molar ratios of 2:1:2:1. GC-MS analysis of lipid fraction showed that hexadecanoic acid and octadecanoic

346 acid were the major fatty acids that account for 89.85% of total fatty acids. Other fatty acids
347 determined at lower extent were decanoic acid (2.38%), dodecanoic (4.25%) acid and tetradecanoic
348 acid (3.52%). The amino acids of the peptides of the bioemulsifier were analyzed and tabulated in
349 Table 3.

350 *3.4. Emulsification activity*

351 Emulsification property is critical for bioemulsifier to be promising in different environmental and
352 industrial applications. The emulsification activity of *Anoxybacillus* bioemulsifier against various oil
353 phases was investigated at room temperature for 24 hours. Results as shown in Fig.5A that
354 *Anoxybacillus* bioemulsifier can effectively emulsify the different hydrocarbons, and formed stable
355 emulsions with pure alkane (C6-C10, C12 and C16), as well as the mixtures (crude oil, kerosene,
356 paraffin). Furthermore, it had better emulsification activity with the hydrocarbon mixtures than pure
357 alkane with crude oil as the best oil phase, and the weaker emulsion was formed along with the
358 increasing of the alkane chain number.

359 Effectiveness of the bioemulsifier over a wide range of temperature, pH and salinity can endow its
360 broad applications. Exposed to different extreme temperature (<10^{o}C and >80^{o}C) for 2 hours, this
361 bioemusifier exhibited the high activity (EI>60%) stably over a wide temperature range (Fig. 5B), and
362 a slight decreasing was detected with the treatment higher than 80°C. In pH evaluation, *Anoxybacillus*
363 emulsifier showed the relative stable emulsification performance at pH from 6 to 12 and was
364 significantly inhibited under the extreme acid conditions (Fig. 5C. Previous literatures reported that the
365 emulsifying activity was significantly inhibited at a NaCl concentration greater than 5 %,[42] whereas the
366 bioemulsifier from *Anoxybacillus sp* WJ-4 showed a better halotolerance, remaining high
367 emulsification (EI>60%) under the salinity up to 20% (Fig. 5D). In contrast, the commercially

368 chemical surfactants SDS (sodium dodecyl sulfate), Triton X-100 or Tween 80 have no emulsifying
369 activity at NaCl concentrations of 100-120 g/l.[60] Although the production cost of
370 bioemulsifier/biosurfactant was still high, it is obvious that the trend of the replacement of chemical
371 surfactant by bio-counterpart is being accelerated due to the unparalleled advantages over the
372 commercial petro-derived chemical surfactant and the rapid development of biological methods and
373 purification technology.

374 *3.5. Alkane hydroxylase gene analysis*

375 Because of the nearest phylogenetic relationship with *Geobacillus* genus,[25,50] the total DNA of the
376 strain WJ-4 grown on alkanes containing medium was used to amplify *alkB* gene fragments with
377 various degenerate primers targeting the most conserved region of the *Geobacillus alkB* gene.[45,46] The
378 selected 50 clones were analyzed by DNA sequencing using plasmid primers. The obtained nucleotide
379 sequences were compared to the known sequences of *alkB* geo homologs, as well as with *alkB* genes of
380 various bacteria deposited in GenBank. Four homologs of alkB gene were revealed in *Anoxybacillus*
381 strain WJ-4, namely, *alkB*-an1 (10 clones), *alkB*-an2 (11 clones), *alkB*-an3 (8 clones), *alkB*-an4 (21
382 clones). The sequenced *Anoxybacillus alk*B fragments were blastned with the homologs available from
383 GenBank. The corresponding phylogenetic trees differ from each other shown in Fig. 6. The genbank
384 access number of four genes is from KR153280 to KR153283.

