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Effect of rhamnolipid solubilization on hexadecane bioavailability: enhancement or reduction?

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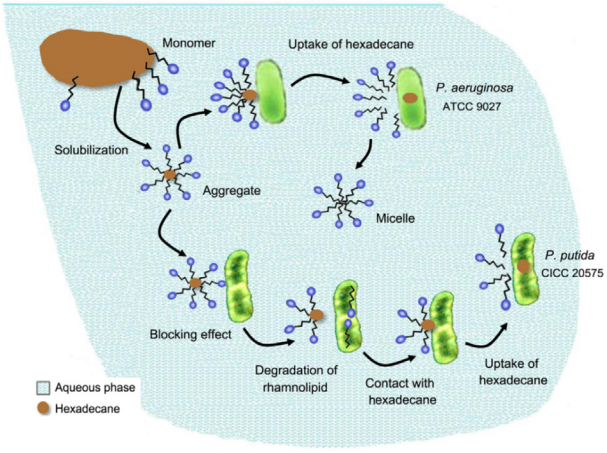
HIGHLIGHTS

- Rhamnolipid-solubilization increases availability of hexadecane for *P. aeruginosa.*
- Rhamnolipid-solubilization reduces bioavailability of hexadecane for *P. putida*.
- Blocking effect of surfactants accounts for reduction in hexadecane bioavailability.
- Bacterial compatibility is important to overcome the blocking effect.

GRAPHICAL ABSTRACT



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ABSTRACT

In this study, liquid culture systems containing rhamnolipid-solubilized, separate-phase, and multi-state hexadecane as the carbon source were employed for examining the effect of rhamnolipid solubilization on the bioavailability of hexadecane. Experimental results showed that the uptake of rhamnolipid-solubilized hexadecane by *Pseudomonas aeruginosa* ATCC 9027, a rhamnolipid producing strain, was enhanced compared to the uptake of mass hexadecane as a separate phase, indicating rhamnolipid solubilization increased the bioavailability of hexadecane for this bacterium. For *Pseudomonas putida* CICC 20575 which does not produce but degrade rhamnolipid, the uptake of either rhamnolipid-solubilized hexadecane or multi-state hexadecane was inhibited. The reduction of bioavailability was assumed to be the consequence of the blocking effect caused by the partition of rhamnolipid molecules at the hexadecane-water interface. The results show that how rhamnolipid solubilization changes the bioavailability of hexadecane depends on the bacterial compatibility to rhamnolipid. The study adds insight into the knowledge of biosurfactant-associated bioavailability of hydrophobic organic compounds (HOCs), and is of importance for application of biosurfactants in bioremediation of HOCs.

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1. Introduction

Water and soil contamination by hydrophobic organic compounds (HOCs) due to anthropogenic activities, e.g., oil exploitation and transportation, downstream petroleum product manufacturing and processing, and improper discharge of industrial wastes, has attracted increasing attention in recent decades. Bioremediation technology, which has high ecological significances, is widely used for cleaning of these contaminations. However, the hydrophobic nature (low water solubility) of HOCs is a major obstacle for their uptake by microbes since most of these compounds either resided in non-aqueous phase liquids (NAPLs) or adsorbed on soil, which greatly limit their bioavailability [1–4]. Unlike bioaccessibility, bioavailability does not only refer to whether the organism has access to the chemical in the environment. Bioavailability is defined as "the compound is freely available to cross the cellular membrane of an organism from the medium the organism inhabits at a given time" [5,6].

Surfactants may play a role in degradation of HOCs by enhancing the apparent solubility and hence greater partitioning of these compounds in aqueous phase [7–9]. Such surfactant-aided solubilization of HOCs is usually based on aggregate-formation mechanism [10,11]. Biosurfactants are promising alternatives to synthesized surfactants due to their advantages such as environmental compatibility and high efficiency [12–14]. For example, biosurfactants generally show stronger hydrocarbon solubilization capacity than synthetic surfactants [15,16], and significant solubilization of alkanes at concentrations below critical micelle concentration (CMC) was observed for rhamnolipid [17,18], which is the most extensively studied and commonly used biosurfactant [19,20].

Petroleum hydrocarbons are some of the most intensively studied HOCs. For hydrocarbon degradation, both positive and negative effects of biosurfactant-solubilization were observed in prior studies. Zhang and Miller [21] observed in a batch liquid degradation experiment that 20% of octadecane as the carbon source was mineralized in 84 h by *Pseudomonas aeruginosa* ATCC 9027 in the presence of rhamnolipid, compared to 5% of mineralization without rhamnolipid. The enhancement of degradation was attributed to rhamnolipid-facilitated octadecane dispersion by forming octadecane/rhamnolipid aggregates (octadecane solubilized in rhamnolipid micelles). These aggregates, as tiny hydrocarbon reservoirs, are likely to enhance mass transfer of hydrocarbon to microbial cells [22,23]. Guha and Jaffé [24] formulated a mathematical model to describe the interaction of the biomass-contaminant-water surfactant system, and the results showed that a fraction of the micellar-phase phenanthrene is directly bioavailable. Further researches showed that surfactant sorption and the formation of hemi-micelles on the bacterial cell surface have a strong influence on the surfactant-enhanced bioavailability of HOCs [10,25,26].

