UPTAKE MODES OF FLUORANTHENE BY STRAIN *RHODOCOCCUS* SP. BAP-1

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ABSTRACT

In the present study, an indigenous biosurfactant-producing bacterium, Rhodococcus sp. BAP-1, with effective fluoranthene-degrading ability was isolated from crude-oil contaminated soil. The biodegradation data indicated that Rhodococcus sp. BAP-1 grew well in the presence of 20 mg·L^{-1} of fluoranthene, whereas in the presence of 150 mg·L^{-1} of fluoranthene, the biodegradation rate almost remained at low levels. During growth on mineral salts medium, the bacterium could produce biosurfactants. As a result of biosurfactant synthesis, the surface tension of the culture broth was reduced, thus facilitating the contact between the bacterium and hydrocarbon. According to the experimental results, emulsification was probably not the leading mechanism that occurred simultaneously during the biodegradation process. Pollutants might be growing as a confluent biofilm and the uptake system might have a high specific affinity; an interfacial uptake mechanism may play a predominant role in the biodegradation process. Fourier transform infrared spectroscopy showed that the biosurfactant contained aliphatic hydrocarbons. A disruption of the cell surface in certain zones was observed by scanning electron microscopy in some of the cells grown in the presence of 150 mg·L^{-1} of fluoranthene, which indicated that drastic changes of the cell surface morphology might occur under different growth conditions. The results of this study collectively suggest that Rhodococcus sp. BAP-1 may be useful in the bioremediation of environments contaminated with polycyclic aromatic hydrocarbons.

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a concern in the whole world because of their toxic, mutagenic, teratogenic and carcinogenic effects. PAHs are a class of petroleum pollutants and are typical persistent organic pollutants (6). PAHs contain a group of potential environmental pollutants which can be found in soils, water, air and sediments, which are considered to be possible hazard to the environment, and their distributions in the environment will cause a significant health risk for human beings (8). They can enter human bodies through inhalation, skin contract and ingestion. Exposure to PAHs has been associated with skin, lung, liver, intestine and pancreas cancers and the toxicity is known to increase with increasing the molecular weight (9). Currently, more than 100 kinds of PAH compounds are known; in November 2013, the US Environmental Protection Agency (US EPA) classified 28 compounds of PAHs as "priority pollutants". PAHs are difficult to degrade under natural conditions due to their distinguishing feature of high hydrophobicity. That is why they persist for long periods of time in soils, water, air and sediments (14).

A lot of bacterial strains such as *Sphingomonas* sp., *Mycobacterium* sp., *Brevibacillus* sp., *Bacillus* sp., *Pseudomonas* sp., *Rhodococcus* sp., and *Burkholderia* sp., are well-known to degrade PAHs (12, 15, 16, 20, 22, 24). Despite the high hydrophobicity and the low bioavailability of PAH compounds, bacteria, via a particular mode to contract, manage to absorb and degrade these compounds. Typically, there are three different regulation mechanisms, depending on the state and size of the oil droplets relative to the size of the microbial cells (11): i. high specific affinity uptake systems for pollutants and growth as a confluent biofilm (34); ii. direct attachment to larger hydrocarbon droplets and pseudosolubilization (2); iii. biosurfactant produced by cells to improve microbial ability to utilize emulsified small compounds (19). Sometimes, more than one mechanism is used simultaneously during the biodegradation process.

Bacteria in the *Rhodococcus* genus are some of the most effective PAH-degrading bacteria, which are able to utilize PAHs such as phenanthrene, anthracene, benzo[a]pyrene, fluoranthene, as carbon and energy sources (4; 30). Adding a non-ionic surfactant such as Tween 80 above the critical micelle concentration (CMC) can stimulate *Rhodococcus* to produce biosurfactant, which will significantly enhance the biodegradation rate (17). Some studies have also investigated the stability of the biosurfactant produced by *Rhodococcus* in high salinity and a wide pH range (28). Although the biosurfactant produced by *Rhodococcus* has been identified as a trehalose glycolipid (18; 31), the precise structure of the biosurfactant has not yet been elucidated.

The aim of the present study was to investigate the potential of *Rhodococcus* sp. BAP-1 to utilize fluoranthene as the sole carbon source. The biosurfactant produced by *Rhodococcus* during the growth on fluoranthene was identified via FT-IR. Additionally, the growth of *Rhodococcus* sp. BAP-1 on different concentrations of fluoranthene in flask cultures was evaluated. During this process, the surface tension changes,

the microbial adhesion to hydrocarbon (MATH) and cell growth conditions were analyzed in details. The morphological changes of *Rhodococcus* sp. BAP-1 cells under different fluoranthene concentrations were investigated as well.