385 The sequences of *alk*B-an*1*, *alk*B-an2, *alk*B-an3 and *alk*B-*an4* belong to the phylogenetic clusters
386 were similar with *Geobacillus alk*B-*geo1, Geobacillus alk*B-*geo2, Geobacillus alk*B-*geo3, and*
387 *Geobacillus alk*B-*geo4* found in *Geobacillus* genus.[45,46,53] The nucleotide sequences similarity to the
388 corresponding *Geobacillus alk*B homologs is 97.0, 95.0, 97.0%, and 98.0% for these four alkB genes
389 respectively. Although the PCR and sequencing of the alkane gene from *Anoxybacillus* were

implemented in this study, it is obviously nonsufficient to explain the alkane degradation mechanism of this novel strain. In order to reveal the metabolic pathway of alkane degradation, the whole genome sequencing, reverse transcription, functional analysis and structure of *alkB*-an series, and the intermediate metabolites will be further investigated.

4. Conclusions

New isolated thermophilic *Anoxybacillus* strain from high temperature petroleum reservoir was evaluated to degrade the hydrocarbon pollutant. The produced oligosaccharide-lipid-peptide bioemulsifier showed the good performance of emulsification (EI_{24}>60%) with various oil phases at different conditions. This bioemulsifier reduced surface tension from 72.22 mN/m to 52.45 mN/m, and increased the cell surface lipophilicity to 65% during hydrocarbon degradation. Four hydrocarbon-hydrolysis genes were detected in this strain, and showed higher sequences similarity (>95%) to the corresponding *Geobacillus* alkB homologs. Although the mechanism of degradation and bioemulsifier production need more studies, it has significantly showed the bioemulsifier, cell surface lipophilicity and hydrocarbon monooxygenase has significant effect on the alkane degradation, even under thermophilic condition.

Acknowledgements

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Fig.4. Chromogenic reaction of the bioemulsifier obtained from *Anoxybacillus sp.*WJ-4 when utilizing alkane hydrocarbons. Point A, B and C were developed with 0.2% phenol-sulfatic acid solution, 0.1% ninhydrin solution and iodine vapor to detect glycoside, peptides and lipids respectively.

Fig.5. Emulsification activity evaluation of bioemulsifier produced from novel thermophilic *Anoxybacillus* strain when utilizing the different hydrocarbons. a: the bioemulsifier solution against the pure alkane and hydrocarbon mixture; b-d: the bioemulsifier solution against the crude oil of Daqing oilfield. The values are mean ± standard deviations (n = 3).

Fig.6. Position of the *Anoxybacillus alk*B homologs on phylogenetic trees based on the nucleotide sequences. Sequences obtained in our study are labeled with bold circle. Numerals indicate the statistical reliability of branching order as determined by bootstrap analysis of 1000 alternative trees.

542 Values exceeding 90% are considered significant.

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Table 1

Comparison of the phenotypic characteristics of *Anoxybacillus sp.* WJ-4 and related species

Characteristic	WJ-4	AB04	R270	MR3C	GS5-97
Cell length (um)	3.9-5.6	4.6	3.3-7.0	2.0-2.5	3.9-4.7
Oxygen	Facultative	Aerobic	Aerobic	Facultative	Aerobic
Motility	+	+	+	+	+
Spore shape	Spherical	Spherical	Spherical	Spherical	Oval
Optimal Temperature	68-72	50	55	61	55
Optimal pH	6.5-8.0	7.5-8.5	6.0-6.5	5.6	7.0
Catalse	+	+	+	+	
oxidase	+	+	+	+	
Arabinose	+	+	+	+	ND
Ribose	ND	ND	+	+	ND
Xylose	+	+	+	+	+
Fructose	+	+	+	+	+
Galactose	ND	ND	-	+	+
Mannose	+	+	ND	+	+
Rhamnose	+	-	+	+	-
Sucrose	+	+	-	+	+
Lactose	-	-	-	ND	w
Starch	+	+	+	+	ND
G+C(mol/mol)	44.3	54	41.7	43.5	42.4

+positive; -negative; w weak response; N.D. not determined.

Anoxybacillus ayderensis AB04T;[47] *Anoxybacillus amylolyticus* MR3C;[48]

Geobacillus tepidamans GS5-97;[49] *Anoxybacillus rupiensis* R270.[50]

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Table 2

Cellular fatty acid composition (%, w/w) of strain WJ-4 and some relationship closest strains in phylogenetic tree shown in Fig. 1.