A major concern regarding this aggregate-based solubilization mechanism is that the biosurfactant layer on the surface of aggregates could be a barrier, which blocks microbial cells from capturing the inside hydrophobic compounds. In our prior study it was observed that a *Pseudomonas aeruginosa* strain failed to grow on the hexadecane in thoroughly solubilized form in the presence of rhamnolipid at a concentration higher than CMC [27]. Similar observation that microbial cells were not able to directly ingest compounds closed in surfactant micelles was also reported by Pęziak et al. [28]. Variation of the results of these studies indicates uncertainty for biosurfactant solubilization to enhance hydrocarbon bioavailability. An insight into such uncertainty is required for better application of biosurfactants in bioremediation.

This study is focused on examining whether biosurfactant solubilization of hydrocarbons enhances the bioavailability of hydrocarbons, and why if it does. Hexadecane in various forms was used as the carbon source for degradation by *Pseudomonas aeruginosa* ATCC 9027 and *Pseudomonas putida* CICC 20575. These forms included: a) rhamnolipid-solubilized hexadecane, b) mass hexadecane, and c) multi-state hexadecane (including rhamnolipid-solubilized, emulsified and mass hexadecane). Dur-

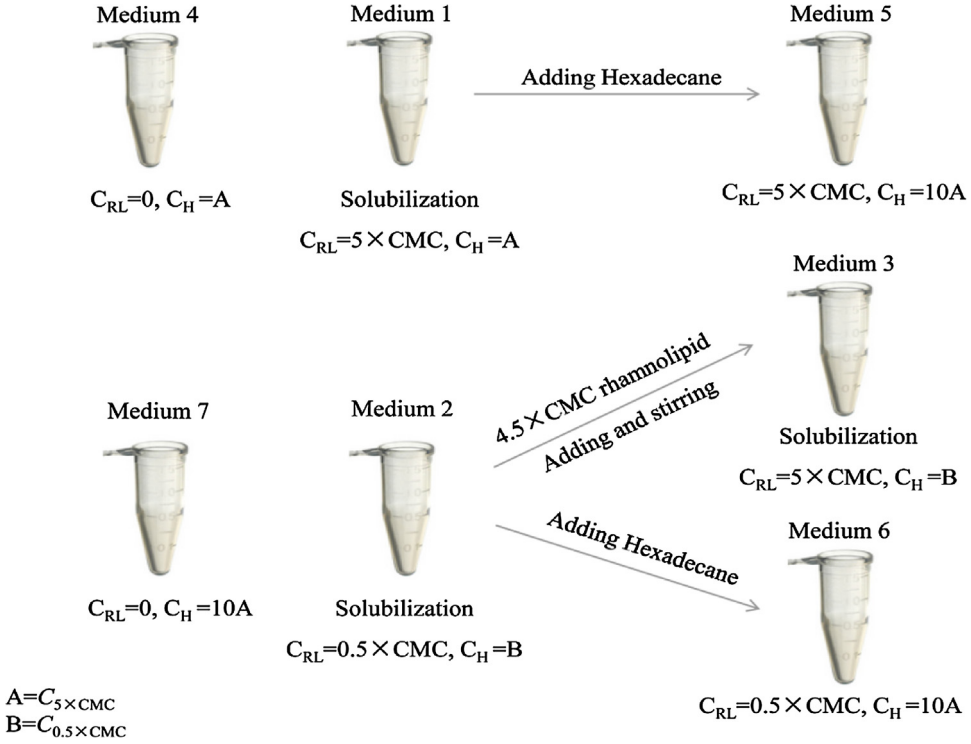

Fig 1. Preparation of seven sets of MSM-based culture medium.

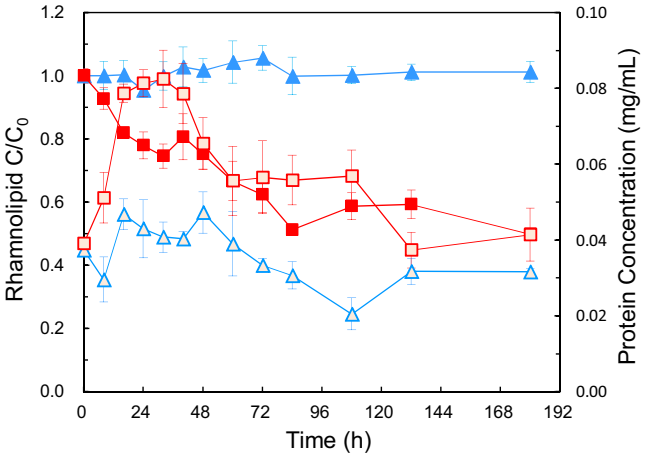

Fig. 2. Changes in concentration of rhamnolipid and protein during 180 h of biodegradation experiment with the rhamnolipid as sole carbon source. (▲, degradation of the rhamnolipid by *P. aeruginosa* ATCC 9027; △, protein concentration of *P. aeruginosa* ATCC 9027; ■, degradation of rhamnolipid by *P. putida* CICC 20575;□, protein concentration of *P. putida* CICC 20575). C_0 represents initial concentration of hexadecane (0 h). Symbol and error bar represent mean ± standard deviation of triplicate experiments.

