Front. Environ. Sci. Eng. 2013, 7(4): 539–551
DOI 10.1007/s11783-013-0498-z

RESEARCH ARTICLE

Selective pseudosolubilization capability of *Pseudomonas* sp. DG17 on *n*-alkanes and uptake mechanisms analysis

Fei HUA, Hongqi WANG (✉)

College of Water Sciences, Beijing Normal University, Beijing 100875, China

Abstract Pseudosolubilized ability of *Pseudomonas* sp. DG17 on *n*-alkanes, role of biosurfactants in *n*-octadecane uptake and trans-membrane transport mechanism of *n*-octadecane were studied by analyzing amount of pseudosolubilized oil components in water phase, and the fraction of radiolabeled ^{14}C *n*-octadecane in the broth and cell pellet. GC-MS results showed that pseudosolubilized oil components were mainly C_{12} to C_{28} of *n*-alkanes. In *n*-octadecane broth, pseudosolubilized *n*-octadecane could be accumulated as long as pseudosolubilized rate was faster than mineralization rate of substrate, and the maximum concentration of pseudosolubilized *n*-octadecane achieved to 45.37 $mg \cdot L^{-1}$. All of these results showed that *Pseudomonas* sp. DG17 mainly utilized alkanes by directly contacting with pseudosolubilized small oil droplets in the water phase. Analysis of ^{14}C amount in cell pellet revealed that an energy-dependent system mainly controlled the trans-membrane transport of *n*-octadecane.

Keywords *Pseudomonas*, alkane, uptake, pseudosolubilization, trans-membrane transport

1 Introduction

The ability to utilize saturated hydrocarbons (*n*-alkanes) is a precious character of diverse microbial populations [1,2]. As *n*-alkanes are major constituents of crude oil, these microbes play important roles in bioremediation of crude oil contaminated soil. Bioavailability of hydrophobic organics in non- aqueous phase liquids, including crude oil, alkane and aromatic hydrocarbons, is often a major barrier for biodegradation [3]. In the case of water-insoluble substrates like *n*-alkanes, hydrophobic nature of the bacterial cell surface has been reported to play an important role in the bioavailability of hydrocarbons [4–7]. On the other hand, it was found that secreted glycolipids did not contribute to the change of cell surface hydrophobicity of *Antartic Nocardioides* A-2 cells, while, it did decrease the hydrophobicity of *Antartic Nocardioides* A-3 cells [8]. Thus, understanding the relationship between biosurfactants and surface properties of microbial degraders is necessary to establish proper modes of biosurfactant utilization in bioremediation [9]. Studies have shown that cell contact with hydrophobic substrates is crucial, because the initial step in aliphatic and aromatic hydrocarbon degradation is often mediated by oxidation reactions catalyzed by cell-surface-associated oxygenases [10]. Three types of hydrocarbon uptake mechanisms during biodegradation have been described earlier [11]: Uptake of dissolved hydrocarbons in aqueous phase [12]; direct adhesion to oil [13]; and a hypothesized pseudosolubilization in which the hydrocarbon degrading bacteria assimilate small droplets of emulsified oil.

Available reports are in favor of the hypothesis that microbial cells attach to the surface of hydrocarbon drops which are smaller than the cells [14]. Biosurfactant secreted by microorganisms can help, by solubilization or emulsification, to release hydrocarbons adsorbed to soil particles and increase the aqueous concentrations of hydrophobic compounds, resulting in higher mass transfer rate [15]. However, this mechanism was not always the major mode of hydrocarbons uptake in other cases. Take *Pseudomonas* sp. PP2 as an example, early- to mid-log-phase cells showed highest cell surface hydrophobicity compared to stationary phase cells. The emulsification activity increased with cell growth and reaches a maximum at late-log-phase to early-stationary-phase. Thus, cells of *Pseudomonas* sp. PP2 showed growth-dependent changes in cell surface hydrophobicity and biosurfactant production [16]. These evidences suggested that microorganisms can directly contact with big hexadecane droplets and emulsify small droplets in different growth phase [17,18].

On the other hand, although interaction between

Received January 20, 2012; accepted February 28, 2013

E-mail: whongqi310@sohu.com

hydrocarbons, biosurfactant and microorganisms is well characterized, how these compounds are taken into cell is not clear. A few studies found that after microbial cells attach to soluble hydrocarbon drops that are much smaller than the cells, substrate uptake presumably takes place through passive diffusion or energy-dependent active transport at the point of contact [19–22]. For cells uninduced by hydrocarbons, passive diffusion transport, a linear process, is very quick. In this case, trans-membrane transport of substrate would be governed by Fick's first law. Thus, it would not display energy dependence. Since a protein-mediated mechanism is not involved in the transport, the entry would not display substrate specificity or exhibit saturation kinetics [21]. For instance, for the uninduced cells of *Mycobacterium* sp. strain RJG II −135, immediate uptake did not show saturation kinetics and was not susceptible to inhibitors [22]. On the other hand, an energy-dependent transport system, which is controlled by the membrane proteins, is the major transport mechanism during substrate influx for cells induced by substrate [23]. For example, phenanthrene uptake by induced cells of *Mycobacterium* sp. strain RJG II −135 s followed saturation kinetics, and was strongly inhibited by energy inhibitorscyanide and carbonyl cyanide m-chorophenylhydrazone [22]. Meanwhile, since the rate of solute diffusion is first order with respect to the difference in concentration $C_{in\text{-}out}$ of a solute across a membrane, the rate of cellular transport of pollutants with limited water solubility and low aqueous diffusion coefficients could limit the rate and extent of pollutant degradation [24]. Moreover, trans-membrane transport of hydrocarbons might be also related with substrate concentration. Shishido and Toda [25] found that an active or facilitated transport mechanism might exist in phenol uptake at aqueous substrate concentrations below 50 $mg \cdot L^{-1}$; while at high phenol concentrations, cell entry would be through passive diffusion. Thus, low aqueous-phase concentration of hydrocarbons might be incapable to drive the diffusional process required for cellular transport.

Previous studies mainly focused on the demulsification capacity of bacteria on crude oil by monitoring demulsification, cell surface hydrophobicity and microphotography of emulsion [26]. However, bacteria must physically contact with these hydrophobic compounds before demulsification process. Moreover, limited studies have reported the trans-membrane transport mechanism of alkanes. The aim of this study was to investigate the crude oil uptake mode, analyze the pseudosolubilization ability of *Pseudomonas* sp. DG17 on *n*-alkanes which can serve preferential uptake carbon and energy source under the demulsification effect of microorganism, which was different from other studies that concentrated on single oil components. For aqueous *n*-octadecane, isotope tracer technique was used to analyze fraction of ^{14}C *n*-octadecane in the broth and cell pellet, which was helpful in studying octadecane transport mechanism.

2 Materials and methods

2.1 Culture and growth conditions

Pseudomonas sp. DG17 (CGMCC: NO. 5052; NCBI accession No. JN 216878), used in this study, was isolated from petroleum contaminated soil (Da Gang oilfield, China). Screening was done by monitoring cell growth of different kinds of microorganisms (plate count method) on crude oil. As for DG17, culture medium became brown after incubation for eight days, which showed that this strain could emulsify crude oil well. Pure colony of DG17 was maintained at 4°C on crude oil solid medium. Before assays, colony of DG17 was transferred into LB culture medium, and cells of DG17 were collected after incubation for 48 h at 30°C. In the domesticated experiment, cells of DG17 were inoculated in mineral salt medium (MSM) culture medium with *n*-octadecane as the sole carbon source. The domesticated experiment was carried out for three times in 30 days. For each time, 5 mL of fermentation broth was transferred into new MSM culture medium with *n*-octadecane as carbon source. Then, the culture was maintained at 4°C on octadecane solid medium and used for the other experiments. Previous studies have showed that rhamnolipids biosurfactant were produced by *Pseudomonas* sp. DG17 when growing on octadecane [27].

