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Uptake and Efflux of Octadecane by *Bacillus* sp. DG24 during the Metabolism Process

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The uptake process, cellular and extracellular distribution of *n*-octadecane during the metabolic process as well as substrate metabolic kinetic were studied by using isotope tracer technique. Bacteria was isolated from crude oil contaminated soil and identified. The results showed that uptake of [^{14}C] *n*-octadecane was immediate when no metabolic process occured. Cellular [^{14}C] increased non-linearly to maximum value at 0.19±0.0022 μmol·L^{-1}, which taken account of 53% of total octadecane in the broth. However, efflux that remove accumulated octadecane from bacterial cell membranes caused the amount of cellular [^{14}C] declined to steady level. Supplement of NaN_3 prevented the influx and efflux of octadecane which indicated that uptake of octadecane was ATP dependent. During metabolic process, total [^{14}C] *n*-octadecane in the broth decreased as time went on, and cellular [^{14}C] took account of 40% to 50% of total [^{14}C]. Kinetic analysis showed that the metabolic of *n*-octadecane was non-linear with an apparent dissociation constant K_m of 9.9926 μmol·L^{-1} and V_{max} of 0.1612 μmol·L^{-1}·h^{-1}. Meanwhile, no efflux of cellular octadecane was observed during the metabolism process for accumulated [^{14}C] *n*-octadecane might be nontoxic to cells of *Bacillus* sp.DG24. All of these findings suggested that *Bacillus* sp.DG24 could mediate cellular *n*-octadecane through efflux to avoid the toxic effect of substrate, which influenced the cellular and extracellular *n*-octadecane distribution obviously before metabolic process begin.

Key words: *Bacillus*, *n*-octadeane, uptake, metabolic, cellular/extracellular, efflux.

Alkanes are saturated hydrocarbons representing the main constituents of mineral oil. They can be linear (*n*-alkanes), circular (cyclo-alkanes), and branched (iso-alkanes), and are virtually insoluble in water. As main components of fuels and oils, they are of outstanding value for modern life, but the relative inertness of alkanes poses ecological problems upon their release to the environment (Wentzel *et al.,* 2007).Studies have shown that some kinds of microorganisms could transport extracellular hydrocarbons across cell membrane before the oxidase became functional. Once inside, the hydrocarbon droplet appeared[1] to be broken down by means of internal cellular projections possibly to increase the surface area for further hexadecane metabolism (Swaranjit *et al.,* 2009). Although how was the hydrophobic compound taken into cell membrane, some studies have shown that there are two uptake types of petroleum hydrocarbons: the passive diffusion without energy and active transport depend on cellular ATP (Miyata *et al.,* 2004). In these studies, different inhibitors like sodium azide, carbonyl cyanide m-chlorophenylhydrazone (CCCP), 2, 4-dinitrophenol, and cyanide were used to study if the uptake process of these hydrocarbons were active or passive diffusion process, and results showed that these structural different inhibitors could reduce the accumulative substrate, which indicated that an energy dependent substrate influx process might be exist in the uptake (Beal *et al.* , 2000; Bugg *et al.,* 2000; Miyata *et al.,* 2004;

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Aristeidis *et al.,* 2007). However, uptake of hydrocarbon was not influenced by ATP inhibitor was also observed which shown that influx process of hydrocarbon could be passive diffusion (Bateman *et al*. 1986). Until now, the growth of microorganisms on hydrocarbons, like hexadecane (Coimbra *et al.,* 2009; Puntus *et al.,* 2005; Kim *et al.,* 2002), phenanthrene (Tecon *et al.,* 2010), octadecane (Chrzanowski *et al.,* 2005), have been much studied well, little is known about the uptake process and cellular/extracellular distribution of alkanes during the whole metabolic process.