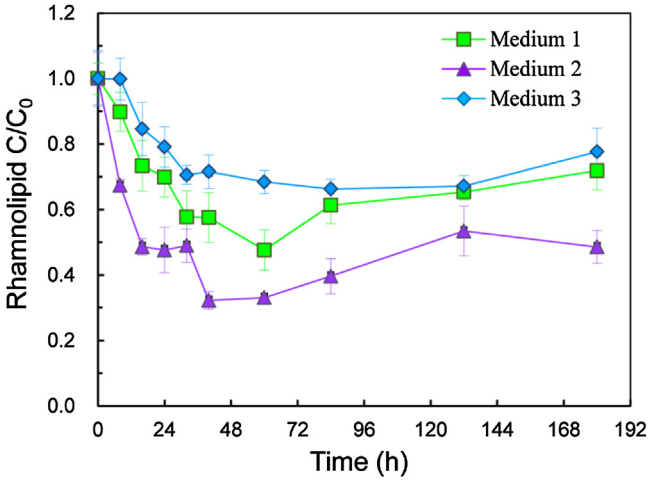

Fig. 4. Degradation of rhamnolipid by *P. putida* CICC 20575 in the systems with rhamnolipid-solubilized hexadecane. C_0 represents the concentration of rhamnolipid (0 h). The bars represent the standard deviations of triplicate experiments. See "Materials and Method" for definitions of Medium 1 to 3.

ing the experiments, the changes of hexadecane, rhamnolipid and protein concentration were determined and analyzed.

2. Materials and methods

2.1. Chemicals

The monorhamnolipid (rhamnolipid) biosurfactant (purity 99.9%) was purchased from Zijin Biological Technology Co. Ltd (Huzhou, China). It has a chemical structure of α-L-rhamnopyranosyl-β-hydroxyalkanoyl-β–hydroxyalkanoate [29]. According to our previous studies, this rhamnolipid mixture consists of Rha–C_{10}–C_8, Rha–C_{10}–$C_{10:1}$, Rha–C_{10}–C_{10}, Rha–C_{10}–$C_{12:1}$ and Rha–C_{10}–C_{12} with approximate molar ratio of 7.8: 0.9: 75.5: 3.1: 12.7 based on the peak area of the species in HPLC–MS chromatogram [30]. The molecular weights of five rhamnolipid homologues are 476, 502, 504, 530 and 532, respectively [30]. Based on above data, the molar mass of this rhamnolipid mixture is determined to be 506 g/mol. The CMC value of this rhamnolipid in mineral salt medium (MSM) solution is determined to be 83 μM (42 mg/L) based on the dependence of MSM surface tension on rhamnolipid concentration [31]. Hexadecane of analytical

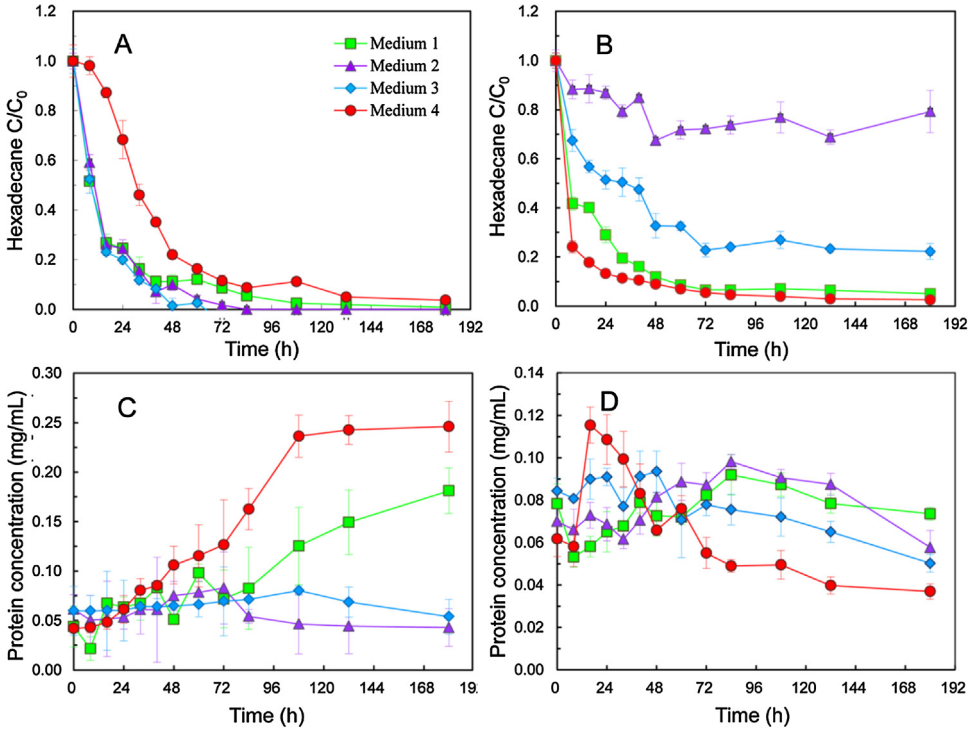

Fig. 3. Changes of rhamnolipid-solubilized hexadecane and protein concentration. A) Hexadecane concentration in *P. aeruginosa* ATCC 9027 system, B) Hexadecane concentration in *P. putida* CICC 20575 system, C) Protein concentration in *P. aeruginosa* ATCC 9027 system, D) Protein concentration in *P. putida* CICC 20575 system. C_0 represents the initial concentration of hexadecane (0 h). The bars represent the standard deviations of triplicate experiments. See "Materials and Methods" for definitions of Medium 1 to 4.