Bacterial inoculum from the octadecane solid medium was transferred into 100 mL of LB culture medium with 0.5% yeast extract in a 250-mL Erlenmeyer flask and enriched at 30±1°C on a gyratory shaker at 140 $r \cdot min^{-1}$ for 48 h. Cells were collected by centrifuge at 4000 $r \cdot min^{-1}$ for 10 min, washed twice by mineral salts medium (MSM, pH 7.0)), and re-dissolved in sterile MSM with equal volume. The final A_{600} value measured at 600 nm on a spectrophotometer (Varian, Palo Alto, California, USA) was controlled at 1.5. The MSM was composed of 0.4 g Na_2HPO_4, 0.15 g KH_2PO_4, 0.1 g NH_4Cl, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.0015 g $CaCl_2$, 0.1 g $NaNO_3$, and 1 mL trace medium (per 100 mL solution containing 0.5 mg $CuSO_4 \cdot 5H_2O$, 1.0 mg H_3BO_3, 1.0 mg $MnSO_4 \cdot 5H_2O$, 7.0 mg $ZnSO_4$) in 1000 mL deionized water. The liquid culture medium was sterilized by autoclaving at 121°C for 20 min.

The MSM medium was supplemented with 0.2 g of crude oil as the sole carbon source. Stock solution of crude oil (200 $mg \cdot mL^{-1}$) dissolved in dichloromethane was filtered by 0.2 μm filter membrane into the flask, then the flask was shaken for 6 h to remove dichloromethane. The biodegradation process began with the addition of cells of DG17, and final cell density (A_{600}) was 0.15. The flasks were incubated at 150 $r \cdot min^{-1}$ and 25°C.

2.2 GC-MS analysis of pseudosolubilized *n*-alkanes components in the water phase

After incubation for 20 d and 35 d, 20 mL of water phase in the flask which became brown was transferred into 250 mL

separating funnel, and extracted by equal volume of dichloromethane for three times in order to extract the whole crude oil components in the water phase. The organic phase was ultrasonic- extracted for three times and 10 min each time, and transferred into a beaker (treated by nitric acid) in order to volatilize dichloromethane. Then, 2 mL of dichloromethane was supplemented into amber gas chromatotraphy vials possessing teflonlined crimp-top seals and analyzed by Gas Chromatography–Mass Spectrometry (GC-MS) (450GC-320MS, Bruker Daltonics, Massachusetts, USA).

The GC-MS instrument was equipped with split injector, and a VF-5 column was used for separation (30 m, 0.25 mm id, 0.25 μm film thickness). The temperature program started at 60°C and was held for 5 min. Oven temperature was standardized at 290°C with injector and detector at 350°C. Mass spectra were recorded at 1 scan/s under electron impact at 70 eV, mass range 40–650 amu. Standard sample which contains different carbon chain length from C_{10}–C_{30} of *n*-alkanes (Sigma, Santa Clara, California, USA) was measured at the same time.

2.3 The pseudosolubilization ability assay

For pseudosolubilization ability assay, MSM medium was supplemented with 400 $mg \cdot L^{-1}$ of *n*-octadecane as the sole carbon source. Stock solution of octadecane (1000 $mg \cdot L^{-1}$) that dissolved in hexane was injected into 250-mL Erlenmeyer flasks filtered by 0.2 μm filter membrane. The inoculum of DG17 was 5% (v/v), and cell density at A_{600} was about 0.127. After incubation for 6 h to remove hexane, biodegradation process began and the flasks were incubated at 140 $r \cdot min^{-1}$ and 25°C for 6 d. At different time intervals, cell density at A_{600} and surface tension of the medium were monitored. Two duplicate samples were also conducted for standard deviation analysis.

Three groups of flasks were used for pseudosolubilization of *n*-octadecane. The control group that not supplemented with cells was used for abiotic loss analysis of *n*-octadecane. The second group was used for total biodegradation analysis of *n*-octadecane in the flasks. The whole culture medium was extracted with *n*-hexane. The third group was used for aqueous *n*-octadecane analysis. At different time intervals, 10 mL of culture medium in the water phase was transferred into centrifuge tube, and cell pellets were collected by centrifugation at 4,000 $r \cdot min^{-1}$. The supernatant was transferred into a separating funnel containing equal volume of *n*-hexane. Then, the organic phase was ultrasonic extracted for 3 times, and transferred into a beaker (treated by nitric acid) in order to volatilize hexane. Lastly, 2 mL of hexane was used to dissolve the hydrocarbon for GC-MS analysis. Standard curve of hydrocarbon concentration was as follows: $Ai = 7 \times 10^7 X$ for octadecane, in which Ai was peak area and X was hydrocarbon concentration ($mg \cdot L^{-1}$). GC-MS condition was described as above. At different time intervals, three different samples were conducted simultaneously for standard deviation analysis.

2.4 Surface tension measurement

According to the ring method, surface tension against *n*-octadecane was determined on the cell-free broth obtained by centrifuging cultures at 4000 $r \cdot min^{-1}$ for 10 min using the surface tension meter (JYW-200, Chengde, Hebei Province, China) at room temperature. Stabilization was allowed to occur until the standard deviation of 10 successive measurements was less than 0.4 $mN \cdot m^{-1}$. For a given sample, three independent determinations were made, with the standard deviation being within 4%.

2.5 Phase-contrast micrograph

At different time intervals, after the flask was standing for 15 min in order to eliminate the disturbance of oil phase droplets, part of medium in the water phase was used for phase-contrast micrograph analysis. Meanwhile, oil droplets in the oil membrane (crude oil and octadecane that suspend on the water phase) were also observed as controls. For analysis of oil phase droplets, surface liquid was transferred by pipettor, and oil droplet was analyzed by phase contrast micrograph (Axio Imager.M1, Zeiss, Germany) with a camera (AxioCamMRc5; Zeiss, Jena, Germany; Objective Magnification: 40 and 10).

2.6 Scanning electron microscopy observation

Scanning Electron Microscopy (SEM) was performed on *Pseudomonas* sp. DG17 grown in minimal medium with *n*-octadecane as carbon source. After 120 h of growth, cells were spinned down and washed twice with PBS (phosphate buffer saline, pH 7.2), fixed in 2.5% glutaraldehyde made in PBS for 2 h at 4°C. After three successive gentle washings in PBS, cells were washed with isoamyl acetate buffered salt solution for three times, dehydrated through a series of alcohol dehydration steps (30%, 50%, 70%, and 90% [v/v]) and washed by absolute ethanol for 15 min twice. The samples were critical point drying (HCP-2, Hitachi, Tokyo, Japan) and sputter-coating with ion coater instrument (IB-3, Eiko, Japan) in argon atmosphere to an approximate thickness of 10–20 nm, and examined by scanning electron micrograph (S-4800, Hitachi, Tokyo, Japan) using an acceleration voltage of 8000 and 5000 Volt.

2.7 ^{14}C *n*-octadecane transport assay

Uptake of *n*-octadecane by *Pseudomonas* sp. DG17 was assayed by the methods of Miyata [23]. Cells were harvested at exponential phase, washed with phosphate buffer (0.1 $mmol \cdot L^{-1}$, pH 7.0), and re-suspended in the same MSM essentially. ^{14}C *n*-octadecane (ARC 1261, 99%

pure, American Radiolabeled Chemicals Inc., St. Louis, Missouri, USA; 55 mCi·mmol^{-1}, 100 μCi·mL^{-1}) was added as methanol stock. For uptake assay, 30 μL of methanol solution of ^{14}C *n*-octadecane (25μCi·mL^{-1}) was added to 100 mL of flasks containing 60 mL of cell suspended MSM with a final substrate concentration at 0.227 μmol·L^{-1}. For energy dependent experiment, the medium was additionally supplemented with 30 mmol·L^{-1} of sodium azide (NaN_3) as inhibitor. Control group flask that was not added with cells of DG17 was also conducted to determine the abiotic loss of *n*-octadecane. Flasks were incubated at room temperature (30°C –35°C) with shaking at 140 r·min^{-1}. During the incubation time, no significant loss of radioactivity due to volatilization of *n*-octadecane was observed. Samples were taken at regular intervals over 96 h. For each time, 1 mL aliquots were filtered under vacuum by using Whatman GF/C glass fiber filters, and filtered cells were washed with 1 mL of PBS for three times. The fiber filter was added to scintillation fluid (Nonylfenolethoxylate, 9016-45-9, perkinelmer Waltham, Massachusetts, USA) and the radioactivity was measured by PerkinElmer liquid scintillation counter (Wallac Oy 1450 MicroBeta). This part of ^{14}C *n*-octadecane was taken as cellular *n*-octadecane. Meanwhile, another 1 mL aliquot was directly removed for radioactivity analysis as total ^{14}C *n*-octadecane in the culture broth. The value of extracellular ^{14}C level (nmol·mL^{-1}) was calculated as follows: Total ^{14}C *n*-octadecane in the broth (nmol·mL^{-1}) - cellular ^{14}C *n*-octadecane (nmol·mL^{-1}). Cellular ^{14}C *n*-octadecane in 1 μg of cells of DG17 was also demonstrated as nmol ·μg^{-1}. For soluble ^{14}C *n*-octadecane biodegradability analysis, the residue ^{14}C *n*-octadecane in 1 mL aliquot of culture broth was analyzed at different time intervals. For each time, three independent determinations were made for standard deviation analysis.

