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Isolation, Identification, and Performance Studies of a Novel Paraffin-degrading Bacterium of *Gordonia amicalis* LH3

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Abstract In this study, we describe the isolation and identification of a novel long-chain n-alkane degrading strain, *Gordonia amicalis* LH3. Under aerobic conditions, it utilized approximately 18.0% of paraffin (2% w/v) after 10 day of incubation, and the paraffin compositions of C_{18}~C_{24} alkalines were utilized preferentially. Under anaerobic conditions, paraffin utilization was approximately 1/8 that seen under aerobic conditions, and the compositions of C_{34} and C_{36} alkalines were utilized preferentially. The effects of salinity, temperature, and biosurfactants on paraffin degradation were also evaluated. The strain was also demonstrated to grow on oil, and decreased oil viscosity by 44.7% and degraded oil by 10.4% under aerobic conditions. Our results indicated that *G. amicalis* LH3 has potential applications in paraffin control, microbial enhanced oil recovery (MEOR), and the bioremediation of hydrocarbon-polluted environments. © KSBB

Keywords: Gordonia amicalis, *paraffin*, *oil*, *biodegradation*, *MEOR*, *bioremediation*

INTRODUCTION

The high wax contents of oil complicate the processes of oil production and transportation. The wax content of the majority of oil resources in China is generally over 20% and as high as 40~50% in some oil fields [1]. These waxes occur as two general types: paraffin and macrocrystalline waxes [2]. Paraffin is solid, and is principally composed of straight-chain saturated hydrocarbons comprised of 16 or more carbon atoms, and also tends to be crystallized and deposited at room temperature; whereas microcrystalline waxes harbor more branched and cyclic hydrocarbons, and are thus relatively infrequently solidified and deposited. The existence of paraffin results in increases in the freezing point and viscosity and a decrease in the fluidity of oil, consequently resulting in low recovery and pipeline blockades in both oil production and transportation, thus increasing production and maintenance costs [3-5].

In the production of highly viscous oils, large quantities of chemical surfactants and viscosity-reducing agents are employed [6,7]. In overcoming pipeline blockade, washing methods utilizing hot fluids such as oil or water, air, or chemical cleaning agents can be used [8]. However, these methods tend to be costly, and the chemicals used are generally both flammable and toxic. By way of contrast, microbial methods have, over the last decade, proven effective in MEOR and in the prevention of oil pipeline blockade [3,8,9], and these methods are inexpensive, easily applied, and environmentally safe. In addition, microbial tenchniques have also been successfully employed in the bioremediation of oil- and paraffin hydrocarbon-contaminated soil or water [10-12].

Microbial paraffin control is principally conducted via three mechanisms: (a) direct microbial degradation of paraffin (b) microbial products, including fatty acids and biosurfactants, interact with crystallized paraffin to prevent its deposition (c) the biofilm formed by the adsorption of the microorganisms on the surface of paraffin prevents paraffin crystallization and deposition [8,13]. Facultative anaerobic hydrocarbon-degrading bacteria effect significant advantages in actual applications, as they allow for the maintenance of bioactivity without oxygen in underground oil reservoirs during oil production or in oil pipelines during transportation, and the maintenance of bioactivity without sugars via the use of paraffin or oil as the energy and carbon sources, which renders the microbial method

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both simple and economical.

Until now, the commonly used hydrocarbon-degrading bacteria species are limited principally to *Pseudomonas* sp., *Bacillus* sp., *Acinetobacter* sp., *Rhodococcus* sp., *et al*. [12,14-16]. Therefore, it is very important to detect and research more novel hydrocarbon-degrading microorganism species for both scientific research and applications. In this paper, we describe the isolation and properties of a novel facultative paraffin-degrading strain of *Gordonia amicalis*.