Fatty acid	WJ-4	5965	GS5-97	MR3C	R270	LGM	3721
13:0 iso	-	-	0.4	-	-	-	-
14:0 iso	-	0.20	0.60	-	-	-	0.30
14:0	2.87	0.20	4.10	-	0.30	3.00	1.10
15:0 iso	48.79	22.90	44.33	41.2	52.81	52.00	16.90
15:0 ai	1.32	5.10	6.60	2.13	1.64	7.00	2.40
15:0	0.32	1.70	-	0.10	0.31	-	2.90
16:0 iso	5.68	7.30	3.20	7.00	2.01	5.00	15.20
16:0 ai	-	-	-	0.12	-	-	-
16:0	12.65	14.0	15.10	6.3	5.44	11.00	18.40
17:0 iso	28.45	25.40	15.00	31.60	33.55	12.00	29.90
17:0 ai	4.02	8.10	6.10	0.70	3.94	7.00	6.40
17:0	-	6.30	-	-	-	-	2.50
18:0 iso	0.30	0.7	0.60	1.30	-	-	1.30
18:1	-	-	-	0.70	-	-	0.10
18:0	-	2.2	-	1.90	-	-	1.40
19:0 iso		0.3					0.3
19:0		-					0.1

Anoxybacillus amylolyticus MR3C;[48] *Geobacillus tepidamans* GS5-97;[49] *Anoxybacillus rupiensis* R270, *Anoxybacillus contaminans* LGM, *Geobacillus Stearothermophilus* 5965, *Geobacillus Stearothermophilus* 3721.[42]

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Table 3

Protein composition of the bioemulsifier from *Anoxybacillus* strain

Amino acid	Concentration (mg/ml)	Amino acid	Concentration (mg/ml)
Asp	6.126	Gly	3.236
Thr	2.781	Ala	6.519
Ser	2.031	Leu	2.773
Glu	7.231	Phe	1.121
Pro	1.142	Lys	2.452
Val	2.828	Ile	1.859

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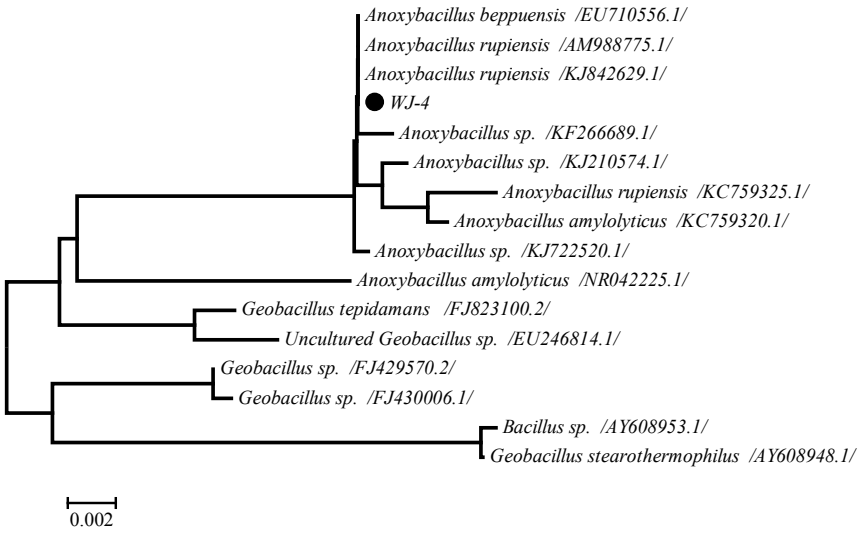