2.8 Energy dependent trans-membrane transport assay

To examine whether trans-membrane transport of *n*-octadecane by *Pseudomonas* sp. DG17 was energy dependent (active transport) or passive diffusion process, transport assays were performed in the presence of sodium azide (30 mmol·L^{-1}), an inhibitor of the electron-flow chain in oxidative phosphorylation, and carbonyl cyanide m-chlorophenylhydrazone (CCCP) (0.1 mmol·L^{-1}), uncoupler of oxidative phosphorylation. For trans-membrane transport assay, 10 μL of methanol solution of ^{14}C *n*-octadecane (25μCi·mL^{-1}) was added to 100 mL of flasks containing 20 mL of cell suspended MSM with a final concentration at around 0.23 μmol·L^{-1}. Final cell density (A_{600}) was controlled at 0.35. Transport assay began when inhibitors were added in the medium at 0 min. Samples were taken at 1, 3, 5, 8, 11, 14, 17, 20, 25, 30, 40, 50, 60 min. For each time, 0.5-mL aliquots were filtered under vacuum using Whatman GF/C glass fiber filters, and filtered cells were washed with 1 mL of PBS for six times. The fiber filter was added to scintillation fluid, and this part of ^{14}C *n*-octadecane was cellular *n*-octadecane. During this time, biodegradation of ^{14}C *n*-octadecane in the culture broth did not happen.

3 Results

3.1 Pseudosolubilized crude oil components in the water phase

GC analyses of extracted crude oil in the water phase revealed the high emulsify ability of *Pseudomona*s sp. DG17, as shown in Table 1 (35 d). The data showed that long chain alkanes were the major components of emulsified oil droplets in the water phase, including C_{12} to C_{28} of *n*-alkanes. Other oil components such as decane, heptadecane, eicosane and octacosane were also detected. Furthermore, some branched paraffin, including hexadecane, 2,6,10,14-tetramethyl, heptadecane, 4-methyl and undecane, 4,8-dimethyl, were also found in the water phase. These compounds might be biodegradation or mineralization products or the initial crude oil components. At the same time, some alkane biodegradation products were also examined, such as 1-decanol, 2-hexyl, 1-dodecanol, 3,7,11-trimethyl and 1-heptacosanol. The results showed that *Pseudomonas* sp. DG17 could uptake alkane and branched paraffin in the water phase preferentially. Furthermore, 1,2-benzenedicarboxylic acid, disooctyl ester, which might be biodegradation product of high ring aromatic hydrocarbon, was also detected in the crude oil medium.

As shown in Table 2, concentration changes of *n*-alkanes at 20 d and 35 d were analyzed by comparing the peak area of each compound. Under the emulsification effect of DG17, concentrations of some long and middle long carbon chain length alkanes, like hexacosane ($C_{26}H_{54}$), octacosane ($C_{28}H_{58}$), triacontane ($C_{30}H_{62}$) heptadecane ($C_{17}H_{36}$), and octadecane ($C_{18}H_{38}$) increased by 100.20%, 172.94%, 136.18%, 574.57%, and 183.23%, respectively, which showed that *Pseudomonas* sp. DG17 could emulsify high molecular weight hydrophobic compounds well. In this case, it was inferred that increased peak areas of middle long chain length alkanes might come from the emulsified crude oil or the mineralized long chain alkanes as metabolism products.

3.2 Components change of oil film

To completely compare the components change of oil film after incubation for 35 d, a spot of oil film that still suspended on the water-oil interface and fresh crude oil film were both analyzed by GC-MS to compare the peak area of each compound. As shown in Table 3, major components in the crude oil film were *n*-alkanes, the chain length of which were from dodecane ($C_{12}H_{26}$) to

Table 1 Pseudosolubilizd crude oil components in the water phase by *Pseudomonas* sp. DG17

No.	retention time /min	name	molecular formula	MW	struture
1	9.606	dodecane	$C_{12}H_{26}$	170	
2	12.415	tetradecane	$C_{14}H_{30}$	198	
3	13.690	pentadecane	$C_{15}H_{32}$	212	
4	14.465	naphthalene, 2,3,6-trimethyl	$C_{13}H_{14}$	170	
6	14.903	hexadecane	$C_{16}H_{34}$	266	
7	15.724	heptadecane, 4-methyl	$C_{18}H_{38}$	254	
8	16.053	heptadecane	$C_{17}H_{36}$	240	
10	17.139	octadecane	$C_{18}H_{38}$	254	
11	17.880	nonadecane, 2-methyl	$C_{20}H_{42}$	282	
12	17.927	erucic acid	$C_{22}H_{42}O_2$	338	
13	18.182	nonadecane	$C_{19}H_{40}$	268	
14	18.802	1-hexadecanol, 2-methyl	$C_{17}H_{36}O$	256	
15	19.173	eicosane	$C_{20}H_{42}$	296	
16	20.115	heneicosane	$C_{21}H_{44}$	296	
17	21.016	docosane	$C_{22}H_{46}$	282	
18	21.888	tricosane	$C_{23}H_{48}$	296	
19	22.719	tetracosane	$C_{24}H_{50}$	338	
20	23.516	heneicosane	$C_{25}H_{52}$	350	
21	23.836	1,2-benzenedicarboxylic acid, diisooctyl ester	$C_{24}H_{38}O_4$	390	
22	24.285	hexacosane	$C_{26}H_{54}$	366	
23	25.026	heptacosane	$C_{27}H_{56}$	380	
24	25.827	octacosane	$C_{28}H_{58}$	394	

octacosane ($C_{28}H_{58}$). Under the emulsification effect of *Pseudomonas* sp. DG17, area percentages of alkanes, including tridecane ($C_{13}H_{28}$), tetradecane ($C_{14}H_{30}$), pentadecane ($C_{15}H_{32}$), hexadecane ($C_{16}H_{34}$), heptadecane ($C_{17}H_{36}$), octadecane ($C_{18}H_{38}$), and nonadecane ($C_{19}H_{40}$) decreased during the incubation time. For instance, peak area of heptadecane ($C_{17}H_{36}$) declined from 5.045×10^9 to 2.937×10^9, which also demonstrated that some long chain alkanes might be biodegraded and transferred into middle chain length alkanes or pseudosolubilized into water phase before biodegradation process happened. Meanwhile, peak areas of some long carbon chain alkanes, like hexacosane ($C_{26}H_{54}$), heptacosane ($C_{27}H_{56}$), octacosane ($C_{28}H_{58}$), and triacontane ($C_{30}H_{62}$), decreased more or less, which suggested again that *Pseudomonas* sp. DG17 could emulsify high molecular weight hydrophobic compounds well. For triacontane ($C_{30}H_{62}$), on the other hand, the peak area did not have obvious change. Normally, high molecular weight hydrophobic compounds have prolonged persistence due to their low water solubility, which increases their sorption to surfaces and limits their availability to biodegrading microorganisms [28].