MATERIALS AND METHODS

Samples, Microorganisms, Media and Cultivation Conditions

The oil-contaminated water samples utilized for microbial strain isolation were collected from Jidong Oilfield Co. Ltd., in Tangshan, China. Solid paraffin (C_{18}~C_{36}) and crude oil (C_{15}~C_{28} with viscosity of 128 mPa·s at 50°C) were acquired from Shengli Oilfield Co. Ltd., in Dongying, China. *P. aeruginosa* SS-18 and *Bacillus* sp. SZ-32 were isolated from oil-contaminated soil from Shengli Oilfield Co. Ltd., in Dongying, China. The composition of the basal mineral salt solution (BMSM) was (g/L): K_2HPO_4, 5.0; $(NH_4)_2SO_4$, 1.0; $MgSO_4·7H_2O$, 0.25; $NaNO_3$, 2.0; NaCl, 5.0; $K_2HPO_4·3H_2O$, 1.0; pH 7.0~7.2. Enriched paraffin medium: 2% (w/v for all if not specifically indicated) liquid paraffin and 0.2% yeast extract in BMSM. Paraffin medium: 2 or 0.1% solid paraffin in BMSM. Oil medium: 0.1 or 60% oil in BMSM. Seed medium: 2% glucose and 0.05 % yeast extract in BMSM.

Anaerobic cultivation was conducted as follows: the water for media preparation was boiled for 20 min in order to dispel all dissolved oxygen prior to use. L-Cysteine and resazurin as an oxygen indicator were added to the medium to final concentrations of 0.05 and 0.01%, respectively. The anaerobic culture bottles were sealed with rubber caps, sterilized, and filled with filter-sterilized pure nitrogen gas until the oxygen indicator in the medium become achromatic. The culture was then inoculated via injection. The anaerobic and aerobic liquid medium cultivations were conducted at 37°C and 150 rpm.

Isolation of Facultative Anaerobic Paraffin-degrading Microorganisms

Oil-contaminated water samples were enriched via anaerobic cultivation using enriched paraffin medium for 3 day. The culture broth was then streaked onto BMSM plates covered with sterilized liquid paraffin-soaked filter paper, and cultivated at 37°C. The colonies on these plates were reiteratively streaked on LB plates and the pure isolates were confirmed via re-cultivation in paraffin medium under aerobic or anaerobic conditions.

Identification of Microorganisms

Colony morphology was observed using an optical microscope, and the cell morphology was determined using a JEM-100CXII type transmission electron microscope (JEOL, Tokyo, Japan). Physiological and biochemical tests were conducted [17]. The chromosomal DNA of the isolate was extracted and the 16S rDNA was amplified via PCR with upstream primer (5′-AGAGT TTGAT CCTGG CTCAG-3′) and downstream primer (5′-AAGGA GGTGA TCCAG CCGCA-3′). The purified PCR product was sequenced and similarity research was conducted via BLAST search.

Analytical Methods

Paraffin was measured via the extraction of the residual solid paraffin in the culture broth using an equal volume of hexane, and the extract was dried and weighed accurately. The uninoculated control was prepared via the same method, and the percentage (w/w) of solid paraffin degradation was calculated.

Oil viscosity was determined using a Brookfield LV type viscometer (Brookfield Engineering Laboratories Inc., Newington, NH, USA) at 50°C. After cultivation in the oil medium, the samples were centrifuged for 30 min at 4,000 × g in order to separate and obtain the oil phase for measurement. An uninoculated control was prepared using the same method and the percentage of oil viscosity reduction was calculated.

The oil content was measured via the extraction of the residual oil in culture broth using 30 mL of hexane after the culture broth had been thoroughly mixed with 5 mL of H_2SO_4 and 20 g of NaCl, and the crude oil content was measured with a Unico 2000 spectrophotometer (Unico Shanghai Instruments Co. Ltd., Shanghai, China) at a wavelength of 256 nm [18]. An uninoculated control was prepared using the same method and the percentage (w/w) of oil degradation was calculated.

The surface tension of the culture broth was measured with a Jzhy1-180 type tensiometer (Chengde Experimentation Equipment Co., Chengde, China) using the samples of the supernatant separated via the centrifugation of the culture broth.