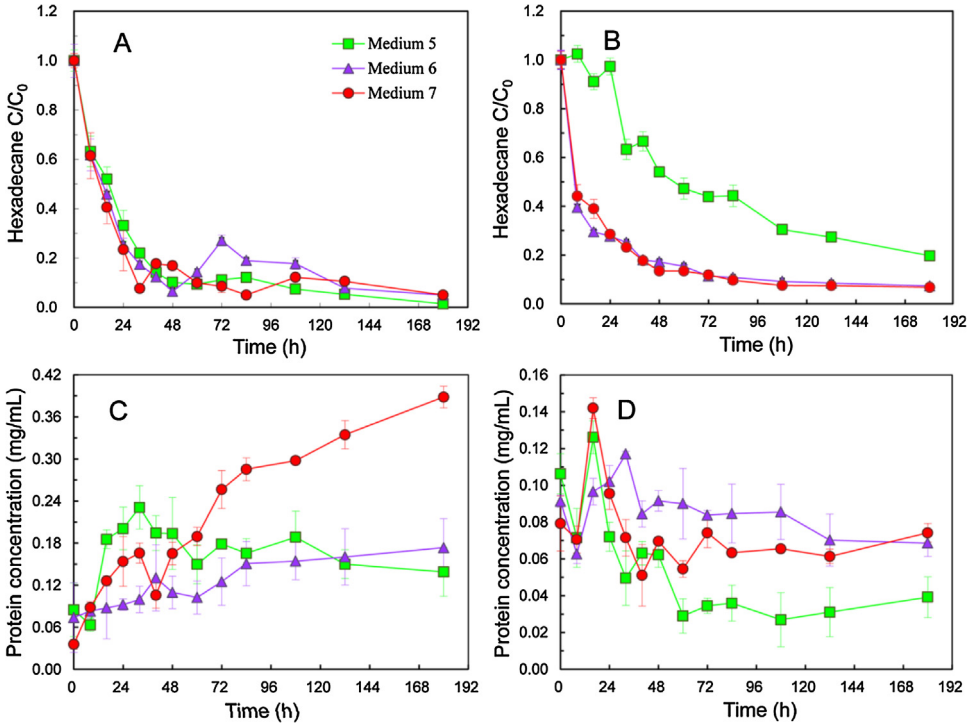

Fig. 5. Changes of concentrations of multi-state hexadecane and protein. A) Hexadecane concentration in *P. aeruginosa* ATCC 9027 system, B) Hexadecane concentration in *P. putida* CICC 20575 system, C) Protein concentration in *P. aeruginosa* ATCC 9027 system, D) Protein concentration in *P. putida* CICC 20575 system. C_0 represents the concentration of hexadecane (0 h). The bars represent the standard deviations of triplicate experiments. See "Materials and Method" for definitions of Medium 5 to 7.

grade (purity ≥99%) was purchased from Sigma-Aldrich (St. Louis, MO., U.S.). NaOH and HCl (analytical reagent grade) were obtained from Damao Chemicals (Tianjin, China). All other reagents were of analytical reagent grade and purchased from Sinopharm (Beijing, China). The water used throughout the whole experiment was produced by UPT-II-40 (Ulupure, Chengdu, China) with an initial resistivity of 18.3 MW·cm.

2.2. Microorganisms

P. aeruginosa ATCC 9027 and *P. putida* CICC 20575 were used as the degrader of the hexadecane due to their strong potential for hydrocarbon degradation [32,33]. These two strains were purchased from the American Type Culture Collection (ATCC, Rockville, Md.) and the China Center for Industrial Culture Collection (CICC, Beijing, China), respectively. They were received as lyophilized stock, revived on peptone agar slants, and then maintained at 4 °C. Before use they were incubated at 37 °C for at least 24 h for activation.

P. aeruginosa ATCC 9027 and *P. putida* CICC 20575 from slants were respectively enriched in 50 mL of Kay's minimal medium with gyratory agitation at 37 °C, 200 rpm for 12 h. The Kay's minimal medium consisted of $NH_4H_2PO_4$ 3 g/L, K_2HPO_4 2 g/L, glucose 2 g/L, $FeSO_4$ 1.4 mg/L, $MgSO_4{\cdot}7H_2O$ 1 g/L [34]. Then the cultures obtained were inoculated at the ratio of 2.5% (v/v) into MSM containing 20 g/L hexadecane as the sole carbon source. The inoculated cultures were incubated under gyratory shaking at 200 rpm and 30 °C for production of bacterial cells used as the inoculum in the degradation experiment. The MSM is consisted of 0.5% NH_4Cl, 0.5% Na_2HPO_4, 0.25% KH_2PO_4, and 0.05% $MgSO_4{\cdot}7H_2O$, with a pH of 6.8 adjusted by NaOH or HCl.

2.3. Solubilization of n-hexadecane by rhamnolipid

Solutions of hexadecane solubilized by rhamnolipid were prepared using the following procedures. 2 mL of *n*-hexadecane was pipetted into a 500-mL conical flask, and the flask was slowly rotated to spread hexadecane on the bottom of the glass flask. Then 100 mL of sterilized MSM containing rhamnolipid at concentration of 21 mg/L (0.5 × CMC) or 210 mg/L (5 × CMC) was added into the conical flask. The flasks were incubated on a reciprocal shaker running at 30 °C, 100 rpm for 72 h. The results of our previous study showed that the equilibrium of hexadecane solubilization can be reached within 72 h [27]. Then the solution in the flasks was transferred into separatory funnels and allowed to stand overnight for phase separation. After that, the bottom aqueous phase was released at a slow and steady flow rate (approximate one drop per 10 s) and collected. The above separation procedure was repeated and rhamnolipid-solubilized hexadecane solutions with rhamnolipid at concentrations of 0.5 × CMC and 5 × CMC were obtained. The concentration of hexadecane was determined using gas chromatography.

