Biodegradation of n-hexadecane by bacterial strains B1 and B2 isolated from petroleum-contaminated soil

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Two hydrocarbon-biodegrading bacterial strains, B1 and B2, were isolated from petroleum-contaminated soil collected from Tianjin, China. The strains were identified as *Pseudomonas aeruginosa* (B1) and *Acinetobacter junii* (B2). The degradation rate of n-hexadecane by B1 and B2 reached 96% and 78% respectively after 7 days, though the strains employed different mechanisms of degradation. The results showed that B2 was not able to use glucose as carbon source. B1 could produce glycolipid surfactants using glucose as the carbon source, according to the results of blue agar plate analysis and thin layer chromatography (TLC), and the bacterial culture of B1 had a high oil discharge and emulsification activity. Both B1 and B2 could produce biosurfactants with hexadecane as the sole carbon source, but their modes of action were different. The carbon source was found to affect the cell surface hydrophobicity. Cell surface hydrophobicity was poor with glucose as the carbon source, but enhanced when hexadecane was used as the carbon source.

hexadecane, biosurfactant, hydrophobicity, emulsification, bacteria

1 Introduction

Petroleum, which is widely used in everyday life, is one of the most important energy sources for industry and transport. However, hydrocarbon pollutants (such as oils) released by the extraction, processing and use of petroleum accumulate frequently in soil, marine, groundwater, and other aqueous environments, threatening the ecosystem [1–3]. The insoluble characteristics of hydrocarbons make their clean-up extremely difficult. Conventional physical and chemical approaches to the remediation of hydrocarbons are usually expensive and laborious, and require the use of hazardous

solvents, which can lead to secondary environmental pollution [4, 5]. Conversely, the bioremediation of soils contaminated by petroleum has been shown to be an efficient, economical, versatile, and environmentally friendly way of mopping up hydrocarbon pollutants [4–6].

Despite the highly efficient hydrocarbon-degrading properties of some bacterial strains, one of the key limiting steps in bioremediation is the inadequate bioavailability of hydrocarbons to microorganisms due to their low solubility, which limits the uptake of hydrocarbons by various organisms [7–10]. Uptake and use of water-insoluble substrates by organisms requires specific physiological adaptations, and various strategies had been developed by different bacteria to interact with these hydrophobic compounds. Two

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general types of hydrocarbon-cell interactions have been postulated, depending on the state and size of the oil droplets relative to the size of microbial cells: specific adhesion of cells to larger oil drops, and pseudo-solubilization involving the cellular assimilation of emulsified smaller hydrocarbon droplets [11]. Most hydrocarbon-degrading bacteria excrete biosurfactants, amphiphilic molecules of diverse chemical nature, which improves the ability of microbial cells to use hydrophobic compounds as growth substrates [12, 13]. Surfactants not only affect substrates, increasing or in some cases decreasing their bioavailability [14–16], but also have an impact on microbial cells, evoking changes in their hydrophobicity and surface charge matrices [17]. Zhang and Miller [18] found that surfactant bound to bacteria changed the surface cell hydrophobicity and thus altered cells' affinity to the hydrophobic substrate, increasing uptake and biodegradation.

Hexadecane is present in the aliphatic fraction of crude oil and is one of the major components of diesel [19]. Due to its presence at many oil-contaminated sites and its well-characterized biodegradability [20], this compound has been used as a model molecule to study aliphatic hydrocarbon biodegradation [21]. In the present study, two bacterial strains, B1 and B2, were isolated from petroleum-contaminated soil from Tianjin, China, and characterized as *Pseudomonas aeruginosa* (B1) and *Acinetobacter junii* (B2). Both were able to use hydrocarbons as the carbon and energy source and produce biosurfactants when grown on hydrocarbons and other water-miscible and immiscible carbon substrates. Despite numerous reports of isolated strains which can produce biosurfactants and have high degradation ability, most studies have looked at only one kind of strain. This study investigated and compared the different mechanisms of hydrocarbon degradation by two bacterial strains, B1 and B2, including the role of biosurfactants and the relationship between cell surface hydrophobicity and the ability of such bacteria to degrade hydrocarbons.