The pH was determined using an 818 type pH meter (Thermo Electron/Orion Co., USA).

The paraffin composition was measured using a GCMS-QP2010 type GC-MS (Shimadzu Co., Kyoto, Japan), equiped with a DB-5 quartz capillary column (30 m × 0.25 mm × 0.25 μm). Split injections were conducted using helium as the carrier gas. The column temperature was maintained for 2 min at 70°C and then increased to 290°C at a rate of 7 °C/min. An injection temperature of 300°C, an interface temperature of 250°C, and an ion source temperature of 200°C were utilized. The samples were prepared via the extraction of 100 mL of the paraffin medium culture broth using 50 mL of hexane, and 0.6 μL of extract solution was injected.

A 5% inoculum was employed for each experiment and all measurements were conducted in triplicate.

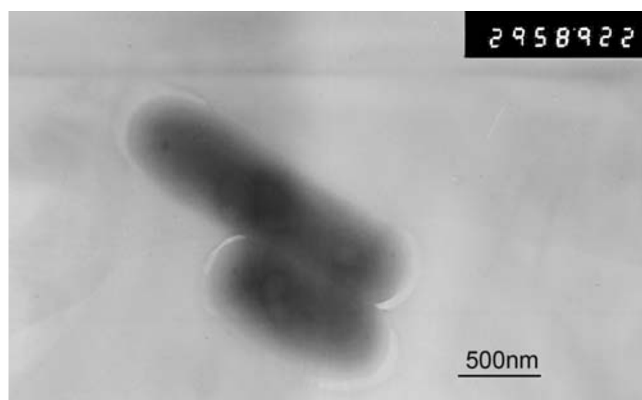

Fig. 1. Transmission electron micrograph of *G. amicalis* LH3 grown in liquid basal mineral salt medium containing 2% (w/v) glucose and 0.05% yeast extract for 36 h (29,000 ×).

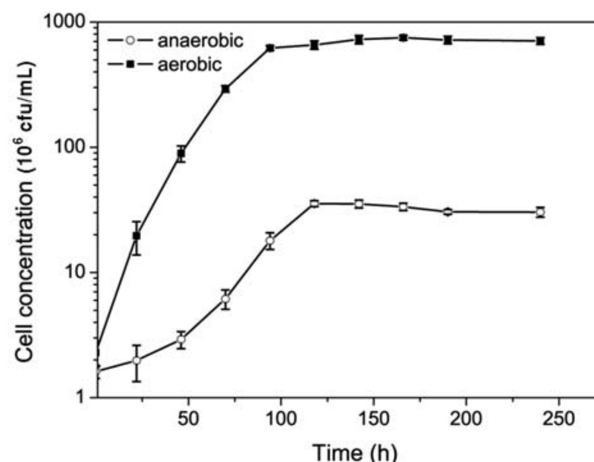

Fig. 2. Growth of *G. amicalis* LH3 in 2% (w/v) solid paraffin medium for 10 day under aerobic and anaerobic conditions.

RESULTS AND DISCUSSION

Isolation and Identification of Paraffin-degrading Microorganisms

Fifteen paraffin degradable facultative anaerobic bacteria strains were isolated and the final cell concentration (OD_{600}), pH, surface tension, and paraffin degradation were assessed following cultivation under aerobic conditions using solid paraffin medium for 10 day. The results indicated that the final cell concentrations (OD_{600}) ranged from 0.356 to 5.208, the pH values ranged from 6.6 to 7.5, the surface tensions ranged from 58.7 to 70.3 mN/m, and the paraffin degradation (by percentage) ranged from 0.2 to 16.7% for the 15 isolated strains. The strain LH3 evidenced the highest OD_{600} value (5.208), the highest percentage of paraffin degradation (16.7%) and the second lowest surface tension (60.4 mN/m), and was selected for further studies.