Materials and Methods

Cultivation media and conditions

Rhodococcus sp. BAP-1 inoculum from the nutrient agar plate was enriched in 100 mL Luria–Bertani (LB) culture medium (5 $g \cdot L^{-1}$ of NaCl, 5 $g \cdot L^{-1}$of yeast extract, 10 $g \cdot L^{-1}$ of tryptone) in a 250 mL Erlenmeyer flask at 30 °C ± 1 °C on a shaking table at a rotating speed of 120 $r \cdot min^{-1}$, and incubated for 48 h. Bacterial cells were collected by centrifugation (6000×*g*, 10 min), washed twice in mineral salts medium (MSM, pH = 7.0), redissolved in sterile MSM, and then the absorbance was measured by a UV-Visible spectrophotometer (Varian, Palo Alto, CA, USA) at 600 nm. The final cell optical density (OD_{600}) value was controlled at 1.5. Then, dissolved cells were used as inoculum at 5 % (v/v) in 100 mL Erlenmeyer flasks containing 50 mL of MSM. Stock solution of fluoranthene (1 $g \cdot L^{-1}$) with acetone as a solvent was made before inoculum as the sole carbon source. The composition of MSM was as follows: 4.0 $g \cdot L^{-1}$ of Na_2HPO_4; 1.5 $g \cdot L^{-1}$ of KH_2PO_4; 1.0 $g \cdot L^{-1}$ of NH_4Cl; 1.0 $g \cdot L^{-1}$ of $NaNO_3$; 0.2 $g \cdot L^{-1}$ of $MgSO_4 \cdot 7H_2O$; 0.02 $g \cdot L^{-1}$ of $CaCl_2$; 0.03 $g \cdot L^{-1}$ of $FeSO_4 \cdot 7H_2O$, and 1 mL of micronutrients (0.005 $g \cdot L^{-1}$ of $CuSO_4 \cdot 5H_2O$; 0.01 $g \cdot L^{-1}$ of H_3BO_3; 0.01 $g \cdot L^{-1}$ of $MnSO_4 \cdot 5H_2O$; 0.07 $g \cdot L^{-1}$ of $ZnSO_4$; 0.005 $g \cdot L^{-1}$ of $CoCl_2 \cdot H_2O$. The pH of the medium was adjusted to 7.0. Both the LB and MSM liquid culture media were sterilized by autoclaving at 121 °C for 20 min.

Biodegradation assay

Fluoranthene biodegradation. Erlenmeyer flasks (100 mL) and MSM were autoclaved for 20 min at 121 °C. Different volumes of acetone (0.25 mL, 0.5 mL, 1.25 mL, 2.5 mL, 5 mL, 15 mL) containing the required fluoranthene concentration were added to autoclaved flasks in order to allow the acetone to evaporate. After the acetone evaporated completely, 50 mL of sterile MSM was added, and different concentrations of fluoranthene (5 $mg \cdot L^{-1}$, 10 $mg \cdot L^{-1}$, 25 $mg \cdot L^{-1}$, 50 $mg \cdot L^{-1}$, 100 $mg \cdot L^{-1}$, 150 $mg \cdot L^{-1}$) were used for the subsequent studies. The flasks were inoculated with BAP-1 and incubated at 30 °C, 120 $r \cdot min^{-1}$ in a rotary shaker. Samples were aseptically collected at a regular interval; 4 mL of the culture medium was used for analyzing the cell growth at 600 nm (OD_{600}), 1 mL of broth without cells was used for glycolipid production analysis, and 10 mL of broth was used for measurement of the surface tension.

High performance liquid chromatograph (HPLC) analysis. To extract the remaining fluoranthene from the culture broth, the whole flask of broth (50 mL) was subjected to extraction with an equal volume of n-hexane, using a ultrasonic cleaner machine under a 40 Hz frequency for 30 min. The separated solvent layer was removed and the lower aqueous phase was re-extracted as before. Two milliliters of the combined extracts were filtrated through a 0.2 μm membrane filter and transferred into Agilent vials. Quantitative analysis of residual fluoranthene in the culture media was performed by HPLC (U3000, DIONEX, USA) with UV detection at 254 nm. An aliquot (20 μL) was analyzed using an Agilent TC-C18 column (250 mm × 4.6 mm, 5 μm). The mobile phase (water/acetonitrile = 40:60 v/v) was adjusted to 1.2 $mL \cdot min^{-1}$ at 25 °C.

Surface tension measurements. The surface tension was measured by the Du Noüy ring method (21), using a JWY-200 interfacial tensiometer (Puhui, Chengde, Hebei Province, China) with a 2 cm diameter platinum ring at room temperature. The stability of the surface tension readings were checked with pure water (70.25 $mN \cdot m^{-1}$ ± 0.2 $mN \cdot m^{-1}$) before each experiment. For each concentration gradient, three independent samples were made, with the standard deviation being within 5%.