On the other hand, many microorganisms can protect themselves from cell injury caused by toxicant by flushing toxicant out of the cell, and this mechanism which is called efflux is a kind of mechanism that enables microorganisms to survive in an adverse environment (Kieboom *et al.,* 1998; Isken *et al.,*1998). In living cells, however, transport of solutes across the cell membrane can be assisted by "pump" proteins that use metabolic energy to transport components against the local concentration gradient (Sonja *et al.,* 1998). Fumiyasu *et al*. (Fumiyasu *et al.,*2008) have shown that a proton motive energy-dependent toluene-transporting system which could pump toluene out from the cytoplasmic membrane into the environment was present in toluene-adapted cells of *Pseudomonas putida*. However, litter is known about the efflux process of octadecane, and whether efflux process existed in the uptake process of hydrocarbon was not clear.

Until now, fewer studies have concentrated on the uptake of alkane into bacterial cells, but some results still can explain the phenomena. In the present study, the uptake and cellular /extracellular distribution of octadecane during substrate metabolic process were studied to investigate the internal relationship between uptake and metabolism of *n*-octadecane as well as the effect of ATP inhibitors on the cellular uptake.

MATERIALS AND METHODS

Microorganisms and 16S rDNA identification

Microbial strains DG24 (CGMCC: NO. 5051; NCBI accession NO.: JN 216879) used in this study were previously isolated from crude oil contaminated soils nearby Da Gang oilfield in China. In the domesticated experiment, which was carried out for 3 times in 30 days as described by Hua and Wang (Hua *et al.,* 2012), cells of DG24 were inoculated in mineral salt medium (MSM) culture medium with crude oil as the sole carbon source. The MSM, sterilized by autoclaving at 121 ! for 20 min, was supplemented with 0.4g Na_2HPO_4, 0.15g KH_2PO_4, 0.1gNH_4Cl, 0.05g $MgSO_4 \cdot 7H_2O$, 0.0015g $CaCl_2$, 0.1g $NaNO_3$, 1 mL trace medium (per 100mL solution containing 0.5mg $CuSO_4 \cdot 5H_2O$, 1.0mg H_3BO_3, 1.0mg $MnSO_4 \cdot 5H_2O$, 7.0mg $ZnSO_4$) in 250 ml capacity baffled flasks. The culture were maintained at 4° on crude oil solid medium and transferred monthly.

The DNA of strain DG24 was extracted using DNA extracted kit (Biomand, Beijing, China), and quantified by comparing with the DNA marker (Dingguo, Beijing, China), and observed by Gel documentation system (G: Box, syngene, British). Then, the DNA product was used as template for PCR amplification of 16S rDNA as described by Hua and Wang (2012). The product of garose gel electrophoresis was observed by Gel documentation system (G: Box, syngene, Britain). The phylogenetic relationship of the strain has been shown in Fig. 1 with MEGA 4.0 software (Tamura *et al.,* 2007).

Intra/extra cellular [^{14}C] hydrocarbon distribution

Uptake of *n*-octadecane by DG24 was assayed by the methods of (Miyata *et al.,* 2004; Whitman *et al.,* 1998). Cells were harvested at the exponential phase, washed with phosphate buffer (0.1 M, pH 7.0), and re-suspended in the same MSM essentially as described above. [^{14}C] *n*-octadecane (ARC 1261, 99% pure, S.A. 55 mCi·mmol^{-1}, 100μCi·ml^{-1}) was added as methanol stock. For metabolic assay, 15 μL of methanol solution of [^{14}C] *n*-octadecane (25μCi·ml^{-1}) was added to 100 mL of flasks containing 30 mL of cell suspend MSM with a final concentration at 0.227μmol·L^{-1}. Control group that was not added with cells of DG24 was also conducted to determine the extent of abiotic loss of *n*-octadecane. The flasks were incubated at room temperature with shaking at 140 rpm. Samples were taken at regular intervals over 96 hours. 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 phosphate buffer for 3 times. The filter with cells was added to scintillation fluid (Nonylfenolethoxylate, 9016-45-9, perkinelmer) and

the radioactivity was measured by a PerkinElmer liquid scintillation counter (WallacOy 1450 MicroBeta). This part of [^{14}C] was taken as cellular *n*-octadecane in the supernatant. Meanwhile, another 0.5-mL aliquot was directly removed for radioactivity analysis as total [^{14}C] *n*-octadecane in the broth. Extracellular ^{14}C level was calculated as follows: Total [^{14}C] in the broth- cellular [^{14}C] in the filters. For [^{14}C] *n*-octadecane metabolism analysis, the residue [^{14}C] *n*-octadecane in 0.5-mL aliquot of culture broth was analyzed. For each time, three independent determinations were made for standard deviation analysis.