The colony of strain LH3 was circular, smooth, orange-red, and evidenced a convex elevation. The cells were short and rod-shaped and occurred singly, in pairs or in small clusters, occasionally in short chains. No spores or polar flagella were observed. A flat transparent sheath around the cells and granular inclusions were observed in the cells via transmission electron microscopy (Fig. 1). The cells were Gram-positive, oxidase-negative, catalase-positive, nitrate reduction-negative, and indole test-negative. The partial 16S rDNA (1,511 bp, EF424581) of the LH3 strain was sequenced and the homology with other species of microorganisms was assessed via the construction of a distance tree. The results indicated that it was in the cluster comprising members of the genus *Gordonia*, with sequence similarity values of over 99% between strain LH3 and the type strains of *G. amicalis*. According to the above results, the LH3 strain was identified as *G. amicalis*.

Gordonia was first identified in 1971, and ultimately subsumed within the suborder of *Corynebacterineae* of order *Actinomycetales* [19,20], and has been thought in recent years to have some potential in bioremediation applications. 16S rDNA sequence comparisons revealed that it was closely related to the genus *Acinetobacter* and *Rhodococcus*. Until now, there were 21 species in the genera *Gordonia*, many of which were discovered in recent years [17,21]. Members of the genus *Gordonia* evidenced abundant metabolic diversity and were capable of degrading toxic environment-contaminating compounds [22]. *G. paraffinivoranset* and *G. alkanivorans* on alkane degradation [22,23]; *G. amicalis* type strain $IEGM^T$ isolated in 2000 on dibenzothiophene desulfuration [24]; *Gordonia* sp. SP72-3, with 99% 16S rDNA similarity with *G. amicalis* $IEGM^T$, on hexane degradation [25] were reported. However, only a few reports have thus far been filed concerning the long-chain alkane degradation of *G. amicalis*.

Although it has been theorized that bacterial consortia are more effective than single pure cultures in MEOR and bioremediation applications [8,16,26], the isolation and identification of a pure culture is important with regard to the confirmation of the performance, advantage, and proportion of each member of the bacterial consortium, as well as in the construction of the efficient bacterial consortium via the mixing of several identified strains. *G. amicalis* LH3, a novel paraffin-degrading bacterium, enriches the species of long-chain alkane-degrading bacteria and evidences enlarged metabolic abilities of *G. amicalis*.

Growth and Paraffin Utilization under Aerobic and Anaerobic Conditions

Aerobic and anaerobic cultivations were conducted using 100 mL of 2 and 0.1% paraffin medium, respectively, for 10 day in order to determine the total quantity and compositions of the paraffin utilized. Samples were prepared, the live cell numbers were determined via plate counting, and the total paraffin and paraffin compositions were evaluated. The results are provided in Figs. 2~3 and Table 1.

Under aerobic conditions, the highest live cell concent-

Table 1. Relative degradation of each paraffin compositions by *G. amicalis* LH3 under aerobic and anaerobic conditions

n-Alkane	Aerobic degradation (%)	Anaerobic degradation (%)	n-Alkane	Aerobic degradation (%)	Anaerobic degradation (%)
n-C18	100.0 ± 0.0	7.6 ± 2.3	n-C25	51.6 ± 0.5	12.6 ± 2.3
n-C19	99.3 ± 0.7	6.3 ± 2.3	n-C26	37.1 ± 1.7	9.0 ± 1.6
n-C20	97.7 ± 0.4	15.5 ± 2.0	n-C27	37.1 ± 1.6	13.2 ± 0.8
n-C21	92.2 ± 0.3	15.1 ± 1.1	n-C28	30.3 ± 1.6	17.3 ± 0.9
n-C22	83.3 ± 1.8	14.3 ± 1.2	n-C30	3.2 ± 0.7	0.2 ± 0.3
n-C23	71.9 ± 0.9	13.0 ± 2.3	n-C34	39.1 ± 0.6	34.9 ± 2.3
n-C24	61.4 ± 1.1	13.8 ± 2.8	n-C36	59.1 ± 1.9	41.2 ± 0.7

0.1% (w/v) solid paraffin medium used, cultivated for 10 day under aerobic and anaerobic conditions. The residual paraffin compositions were extracted using hexane and analyzed using GC/MS. The relative degradation of each paraffin compositions was calculated by a peak area compared to the control without inoculum. The ± indicates one standard deviation of three replicates.