3.3 Pseudosolubilized *n*-octadecane in the water phase

As shown in Fig. 1, cells that grew on octadecane entered into exponential growth phase at around 12 h. Surface tension did not decline which showed that no biosurfactant was produced. Pseudosolubilization capability of *Pseudomonas* sp. DG17 that grew on *n*-octadecane was shown in Fig. 2. After incubation for 12 h, residue octadecane in the

Table 2 Pseudosolubilized *n*-alkanes components in the water phase under the effect of *Pseudomonas* sp. DG17

retention time /min	molecular formula	20d		35d	
		area	total/%	area	total/%
9.603	$C_{12}H_{26}$	1.059E + 7	2.468	1.118 E + 7	1.286
12.415	$C_{14}H_{30}$	1.241 E + 7	3.024	1.243 E + 7	1.467
13.821	$C_{15}H_{32}$	1.074E + 7	2.504	1.526E + 7	1.903
14.899	$C_{16}H_{34}$	4.292E + 6	0.806	1.966E + 7	2.452
16.062	$C_{17}H_{36}$	9.865E + 6	2.226	6.658E + 7	8.304
17.193	$C_{18}H_{38}$	2.672E + 6	0.721	4.732E + 7	5.902
18.171	$C_{19}H_{40}$	1.240E + 6	0.291	3.023E + 7	3.771
19.157	$C_{20}H_{42}$	2.924E + 6	0.922	3.675E + 7	4.584
20.103	$C_{21}H_{44}$	4.490E + 6	1.052	3.868E + 7	4.824
21.873	$C_{23}H_{48}$	6.746E + 6	1.581	3.817E + 7	4.761
22.704	$C_{24}H_{50}$	8.715E + 6	2.043	3.507E + 7	4.374
23.506	$C_{25}H_{52}$	1.077E + 7	2.525	2.975E + 7	3.711
24.273	$C_{26}H_{54}$	1.002E + 7	2.346	2.226E + 7	2.776
25.015	$C_{27}H_{56}$	1.207E + 7	2.829	2.218E + 7	2.766
25.816	$C_{28}H_{58}$	7.968E + 6	2.106	2.175E + 7	2.713
26.740	$C_{29}H_{60}$	7.804E + 6	1.947	1.704E + 7	2.125
28.023	$C_{30}H_{62}$	7.527 E + 6	1.746	1.778E + 7	2.217

Table 3 *n*-alkanes components changes in oil film by *Pseudomonas* sp. DG17

retention time /min	molecular formula	residue crude oil components		fresh crude oil components	
		area	total/%	area	total/%
9.606	$C_{12}H_{26}$	9.221E + 08	2.481	1.501E + 09	1.973
11.233	$C_{13}H_{28}$	1.858E + 09	4.324	3.403E + 09	4.463
12.408	$C_{14}H_{30}$	2.114E + 09	4.923	3.841E + 09	5.043
13.690	$C_{15}H_{32}$	1.982E + 09	4.616	3.657E + 09	4.798
14.899	$C_{16}H_{34}$	2.219E + 09	5.176	3.327E + 09	4.374
16.053	$C_{17}H_{36}$	2.937E + 09	6.846	5.045E + 09	6.614
17.136	$C_{18}H_{38}$	2.219E + 09	5.121	2.960E + 09	3.887
18.177	$C_{19}H_{40}$	3.39E + 09	7.903	3.318E + 09	4.351
19.164	$C_{20}H_{42}$	1.204E + 09	2.795	2.529E + 09	3.315
20.110	$C_{21}H_{44}$	1.363E + 09	3.159	2.552E + 09	3.348
21.876	$C_{23}H_{48}$	1.201E + 09	2.801	2.584E + 09	3.392
22.709	$C_{24}H_{50}$	1.168E + 09	2.731	2.357E + 09	3.094
23.507	$C_{25}H_{52}$	8.827E + 08	2.058	2.109E + 09	2.768
24.235	$C_{26}H_{54}$	8.254E + 08	1.683	1.701E + 09	2.233
25.017	$C_{27}H_{56}$	8.084E + 08	1.391	1.284E + 09	1.676
25.827	$C_{28}H_{58}$	7.763 E + 08	1.081	8.421 E + 08	1.134
26.740	$C_{29}H_{60}$	7.534E + 08	0.821	7.307E + 08	0.959
28.023	$C_{30}H_{62}$	7.521E + 08	0.814	6.958E + 08	0.914

medium was 18.78 mg with no pseudosolubilized (PS) octadecane detected. During this time, cell density at A_{600} increased from 0.126 to 0.181. It was found that cell growth entered into exponential phase at 12 h, after which

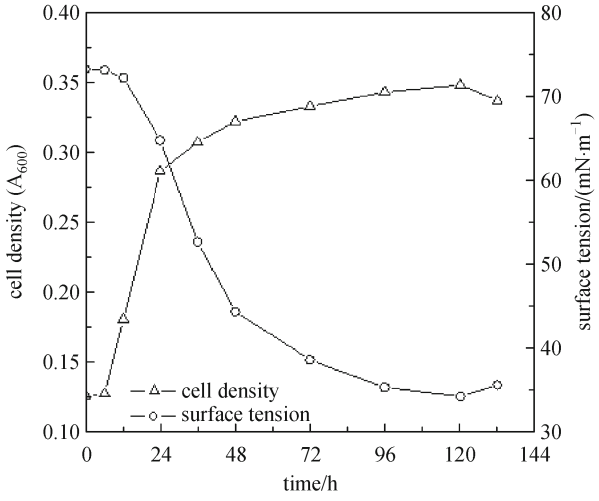

Fig. 1 Cell growth of *Pseudomonas* sp. DG17 on *n*-octadecane (open triangles) and surface tension changes of culture medium (open circles)

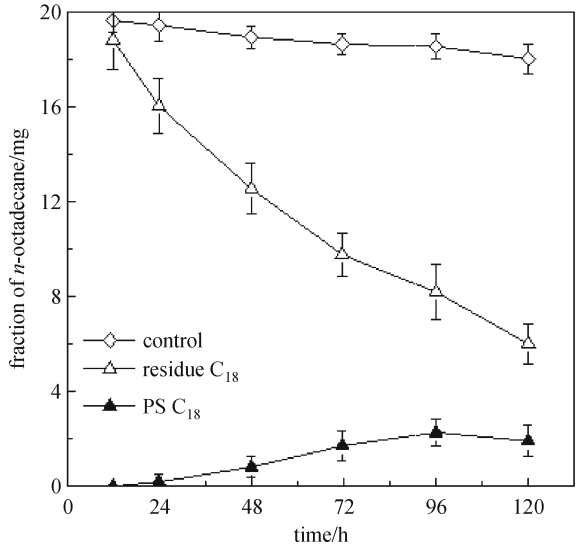

Fig. 2 Content distribution of *n*-octadecane in the medium. Total *n*-octadecane in the broth (open triangles); pseudosolubilization of *n*-octadecane (filled triangles); Control group(open diamonds)

surface tension begun to decline. For example, cell density (A_{600}) increased to 0.322 after incubation for 48 h with a surface tension value of 44.32 mN·m^{-1}. Meanwhile, mineralizaition of octadecane achieved to 34.51%, and pseudosolubilized octadecane increased to 0.82 mg in the water phase with a concentration at 16.30 mg·L^{-1}. The results indicated that pseudosolubilization of octadecane might be dependent on production of biosurfactant. Surface tension decline might facilitate pseudosolubilization process of octadecane. It was also inferred that mineralization rate of octadecane was slower than its pseudosolubilization rate during the exponential growth phase. If mineralization rate was equal to or faster than pseudosolubilization rate, aqueous-phase concentration of octadecane might be at stable level or no pseudosolubilized octadecane could be found. After incubation for 48 h, cell growth entered into stationary phase, and the highest pseudosolubilized mass of octadecane achieved to 2.27 mg at 96 h with a concentration of 45.37 mg·L^{-1}. During later stationary phase, *Pseudomona*s sp. DG17 could still utilize substrate, and the mineralizaition rate might be higher than the pseudosolubilization rate. Meanwhile, decrease of pseudosolubilization rate might result from substrate consumption throughout cell growth phase. After incubation for 120 h, residue substrate was around 6.02 mg with only 1.91 mg in the water phase. Part of octadecane still suspended on the water-oil interface. Pseudosolubilization rate of this part of octadecane might be limited for limited substrate could be transferred into water phase.

3.4 Culture medium changes of both the water and oil phases

After incubation for 35 d, oil droplets with different sizes suspended in the water phase as shown in Fig. 3(a). Observation results directly showed that some crude oil components entered into water phase because of the pseudosolubilization effect of *Pseudomonas* sp. DG17. Moreover, this phenomenon indicated that microorganism that used here could uptake pseudosolubilized crude oil droplets. Meanwhile, octadecane droplets in the culture medium were also observed by phase-contrast micrograph. After incubation for 48h, it was found that octadecane droplets were floating in the water phase (Fig. 3(b)). Droplets with size from about 2 to 13 μm were the major components. In this process, uptake of pseudosolubilized small oil droplets existed in the octadecane uptake system. Role of biosurfactant was to increase the direct contact chance between bacteria and oil droplets.