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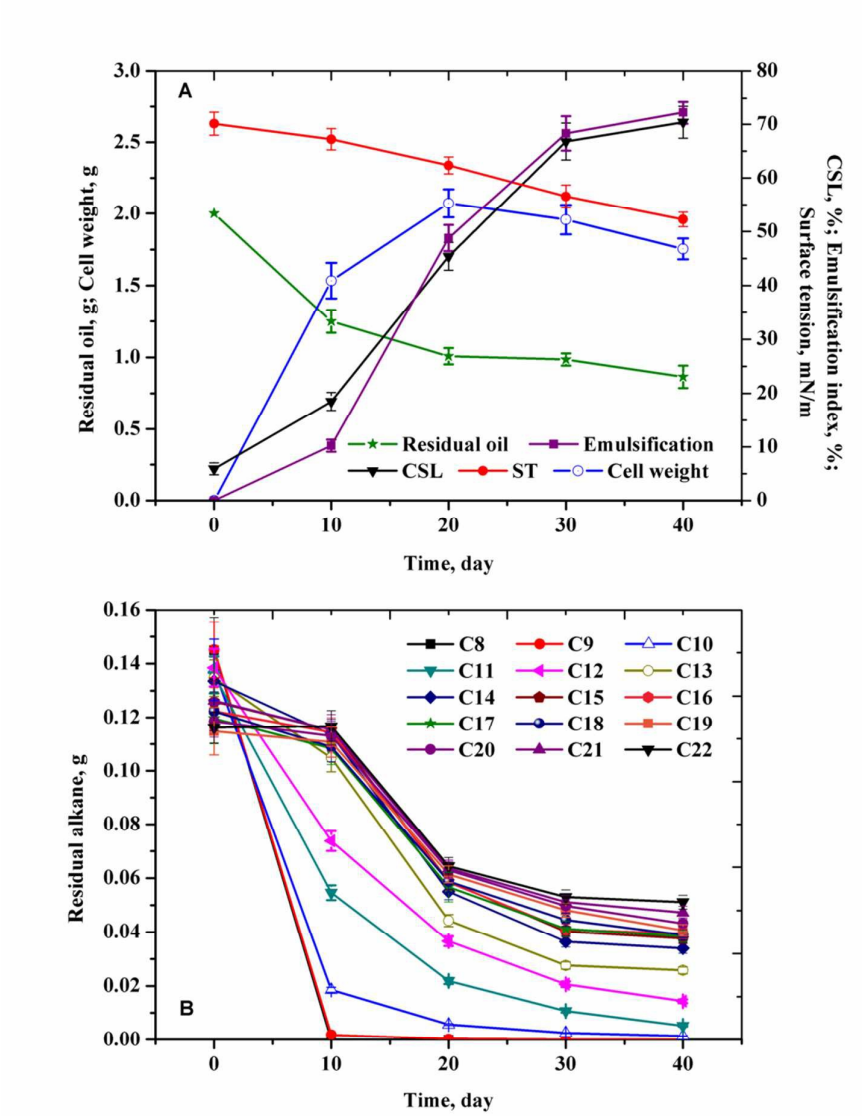