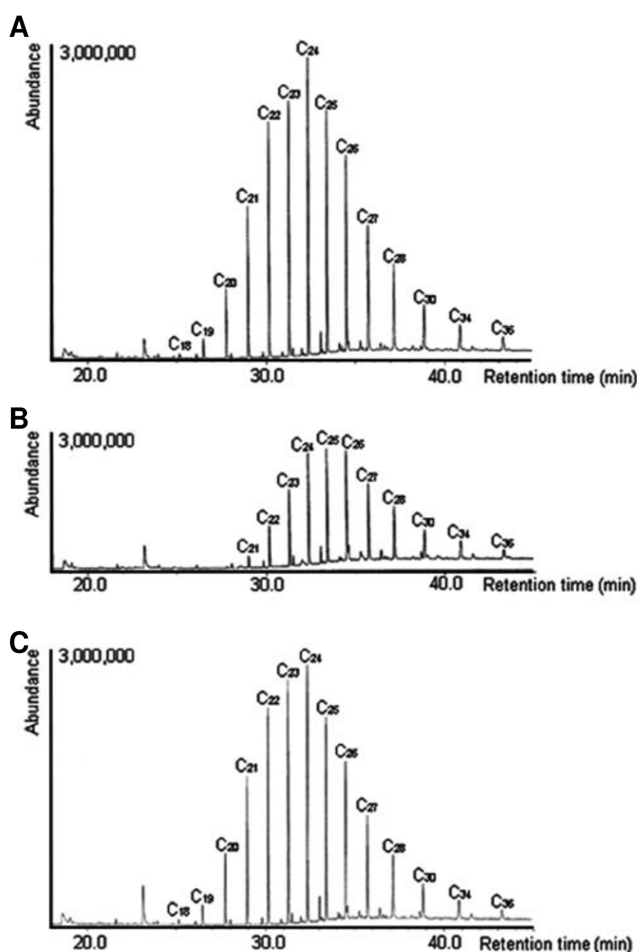

Fig. 3. GC/MS analysis of residual paraffin compositions after growth of *G. amicalis* LH3 in 0.1% (w/v) solid paraffin medium for 10 day under aerobic and anaerobic conditions. (A) Control without inoculum; (B) aerobic condition; (C) anaerobic condition.

ration was 7.5×10^8 cfu/mL and the paraffin utilized was 17.6% (w/w) whereas under anaerobic conditions, they were 3.6×10^7 cfu/mL and 2.3% (w/w), respectively (Fig. 2). A wide range of alkanes (C_{18}~C_{36}) could be utilized under both aerobic and anaerobic conditions. However, the alkanes of C_{18}~C_{24}, and particularly C_{18}~C_{21}, were utilized preferentially under aerobic conditions, whereas the long-chain alkanes of C_{34} and C_{36} were utilized preferentially under anaerobic conditions (Fig. 3 and Table 1).

Under aerobic conditions, the paraffin degradation rate of *G. amicalis* LH3 was 36 mg/d, which was consistent with the reports of 20~38 mg/d by the single strain [16,27,28] and 39 mg/d by a mixed bacterial consortium [16]. Under anaerobic conditions, the paraffin degradation rate of *G. amicalis* LH3 was 4.4 mg/d, which was also at the same level as described in other reports [29,30]. The majority of reports on *Gordonia* sp. have been conducted under aerobic conditions [17,22,24]. However, the growth of *G. sihwensis* SPR2^T has been reported under aerobic or anaerobic conditions [31] and the growth of *G. alkanivorans* has been reported under microaerophilic conditions [23]. The anaerobic biodegradation of paraffin is ideal for MEOR applications, the prevention of oil pipeline blockade, and bioremediation, as the input of air into underground oil reservoirs, pipelines, soil, or water will greatly increase the cost and technical difficulties, as was mentioned in the introduction section of this report. Attempts to augment the anaerobic biodegradation of hydrocarbons have been made by adding electron acceptors [29,30].