3.5 Morphological changes of bacteria cell

As shown in Fig. 4, morphological changes of cells grown on crude oil and *n*-octadecane (stationary phase) were examined by SEM. Ultrastructural studies revealed that most cells grown on crude oil were connected to each other by means of numerous fiber like projections and clustered in the network formed by the extracellular secretion (Fig. 4 (a)). The reason might be related to biosurfactant that produced by *Pseudomonas* sp. DG17. Meanwhile, both the surface structures of majority cells were smooth and distinct. For the cells grown on octadecane, however, a disruption of the surface membrane in certain zones was observed in some of the cells (Fig. 4(b)). The reason might be that some of the cells in the stationary phase were old or died, and the same membrane change was also observed for cells grown on crude oil. Meanwhile, rhamnolipid that produced by *Pseudomonas* sp. DG17 might have damage

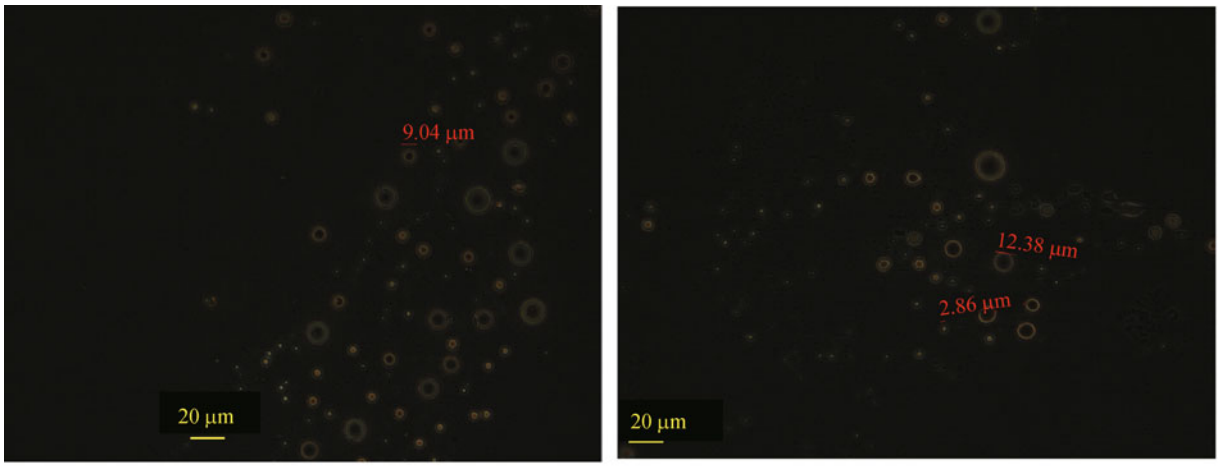

Fig. 3 Phase-contrast micrograph of oil droplets size in the culture medium (a: crude oil droplets in the water phase after incubation for 35d-40 magnification; b: *n*-octadecane droplets in the water phase at 48h-40 magnification). Bar represents 0.02 mm

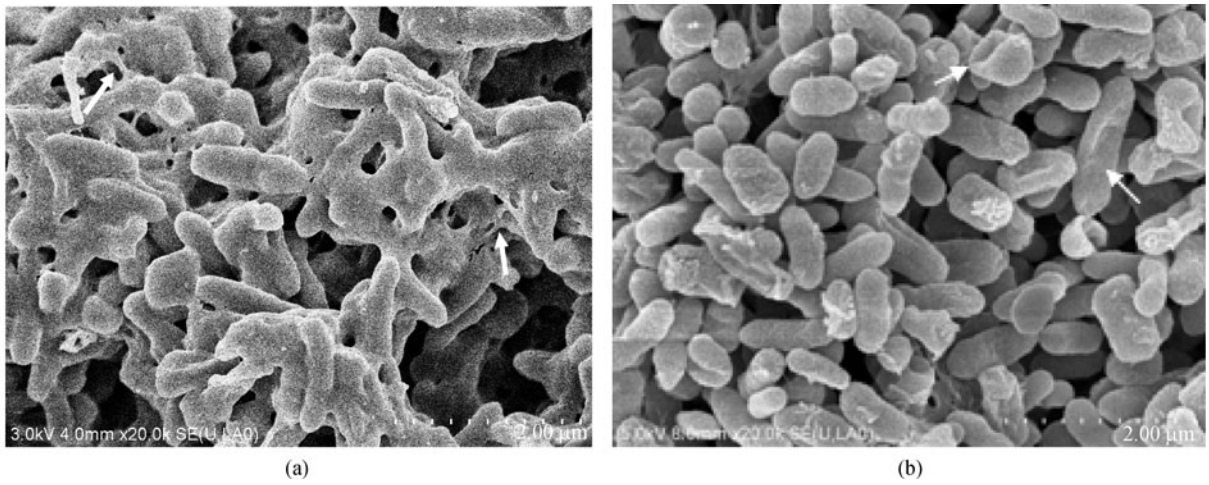

Fig. 4 Morphological changes of *Pseudomonas* sp. DG17 (a) Cells grown on crude oil at 35d-30000 magnification; (b) Cells grown on 400 mg·L^{-1} of octadecane at 120h-20000 magnification). Bar represents 2 μm

effect on the cell membrane due to its amphiphilic property which needs further studies.

3.6 14C *n*-octadecane degradation

^{14}C *n*-octadecane degradation was shown in Fig. 5. Distribution of radiolabeled ^{14}C *n*-octadecane in the broth (Fig. 5(a)) and cell pellet (Fig. 5(b)) were both analyzed. In the control group without cells of DG17, total ^{14}C *n*-octadecane in the broth was held at 0.26 nmol·mL^{-1} with no obvious change. Meanwhile, after incubation for 6 h, total ^{14}C *n*-octadecane without NaN_3 decreased from 0.253±0.00610 to 0.232±0.00327 nmol·mL^{-1}, with a biodegradation rate of 8.30% by *Pseudomonas* sp. DG17. Another group of *n*-octadecane biodegradation assay was conducted on by adding 30 mmol·L^{-1} of NaN_3 after incubation for 12 h. Before NaN_3 was added into the medium, residue *n*-octadecane in the broth declined to 0.186 nmol·mL^{-1} with a biodegradation rate of 25.12%. However, this value was kept at steady-state after the addition NaN_3, which inferred that NaN_3 strongly inhibited the biodegradation process of *n*-octadecane. Moreover, for cells not treated with NaN_3, it was found that the initial cellular ^{14}C *n*-octadecane of *Pseudomonas* sp. DG17 was 0.0341 nmol·mL^{-1} for the first sample at time zero, which accounted for 13.47% of total ^{14}C in the culture. During the first 3h, cellular ^{14}C increased to 0.0562 nmol·mL^{-1} for substrate mineralization did not happen. Then, ^{14}C *n*-octadecane that both in the broth and cellular decreased as time went on. If octadecane was just mineralized into alcohol or acid, total ^{14}C would be at stationary state level. However, our result did not support this assumption, which