Uptake of [^{14}C] *n*-octadecane assay

To study the cellular and extracellular [^{14}C] *n*-octadecane distribution before metabolism and uptake process of [^{14}C] *n*-octadecane by *Bacillus* sp.DG24, effects of ATP inhibitor azide sodium on the uptake of [^{14}C] were assayed by the method of Miyata *et al*. (2004). All glassware was treated with nitric acid to minimize the adsorption of octadecane. Methanol solution of [^{14}C] *n*-octadecane (25μCi·mL^{-1}) was added to 100 mL of flasks containing 30mL of cell suspended MSM at room temperature with final concentration at 0.227μmol·L^{-1}. Control group flask that was not added with inhibitors was also conducted on. For energy dependent uptake assay, 30 mmol·L^{-1}of sodium azide, an inhibitor of the electron-flow chain in oxidative phosphorylation, were added in the flasks at 5 and 14 min, respectively. Final cell density (A_{600}) was both controlled at 0.45. Uptake assay began when cells of *Bacillus* sp.DG24 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. No metabolism was observed during the incubation time. Thus, initial *n*-octadecane supplemented in the medium was considered to be a constan tvalue. Cellular and extracellular [^{14}C] radioactivity were determined as demonstrated above. The standard deviations based on three samples for each time.

Metabolic kinetic analysis of [^{14}C] *n*-octadecane

To verify the metabolism production of [^{14}C] *n*-octadecane, we performed $^{14}CO_2$ detection as described by Whitman *et al.* (1998) with some modifications. The uptake and metabolism were conducted in 16×70 mm teflon-lined screw-capped culture tubes containing 5 mL of cell suspend MSM (final A_{600} was 0.45) and a 6×50mm Durham tube with 800 μL freshly prepared 10 mol·L^{-1} of NaOH simultaneously. Reaction begins with different final concentration of [^{14}C] *n*-octadecane ranging from 0.096 to 45.16 μmol·L^{-1} added in the tubes. The reaction was stopped after 48 h of incubation at room temperature, by injecting 20 μL 95% H_2SO_4. Tubes were kept for 30 min after the acid addition to allow absorption of the $^{14}CO_2$ by the NaOH. Samples were analyzed at 0 and 48h, respectively. Total [^{14}C] *n*-octadecane was analyzed as described above. The biodegradability of [^{14}C] *n*-octadecane was calculated as: (initial total [^{14}C] in the broth-total [^{14}C] in the broth after incubation for 48h) / initial total [^{14}C] in the broth ×100%. For metabolic kinetic analysis, the apparent affinity constant (K_m) and the maximum velocity (V_{max}) were estimated by nonlinear regression analysis based on the fit of the data to the Michaelis-Menten equation: $V=V_{max}\cdot[S]/(K_t + [S])$, where V (μmol·L^{-1}·h^{-1}) is the biodegradation rate, V_{max} (μmol·L^{-1}·h^{-1}) is the maximum value of biodegradation rate, K_m (μmol·L^{-1}) is the equilibrium dissociation constant (substrate concentration yielding 1/2 V_{max}), and [S] (μmol·L^{-1}) is the initial substrate concentration. The standard deviations based on three samples for each time. Previous studies have shown that the abiotic loss of *n*-octadecane for each sample was lower than 6.15%.

RESULTS

Microorganism identification

The almost complete sequence (1435 base) of this strain DG24 was determined, which amounts to more than 90% of the 16S rDNA gene. Initial analysis of sequence was done at NCBI, where relevant sequences from the databases were downloaded for further analysis. The sequence showed 99.9% sequence similarity with *Bacillus* strain. In the phylogenetic analysis, it clustered with the first 20 strains in the NCBI thus confirming its identity as *Bacillus* sp.DG24.This culture is maintained in China General Microbiological Culture Collection Center (CGMCC: NO. 5051; NCBI accession NO.: JN 216879).