G. amicalis LH3 was shown to be able to utilize a wide range of long-chain alkanes (C_{18} to C_{36}) under either aerobic or anaerobic conditions. No single microbial species can degrade all of the hydrocarbon components of crude oil [32]. The strains utilizing a wide range (C_{12} to C_{32}) or narrow range of hydrocarbons [27], as well as those utilizing saturated alkanes or cyclic alkanes [15,33] have been previously described.

The exact mechanism underlying hydrocarbon degradation under anaerobic conditions remains to be precisely elucidated. One possible reason that the relatively long chain (C_{34}~C_{36}) alkaline proved more susceptible to anaerobic degradation than the shorter chain might be the result of adaptation to the energy limitations inherent to anaerobic conditions as compared to aerobic conditions. Alkane uptake is considered to be major pathway of active transportation, which consumes a considerable amount of energy [34-36]. Thus, under anaerobic conditions, it proved economic for the

Table 2. Effects of rhamnolipid concentrations on surface tension of *G. amicalis* LH3 cultivation broth

Surface tension (mN/m)	Rhamnolipid concentration (%)						
	0	0.02	0.04	0.2	0.4	0.6	0.8
0 day	74.3 ± 0.7	56.0 ± 0.2	55.0 ± 2.0	49.3 ± 2.6	45.0 ± 3.7	41.8 ± 2.5	38.4 ± 2.7
10 day	59.1 ± 2.3	58.5 ± 2.5	57.1 ± 3.4	52.0 ± 1.7	53.4 ± 1.5	53.2 ± 2.3	58.1 ± 3.1

2% (w/v) solid paraffin medium supplemented with various amounts of rhamnolipid, cultivated in aerobic condition for 10 day. The surface tension of the supernatant of the culture broth before and after cultivation was measured. The ± indicates one standard deviation of three replicates.

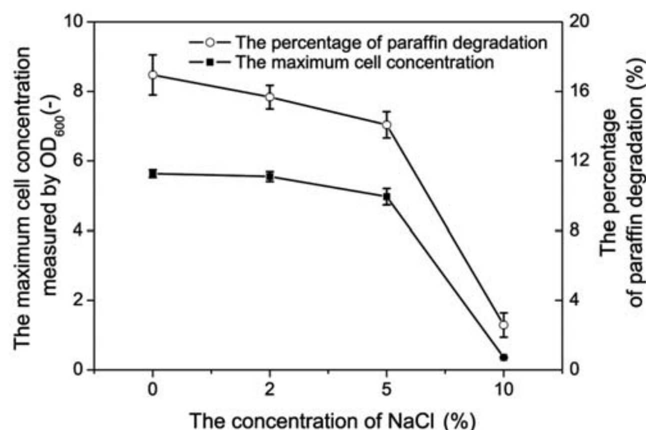

Fig. 4. The maximum cell concentration and paraffin degradation of *G. amicalis* LH3 grown for 10 day in 2% (w/v) solid paraffin medium of various salinity under aerobic conditions.

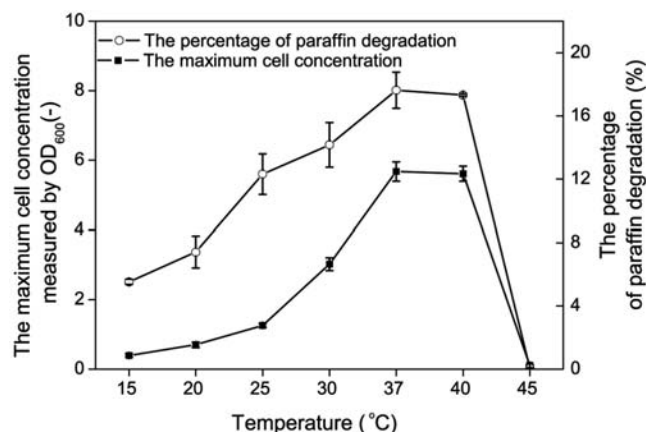

Fig. 5. The maximum cell concentration and paraffin degradation of *G. amicalis* LH3 grown in 2% (w/v) solid paraffin medium at various temperatures under aerobic conditions for 10 day.

cells to uptake long chain alkanes to produce more energy, while consuming the same amount of energy in alkane transportation.