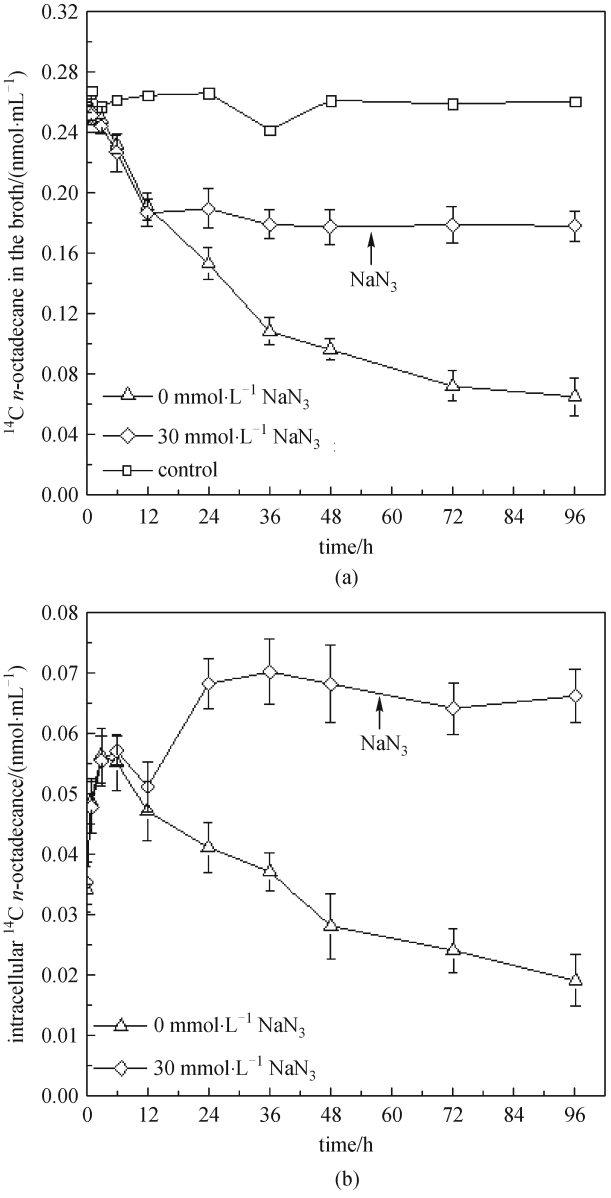

Fig. 5 ^{14}C *n*-octadecane biodegradation by *Pseudomonas* sp. DG17 in the aqueous phase (a) and cellular ^{14}C (b) of *Pseudomonas* sp. DG17. ^{14}C in control group (open squares); Cells in the absence of NaN_3 (open triangles); Cells in the presence of NaN_3 (open diamonds)

showed that cellular substrate was biodegraded and transformed into $^{14}CO_2$. Meanwhile, biodegradation rate of ^{14}C *n*-octadecane was faster than its influx rate which could explain the loss of cellular ^{14}C. If biodegradation rate was equal to or slower than the influx rate of octadecane, cellular ^{14}C might be at stable level or have increased. For example, cell growth entered into exponential phase at 6h with cellular ^{14}C *n*-octadecane at 0.0552 nmol·mL^{-1}. As time went on, this value declined to 0.028 nmol·mL^{-1} at 48 h, and total ^{14}C in the broth also decreased to 0.096 nmol·mL^{-1}. This result indicated that biodegradation rate of octadecane might greater than the influx process of octadecane after incubation for 6 h. As time went on, residue *n*-octadecane in the medium declined to 0.063±0.0121 nmol·mL^{-1} at 96 h, and biodegradability of *n*-octadecane increased to 74.33%. Meanwhile, in the addition of NaN_3 (Fig. 5(b)), cellular ^{14}C *n*-octadecane also declined from 0.0548 nmol·mL^{-1} at 12 h to 0.0181 nmol·mL^{-1}. ATP inhibitor might completely block *n*-octadecane degradation and uptake by *Pseudomonas* sp. DG17. There was another uptake system exhibited during the incubation.

3.7 Effect of inhibitors on the trans-membrane transport of 14C *n*-octadecane

Effect of inhibitors on the transport of ^{14}C *n*-octadecane was shown in Fig. 6. Total ^{14}C *n*-octadecane in the broth was 0.234 nmol·mL^{-1}. It was found that trans-membrane transport of *n*-octadecane uptake was highly sensitive to the presence of either NaN_3 or CCCP. In the absence of inhibitors, initial cellular ^{14}C *n*-octadecane of DG17 was 0.0374±0.00421 nmol·mL^{-1} at 1 min, and increased to maximum value of 0.149±0.00845 nmol·mL^{-1} at 17 min. However, this value declined to 0.111± 0.00428 nmol·mL^{-1} at 60 min. Meanwhile, cellular ^{14}C changed from 0.126±0.00626 nmol·mL^{-1} at 11 min to 0.124±0.00813 nmol·mL^{-1} at 40 min, which accounted for more than 50% of total ^{14}C *n*-octadecane in the broth and higher than extracellular ^{14}C *n*-octadecane. This result indicated that trans-membrane transport of *n*-octadecane during this time was against concentration gradient.

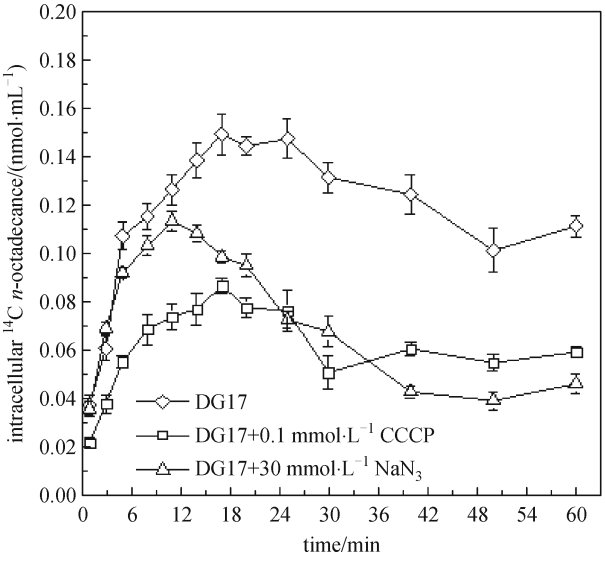

Fig. 6 Effect of different inhibitors on the transport of ^{14}C *n*-octadecane by *Pseudomonas* sp. DG17. Cells in the absence of inhibitor (open diamonds), cells treated with 0.1 mmol·L^{-1}CCCP (open squares), and cells treated with 30 mmol·L^{-1} NaN_3 (open triangles)

During the incubation time, biodegradation of ^{14}C *n*-octadecane did not happen, but cellular *n*-octadecane decreasd after incubation for 17 min. The reason might be that part of cellular ^{14}C *n*-octadecane was transported out of cell membrane for cells of DG17 could mediate cellular concentration of *n*-octadecane. When cells of DG17 were conducted in the presence of azide (30 $mmol \cdot L^{-1}$) and CCCP (0.1 $mmol \cdot L^{-1}$), however, cellular *n*-octadecane were both inhibited obviously. For cells treated with azide, cellular ^{14}C achieved at 0.0736 ± 0.00513 $\mu mol \cdot L^{-1}$ for cells treated with CCCP. Similarly, cellular *n*-octadecane decreased after incubation for about 30min. In the presence of azide and CCCP, after incubation for 60 min, cellular *n*-octadecane declined to 0.0545 ± 0.00402 $\mu mol \cdot L^{-1}$ and 0.0591 ± 0.00217 $\mu mol \cdot L^{-1}$, respectively. During the incubation time, part of cells in the medium might die for the addition of energy inhibitors. The finding showed that trans-membrane transport of *n*-octadecane might be an energy-dependent active transport process.

4 Discussion

Microorganisms are capable of synthesizing biosurfactants from crude oil, pure hydrocarbons and a variety of non-hydrocarbon substrates such as simple carbohydrates, acids and alcohols. In our experiment, visual observation results revealed that emulsions droplets were formed in the water phase in the addition of *Pseudomonas* sp. DG17. Emulsification is a cell-density-dependent phenomenon: that is, the greater the number of cells, the higher the concentration of extracellular product [28]. Biosurfactants are amphipathic molecules consisting of both a hydrophobic and a hydrophilic domain. This structure allows biosurfactants to decrease interfacial tensions between water and non-aqueous phase liquids, thus allowing the formation of pseudosolubilized droplets surrounded by biosurfactants in aqueous phase, which can be utilized by microorganisms [29]. In our experiments, medium carbon chain length *n*-alkanes from C_{14} to C_{19} were the major and prefered carbon source for *Pseudomonas* sp. DG17. As for long chain alkanes, bacteria must made some changes to utilize these hydrophobic compounds. Report from Maria et al. [30] indicated that applying biosurfactant secreted by *Bacillus subtilis* O9 accelerated the biodegradation of aliphatic hydrocarbons, while, aromatic hydrocarbon degradation was not stimulated. Because of the amphiphilic property of these hydrophobic compounds, biosurfactants, secreted into the culture medium during the growth of the microorganisms, can emulsify hydrocarbons, thus enhancing their water solubility [16,31,32], decreasing surface tension, increasing the displacement of oily substances from soil particles [33], assisting the transport and translocation of the insoluble substrates across cell membranes [18]. Furthermore, different microorganism could use different type of alkanes. Some bacteria prefer utilize short chain alkanes and leave long chain alkanes in the environment [34]. For example, *Pseudomonas. maltophilia* N246-1 was able to utilize C_8 to C_{14} of *n*-alkanes, *R. sphaeroides* M1 only utilized C_{12} to C_{16} alkanes, and transconjugant strains, *R. sphaeroides* strains with the OCT plasmid were able to utilize C_8 to C_{16} of *n*-alkanes [35]. Similarly, Kim et al. [2] found that *R. erythropolis* S + 14He was able to selectively discriminate and preferentially transport *n*-hexadecane from mixtures of structurally similar alkanes into intracellular inclusions by an energy-driven transport system. Thus, the high bioavalibility of *n*-alkanes by *Pseudomonas* sp. DG17 might be correlated with the plasmid gene sequence [2,35]. Normally, high molecular weight hydrophobic compounds have prolonged persistence due to their low water solubility, which increases their sorption to soil particles and limits their availability to biodegrading microorganisms. In our experiment, however, long chain alkanes could be emulsified and transferred from oil phase to water phase and biodegraded by *Pseudomonas* sp. DG17 which might be related to the cell surface hydrophobicity changes [28].