2 Material and methods

2.1 Bacterial isolation and culture conditions

Two bacteria named B1 and B2 were used in this study. These two strains were isolated from soil collected from petroleum-contaminated sites in Tianjin, China by enrichment culture, and were found to exhibit a relatively strong potential to biodegrade petroleum hydrocarbons. Both strains could grow in a 100 mL Erlenmeyer flask containing 40 mL of mineral salt medium (MSM) with 5,000 mg/L hexadecane as the carbon source.

Culture mediums were sterilized in an autoclave at 121°C for 20 min. The mineral salt medium contained 0.05% KH_2PO_4 , 0.05% K_2HPO_4 , 0.3% NH_4NO_3 , and 0.2% (V/V) trace elements solution. The trace elements solution contained 0.4% MgSO_4 , 0.1% FeSO_4 , 0.1% CuSO_4 , 0.1%

CaCl_2 , and 0.1% MnSO_4 . The pH value was adjusted to 7.0–7.2 using 0.1 mol/L NaOH or 0.1 mol/L HCl. Flasks containing culture media were incubated at 30 °C on a rotary shaker (150 r/min) for 4 days. At the end of the incubation period, 1.0 mL of the enriched culture was transferred to a fresh medium containing 5,000 mg/L hexadecane and re-incubated for another 4 days.

2.2 Identification of bacteria

The bacteria were identified by determining the gene sequence coding for 16S ribosomal DNA (16S rDNA). A modification of the sodium dodecyl sulfate (SDS)-based extraction method was used to obtain genomic DNA from the Luria-Bertani (LB) culture. The extracted DNA was stored at –20°C for later use in polymerase chain reaction (PCR). PCR was performed on the extracted DNA to amplify the 16S rDNA genes. The forward primer of 27F (5'-AGAGTTTGATCATGGCTCAG-3', universal primer) and the reverse primer of 1492R (5'-TACGGTTACCTTGTTACGACTT-3', universal primer) were used for the PCR. The PCR mixture contained 5.0 µL buffer, 4 µL deoxynucleoside triphosphate (dNTP), 1.0 µL each of forward primer (27F) and reverse primer (1492R), 1.0 µL of DNA template, and 0.25 µL of Taq DNA polymerase in a final volume of 50 µL. Amplification was performed using an automated thermal cycler. PCR amplification was carried out under the following conditions: initial denaturation at 95 °C for 5 min; 30 cycles of denaturation for 1 min at 95 °C, annealing for 2 min at 52 °C, extension for 2 min at 72 °C respectively, and a final extension at 72 °C for 10 min. The PCR products were separated by electrophoresis using 0.1% agarose gel in TAE buffer. To identify the isolated bacteria, the 16S rDNA consensus sequence obtained from Shanghai Sangon Biological Engineering Technology & Services Company Limited was then compared with 16S rDNA gene sequences from the public GenBank (US), EMBL (Europe), and DDBJ (Japan) databases using the advanced gapped n-BLAST program, version 2.1. The program was run via the Internet through the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/blast/>). Sequences found to be more than 98% identical to a GenBank sequence were considered to be the same species as the highest score-matching sequence on the public databases.

2.3 Hexadecane and extracellular fatty acid analysis

2.3.1 Hexadecane analysis

Residual hexadecane in the culture medium was extracted three times with an equal volume of hexane as the extraction solvent. The quantity of hexadecane in the extract was determined using a gas chromatograph with a flame ionization detector (GC-FID, Agilent 6820, USA). The GC-FID was operated with nitrogen as the carrier gas at a flow rate

of 5.0 mL/min, inlet temperature of 250 °C, detector temperature of 300 °C, and an oven temperature program rising from 100 °C (held for 2 min) to 300 °C (held for 10 min) at a rate of 10 °C/min. The injection volume for all samples was 1 μ L.