Uptake of [^{14}C] *n*-octadecane

Uptake of *n*-octadecane by *Bacillus* sp. DG24 in the presence or absence of ATP inhibitor when metabolic of octadecane did not begin was shown in Fig. 2. In the absence of inhibitor, an immediate uptake was observed at 1 min with

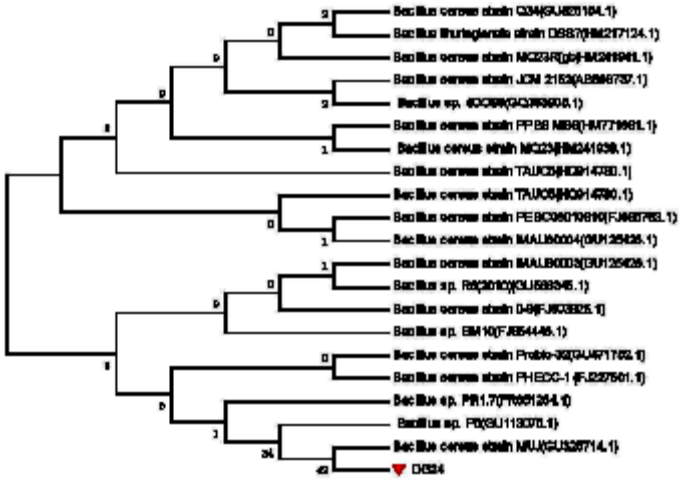

Fig. 1 Phylogenetic tree of *Bacillus* sp.DG24 and related strains based on 16S rDNA sequences with MEGA 4.0 software

cellular [^{14}C] *n*-octadecane at 0.082±0.0015 µmol·L^{-1}, and accumulative *n*-octadecane increased to 0.19±0.0022 µmol·L^{-1} at 14 min, which indicated that cellular octadecane taken account of 53% of total octadecane in the broth. From 8 to 17 min, cellular *n*-octadecane was more than extracellular ones, and uptake of *n*-octadecane was against concentration gradient. However, cellular *n*-octadecane declined dramatically at 20min, and achieved at stable level at around 0.12±0.0072 µmol·L^{-1} at 60 min. Finally, cellular and extracellular took account of 33.33% and 66.67% of total [^{14}C] *n*-octadecane, respectively. During the incubation time, substrate that accumulated cellular was transported out of cell membrane against concentration gradient. On the other hand, it was found that uptake of substrate by *Bacillus* sp. DG24 was inhibited after 30mmol·L^{-1} of azide was supplemented at 5 min. After incubation for 5min, cellular *n*-octadecane increased to 0.12±0.0042 µmol·L^{-1} after which accumulated [^{14}C] was almost kept at stable level. Cellular [^{14}C] *n*-octadecane was 0.12±0.0026 µmol·L^{-1} which accounted for 33.33% of total [^{14}C] *n*-octadecane in the medium after incubation for 60 min. This result showed that influx of [^{14}C] *n*-octadecane was related with cellular and extracellular distribution of octadecane. In this case, ATP was needed to avoid substrate concentration gradient. Meanwhile, cellular [^{14}C] *n*-octadecane seemed was kept at constant value and efflux process was not obviously observed after NaN_3 was added at 14 min. This result suggested that efflux of octadecane was also dependent on ATP.