Effects of Salinity, Temperature, and Rhamnolipid Biosurfactants on Growth and Paraffin Degradation

The experiments conducted to assess the effects of salinity, temperature, and rhamnolipids were done under aerobic conditions using 2% paraffin medium. In the salinity effect experimets, final NaCl concentrations of 0, 2, 5, and 10% were employed. In the temperature effect experiments, temperatures of 15, 20, 25, 30, 37, 40, and 45°C were employed. In the rhamnolipid effect experiments, final rhamnolipid concentrations of 0, 0.02, 0.04, 0.2, 0.4, 0.6, and 0.8% were utilized. The samples were made after 10 day cultivation, after which the cell growth (OD_{600}), paraffin concentration, and surface tension of the culture supernatants were assessed. The results are provided in Figs. 4~6 and Table 2.

The results of the salinity effect experiments indicated that in cases in which the NaCl concentration was lower than 5%, the inhibition of cell growth and paraffin degradation was not severe, but when the NaCl concentration was increased from 5 to 10%, the inhibition effects became severe (Fig. 4). At 10% NaCl, the cell growth and paraffin degradation were severely inhibited and only 2.6% of paraffin was degraded (Fig. 4). The surface tension was not affected by salinity and it was reduced from 74.2 to approximately 60.0 mN/m for all tested NaCl concentrations. High salinity, which may occur in MEOR applications, inhibits the microbial degradation of hydrocarbons [37]. A diesel oil degrading microbial consortia was reported to tolerate NaCl concentrations of up to 5% [29]. Members of *Gordonia* sp. evidenced differing salinity tolerance. The growth of *G. amarae*, *G. hirsute*, and *G. paraffinivorans* was inhibited completely by 7% NaCl; the growth of *G. alkanivorans*, *G. hydrophobica*, and *G. terrae* was significantly inhibited by 10% NaCl [22,23]. However, no reports have yet been filed on the salinity tolerance of *G. amicalis*.

The results of the temperature effect experiments indicated that *G. amicalis* LH3 could grow on paraffin at temperatures from 15 to 40°C with the optimal temperature ranging between 37~40°C (Fig. 5). The growth was almost completely inhibited and little paraffin was degraded at 45°C (Fig. 5). These results were consistent with other reports regarding *Gordonia* sp. [17,22,24]. The results indicated that *G. amicalis* LH3 is not applicable to very deep underground oil reservoirs in MEOR applications, as the temperature increases by 4°C when the underground depth increases by 100 m.

The results of the rhamnolipid biosurfactant effect experiments indicated that a rhamnolipid concentration of 0.2% was optimal for both cell growth and paraffin degradation, with an 11.7% increase in the maximum cell concentra-

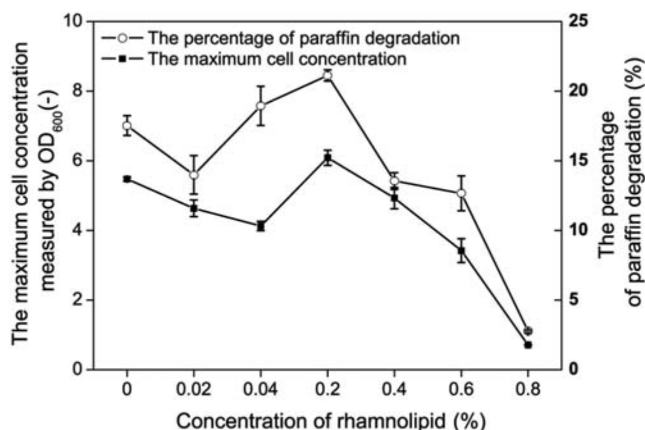

Fig. 6. The maximum cell concentration and paraffin degradation of *G. amicalis* LH3 grown for 10 day in 2% (w/v) solid paraffin medium supplemented with various quantities of rhamnolipid under aerobic conditions.