2.3.2 Extracellular fatty acid analysis

The growth culture medium (5 mL) was subjected to liquid-liquid extraction following basification to pH > 11 using 0.1 mol/L NaOH solution, then extracted using 10 mL dichloromethane (CH_2Cl_2) three times. The remnant aqueous phase was acidified to pH < 2 using HCl and extracted using 10 mL CH_2Cl_2 twice. All extractions were dried with sodium sulphate and concentrated by nitrogen blowing. The fatty acid fraction of the extract was analyzed using gas chromatography-mass spectrometry (GC-MS) (Agilent GC/MS, GC 7890A, MSD 5975C). The mass spectrometry conditions were as follows: ion source, 250 °C, 1200 V; scanning mode, completely scan 35–500 amu. The conditions for GC were as follows: the temperature program rose from 60 to 280 °C (held for 5 min) at a rate of 10 °C/min, while the inlet temperature was 280 °C, and nitrogen gas was the carrier gas at a flow rate of 25 mL/min. The injection volume for all samples was 1 μ L.

2.4 Physicochemical characterization of biosurfactant produced by B1 and B2

2.4.1 Oil displacement test

The oil displacement test was done using the method described by Rodrigues *et al.* [22] with some amendments. Firstly, 50 mL of redistilled water was added to a Petri dish with a diameter of 15 cm. Then, 30 μ L of hexadecane was pipetted into the dish to form a thin oil layer on the surface of the water, followed by 100 μ L of bacterial culture solution deposited onto the surface of the oil making a clear zone in the center of the oil layer. The maximum diameter of the clear zone was recorded under daylight. The higher the surface activity of the test solution, the larger the diameter of the clear zone [23].

2.4.2 Measurement of emulsification activity

Emulsification activity was determined according to the method described by Cooper and Goldenberg [24] with some modifications. A mixture of 3 mL of the studied hexadecane or diesel oil and 2 mL of bacterial culture solution was vortexed at high speed for 2 min. The emulsification activity was determined after 24 h and the emulsification index (E_{24}) was calculated using the following equation:

$$E_{24}(\%) =$$

$$\frac{\text{Height of emulsified zone}}{\text{Height of total liquid (sum of oil, emulsified, and aqueous zones)}} \times 100\%$$

The higher the emulsification index (E_{24}), the higher the emulsification activity of the tested surfactant.

2.4.3 Blue agar culture analysis

Extracellular glycolipid production was detected by blue agar culture analysis according to the method described by Siegmund *et al.* [25]. After 3 days incubation in a 30 °C rotary shaker, the culture solution was plated onto blue agar plates, and the blue agar plates were incubated at 30 °C for 2–3 days in the dark. The plates contained 0.5% cetyltrimethylammonium bromide (CTAB), 0.002% methylene blue, 0.5% beef extract, 1.0% peptone, 0.5% sodium chloride (NaCl), and 2.0% agar. Biosurfactant production could be observed by the formation of dark blue halos around the colonies.

2.4.4 Analysis of cell surface hydrophobicity

The microbial adherence to hydrocarbons (MATH) assay was used to test the hydrophobicity of the strains. The general protocol of MATH is described by Zhang and Miller [18] in detail. In this experiment, cells were washed with MSM three times from the cell pellet, then resuspended in MSM and adjusted to an optical density measured at a wavelength of 600 nm (OD_{600}) of 0.4–0.6. Diesel oil (5 mL) was added to 5 mL of the adjusted cell suspension in a test tube and was vortexed for 5 min. After that, the mixture was allowed to settle and separate for 5 min, and then the OD_{600} of the aqueous phase was measured. The MATH value was calculated according to the following equation.

$$\% \text{MATH} =$$

$$\left[1 - \frac{\text{OD}_{600} \text{ of aqueous phase of culture after the addition of diesel oil}}{\text{OD}_{600} \text{ of culture before the addition of diesel oil}} \right] \times 100\%$$

3 Production and purification of biosurfactant

To investigate the production and activity of biosurfactant produced by the strains in different mediums, the strains were grown on MSM containing only water-soluble (glucose) or insoluble (hexadecane) carbon sources, or a mixture of glucose and hexadecane, at 30 °C on a rotary shaker (150 r/min) for 7 days.