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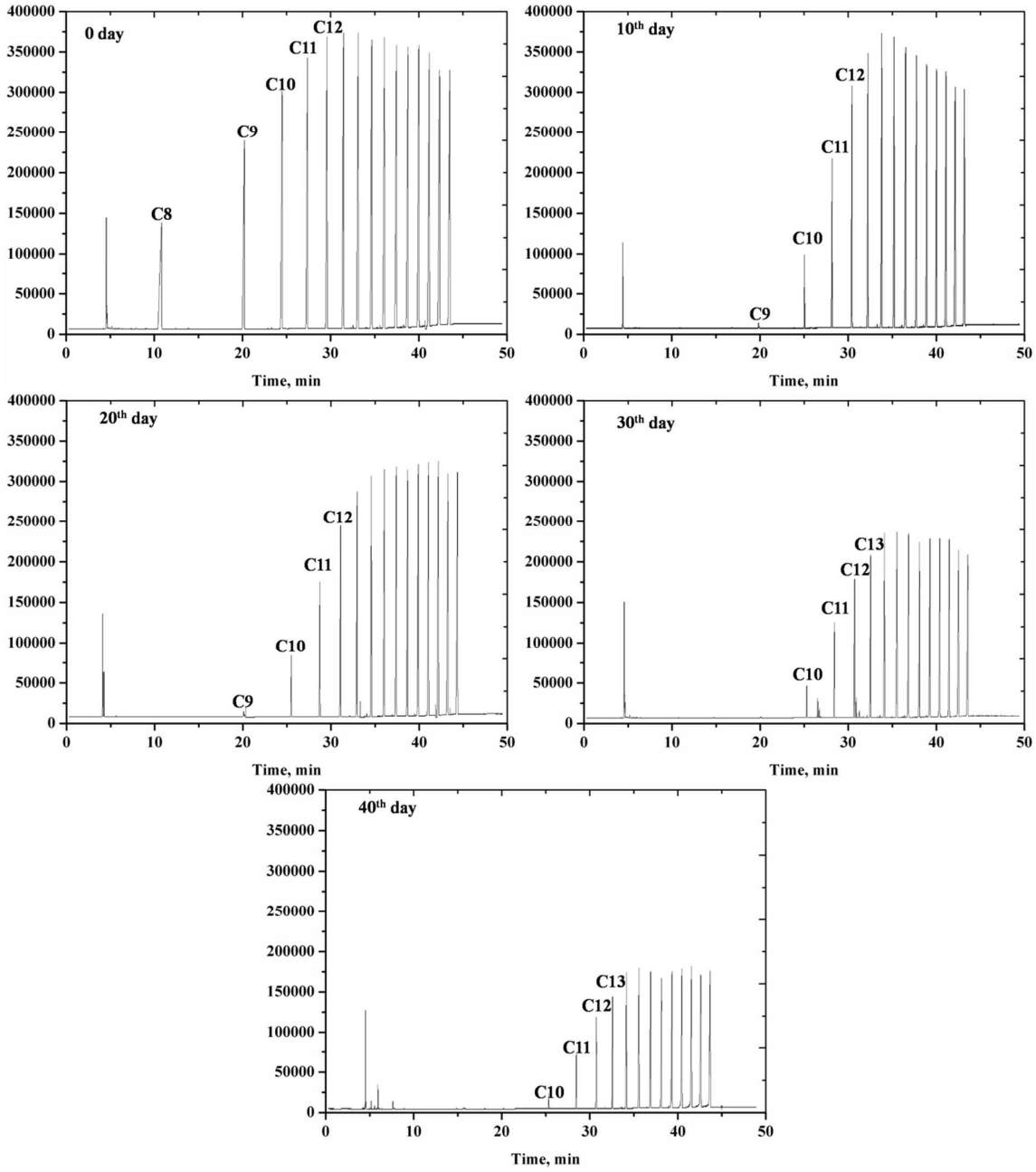

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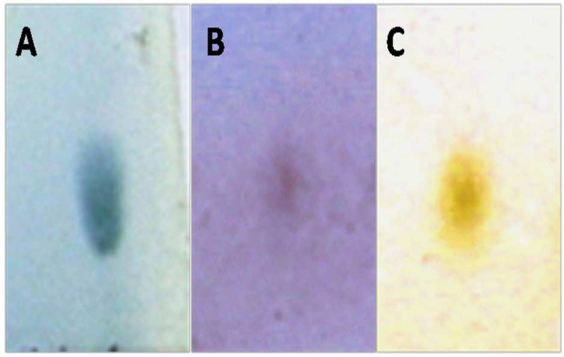