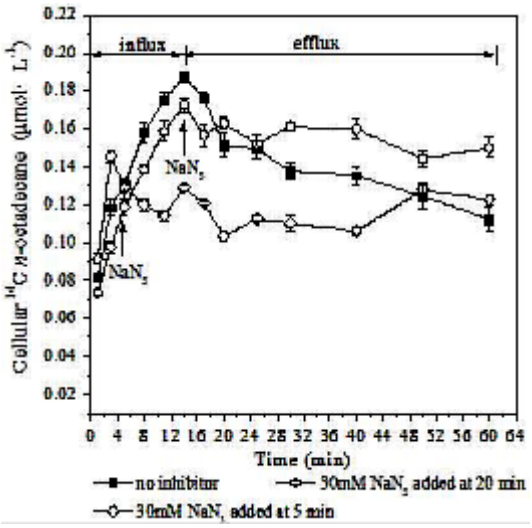

Fig. 2 Effect of ATP inhibitor on the uptake of [^{14}C] *n*-octadecane by *Bacillus* sp. DG24.Substrate concentration was 0.36 ìmol·L^{-1}. Cells in the absence of inhibitor (*filled squares*), cells treated with 30mmol·L^{-1}of NaN_3 added at 5 min (*filled diamonds*), and cells treated with 30mmol·L^{-1}of NaN_3 added at 0 min (*filled circles*). Cell protein was 15.36 µg·mL^{-1} during the incubation time (60 min). Standard deviations were lower than 0.0064 µmol·L^{-1}

[^{14}C] distribution during metabolic process

[^{14}C] *n*-octadecane metabolism was shown in Fig. 3. The distribution of radiolabel cellular [^{14}C] and extracellular [^{14}C] were analyzed during the metabolism process. During the first 1h, , extracellular octadecane was transported across cell membrane, and total [^{14}C] *n*-octadecane in the broth was considered to be not change for metabolism of octadecane did not happen. For instance, cellular [^{14}C] octadecane increased to 0.100±0.00351 umol·L^{-1} after incubation for 0.5h, and took account of 42.74% of total [^{14}C] *n*-octadecane was. After incubation for 3h, ttotal [^{14}C] *n*-octadecane in the culture broth decreased from 0.231±0.0185 to 0.22±0.013 umol·L^{-1} with a metabolic rate of 0.367%. Moreover, it was found that cellular [^{14}C] *n*-octadecane decreased along with the metabolic of total [^{14}C] in the broth. For example, when cell growth entered into exponential phase at 24h, cellular [^{14}C] *n*-octadecane decreased to 0.0475±0.00281 umol·L^{-1}. After incubation for 96h, the residue *n*-octadecane in the medium declined to 0.0498±0.00241 umol·L^{-1} with a metabolic rate of 78.42%, and cellular [^{14}C] octadecane declined to 0.021±0.010 umol·L^{-1}, which took account for 42% of total ^{14}C in the broth. It must be pointed out that concentration of extracelluar [^{14}C] was always higher than that of cellular [^{14}C]. In general, percentage of cellular [^{14}C] *n*-octadecane which took account of total substrate in the broth changed from 40% to 50%. Ratio between cellular and extracellular substrate did not have obvious change during metabolic process. Thus, it was inferred that octadecane was cellularly metabolized along with the uptake of extracellular octadecane by cells of DG24 at the same time. This result revealed that metabolic rate was faster than the uptake rate of octadecane, which could explain the loss of cellular [^{14}C] well.

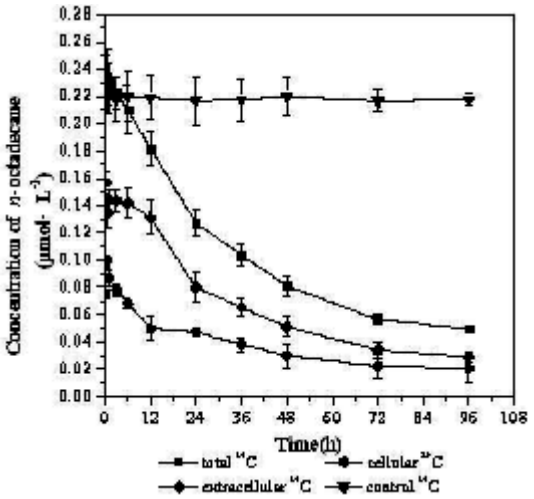

Fig. 3. Distribution of cellular and extracellular [^{14}C] *n*-octadecane in cells of *Bacillus* sp. DG24. Total [^{14}C] in the broth (*filled squares*); total [^{14}C] in the control group (*filled triangles*); cellular [^{14}C] (*filled circles*); extracellular [^{14}C] (*filled diamonds*).Cell protein was 15.36 μg·mL^{-1}. Standard deviations were lower than 0.0204 μmol·L^{-1}