Microbial adhesion to hydrocarbons (MATH). The microbial adhesion to hydrocarbons (MATH) was proposed as an assay for analyzing the microbial cell surface hydrophobicity of *Rhodococcus* sp. BAP-1 (25). Bacterial cells were collected by centrifugation (6000×*g*, 20 min) at different intervals, washed twice and then resuspended in PBS buffer (K_2HPO_4 16.9 $g \cdot L^{-1}$ and KH_2PO_4 7.3 $g \cdot L^{-1}$; pH = 7.1; diluted water 1000 mL). Acid-washed test tubes (7 mL) containing 4 mL of the washed cell suspension in PBS buffer, were added 1.5 mL of liquid *p*-xylene. Following 30 min preincubation at 30 °C, the turbid mixtures were vortexed at high speed for 2 min. After allowing 30 min for the aqueous phase and *p*-xylene to separate completely, the aqueous phase was carefully removed with a disposable syringe (5 mL) and transferred to a cuvette (4 mL). Light absorbance was measured at 400 nm spectrophotometrically (Varian, USA). The cellular hydrophobicity was expressed by the difference between the optical density of the bottom aqueous phase before and after mixing with *p*-xylene, which was calculated as follows: 100 × (1 - OD_{400} of aqueous phase of culture after the addition of *p*-xylene / OD_{400} of the initial cell suspension). For each concentration gradient, three independent samples were measured, with the standard deviation being within 5%.

Quantitative analysis of crude biosurfactant. The concentration of the crude biosurfactant was determined by estimating the concentration of trehaloselipids, using the phenol sulphuric acid method (5). Culture supernatant (2 mL) obtained by centrifuged (6000×*g*, 20 min) of the broth (4 mL) was removed to a glass test tube (50 mL). One milliliter phenol (prepared by adding 20 mL of distilled water to 80 g of redistilled phenol, then added as a 5 % solution in distilled water, prepared before use) was added into the supernatant and mixed immediately. After that, 5 mL of sulfuric acid were added rapidly against the side of the test tube and the test tube was allowed to stand for 10 min in order to obtain good mixing. The absorbance of the mixture was measured at 480 nm (Varian, USA); trehalose was used as the standard (32). For each concentration gradient, three independent measurements were made, with the standard deviation being within 5%.

Biosurfactant identification

Properties of crude biosurfactant extract. The crude biosurfactant that *Rhodococcus* sp. BAP-1 secreted was collected from the culture broth, using the method described by Sim et al. (29). Samples grown on fluoranthene (50 mg·L^{-1}) were separated from the bacterial cells by centrifugation at 6000×*g* for 20 min. The pH of the combined supernatant (500 mL) was adjusted to approximately 2.0 with 2 mol·L^{-1} HCl; and the glycolipid was less soluble in the aqueous phase. The glycolipid was extracted twice with an equal volume of chloroform/methanol (2:1 v/v) from the combined supernatant fluids. The extracts were concentrated at room temperature, after the pooled organic extracts were evaporated, brownish yellow pellets were found in the bottom of the tube. Part of the solid sample was used for the Fourier transform infrared spectroscopy (FT-IR) analysis.

Fourier transform IR spectroscopy (FTIR). FTIR transmittance spectrum is a rapid and simple method for qualitative analysis of chemical structure. The biosurfactant product obtained from *Rhodococcus* sp. BAP-1 was analyzed on a Nexus-670 FTIR spectrometer (Nicolet, Madison, WI, USA) for the identification of functional groups by neat KBr pellet method. Dry nitrogen was used to purge for an hour, in order to remove carbon dioxide. The resolution yielding IR traces were over the range of 400 cm^{-1} to 4000 cm^{-1} and the measurements were carried out in absorbance mode.

Morphological change observation

Rhodococcus sp. BAP-1 cells (in the form of pellet) cultivated on LB and MSM liquid culture medium (the concentrations of fluoranthene were 25 mg·L^{-1} and 50 mg·L^{-1}, respectively) for 96 h were collected by centrifugation at 4500×*g* for 10 min at 4 °C; and the morphological changes were observed by scanning electron micrograph (SEM). The cells were fixed with 2 % (w/v) glutaraldehyde for 3 h at 4 °C, then washed in PBS buffer three times (each time for 15 min). After that, the cells were fixed again with 1 % (w/v) osmic acid for 2 h at 4 °C, then rinsed in PBS buffer six times (each time for 15 min) and then, dehydrated by sequential immersion in increasing concentrations of ethanol (30 %, 50 %, 70 %, 80 % and 90 %) for 15 min each and washed in absolute ethanol for 15 min twice. Finally, cells were immersed into isoamyl acetate for 15 min twice. Subsequently, the samples were freeze-dried (HCP-2, Hitachi, Tokyo, Japan) and then coated with an approximate thickness of 10nm to 20 nm gold by an ion coater instrument (IB-3, Eiko, Japan) in argon atmosphere to be observed with a S-4800 SEM (Hitachi, Tokyo, Japan) at an acceleration voltage of 3000 V.