Biosurfactant was produced when cells growth entered into exponential phase. Meanwhile, pseudosolubilized *n*-octadecane droplets were also detected during the incubation time. In our experiment, the highest amount of *n*-octadecane in water phase was 45.37 $mg \cdot L^{-1}$ which was related with biosurfactant produced by *Pseudomonas* sp. DG17. In other reports, biosurfactants produced by *Pantoea* sp. A-13 when grown on kerosene or *n*-paraffins increased cell hydrophobicity and enhanced both the surface tension–lowering capacity and the emulsifying potential [36]. Cell surface of *Pseudomonas aeruginosa* AP02-1 was highly hydrophobic, and the BATH (bacterial adherence to hydrocarbon) test showed that 71% of cells adhered to hydrocarbons when grown on diesel oil [37]. Meanwhile, some microorganisms can get in contact with the petroleum components without the help of emulsifier or biosurfactant. For example, extracellular biosurfactants produced by strain *Rhodococcus equi* Ou2 had only a minor role in hexadecane degradation, and direct interfacial accession appeared to be the main mechanism for hexadecane uptake. On the contrary, biosurfactants produced by *P. aeruginosa* GL1 were required for growth on hexadecane, and their pseudosolubilization capacity rather than their emulsification capacity was involved in substrate degradation [8]. Therefore, role of biosurfactants was different in various strains, and was directly related to the hydrophobicity of the bacterial cells concerned. The detachment of bacteria from the depleted oil drop enables them to move to other droplets where they metabolize the specific group of utilizable hydrocarbons. Therefore, detachment of bacteria from oil drops results in a more efficient bioremediation [38]. In this work, direct contact with big oil droplets and interaction of cells with

"solubilized/pseudosolubilized" oil droplets happened in the process of oil uptake by *Pseudomonas* sp. DG17. Meanwhile, invaginations of some cells of *Pseudomonas* sp. DG17 occurred during the uptake and biodegradation process of *n*-octadecane. Microbial cells are adapted to respond rapidly to environmental stress stimuli by regulating the fluidity of their membrane, such as invaginations of cell membranes [39], their membrane lipid composition [40] and increased lipopolysaccharide [41] have been observed. Biosurfactant can provoke changes in membrane-phospholipid composition, and lead to increased membrane rigidity, thus, bacteria alter their membrane fluidity by way of phospholipid headgroups to overcome the damaging effects of crude oil [42]. Sotirova et al. [13] found that when cells grew in the presence of rhamnolipid-biosurfactantat concentrations below CMC, cell surface structure became smooth, thin and indistinct. Meanwhile, a disruption of the surface membrane in certain zones was observed when the concentration of rhamnolipid-biosurfactantat was above CMC, and the cytoplasmic content in contact with the disrupted cell membrane became translucent. Actually, role of biosurfactants in hydrophobic hydrocarbon biodegradation need further analysis which might be good for establishing proper modes of biosurfactant utilization in bioremediation.

For the uptake and biodegradation analysis of ^{14}C *n*-octadecane, partitioning of *n*-octadecane into bacterial cell components occurs very quickly. Loss of majority ^{14}C indicated that substrate was cellular mineralized and transformed into $^{14}CO_2$. Similarly, for cells of *Streptomyces* strains, the released radioactive CO_2 was measured after 6 h incubation. The rationale of this experiment was that the appearance of radioactively labeled CO_2 should come from the metabolised hexadecane [43]. The ratio of extracelluar and cellular ^{14}C *n*-octadecane analysis indicated that this immediate trans-membrane transport was caused by passive diffusion based on concentration gradient. It was assumed that if *n*-octadecane transport was just a linear passive diffusion process, cellular *n*-octadecane would increase linearly and not influenced by any inhibitor. However, in our experiments, cellular influx of ^{14}C *n*-octadecane was blocked when sodium azide and CCCP were added in the medium at 0 min, which revealed that there was another energy related influx process in trans-membrane transport of ^{14}C *n*-octadecane. Similarly, for cells of *Pseudomonas aeruginosa* UO299, addition of proton conductor carbonyl cyanide m-chlorophenylhydrazone resulted in a reduction of *n*-hexadecane uptake, which indicated that an energy-dependent uptake was driven by proton motive system [17]. Miyata et al. [23] presented a saturable, energy-dependent system for PAH transport in *Mycobacterium* sp. strain RJGII-135 as well as the initial passive diffusion process. Results from Whitman et al. [21] indicated that naphthalene uptake by *Pseudomonas. fluorescens* Uper-1 was also an energy-linked transport systems. Furthermore, for cells of *Arthrobacter* sp. Strain Sphe3 grown on phenanthrene, substrate was transported into membrane by inducible active mechanism that followed Michaeis-Menten kinetics; while, phenanthrene was transported by diffusion when cells grew on glucose [18]. All of these studies indicated that this energy-dependent transport was mediated by cell membrane proteins. Further studies needs to find the correlated proteins and genes to understand the uptake mechanism better.

5 Conclusions

In this study, *Pseudomonas* sp. DG17 could utilize crude oil components through two modes: uptake of pseudosolubilized oil droplets and directly contact with big oil droplets. Phase-contrast microscope indicated that part of crude oil components, mainly C_{12} to C_{28} alkanes, was transferred into water phase. Thus, *Pseudomonas* sp. DG17 could uptake medium and long chain length *n*-alkanes, including tetradecane, hexadecane, octadecane, and tetracosane preferentially. Meanwhile, isotope ^{14}C assay indicated that these hydrophobic compounds were transported into cell membrane of *Pseudomonas* sp. DG17 through passive diffusion and energy-dependent active transport.

Acknowledgements This paper is sponsored by the Research Fund for National Natural Science Foundation of China (Grant No. 41072177).

References

1. Wentzel A, Ellingsen T E, Kotlar H K, Zotchev S B, Throne-Holst M. Bacterial metabolism of long-chain *n*-alkanes. Appl Microbiol Biotechnol, 2007, 76(6): 1209–1221
2. Kim I S, Foght J M, Gray M R. Selective transport and accumulation of alkanes by *Rhodococcus erythropolis* S + 14He. Biotechnol Bioeng, 2002, 80(6): 650–659
3. Woo S H, Park J M. Microbial degradation and enhanced bioremediation of polycyclic aromatic hydrocarbons. J Ind Eng Chem, 2004, 10(1): 16–23
4. Al-Tahhan R, Sandrin T R, Bodour A A, Maier R M. Rhamnolipid-induced removal of lipopolysaccharide from *Pseudomonas aeruginosa*: effect on cell surface properties and interaction with hydrophobic substrates. Appl Environ Microbiol, 2000, 66(8): 3262–3268
5. Herman D C, Zhang Y, Miller R M. Rhamnolipid (biosurfactant) effects on cell aggregation and biodegradation of residual hexadecane under saturated flow conditions. Appl Environ Microbiol, 1997, 63(9): 3622–3627
6. Zhang Y, Miller R M. Effect of a *Pseudomonas* rhamnolipid