After bacterial cells were removed by centrifugation at 3,700 r/min for 30 min at 4 °C, the supernatant was acidified to pH 2.0 using 1 mol/L HCl, and the acidified supernatant was left overnight at 4 °C for complete precipitation of the biosurfactant. For purification of surface-active component(s), the supernatant was extracted with an equal volume of solvent made up of chloroform: methanol (2:1, V/V) [26]. The organic solvent was transferred to a round-bottom flask connected to a rotary evaporator to remove the solvent, and the residual material was considered to be crude biosurfactant. The crude product was dissolved in dichloromethane and after being filtered to remove any coarse material, the solvent was removed with the aid of rotary evaporator and the solid was washed with triplicate volumes of hexane to remove alkenes and free fatty acids. Hexane was then evaporated and the resulting material was considered to be purified.

fied biosurfactant.

The purified extract was dissolved in dichloromethane and analyzed by thin layer chromatography (TLC) on silica gel plates. The samples were separated on silica gel plates using chloroform: methanol: distilled water (65:25:4, V/V/V) as the solvent system. The components were detected by spraying the plate with ninhydrine reagent for lipopeptide and anthrone reagent for glycolipide detection respectively [27].

4 Results

4.1 Characterization of bacterial strains B1 and B2

The PCR products were separated by electrophoresis using 0.1% agarose gel in TAE buffer (Figure 1). Taxonomical identification of these two strains, designated B1 and B2, was performed by amplifying and sequencing the 16S rDNA genes and comparing them to the database of known 16S rDNA sequences. Alignment of the 16S rDNA gene sequences of B1 and B2 with sequences obtained by doing a blast search revealed 99% similarity to *Pseudomonas aeruginosa* and 98% similarity to *Acinetobacter junii* respectively. Thus, the two strains were designated as *Pseudomonas* sp. (B1) and *Acinetobacter* sp. (B2).

4.2 Hexadecane and extracellular fatty acid analysis

After 7 days, biodegradation of 500 mg/L hexadecane by bacterial strains B1 and B2 was around 96% and 78%, respectively; an increase in bacterial growth was concomitant with a decrease in hexadecane concentration. During the growth of B1 and B2 with addition of n-hexadecane, the pH of the bacterial culture decreased from 6.8 to 4.8 and 4.3 respectively (Figure 2). This change in culture medium pH may have been induced by fatty acid generation from the degradation of hexadecane. After extraction, the ectocytic fatty acid content was determined using GC-MS. Strain B1

Figure 1 The picture of 1% agarose gel electrophoresis of PCR products.

Figure 2 The change of pH during the growth of bacteria.

was found to produce 2,3-dihydroxystearic acid ($C_{18}H_{38}O_4$), hexadecanoic acid ($C_{16}H_{32}O_2$), propanoic acid ($C_3H_7NO_2$), dodecanoic acid ($C_{12}H_{24}O_2$), quinaldic acid ($C_{10}H_9NO_4$), and so on when grown on hexadecane (Figure 3). Other low molecular weight fatty acids identified included nonenoic acid ($C_9H_{18}O_2$), pentanoic acid ($C_5H_{10}O_2$), and butyric acid ($C_4H_8O_2$). Similar products were detected in the culture solution of B2. The decrease in the pH of the culture solu-

Figure 3 The GC-MS chromatogram of B1 ectocytic fatty acid.

tion may have been the result of the acids produced.

4.3 Physicochemical characterization of the biosurfactant produced by B1 and B2

4.3.1 Oil displacement test and emulsification activity determination

The results of the oil displacement test and emulsification activity (E_{24}) determination are shown in Table 1. When B1 was grown on glucose, we measured the largest diameter of the clean zone (about 10.2 cm), and the highest E_{24} , up to 60% (hexadecane) and 71% (diesel oil) respectively. When B1 was grown on a dual carbon source (glucose and hexadecane), the diameter of the clean zone was about 4.5 cm and E_{24} measured about 18% (hexadecane) and 20% (diesel oil) respectively. However, when B1 was grown on hexadecane, the diameter of the clean zone was only 4.1 cm and the E_{24} was only 6.7% (hexadecane) and 8.0% (diesel oil) respectively. B2 did not grow on glucose, but showed similar results of a larger diameter of clean zone and higher emulsification activity when grown on a dual carbon source rather than on hexadecane alone.