Results and Discussion

Biodegradation assay

A well-known property of the gram-positive bacterium *Rhodococcus*, is the capability to degrade PAHs. Previous studies by Mutalik et al. (23) demonstrated that among *Rhodococcus* sp. this ability is often accompanied by the production of biosurfactant and trehaloselipids are the most important group in the process of PAHs utilization (7). However, studies by Bouchez et al. (3) indicate that, when no evidence for the presence of biosurfactants is obtained, an interfacial uptake mechanism is predominant in the degradation process of pyrene and fluoranthene.

Cell growth of *Rhodococcus* sp. BAP-1 was studied in detail in the presence of different concentrations of fluoranthene as the sole carbon and energy source in the culture medium in order to analyze the mode of fluoranthene uptake. The growth curves shown in **Fig. 1** indicate that *Rhodococcus* sp. BAP-1 grew well at low concentrations (5 mg·L^{-1}, 10 mg·L^{-1} and 25 mg·L^{-1}) of fluoranthene, whereas in the absence of fluoranthene, the biomass decreased from OD 0.05 to 0.01. At low fluoranthene concentrations (5 mg·L^{-1}, 10 mg·L^{-1} and 25 mg·L^{-1}) the biomass was more than that at higher concentrations (50 mg·L^{-1}, 100 mg·L^{-1} and 150 mg·L^{-1}) and an exponential growth phase was observed within 120 h, followed by a downward trend. The maximum biomass was observed at 120 h of cultivation in the presence of 10 mg·L^{-1}of fluoranthene. During the exponential phase, the OD_{600} increased from 0.05 to the highest level of 0.69; then, in the downward trend, the OD_{600} decreased from 0.69 to 0.38 within 72 h and stabilized. At higher fluoranthene concentrations (50 mg·L^{-1}, 100 mg·L^{-1} and 150 mg·L^{-1}) the biomass of *Rhodococcus* sp. BAP-1 increased a little, but the typical growth phases were not obvious.

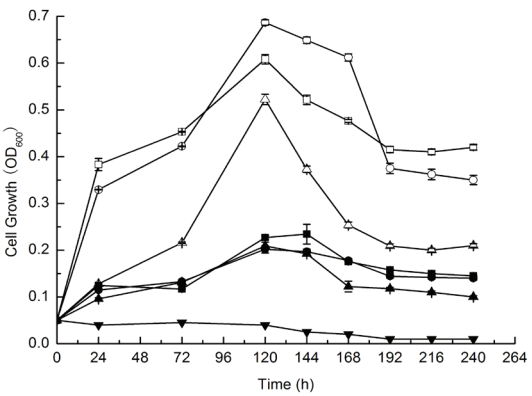

Fig. 1. Growth curves of *Rhodococcus* sp. BAP-1 in MSM containing fluoranthene as a sole carbon source. Error bars represent standard deviation. Concentration of fluoranthene: (□) 5 mg·L^{-1}, (○) 10 mg·L^{-1}, (Δ) 25 mg·L^{-1}, (●) 50 mg·L^{-1}, (■) 100 mg·L^{-1}, (▲) 150 mg·L^{-1} and (▼) 0 mg·L^{-1}.

In addition, there were obvious differences in the MSM culture medium between the lower and higher concentrations of fluoranthene. In the culture medium supplemented with lower concentrations of fluoranthene, especially 5 mg·L^{-1} and 10 mg·L^{-1}, there was a change in opacity and the culture medium appeared uniform ivory in color. In the flasks supplemented with higher concentrations of the carbon source (50 mg·L^{-1}, 100 mg·L^{-1} and 150 mg·L^{-1}), the culture medium was different in appearance. It was non-uniform, a large amount of white suspended substance accumulated and some yellow aggregate appeared at the bottom of the flask. These results indicated that lower fluoranthene concentrations were more suitable

for the growth of *Rhodococcus* sp. BAP-1, whereas high concentrations showed high toxicity, resulting in cell death.

Normally, bacteria can utilize PAHs as a carbon and energy source after adaptation of the cell membrane to the changed cultivation conditions. In the case of our experiments, probably because of the toxicity of fluoranthene, higher concentrations of fluoranthene decreased the chance for contact before the uptake process began.