2.4. Degradation experiment

n-hexadecane (C_{16}) is selected as the hydrocarbon because it is a typical constituent of petroleum hydrocarbons with very low solubility [35]. Seven sets of MSM-based culture medium (Fig. 1) were prepared based on different states of hexadecane in the media. These culture media are: (1) Hexadecane in rhamnolipid-solubilized state (hexadecane in the form of hexadecane/rhamnolipid aggregates evenly distributed in water) with rhamnolipid at a concentration of 5 × CMC ($C_{5\times CMC}$). (2) Hexadecane in rhamnolipid-solubilized state with rhamnolipid at a concentration of 0.5 × CMC ($C_{0.5\times CMC}$). (3) Hexadecane in rhamnolipid-solubilized state with the concentration equals to $C_{0.5\times CMC}$, but the rhamnolipid concentration is 5 × CMC. This

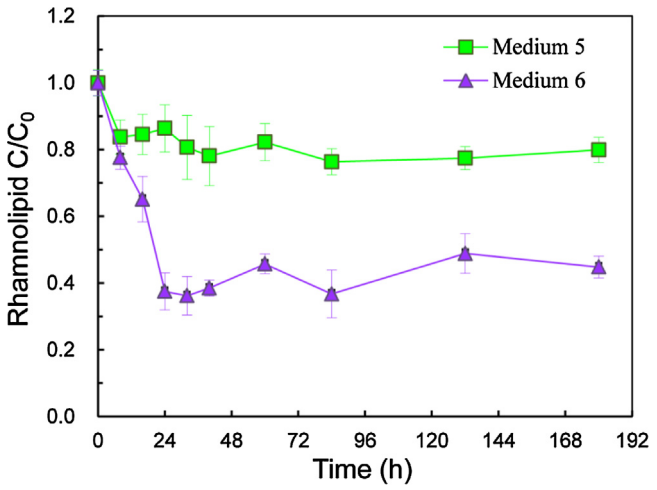

Fig. 6. Degradation of rhamnolipid by *P. putida* CICC 20575 in multi-state hexadecane system. C_0 represents the concentration of rhamnolipid when the incubation started (0 h). The bars represent the standard deviations of triplicate experiments. See "Materials and Method" for definitions of Medium 5 to 6.

is achieved by adding extra rhamnolipid into the hexadecane solution described in (2). (4) Hexadecane in the absence of rhamnolipid (hexadecane as a separate phase). The homogeneous-equivalent concentration of hexadecane is $C_{5\times CMC}$. This is obtained by adding pure hexadecane directly into MSM. This medium was used as a control. (5) Rhamnolipid concentration is $5\times$ CMC and homogeneous-equivalent concentration of hexadecane is $10\times C_{5\times CMC}$. This is obtained by adding extra hexadecane into the hexadecane solution described in (1). (6) Rhamnolipid concentration is $0.5\times$ CMC and the homogeneous-equivalent concentration of hexadecane is $10\times C_{5\times CMC}$. This is obtained by adding extra hexadecane into the hexadecane solution described in (2). (7) Hexadecane in the absence of rhamnolipid. The homogeneous-equivalent concentration of hexadecane is $10\times C_{5\times CMC}$. In additional to these 7 sets of hexadecane-containing culture medium, MSM containing only rhamnolipid as the carbon source with a concentration of $5\times$ CMC was prepared as the culture medium 8 to examine degradability of rhamnolipid (Medium 1 to Medium 8 was used to represent these eight culture media in following text).

For preparation of these culture media, rhamnolipid-solubilized hexadecane solutions, pure hexadecane, and rhamnolipid solutions were autoclaved at 105 °C for 30 min. Then the culture media were prepared under aseptic conditions. No influence of autoclave on the concentration of rhamnolipid-solubilized hexadecane or rhamnolipid was observed in our previous test [27].

10-mL plastic centrifuge tubes were used as the disposable bioreactors for the degradation experiment. Typically, 2 mL of one of the eight culture media described above was placed in the tube, and the inoculum of *P. aeruginosa* ATCC 9027 or *P. putida* CICC 20575 was added at a ratio of 2.5% (v/v). The tubes were stopped with sterile cotton plugs, which were porous and allowed oxygen exchange between inside and outside of the tubes. The tubes were then placed in a gyratory shaker at 30 °C, 200 rpm to start the degradation process. For every 24 h, the cotton plugs were removed and the cultures were exposed to sterile fresh air to enhance the supply of oxygen. At predetermined time intervals, some tubes were sacrificed for analysis of protein content, hexadecane concentration, and rhamnolipid concentration (samples withdrawn immediately after inoculation (0 h) were used for determination of C_0 for hexadecane or rhamnolipid). These tubes were centrifuged at $5600\times g$ for 10 min, and the supernatants were decanted into new tubes. The inner wall of the tube was washed with ethanol and the ethanol was combined into the supernatant which was collected in the new tube. By this means it was guaranteed that the attached hexadecane was also transferred into the supernatant collected. The supernatant collected was used for the analysis of hexadecane concentration. The cell pellet on the bottom of the reactor tube after centrifugation was used for protein content analysis. Rhamnolipid concentration in the supernatant of the reactor tube after centrifugation (but before transference to the new tube) was also determined periodically.