The culture solution of B1 mixed with glucose was effective in emulsifying other hydrocarbons, which indicated that the emulsification activity of the test solution was not substrate-specific. Although both strains grew well on hexadecane, they showed low emulsifying activity, especially for B2. Thus, the level of emulsifying activity is related to the carbon source.

4.3.2 Blue agar culture analysis

The formation of blue halos around the colonies on the blue agar plate indicated the production of glycolipid biosurfactant. In this study, the formation of dark blue halos around colonies was observed only on the blue agar plate hosting the B1 strain (Figure 4), which indicated that B1 could produce extracellular glycolipid biosurfactant, while B2 could not.

4.3.3 Analysis of cell surface hydrophobicity

The results obtained from the MATH assay (Table 2) showed that the cell surface hydrophobicity was dependent on carbon source. When B1 was grown on MSM supplied

Figure 4 Blue agar culture analysis of B1 and B2.

Table 2 Effects of different carbon sources on hydrophobicity (microbial adherence to hydrocarbons or MATH) of hydrocarbon-degrading bacteria

Medium	MATH
B1 (0.5% C16)	0.886
B2 (0.5% C16)	0.852
B1 (0.2% G + 0.5% C16)	0.739
B2 (0.2% G + 0.5% C16)	0.713
B1 (0.2% glucose)	0.038

0.5% C16: The carbon source is 5,000 mg/kg hexadecane; 0.2% G+0.5% C16: the carbon source is 0.2% glucose and 5,000 mg/kg hexadecane; 0.2% Glucose: the carbon source is 0.2% glucose

with glucose, a dual carbon source (glucose and hexadecane) or hexadecane, the values of MATH were 0.04, 0.74, and 0.89 respectively. Because B2 could not grow on glucose, B2 was only tested in MSM containing hexadecane and the carbon source mixture. The values of MATH were 0.85 and 0.71 when B2 was grown on hexadecane and the carbon mixture respectively. Thus, when the substrate becomes more hydrophobic, cells are more likely to change their surface hydrophobicity to adapt to the new environmental conditions.

As shown in Figure 5, the cell hydrophobicity of B1 remained unchanged during its growth on glucose, and the value of MATH was maintained at about 0.04. When cultured with hexadecane, cell hydrophobicity of B1 correlated with the age of the culture. B1 cells in the early or mid-exponential growth phase were less hydrophobic than cells in the stationary growth phase, while the value of MATH increased from 0.71 to 0.91. However when B2 was grown on hexadecane, the cell hydrophobicity of B2 remained unchanged and the value of MATH was about 0.66.

4.4 Production and purification of biosurfactant

Both B1 and B2 produced biosurfactants during their growth on hexadecane and on glucose according to the results of the oil displacement test, blue agar culture analysis, and cell surface hydrophobicity analysis. We carried out the extraction and purification of the biosurfactant. However, the biosurfactant could be extracted only when B1 was grown on MSM with glucose, and the extracted biosurfactant took on a brownish color. TLC analysis of the extracted

Table 1 Oil displacement and emulsification activity (E_{24} , with hexadecane and diesel oil respectively) of culture broth during growth of B1 and B2 in MSM containing different carbon sources

Medium	Oil displacement test (cm)	E_{24} (%) (average $\pm$ SD)	
		hexadecane	diesel oil
B1 (0.2% G)	10.2	60 $\pm$ 3	71 $\pm$ 2
B1 (0.5% C16)	4.1	6.7 $\pm$ 2	8.0 $\pm$ 4
B2 (0.5% C16)	3.2	ND	3.3 $\pm$ 3
B1 (0.2% G+0.5% C16)	4.5	18 $\pm$ 4	20 $\pm$ 3
B2 (0.2% G+0.5% C16)	3.8	6.3 $\pm$ 3	7.1 $\pm$ 2

Figure 5 Difference of the cell-surface hydrophobicity of strains B1 and B2 during their growth.

biosurfactant from B1 indicated the presence of a glycolipid fraction, identified on the basis of yellow spots on the silica gel. Lipopeptides were undetectable in the extracted biosurfactant. Neither glycolipids nor lipopeptides were detected in the culture of B2.