Surface tension was used as an indication of the production of biosurfactant by *Rhodococcus* sp. BAP-1 in the biodegradation process of fluoranthene, which was shown in **Fig. 2**. Unlike the trends of cell growth, the change range of the surface tension correlated with the addition of fluoranthene. The minimum surface tension was observed when *Rhodococcus* sp. BAP-1 was grown in the presence of the highest tested concentration of fluoranthene (150 mg·L^{-1}). A drastic decrease in the surface tension was observed withing 24 h (from 72.35 mN·m^{-1} to 49.64 mN·m^{-1}), then a more gradual decrease followed (from 49.64 mN·m^{-1} to 46.63 mN·m^{-1}) within 120 h. After that, the surface tension of the medium increased and stabilized at 56.63 mN·m^{-1} at 192 h. Furthermore, the surface tension in the samples supplemented with 5 mg·L^{-1}, 10 mg·L^{-1} and 25 mg·L^{-1} of fluoranthene did not change considerably and was in the range from 72.35 mN·m^{-1} to 65.32 mN·m^{-1}, from 72.35 mN·m^{-1} to 61.69 mN·m^{-1}, and from 72.35 mN·m^{-1} to 59.25 mN·m^{-1}, respectively. When no fluoranthene was provided, the surface tension remained around 72.35 mN·m^{-1}. These results suggested that the white suspended substance and yellow aggregate which occurred in the flasks might be the the produced biosurfactant to be analyzed later.

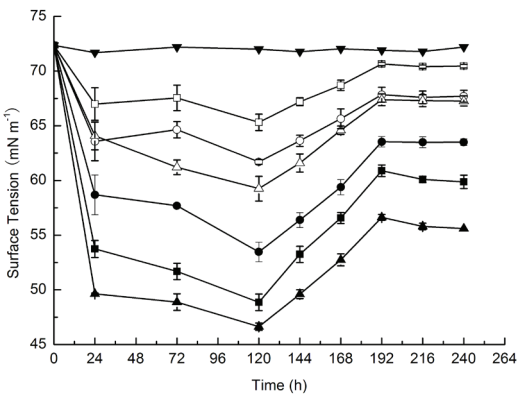

Fig. 2. Surface tension changes of MSM containing fluoranthene as a sole carbon source. Error bars represent standard deviation. Concentration of fluoranthene: (□) 5 mg·L^{-1}, (○) 10 mg·L^{-1}, (Δ) 25 mg·L^{-1}, (●) 50 mg·L^{-1}, (■) 100 mg·L^{-1}, (▲) 150 mg·L^{-1} and (▼) 0 mg·L^{-1}.

Quantitative analysis of trehaloselipids was conducted at the same time in order to study the biosurfactant production under different concentrations of fluoranthene. As shown in **Fig. 3**, no trehaloselipids were produced when there was no fluoranthene in the culture medium. In the presence of higher fluoranthene concentrations (25 mg·L^{-1}, 50 mg·L^{-1}, 100 mg·L^{-1} and 150 mg·L^{-1}), the trehaloselipids production increased continuously and reached the highest levels at 120 h. The maximum value of 360 mg·L^{-1} was observed at 120 h of cultivation in the presence of 150 mg·L^{-1} fluoranthene. When the medium was supplemented with lower concentrations of fluoranthene (5 mg·L^{-1} and 10 mg·L^{-1}), the trehaloselipids production increased rather gradually, reaching a small peak at 144 h. After that, the concentration of biosurfactants decreased drastically and reached a stationary level at 192 h.

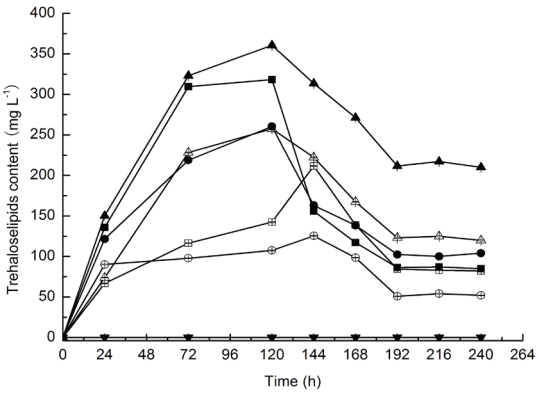

Fig. 3. Content of biosurfactant produced by *Rhodococcus* sp. BAP-1 in the culture medium. Error bars represent standard deviation. Concentration of fluoranthene: (□) 5 mg·L^{-1}, (○) 10 mg·L^{-1}, (Δ) 25 mg·L^{-1}, (●) 50 mg·L^{-1}, (■) 100 mg·L^{-1}, (▲) 150 mg·L^{-1} and (▼) 0 mg·L^{-1}.

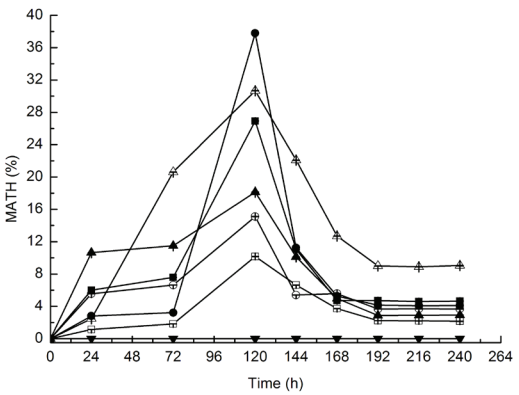

Fig. 4. Cell surface hydrophobicity changes of *Rhodococcus* sp. BAP-1. Error bars represent standard deviation. Concentration of fluoranthene: (□) 5 mg·L^{-1}, (○) 10 mg·L^{-1}, (Δ) 25 mg·L^{-1}, (●) 50 mg·L^{-1}, (■) 100 mg·L^{-1}, (▲) 150 mg·L^{-1} and (▼) 0 mg·L^{-1}.