2.5. Sample analysis

Because the hexadecane-rhamnolipid particles associated with the cells may interfere with cell optical density measurement, protein concentration instead was used to represent cell growth. The protein content was measured with an ultra violet (UV) spectrometry method [36,37] with some modifications. In brief, 1 mL of 0.1 M NaOH was added into the centrifuge tube which contained cell pellet at the bottom. The tube was maintained in water bath at 100 °C for 1 min to lyse cells. Then the solution was diluted to 2 mL using water after cooling down to room temperature, and its absorbance at 260 nm and 280 nm was determined using a Shimadzu UV-2552 spectrometer (Tokyo, Japan) with water as the reference. Protein concentration of the sample was calculated with methods described by Layne [37]. The concentration of hexadecane was determined by using a gas chromatography method described by Liu et al. [38]. Phenol-sulfuric acid method was used to measure rhamnolipid concentration after rhamnolipid was separated from the sample using an ethyl acetate extraction technique [39]. All batch experiments were performed in triplicates. At everysampling point, three same samples were withdrawn and the results were presented as the mean ± standard deviation.

3. Results and discussion

3.1. Solubilization of hexadecane by rhamnolipid

The apparent solubilities of hexadecane in the presence of rhamnolipid at concentrations of $5\times$ CMC (210 mg/L, or 415 μM) and $0.5\times$ CMC (21 mg/L, or 42 μM) are 212 ± 1 and 89 ± 1 mg/L (938 ± 3 and 395 ± 3 μM), respectively. According to the model reported by Ansari et al. [40], the molar solubilization ratios (MSR, defined as the increased molar quantity of solubilized hydrocarbons for per mole increase of surfactant concentration) for hexadecane in rhamnolipid solution at the concentrations of $5\times$ CMC and $0.5\times$ CMC in this study were 0.795 and 9.16, respectively. Our prior study on solubilization of alkanes by rhamnolipid showed that rhamnolipid could enhance the solubility of *n*-decane, dodecane, tetradecane, and hexadecane at concentrations both below and above CMC based on an aggregate-formation mechanism [41]. Also the solubilization was more efficient at concentrations lower than CMC, which is shown by a higher MSR at rhamnolipid concentrations lower than CMC [41]. The measured solubility values of hexadecane in this study are consistent with the results of the prior one, and they are much higher than the solubility of hexadecane in water (0.00009 μM, 25 °C) [41]. Thus, the initial concentrations (or homogeneous-equivalent concentrations) of hexadecane are 212 mg/L in Medium 1 and 4, 89 mg/L in Medium 2 and 3, and 2120 mg/L in Medium 5, 6 and 7.

3.2. Degradability of rhamnolipid

Degradability of surfactants has been potentially a factor affecting bioavailability of the hydrocarbon contaminants due to interfacial partitioning of them in hydrocarbon degradation process [32]. Therefore, the degradation of rhamnolipid as the sole energy source (Medium 8) by the two strains used in this study

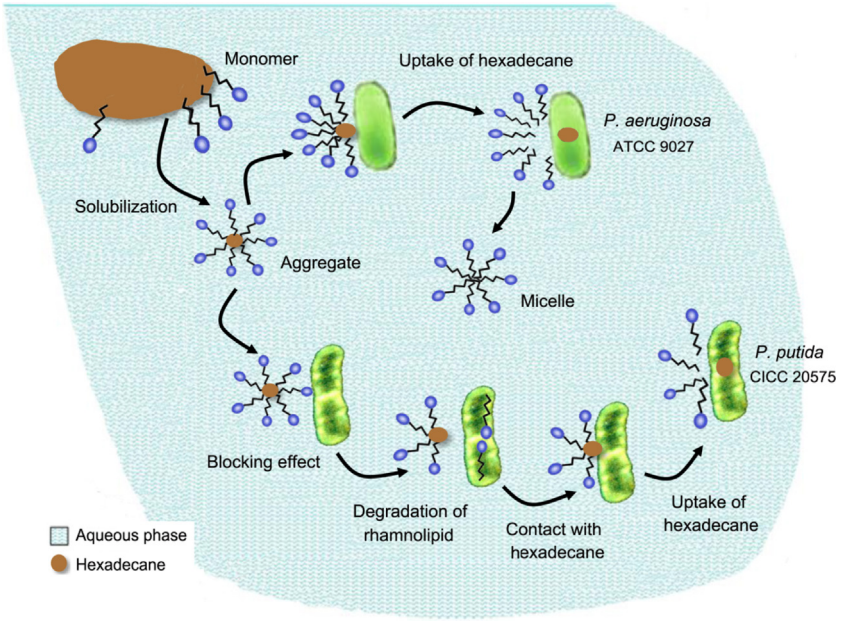

Fig. 7. Schematic diagram of rhamnolipid mediated uptake of solubilized hexadecane by *P. aeruginosa* ATCC 9027 and *P. putida* CICC 20575.