tion and a 20.5% (w/w) increase in paraffin degradation as compared with the controls (Fig. 6). Cell growth was inhibited profoundly at a rhamnolipid concentration of 0.8% (Fig. 6). Rhamnolipid concentrations higher than 0.2% or lower than 0.04% exerted a negative effect on paraffin degradation (Fig. 6). Surface tension was reduced when rhamnolipids were added, but increased again after 10 day of cultivation (Table 2), possibly resulting from rhamnolipid biodegradation. The lowest final surface tension value was obtained at a rhamnolipid concentration of 0.2% (Table 2). Biosurfactants have been demonstrated to increase the bioavailability of hydrophobic compounds and to augment the removal of hydrocarbon pollutants [26,38,39]. However, the optimal amount of rhamnolipid needs to be utilized in applications. High concentrations of biosurfactants may damage cell membranes and inhibit cell activity; however, lower than optimal concentrations still exert negative effects (Fig. 6). The negative effects of low rhamnolipid concentration can be explained in that both the bacterial cell wall and the rhamnolipid-hydroccarbon complexes harbor a net negative charge, and electrostatic repulsion precludes their interaction [40]. The inhibitory effects of biosurfactants on hydrocarbon biodegradation have been reported to be genus-specific [41].

Comparison of *G. amicalis* LH3 with Other Oil-degrading Microbial Strains

Two strains for MEOR applications and the bioremediation of hydrocarbon-polluted environments of *P. aeruginosa* SS-18 and *Bacillus* sp. SZ-32 were used, and were isolated utilizing the same method with *G. amicalis* LH3, except that oil-saturated filter paper covered BMSM plates was used rather than liquid paraffin. Paraffin degradation experiments were conducted using 2% solid paraffin medium, cultivated for 10 day; oil viscosity reduction experiments were conducted utilizing 60% oil medium, cultivated for 7 day; the oil degradation experiments were conducted using 0.1% oil medium, and cultivated for 7 day. Paraffin degradation, oil viscosity reduction, and oil degradation values were determined.

The results indicated that *G. amicalis* LH3, *P. aeruginosa* SS-18, and *Bacillus* sp. SZ-32 reduced oil viscosity by 44.7, 36.8, and 33.3%, respectively, degraded paraffin by 17.8, 13.1, and 11.4% (w/w), respectively, and degraded oil by 10.4, 13.7, and 16.2% (w/w), respectively. *G. amicalis* LH3 evidenced the highest values for both oil viscosity reduction and paraffin degradation but the lowest oil degradation values among the three strains, which were good characteristics for MEOR applications and the microbial prevention of pipeline blockade during oil transportation, allowing for the highest degree of oil fluidity with the least destruction of oil resources. Oil viscosity reduction could be the result of paraffin degradation.

CONCLUSION

The facultative anaerobic paraffin- and oil-degrading strain of *G. amicalis* LH3 was isolated and identified. It degraded paraffin by 18.0% (w/w) at a rate of 36 mg/d under aerobic conditions, and degraded paraffin by 2.3% (w/w) at a rate of 4.4 mg/d under anaerobic conditions after 10 day of cultivation. It reduced oil viscosity by 44.7% and degraded oil by 10.4% (w/w) under aerobic conditions after 7 day of cultivation. The strain was sustained at salinity of up to 5% NaCl and at temperatures higher than 40°C. The addition of 0.2% of rhamnolipids enhanced the growth and paraffin degradation of *G. amicalis* LH3. The results indicated that *G. amicalis* LH3, a novel long-chain alkane-degrading strain, may have potential in paraffin control applications, the bioremediation of hydrocarbon-polluted soil or water, and MEOR applications.

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