The cell surface hydrophobicity of *Rhodococcus* sp. BAP-1 was tested using the MATH assay. The results obtained (**Fig. 4**) showed no change in the MATH values in the absence of carbon source. When the concentration of the carbon source was 50 mg·L^{-1}, there was a slight increase in the hydrophobicity in the first 72 h (from 0 % to 3.22 %), then it increased dramatically to the highest value of 37.78 % at 120 h, and after that, decreased sharply from 120 h to 168 h. During the stationary phase of growth, from 168 h to 216 h, the cellular hydrophobicity remained constant at around 4.15 %. At fluoranthene concentrations higher and lower than 50 mg·L^{-1} the hydrophobicity curves followed a similar trend, but with lower peak values at 120 h: to 30.6 % at 25 mg·L^{-1} of fluoranthene, 26.93 % at 100 mg·L^{-1}, 18.14 % at 150 mg·L^{-1};

and much less obvious peaks at the lowest fluoranthene concentrations tested (5 mg·L^{-1} and 10 mg·L^{-1}).

Exponential degradation of fluoranthene (**Fig. 5**) started in the first 24 h of cultivaton when the concentration of fluoranthene was 10 mg·L^{-1} and 25 mg·L^{-1}, corresponding to a significant increase in the removal rate from 0 % to 37 % and 45 %, respectively. At the other concentration levels, the removal occurred at a lower rate. In contrast to the other variation trends described above, after drastically increasing in the first 24 h, the degradation rate continued to increase more slowly from 24 h to 168 h, and reached a stationary level at about 192 h. Almost 68 % of the initial fluoranthene was degraded after 216 h in the cultivation medium supplemented with 25 mg·L^{-1} of fluoranthene.

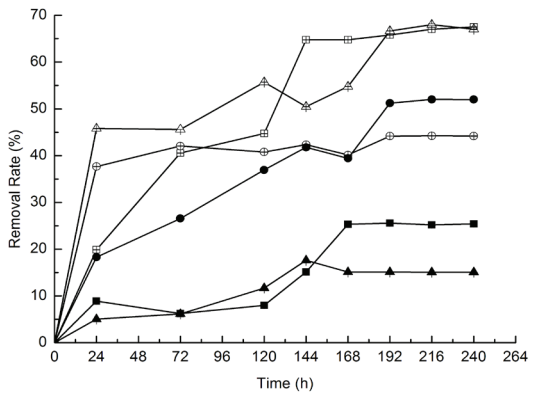

Fig. 5. Biodegradation of fluoranthene by *Rhodococcus* sp. BAP-1. Error bars represent standard deviation. Concentration of fluoranthene: (□) 5 mg·L^{-1}, (○) 10 mg·L^{-1}, (Δ) 25 mg·L^{-1}, (●) 50 mg·L^{-1}, (■) 100 mg·L^{-1}, (▲) 150 mg·L^{-1}

The analysis of the results obtained during the batch cultivation on fluoranthene showed that the surface tension of the culture medium declined with the production of trehaloselipids. For example, the surface tension decreased to the lowest value, 46.63 mN·m^{-1}, when the production of trehaloselipids reached the highest level, 360 mg·L^{-1}, at a carbon source concentration of 150 mg·L^{-1}. In other words, the surface tension is inversely related to the emulsification capacity. This result suggests that the optimal concentration of the carbon and energy source for biosurfactant production is different from that for cell growth. These findings are in agreement with those observed by Shavandi et al. (28) that the carbon source concentration optimal for biomass yield of *Rhodococcus* sp. TA6 was different from that for emulsification capacity of the culture broth.

The lowest surface tension measured in our experiments was 46.63 mN·m^{-1}, which is still much higher than the values reported in our previous study (11). Meanwhile, the highest concentration of biosurfactant production was 360 mg·L^{-1}, which is lower than those previously obtained in the same experimental conditions (11). In addition, almost 68 % of the initial fluoranthene was degraded after 216 h when the medium was supplemented with 25 mg·L^{-1} of fluoranthene, while only 15 % of the initial fluoranthene was degraded when the medium was initially supplemented with 150 mg·L^{-1} of fluoranthene. These results may indicate that during the utilization process of fluoranthene by *Rhodococcus* sp. BAP-1, emulsification is probably not the leading mechanism during the biodegradation process.