was examined in the first place, and the results are shown in Fig. 2. No significant change of rhamnolipid concentration during 180 h for *P. aeruginosa* ATCC 9027, while a rapid decrease was observed in the first 84 h for *P. putida* CICC 20575 and almost 50% of the rhamnolipid was degraded. The result shows that *P. aeruginosa* ATCC 9027 does not utilize rhamnolipid. The remaining unchanged protein concentration also supports this conclusion. This finding is consistent with those of prior studies demonstrating that being a rhamnolipid producer, *P. aeruginosa* ATCC 9027 does not degrade rhamnolipid [33]. In contrast, the decreasing concentration of rhamnolipid shows that *P. putida* CICC 20575 can utilize rhamnolipid. On the other hand, a corresponsive increase of protein concentration was observed in the first 24 h. Similar result was found by Wyrwas et al. [42] who proposed that the use of surfactant molecules as carbon and energy sources can impact the growth of microorganisms. Therefore, for the two strains used in this study, *P. putida* CICC 20575 is a degrader of rhamnolipid (the surfactant) and *P. aeruginosa* ATCC 9027 is not.

3.3. Degradation of hexadecane

3.3.1. Degradation of rhamnolipid-solubilized hexadecane

Surfactant-solubilization is considered a likely method to improve the bioavailability of petroleum hydrocarbons, since good distribution of hydrocarbons in aqueous phase could enhance their interactions with microorganisms [43]. The changes of hexadecane and protein concentration in Medium 1 to 4 are presented in Fig. 3. For *P. aeruginosa* ATCC 9027, the concentrations of hexadecane in Medium 1, 2 and 3 decreased more rapidly than that in control row (Medium 4) (Fig. 3A). More than 70% of hexadecane in solubilized forms (Medium 1, 2 and 3) was removed in the first 16 h. As for *P. putida* CICC 20575, the decrease of hexadecane concentration in Medium 4 was more rapidly than those in Medium 1, 2 and 3 (Fig. 3B). These results show that the solubilization by rhamnolipid can enhance the bioavailability of hexadecane to *P. aeruginosa* ATCC 9027. Such enhancement is not dependent on rhamnolipid concentration for the solubilization, since relative removal rate for the hexadecane solubilized by 0.5 × CMC rhamnolipid (Medium 2) is similar to the rate for the same amount of hexadecane solubilized by 5 × CMC rhamnolipid (Medium 3) (Fig. 3A). There are two commonly recognized mechanisms for surfactant-solubilization to enhance the bioavailability of hydrocarbon: one is the increase of interfacial area facilitating mass transfer of hydrocarbons into aqueous phase [7,44], and the other is direct uptake of hydrocarbons in hydrocarbon/surfactant aggregates [45]. However, rhamnolipid solubilization decreased the availability of hexadecane to *P. putida* CICC 20575.

To further study the effect of rhamnolipid on the biodegradation of hexadecane by *P. putida* CICC 20575. The variation of rhamnolipid concentration during the process was studied. It is observed the changes of rhamnolipid concentration in Medium 1, 2 and 3 (Fig. 4) is similar to the result that obtained with rhamnolipid as the sole carbon source (Fig. 2), showing that *P. putida* CICC 20575 degrades rhamnolipid even in the presence of hexadecane. The rate of the decrease, however, is lower than that of hexadecane (Fig. 3B), indicating that hexadecane is a substrate preferable to the rhamnolipid for *P. putida* CICC 20575. The results show that the inhibitive effect of rhamnolipid on hexadecane degradation for *P.* putida CICC 20575 is not based on the mechanism of preferential use of rhamnolipid as the carbon source.

The growths of *P. aeruginosa* ATCC 9027 in Medium 2 and 3 were insignificant, probably due to the low concentrations of hexadecane. Comparing the cell growths in Medium 2 and 3, a significant increase of protein is observed for the medium with higher hexadecane concentrations (Medium 1 and 4) from 72 h (Fig. 3C). This is in contrast to hexadecane removal from the media, which started at the beginning of cultivation. This result indicates that *P. aeruginosa* ATCC 9027 cells employed a stepwise uptake and intracellular digestion process for hexadecane degradation, which is in consistence with the fact that most enzymes for long-chain alkane degradation are intracellular [46,47]. Profiles of protein concentration for *P. putida* CICC 20575 in the four media are shown in Fig. 3D. Protein concentration in Medium 4 had a significant increase starting at 8 h, indicating fast cell growth at this time, which is in accordance with the strong uptake of hexadecane in the first 8 h. In the late stage of degradation, protein concentration in Medium 4 was lower than those in the media of solubilized hexadecane (Medium 1, 2 and 3). It is probably due to the lack of carbon sources (minimal hexadecane and no rhamnolipid) (Fig. 3B).

3.3.2. Degradation of multi-state hexadecane

Changes of concentrations for hexadecane with multiple forms and protein are shown in Fig. 5. In *P. aeruginosa* ATCC 9027 system, fast decrease in hexadecane mass was observed in the first 32 h in all the three media, and the removal rates were similar regardless of the presence or concentration of rhamnolipid (Fig. 5A). These results indicate that rhamnolipid has minimal influence on the availability of hexadecane in non-solubilized forms for *P. aeruginosa* ATCC 9027. In *P. putida* CICC 20575 system, a rapid decrease of hexadecane concentration was observed in the first 8 h in the absence of rhamnolipid (Medium 7, the control) or in the presence of rhamnolipid at a concentration of 0.5 × CMC (Medium 6) (Fig. 5B). Removal of hexadecane in the presence of rhamnolipid at the high concentration (5 × CMC, Medium 5), however, was delayed and slower compared to Medium 7, causing higher hexadecane concentration level during the whole degradation phase. This result shows that uptake of the hexadecane in the system with excessive mass hexadecane was inhibited when rhamnolipid concentration was sufficiently high, indicating limitation of hexadecane availability.