5 Discussion

In this work, both B1 and B2 bacterial strains showed highly efficient degradation of hexadecane, albeit via different mechanisms. Depending on the state and size of the oil droplets relative to the size of microbial cells, two general types of hydrocarbon-cell interactions have been postulated: specific adhesion of cells to larger oil drops and pseudosolubilization involving cellular assimilation of emulsified small hydrocarbon droplets [11]. Goswami *et al.* [28] reported three mechanisms for the uptake of slightly soluble liquid hydrocarbon by microorganisms: (1) interaction of cells with hydrocarbon dissolved in the aqueous phase; (2) direct contact of cells with hydrocarbon drops considerably larger than the cells; (3) interaction of cells with “solubilized”, “pseudosolubilized”, “accommodated”, “micro-emulsified”, or “submicron” hydrocarbon droplets in entities much smaller than the cells. Therefore, different bacteria adopt different approaches when degrading hydrocarbon.

The isolated strain B1 was designated as *Pseudomonas* sp. B1. The ability of *Pseudomonas aeruginosa* to use hydrocarbons as an energy source has been demonstrated in several studies [18]. During the growth of B1 on n-hexadecane, a floating emulsion could be observed at the air-medium interface. These flocs of B1 were connected by an extracellular polymer, which was a complex mixture of glycoconjugates, suggesting the type of hydrocarbon-cell interaction taking place was specific adhesion of cells to larger oil drops. According to our results (Table 1), strain B1 grew well on hexadecane but showed a lower emulsifying activity than when grown on glucose, suggesting a link with the acid products generated in the culture medium. Belsky *et al.* [29] reported that the cleavage of fatty

acids from emulsan decreased the emulsifying activity by more than 50%. Milva *et al.* [30] suggested that glycolipids with regular long chain fatty acids presumably undergo lipid phase transition more readily, losing their emulsifying ability.

We found that B1 produced a glycolipid type of biosurfactant when grown on hexadecane or glucose. *Pseudomonas* is the best-known bacteria capable of using hydrocarbon as an energy source and producing biosurfactants [31–33]. *Pseudomonas aeruginosa* has been widely studied and is known to produce a glycolipid type of biosurfactant [34, 35]. Rhamnolipids are amphiphilic surface-active glycolipids that are usually secreted in the growth medium [36, 37], but can potentially be used to combat marine oil pollution [8]. Figure 5 shows that the value of MATH increased from 0.71 to 0.91 when B1 was cultured with hexadecane. It appears that biosurfactant-enhanced biodegradation is not simply a function of enhanced solubility, but is also due to changes induced in the cell surface [38]. For example, Zhang and Miller [18] found that rhamnolipids not only increased the apparent solubility of hydrocarbons, but also modified the cell surface resulting in greater hydrophobicity. Herman *et al.* [39] showed that the addition of rhamnolipids induced the formation of multicellular aggregates, implying that the cells forming these aggregates were hydrophobic in nature. These findings indicate that the intrinsic ability of a cell to interact with hydrocarbons in a growth medium increases significantly in the presence of rhamnolipids.

Table 2 illustrates how cell surface hydrophobicity changes when the carbon source is altered. When B1 was grown on MSM supplied with glucose, a dual carbon source (glucose and hexadecane) or hexadecane, the values of MATH were 0.04, 0.74, and 0.89 respectively. Some investigators have reported that changes in the carbon source can affect the cell wall, thus affecting cell surface hydrophobicity [40, 41]. The surface hydrophobicity of some *Pseudomonas* bacteria has been shown to decrease when the bacteria are grown on carbon sources such as glucose and acetate [42, 43].