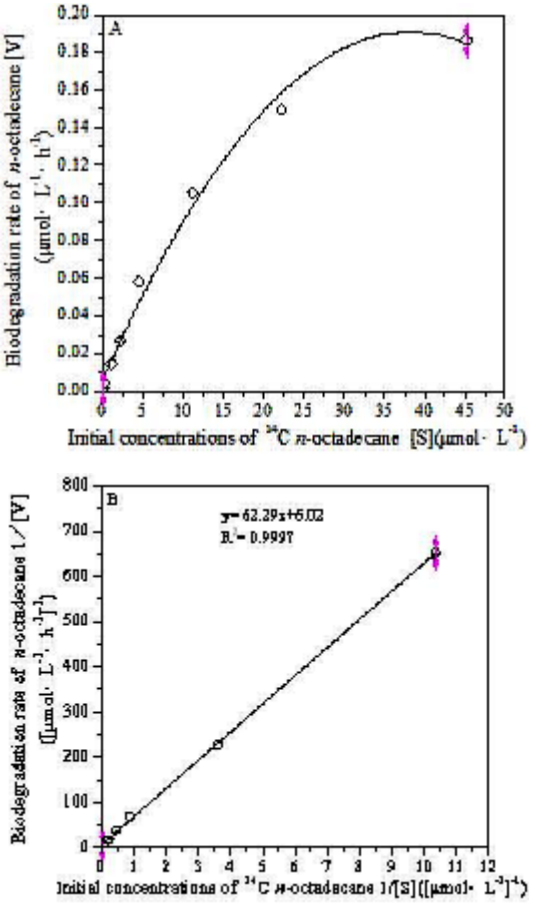

Fig. 4. Relationship between [^{14}C] *n*-octadecane metabolic rate and initial *n*-octadecane concentrations by *Bacillus* sp. DG24 (A). The solid line shows the nonlinear regression of the data for octadecane metabolic kinetic parameter K_m=9.9926 μmol·L^{-1}, and V_{max} =0.1612 μmol·L^{-1}·h^{-1}, R^2=0.9997 (B)

Metabolic Kinetics

Metabolic kinetic of the *n*-octadecane by *Bacillus* sp. DG24 (determined between 0 and 48 h) at external *n*-octadecane concentrations ranging from 0.096 to 45.16 μmol·L^{-1} was shown in figure 4. After incubation for 48h, average metabolic rate of [^{14}C] *n*-octadecane reached to 0.0015 to 0.19 μmol·L^{-1}·h^{-1} (Fig.4-A). The data obtained are presented in the form of a Lineweaver-Burk plot which showed Michaelis-Menten kinetics (R^2= 0.9979) with a K_m value of 9.9926 μmol·L^{-1} and a V_{max} of 0.1612 μmol·L^{-1}·h^{-1} (Fig.4-B).