Approximately 50% of rhamnolipid was degraded when it was used as sole energy source (Fig. 2), while only 20% removal was observed in Medium 5 (Fig. 6). This is probably due to the presence of large amount of hexadecane as the preferable substrate. Partition of rhamnolipid at the interfaces, on the other hand, could block adhesion of cells to mass hexadecane or mass transfer of hexadecane to aqueous phase, and thus cause the decrease in availability of hexadecane. Such blocking effect in Medium 6 may not be significant due to much lower rhamnolipid concentration (0.5 × CMC) and significant loss of rhamnolipid (Fig. 6). As a result, hexadecane uptake rate is close to that in the absence of rhamnolipid (Medium 7).

The beginning of the growth of *P. aeruginosa* ATCC 9027 in these three media (Fig. 5C) was earlier than that in the media with only solubilized hexadecane (Medium 1, 2, 3, and 4), likely due to the large amount of hexadecane supplied in these three media. Compared with the control row in the absence of rhamnolipid (Medium 7), high-concentration rhamnolipid (5 × CMC, Medium 5) caused a rapid increase of protein concentration in the early growth stages, while low-concentration rhamnolipid (0.5 × CMC, Medium 6) had an inhibitory effect. Such inhibitory effect of low-concentration rhamnolipid on cell growth has also been reported elsewhere [27,48,49]. The increase of protein concentration for *P. putida* CICC 20575 was observed during 8–16 h for all the three media (Fig. 5D). The protein concentration in the presence of 5 × CMC rhamnolipid (Medium 5) decreased rapidly after 16 h and remained low in the late degradation stage, which is in accordance with the observed limitation in hexadecane availability.

3.4. Relation between solubility and availability

Results of hexadecane degradation show the difference in bioavailability of rhamnolipid-solubilized hexadecane to the *P. aeruginosa* and the *P. putida* cells. *P. aeruginosa* ATCC 9027 is a strain that produces rhamnolipid, in particular when it is grown on hydrophobic organic compounds, e.g. octadecane [50,51]. Production of rhamnolipid is likely to be a strategy for this bacterium to grow on the substrates with limited water solubility. Based on the results in Section 3.3, the solubilized hexadecane is more available than the mass hexadecane for this bacterium. Meantime this bacterium does not degrade rhamnolipid (demonstrated by results shown in Fig. 2). This means that the cells of this bacterium are able to have good access to the hexadecane enclosed by the rhamnolipid layer in the hexadecane-rhamnolipid aggregates. A hypothesis is that the rhamnolipid molecules at the periphery of the aggregate may fuse with the outer membrane of bacterial cells in a mode similar to that described for the liposome in transferring alkane, which facilitates direct delivery of hexadecane into the bacterial cells [21,52] (Fig. 7). In the media in which mass hexadecane is in great access (Medium 5, 6 and 7), rhamnolipid solubilization is insignificant in enhancing hexadecane availability due to the too small proportion of solubilized hexadecane.

P. putida CICC 20575 is not a rhamnolipid producer and it metabolizes on rhamnolipid (Fig. 2). Thus, it is unlikely that it possesses a strategy to take advantage of rhamnolipid for hexadecane delivery (e.g., cell membrane fusion). As a result, in order to get access to the solubilized hexadecane in the core area of an aggregate, it has to break the rhamnolipid layer (Fig. 7). Degradation of rhamnolipid is slower than uptake of hexadecane, which is indicated by the degradation profiles of rhamnolpid and mass hexadecane (Figs.4 and 3 B). Such blocking effect of rhamnolipid is likely the reason for lower availability of solubilized hexadecane compared to mass hexadecane as a separate phase (Fig. 3B). In the media in which the hexadecane mass is in great access to the solubility, partition of rhamnolipid to the interfaces occurs, which still can block direct contact between cells and mass hexadecane at sufficiently high concentrations and thus result in lower hexadecane availability (Fig. 5B). In all, the difference in the effect of rhamnolipid on hexadecane availability for these two strains indicates the importance of compatibility between biosurfactant and bacteria for the biosurfactant to enhance bioavailability of HOCs.

4. Conclusions

The results of this study show that hexadecane solubilization by rhamnolipid does not always enhance hexadecane bioavailability, and the causal relationship between solubilization and bioavailability is bacterial compatibility dependent. The solubilization with the presence of 0.5 × CMC and 5 × CMC rhamnolipid both enhanced hexadecane uptake by *P. aeruginosa* ATCC 9027, resulting in more than 70% mass loss of hexadecane in the first 16 h. Comparing with the control row, the solubilization had an inhibition effect on the uptake of hexadecane by *P. putida* CICC 20575 since no significant decrease of hexadecane concentration in the first 8 h. Blocking effect of rhamnolipid molecule layer at the organic-water interface is supposed to be the reason for the bioavailability reduction. The insights obtained in this study on the relation between HOCs bioavailability and rhamnolipid solubilization are of importance for design and implementation of rhamnolipid-enhanced bioremediation.

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