B2 was unable to use glucose as the sole source of carbon, similar to the findings with *Acinetobacter* RAG-1 isolated by Luo *et al.* [44]. Given that *Acinetobacter* RAG-1 lacks 6-phosphofructokinase, it may have been unable to harness the energy and primary metabolites from the glycolytic pathway [44]. When B2 grew on hexadecane, large oil drops were emulsified into small hexadecane droplets. This phenomenon suggests that the type of hydrocarbon-cell interaction taking place was pseudosolubilization, involving the cellular assimilation of emulsified small hydrocarbon droplets. This phenomenon was similarly reported by Reisfeld *et al.* for the RAG-1 strain [45]. RAG-1 produced emulsan, which was then absorbed onto the surface of the oil, effectively preventing the aggregation of oil droplets and forming a stable emulsion. This increased the opportunities for bacterial cells to make contact with alkanes, and

subsequently enhanced the bacterial absorption of alkanes.

In general, biodegradation is accompanied by emulsification, resulting in a greater oil-water interface. Further evidence indicates that the greater the oil-water interface, the faster the rate of oil decomposition by microorganisms. During the growth of B2 on hexadecane, fatty acids were produced as evidenced by the decrease in pH (Figure 2). It has been postulated that fatty acids produced through alkane metabolism or triglyceride hydrolysis can aid the uptake of alkanes by emulsifying the substrate [46]. We also found that the B2 strain grown on hexadecane showed a low emulsifying activity, which correlates with the cleavage of fatty acids. One study reported that the cleavage of fatty acids from emulsan decreased the emulsifying activity by more than 50% [29]. Like B1, when B2 grew on hexadecane, it also produced biosurfactants. Various gamma-proteobacteria have the ability to produce biosurfactant molecules. For example, the *Acinetobacter calcoaceticus* RAG-1 can produce emulsan [47–54].

It is not clear why biosurfactants could not be extracted when strains B1 and B2 were grown on MSM with hexadecane. One of the reasons may be that the mechanisms responsible for biosurfactant excretion were controlled by the cells themselves, so that only necessary amounts of biosurfactant were produced when strains were growing on hexadecane. Although it has been possible to force microorganisms to overproduce biosurfactants such as rhamnolipids when cells have been cultivated on non-hydrophobic compounds like glycerol, such overproduction has never observed in the degradation of actual pollutants [50]. A further reason for the apparent lack of biosurfactant may be the decrease in pH. One study found that surfactin (SF) produced by bacteria disappeared rapidly in the medium broth once the pH fell below 5.0 [51]. In a later study, the disappearance of SF was linked to its precipitation triggered by the accumulation of extracellular acidic metabolites [52].

In this study, biosurfactants were found to enhance both the solubility and the solubilization rate of hydrocarbons. Some researchers have reported that biodegradation rates of slightly soluble compounds increase significantly upon the introduction of biosurfactants to a reaction vessel [53]. Cell surface hydrophobicity has also been found to correlate with the degradability of organic pollutants in the environment. Strains with a greater cell surface hydrophobicity can degrade hydrophobic organic compounds faster than strains with a smaller cell surface hydrophobicity.

6 Conclusions

Two efficient hydrocarbon-biodegrading bacterial strains, B1 and B2, were isolated in soil from petroleum-contaminated sites in Tianjin, China, and identified as *Pseudomonas aeruginosa* and *Acinetobacter junii*, respectively. Both B1 and B2 were found to produce biosurfactants, but their

modes of action were different, suggesting their mechanisms of biodegradation are different. Pseudosolubilization was behind the substrate degradation observed here, allowing uptake from hexadecane micelles by the hydrophilic cells of strain B1. Conversely, direct interfacial accession appeared to be the main mechanism for hexadecane uptake by B2. Our results show that different microbial strains employ different degradation mechanisms, where the role of biosurfactants is related to their surface properties. Thus, it may be necessary to fully investigate the modes of action of biosurfactants used in the bioremediation of contaminated land.

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