Changes of the cell hydrophobicity occurred in the presence of biosurfactant combined with a slightly soluble substrate, in this case, fluoranthene. The increasing of the cell hydrophobicity can promote the adhesion between bacterial cells and hydrocarbon droplets, which can stimulate the degradation process (35). The degradation ability of hydrocarbon by bacteria has been shown to depend on cell affinity, indicating that bacteria with high affinity for hydrocarbon utilization are more effective than those with lower affinity (26). In our experiments, similar results were obtained. *Rhodococcus* sp. BAP-1 cells with high hydrophobicity (30.6 % in the sample with 25mg·L^{-1} of fluoranthene) showed an increased degradation rate of fluoranthene (68 %). However, the dynamics of bacterial cell hydrophobicity were not correlated with the biosurfactant production during the biodegradation process. High cell hydrophobicity increased the contact chance before the initiation of the uptake process and thus, corresponded to high degradation rate. In this case, the pollutants may be accumulating as a confluent biofilm and the uptake systems may have a high specific affinity; i.e. an interfacial uptake mechanism may perform predominantly in the biodegradation process. These results are very important and should be further addressed in future studies.

Fourier transform IR spectroscopy (FT-IR)

The resulting transmission of radiation shown in **Fig. 6** was measured over a frequency spectrum from 400 cm^{-1} to 4000 cm^{-1}. This so-called fingerprint area indicates deformation bands characteristic of a specific molecule and allowed for the chemical substances to be identified from the spectrum files. Additionally, the areas from 1500 cm^{-1} to 4000 cm^{-1} were used to analyze the partial structures by the dilation oscillations, because chemical bonds generate distinct valency oscillation bands (10, 11).

The functional groups observed through FT-IR spectroscopy were a strong O–H stretching and C–H stretching at 3381.25 cm^{-1}; the peak from 3065 cm^{-1} to 2850 cm^{-1} was caused by the stretching vibration of saturated C–H. Bands characteristic at 2958.18 cm^{-1} and 2924.48 cm^{-1} resulted from the –CH_3 stretching mode, whereas, the absorption peak at 2853.28 cm^{-1} was caused by deformation vibration of –CH_2–. The characteristic peak at 1731.67 cm^{-1} was caused by the stretching of ester carbonyl (C=O) and that at 1667.30 cm^{-1} indicated the existence of amide. The peak from 1680 cm^{-1} to 1620 cm^{-1} was caused by the vibration of the benzene ring and that between 1380 cm^{-1} and 1470 cm^{-1} was caused by the C–H bending, just as the strong absorption peaks at 1462.85 cm^{-1} and 1377.26 cm^{-1}. The peak around 1260 cm^{-1} showed the deformation vibration of epoxyethane, as did the peak at 1275.53 cm^{-1}. The characteristic peak at 1190.45 cm^{-1}

indicated the existence of propionate. The peak at 1122.20 cm^{-1} reflected R–O–R stretching and the peak that appeared around 1050 cm^{-1} (1027.82 cm^{-1}) indicated that the structure might be symmetric. The characteristic peak around 970 cm^{-1} (966.64 cm^{-1}) indicated that –H might be in the trans-form. The peak from 900 cm^{-1} to 650 cm^{-1}, as those at 776.36 cm^{-1} and 774.32 cm^{-1}, were caused by the bending of aromatic hydrogen. The presence of these characteristic peaks indicated that the biosurfactant produced by *Rhodococcus* sp. BAP-1 had a glycolipid nature (10, 13).

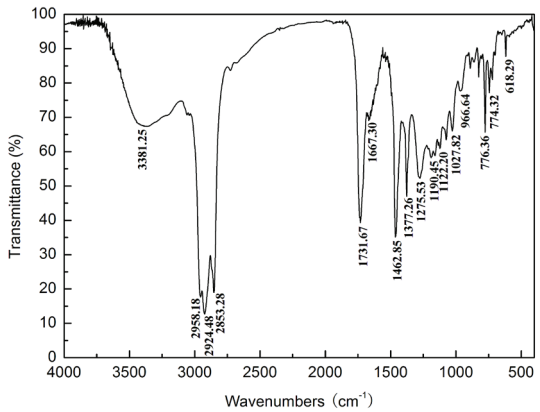

Fig. 6. Fourier transform infrared (FT-IR) transmittance spectrum of the biosurfactant produced by *Rhodococcus* sp. BAP-1.