DISCUSSION

The [^{14}C] isotope results indicated that the initial partitioning of *n*-octadecane into cell was very quick. When no metabolic process occurred, cellular octadecane increased non-linearly even against concentration gradient, and uptake was inhibited by NaN_3. Similarly, for cells of *Arthrobacter* sp. Strain Sphe3, phenanthrene was transported into membrane via phenanthrene-inducible active transport mechanism, and uptake process was saturable followed Michaeis-Menten kinetic (Aristeidis *et al.,* 2007). The addition of proton conductor CCCP resulted in a reduction of *n*-hexadecane uptake by *Pseudomonas aeruginosa* UO299, which indicated that the uptake was driven by an energy-dependent system (Beal and Betts, 2000). What's more, Miyata *et al*. (2004) found that PAH transport in *Mycobacterium* sp. strain RJGa!-135 was a saturable, energy-dependent system. However, other kind of bacteria could uptake the hydrophobic compound through passive transport. For instance, Bateman *et al*. (1986) found that the uptake of naphthalene by *Pseudomonas putida* strain PpG1064 was a process that not associated with an active transport system but with diffusion, for the uptake was not susceptible to ATP inhibitors. Moreover, the lack of saturation of phenanthrene uptake by LP6a-1 in the presence of inhibitory levels of azide suggest that uptake into the cell membranes by diffusion dominates the partitioning process (Bugg, 2000). In our study, *Bacillus* sp. DG24 could still accumulate substrate even against concentration gradient, which could be taken as an evidence for ATP dependent active uptake of octadecane. While, the uptake process stopped after incubation for about 14 min, and this phenomena was not reported in the phenanthrene (Aristeidis *et al.,* 2007), hexadecane (Beal and Betts, 2000) or naphthalene (Miyata *et al.,* 2004) uptake process. In this study, accumulated hydrocarbons might have toxic effect on microorganism. Thus, living cells pumped part of cellular octadecane out of cell membrane to protect themselves. It was inferred that *Bacillus* sp. DG24 could mediate distribution of cellular and extracellular toxic hydrocarbon until metabolism of octadecane did happen. While, for cells treated with NaN_3 at 14 min, cellular and extracelluar octadecane were both at equilibrium level, and no efflux process was observed. Similarly, For *P. fluorescens* LP6a, phenanthrene partitioned into the cell membranes by passive diffusion and was transported back into the medium by an active efflux pump. The efflux pump maintained the intracellular concentration of phenanthrene below its equilibrium level. When this pump was inhibited, the phenanthrene concentrations in the pellet approached equilibrium levels. Similarly, the efflux pump maintained the intracellular concentration of phenanthrene below its equilibrium level (Nikaido, 1996) was also observed. Meanwhile, the ability of *P. putida* to export toluene by an efflux mechanism was inhibited by CCCP and cyanide, so that the levels of toluene in the cell pellet rose to the same levels as those in cells without efflux capability (Isken and de Bont 1996) For LP6a strain, it was found that that there is a degree of selectivity in the active efflux pump. The more hydrophobic PAHs like phenanthrene, fluoranthene, and anthracene were transported out of the cells by efflux, whereas the more water-soluble compound naphthalene was not. The preferred growth substrate for is naphthalene (Foght *et al.,* 1996). All of these studies inferred that efflux might be active and specific.

For total [^{14}C] analysis in the broth, metabolic of *n*-octadecane was not a linear process. In our study, metabolic of *n*-octadecane by *Bacillus* sp. DG24 followed Michaelis-Menten kinetics (R^2= 0.9979) with a K_m value of 9.9926 μmol·L^{-1} and a V_{max} of 0.1612 μmol·L^{-1}·h^{-1} (Fig.4-B). During metabolism process, cellular [^{14}C] octadecane was always lower than extracellular ones. Thus, it was assumed that if the metabolic rate was equal to that of the uptake rate of *n*-octadecane, cell pellet concentration might be