biosurfactant on cell hydrophobicity and biodegradation of octadecane. Appl Environ Microbiol, 1994, 60(6): 2101–2106
7. Zhang Y, Miller R M. Effect of rhamnolipid (biosurfactant) structure on solubilization and biodegradation of *n*-alkanes. Appl Environ Microbiol, 1995, 61(6): 2247–2251
8. Vasileva-T E, Gesheva V. Glycolipids produced by *Antartic Nocardioides* sp. during growth on *n*-paraffin. Process Biochem, 2005, 40(7): 2387–2391
9. Bouchez-Naitali M, Vandecasteele J P. Biosurfactants, an help in the biodegradation of hexadecane? The case of *Rhodococcus* and *Pseudomonas* strains. World J Microbiol Biotechnol, 2008, 24(9): 1901–1907
10. Rosenberg E. Exploiting microbial growth on hydrocarbon: New markets. Trends Biotechnol, 1993, 11(10): 419–424
11. Bouchez-Naitali M, Rakatozafy H, Marchal R, Leveau J Y, Vandecasteele J P. Diversity of bacterial strains degrading hexadecane in relation to the mode of substrate uptake. J Appl Microbiol, 1999, 86(3): 421–428
12. Nakahara T, Erickson L E, Gutierrez J R. Characteristics of hydrocarbon uptake in cultures with two liquid phases. Biotechnol Bioeng, 1997, 19(1): 9–25
13. Wick L Y, de Munain A R, Springael D, Harms H. Responses of *Mycobacterium* sp. LB501T to the low bioavailability of solid anthracene. Appl Microbiol Biotechnol, 2002, 58(3): 378–385
14. Tecon R, van der Meer J R. Effect of two types of biosurfactants on phenanthrene availability to the bacterial bioreporter *Burkholderia sartisoli* strain RP037. Appl Microbiol Biotechnol, 2010, 85(4): 1131–1139
15. Sotirova A, Spasova D, Vasileva-Tonkova E, Galabova D. Effects of rhamnolipid-biosurfactant on cell surface of *Pseudomonas aeruginosa*. Microbiol Res, 2009, 164(3): 297–303
16. Prabhu Y, Phale P S. Biodegradation of phenanthrene by *Pseudomonas* sp. strain PP2: novel metabolic pathway, role of biosurfactant and cell surface hydrophobicity in hydrocarbon assimilation. Appl Microbiol Biotechnol, 2003, 61(4): 342–351
17. Cameotra S S, Singh P. Synthesis of rhamnolipid biosurfactant and mode of hexadecane uptake by *Pseudomonas* species. Microb Cell Fact, 2009, 8(16): 1–7
18. Beal R, Betts W B. Role of rhamnolipid biosurfactants in the uptake and mineralization of hexadecane in *Pseudomonas aeruginosa*. J Appl Microbiol, 2000, 89(1): 158–168
19. Kallimanis A, Frillingos S, Drainas C, Koukkou A I. Taxonomic identification, phenanthrene uptake activity, and membrane lipid alterations of the PAH degrading *Arthrobacter* sp. strain Sphe3. Appl Microbiol Biotechnol, 2007, 76(3): 709–717
20. Bugg T, Foght J M, Pickard M A, Gray M R. Uptake and active efflux of polycyclic aromatic hydrocarbons by *Pseudomonas fluorescens* LP6a. Appl Environ Microbiol, 2000, 66(12): 5387–5392
21. Whitman B E, Lueking D R, Mihelcic J R. Naphthalene uptake by a *Pseudomonas fluorescens* isolate. Can J Microbiol, 1998, 44(11): 1086–1093
22. Miyata N, Iwahori K, Foght J M, Gray M R. Saturable, energy-dependent uptake of phenanthrene in aqueous phase by *Mycobacterium* sp. strain RJGII-135. Appl Environ Microbiol, 2004, 70(1): 363–369
23. Gray R M, Bugg T. Selective biocatalysis in bacteria controlled by active membrane transport. Ind Eng Chem Res, 2001, 40(23): 5126–5131
24. Mihelcic J R, Lueking D R, Mitzell R J, Stapleton J M. Bioavailability of sorbed and separate phase chemicals. Biodegradation, 1993, 4(3): 141–153
25. Shishido M, Toda M. Apparent zero-order kinetics of phenol biodegradation by substrate-inhibited microbes at low substrate concentrations. Biotechnol Bioeng, 1996, 50(6): 709–717
26. Wen Y, Cheng H, Lu L J, Liu J, Feng Y, Guan W, Zhou Q, Huang X F. Analysis of biological demulsification process of water-in-oil emulsion by *Alcaligenes* sp. S-XJ-1. Bioresour Technol, 2010, 101 (21): 8315–8322
27. Hua F, Wang H. Uptake modes of octadecane by *Pseudomonas* sp. DG17 and synthesis of biosurfactant. J Appl Microbiol, 2012, 112 (1): 25–37
28. Ron E Z, Rosenberg E. Biosurfactants and oil bioremediation. Curr Opin Biotechnol, 2002, 13(3): 249–252
29. Goswami P, Singh H D. Different modes of hydrocarbon uptake by two *Pseudomonas* species. Biotechnol Bioeng, 1991, 37(1): 1–11
30. Cubitto M A, Morán A C, Commendatore M, Chiarello M N, Baldini M D, Siñeriz F. Effects of *Bacillus subtilis* O9 biosurfactant on the bioremediation of crude oil-polluted soils. Biodegradation, 2004, 15(5): 281–287
31. de Carvalho C C C R, Poretti A, da Fonseca M M R. Cell adaptation to solvent, substrate and product: a successful strategy to overcome product inhibition in a bioconversion system. Appl Microbiol Biotechnol, 2005, 69(3): 268–275
32. Abalos A, Vinas M, Sabate J, Manresa M A, Solanas A M. Enhanced biodegradation of Casablanca crude oil by a microbial consortium in presence of a rhamnolipid produced by *Pseudomonas aeruginosa* AT10. Biodegradation, 2004, 15(4): 249–260
33. Ivshina I B, Kuyukina M S, Philp J C, Christofi N. Oil desorption from mineral and organic materials using biosurfactant complexes produced by *Rhodococcus* species. World J Microbiol Biotechnol, 1998, 14(5): 711–717
34. Lindley N D, Heydeman M T. The uptake of *n*-alkanes from alkane mixtures during growth of the hydrocarbon-utilizing fungus *Cladosporium resinae*. Appl Microbiol Biotechnol, 1996, 23(5): 384–388
35. Lee M H, Hwang M O, Choi S Y, Min K H. *n*-Alkane dissimilation by *Rhodopseudomonas sphaeroides* transferred OCT plasmid. Microb Ecol, 1993, 26(3): 219–226
36. Vasileva-Tonkova E, Gesheva V. Biosurfactant production by antarctic facultative *anaerobe Pantoea* sp. during growth on hydrocarbons. Curr Microbiol, 2007, 54(2): 136–141
37. Perfumo A, Banat I M, Canganella F, Marchant R. Rhamnolipid production by a novel thermophilic hydrocarbon-degrading *Pseudomonas aeruginosa* AP02-1. Appl Microbiol Biotechnol, 2006, 72 (1): 132–138
38. Rosenberg E, Gottlieb A, Rosenberg M. Inhibition of bacterial adherence to hydrocarbons and epithelial cells by emulsan. Infect Immun, 1983, 39(3): 1024–1028

39. Scott C C L, Finnerty W R. Characterization of intracytoplasmic hydrocarbon inclusions from the hydrocarbon-oxidizing *Acinetobacter* species HO1-N. J Bacteriol, 1976, 127(1): 481–489
40. Heipieper H J, Meinhardt F, Segura A. The cis-trans isomerase of unsaturated fatty acids in *Pseudomonas* and *Vibrio*: biochemistry, molecular biology and physiological function of a unique stress adaptive mechanism. FEMS Microbiol Lett, 2003, 229(1): 1–7
41. Witholt B, de met M J, Kingma J, Vanbeilen J B, Kok M, Lageveen R G, Eggink G. Bioconversion of aliphatic hydrocarbons by *Pseudomonas oleovorans* in multiphase bioreactors: background and economic potential. Trends Biotechnol, 1990, 8: 46–52
42. Chauhan A, Fazlurrahman, Oakeshott J G, Jain R K. Bacterial metabolism of polycyclic aromatic hydrocarbons: strategies for bioremediation. Indian J Microbiol, 2008, 48(1): 95–113
43. Barabas G, Vargha G, Szabo I M, Penyige A, Damjanovich S, Szollosi J, Matko J, Hirano T, Matyus A, Szabó I. *n*-Alkane uptake and utilisation by *Streptomyces* strains. Antonie van Leeuwenhoek, 2001, 79(3–4): 269–276