Ultrastructural investigation of bacterial cells

Rhodococcus sp. BAP-1 cells grown in the LB culture medium and fluoranthene culture medium (5 mg·L^{-1} and 150 mg·L^{-1}) were examined by SEM to observe the surface morphological variation at 168 h of cultivation. The ultrastructural investigation revealed that in the LB culture medium, the length of the cells was between 0.8 μm and 2 μm (**Fig. 7A**), while the length of the cells in the culture media supplemented with 5 mg·L^{-1} and 150 mg·L^{-1} of fluoranthene was from 0.4 μm to 1.2 μm (**Fig. 7B**) and around 1 μm (**Fig. 7C**), respectively. Obviously, the incubation in fluoranthene culture medium appeared to shorten the cells in length, especially when the concentration was 5 mg·L^{-1} (**Fig.7B**). Meanwhile, some blebs could be observed on the surface of cells grown in the presence of 150 mg·L^{-1} of fluoranthene (**Fig. 7C**). Similar changes in the cell morphology have been reported for *Rhodococcus opacus* PD630 treated under water stress and the cells were surrounded by extracellular polymeric substances (EPS) at surfaces (1). Our observations also revealed that the surfaces of cell flocks were relatively smooth in LB culture medium and the 5 mg·L^{-1} fluoranthene culture medium, but those seen in the 150 mg·L^{-1} fluoranthene culture medium were rough. These changes might due to lipopolysaccharide (LPS) release (11), and might be caused by EPS produced by the strains. It could be speculated that the EPS produced in our experiments might be the biosurfactant. It can help the strains to adjust to physiological changes more easily, and allow the bacteria to survive in conditions of dehydration (1), helping to stimulate the uptake process of hydrophobic compounds in a better way.

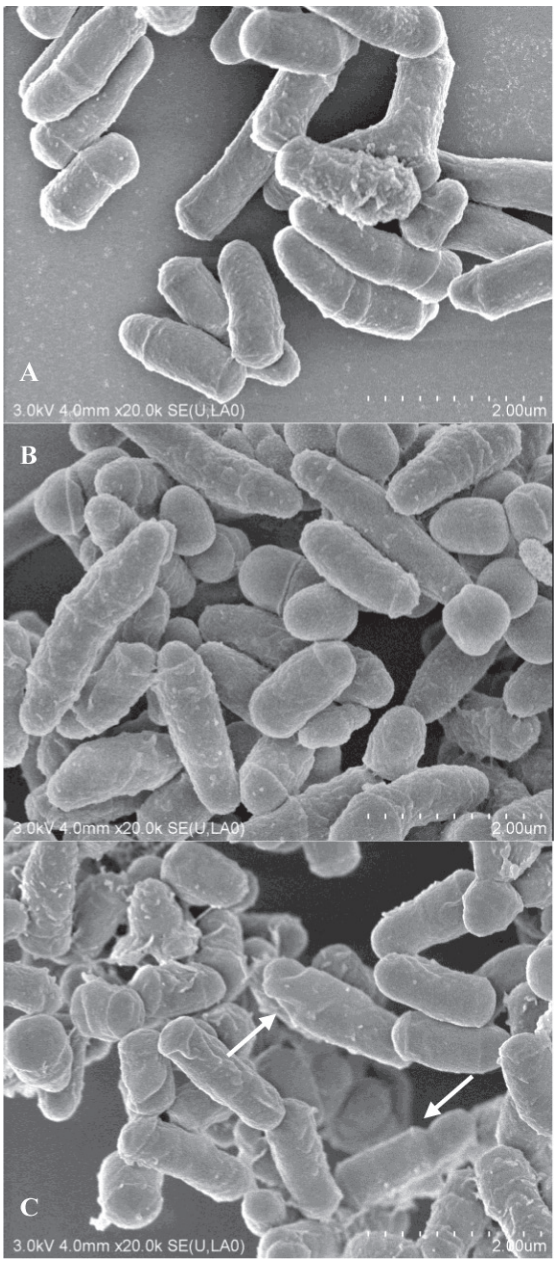

Fig. 7. Scanning electron micrograph of cell morphology of *Rhodococcus* sp. BAP-1 cells grown in: LB culture medium (**A**); medium supplemented with 5 mg·$L^{-1}$of fluoranthene (**B**) and 150 mg·$L^{-1}$of fluoranthene (**C**). Cultivation time: 168 h. Magnification: 20 000 X. Arrows show the disruption of cells.

Disruption of the cell surface in certain zones (**Fig. 7C**) was also observed in some of the cells. These observations indicated that drastic changes of the cell surface morphology might occur under different growth conditions, and cells die in the case of excessive toxicity. Furthermore, *Rhodococcus* cells responded to different growth conditions by changes in the surface ultrastructure, aiming to uptake and assimilate the hydrophobic compounds in a better way (27, 33).

Conclusions

This study aimed to throw more light on the uptake modes of fluoranthene by *Rhodococcus* sp. BAP-1. The strain was capable of growing in a fluoranthene-containing culture medium. High concentrations of the substrates could decrease the contact chance before the uptake process begins. The results also showed that the dynamics trend of the bacterial cell hydrophobicity is not related to the biosurfactant production; however, increasing of the cell hydrophobicity can act to enhance the contact chance between bacteria and hydrocarbon droplets, which can stimulate the biodegradation process. Thus, fluoranthene here may be presented as a confluent biofilm and the interfacial uptake mechanism may perform a predominant role in the uptake process. Further research is required to elucidate the specific structure of the biosurfactant and the mechanism for transport of hydrophobic substrates such as PAHs across the cell membrane.

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