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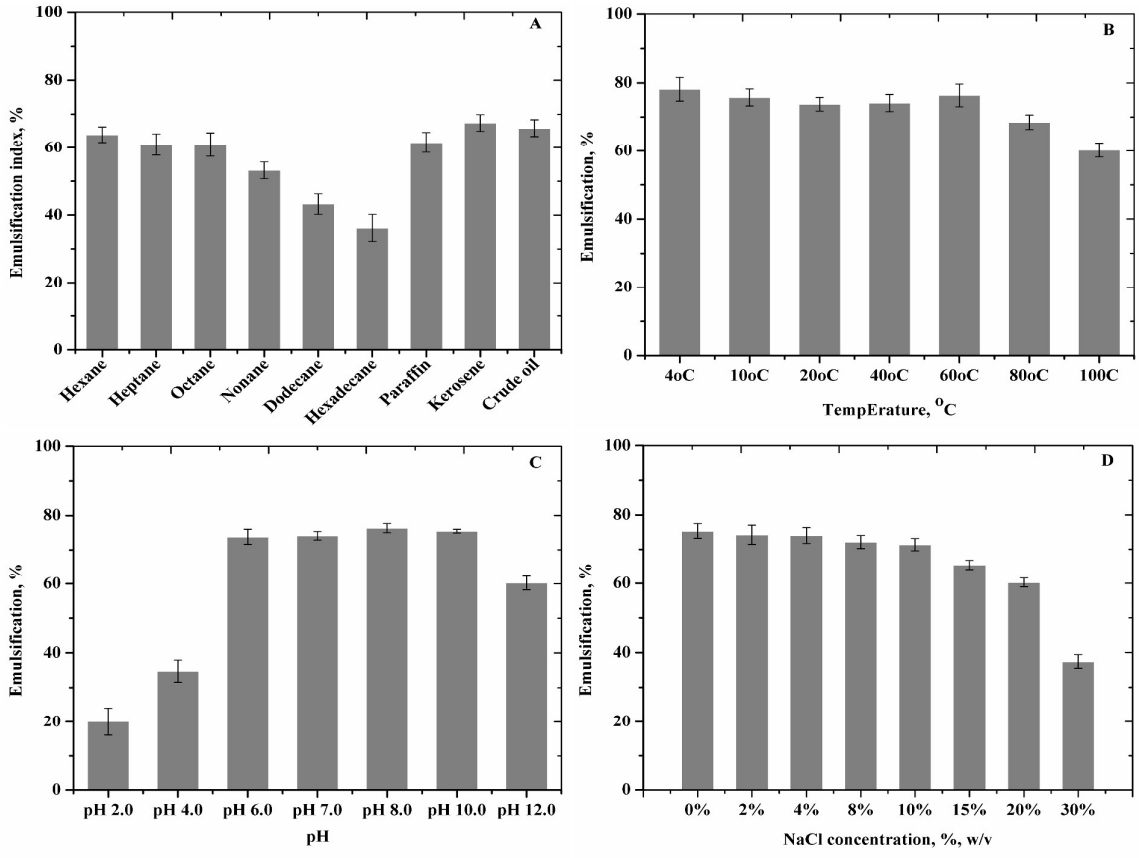

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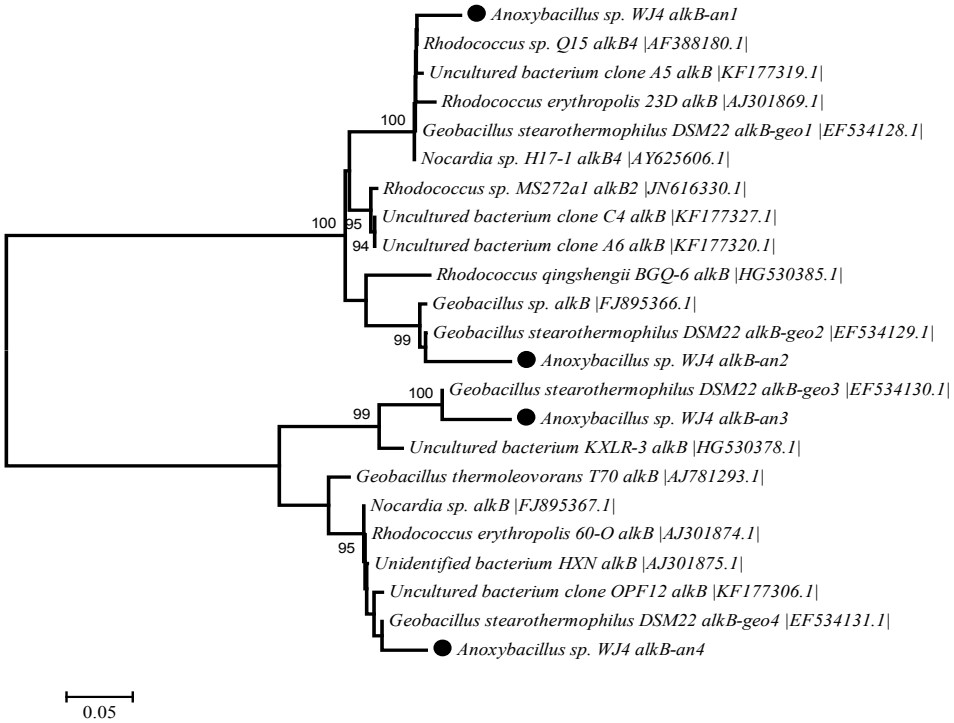

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