maintained at a steady state level. However, the data in our experiment was inconsistent with the assumption. The cell pellet concentration declined with the mineralization of cellular *n*-octadecane, which revealed that metabolism was fast enough to maintain a low cellular *n*-octadecane level (Miyata *et al*. 2004). Alvarez *et al*. (Alvarez *et al.,*1997) found that accumulated hydrocarbons could form transparent inclusions in cell membrane, and composition of the lipid inclusions depended on the compounds provided as carbon source. Alkane-utilizing microorganisms tended to accumulate fatty acids with chains equivalent in length to those of the alkane substrate (Gyorgy *et al.,* 2001; Stelmack *et al.,*1999). Furthermore, percentages of cellular and extracellular octadecane taken accounted for total octadecane did not have obvious changes which demonstrated that uptake of octadecane during metabolic process was also related with concentration of extracellular substrate. For example, phenol could enter the cells of degrading bacteria through active transport when the concentration of phenol was less than 50 $mg \cdot L^{-1}$, but in higher concentrations, the bacteria transported phenol into the cells by passive transport (Shishido at al., 1996). On the other hand, cellular octadecane decreased although biomass of *Bacillus* sp. DG24 increased along with octadecane metabolism. Thus, it was inferred that toxic effect of hydrocarbon on cells decreased also along with the decline of accumulated octadecane, and there was no need to start efflux function of *Bacillus* sp. DG24 during metabolic process. Studies have shown that Gram-negative bacteria, such as *Pseudomonas* sp., have two distinct cellular membranes separated by the periplasm. The efflux protein complex spans the inner and outer cell membranes and serves to remove compounds either from the cytoplasm or from the inner boundary of the inner membrane and to export them into the medium outside the cell (de Bont *et al.,* 1998). Murray *et al*. (Murry *et al.,*2001) found that selective efflux of one compound versus another controls the selectivity of the reaction by intracellular enzymes provided that the rate of efflux is significantly greater than the rate of permeation across the cell membranes. In this case, efflux process of alkanes must be considered when understanding the uptake mechanism. Until now, whether the influx and efflux of octadecane or other hydrocarbons were related with cell membrane of Gram-positive bacteria was not clear. Some studies which have explained the relationship between gen and efflux mechanism of bacteria could provide hits to resolve this problem. Disruption of the *emhB*gene in *P. fluorescens*c LP6a-1 proved that the gene product was responsible for the proton-dependent efflux of phenanthrene. The EmhB protein also is involved in the active efflux of anthracene and fluoranthene, although additional active efflux mechanisms may be present in *P. fluorescens*c LP6a to transport these hydrocarbons since disruption of *emhB*did not completely eliminate the efflux (Elizabeth *et al.,* 2003). Furthermore, Laila *et al*. (Laila *et al.,*2009) found that fluorinated lipids were capable of entering living cells through endocytosis. They are furthermore able to facilitate the entry of non-covalently bound proteins. The delivery agents were nontoxic and were distributed both on the surface and in the cytoplasm of cells but not in the nucleus. The report of time dependent of the influx and efflux of phenol indicated that the outer membrane has narrow channels made up of protein molecules. The efflux transporter molecule connects with the outer membrane channel protein via an accessory protein present in the periplasm. The phenol molecules can be expected to enter the bacterial cell through the porin channels resulting in their uptake. Once phenol starts accumulating inside the cell, the efflux protein flushes them out into the periplasm and then to the extra-cellular medium with the help of the accessory proteins and the porin proteins (Sharma *et al.,* 2002).

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REFERENCES

1. Alvarez HM, Pucci OH, Steinbuchel A., Lipid storage compounds in marine bacteria. *Appl. Microbiol. Biotechnol.* 1997; **47**: 132-139.
2. Aristeidis K, Stathis F, Constantin D, Anna IK., Taxonomic identitication, phenanthrene uptake activity, and membrane lipid alterations of the PAH degrading *Arthrobater* sp. Strain Sphes.

Applied Microbial and Cell Physiology.2007; **76**: 709-717.

3. Bateman JN, Speer BL, Feduik L, Hartline RA., Naphthalene association and uptake in *Pseudomonas putida*. *J. Bacteriol.*1986; **166**: 155-161.
4. Beal R, Betts WB., Role of rhamnolipid biosurfactants in the uptake and mineralization of hexadecane in *Pseudomonas aeruginosa*. *J. Appl. Microbiol*. 2000; **89**: 158-168.
5. Bugg T, Foght JM, Pickard MA, Gray MR., Uptake and active efflux of polycyclic aromatic hydrocarbons by *Pseudomonas fluorescens* LP6a. *Appl. Environ. Microbiol*. 2000; **66**: 5387-5392.
6. Chrzanowski L, KaczorekE, Olszanowski A., Relation between Candida maltosa hydrophobicity and hydrocarbon biodegradation. World *Journal of Microbiology and Biotechnology.* 2005; **21**: 1273-1277.
7. Coimbra CD, Rufino RD, Luna JM, Sarubbo LA., Studies of the Cell Surface Properties of Candida Speciesand Relation to the Production of Biosurfactants. *Curr. Microbiol*. 2009; **58**: 245–251.
8. de Bont, Jan AM., Solvent-tolerant bacteria in biocatalysis. *Trends in Biotechnology*, 1998; **16**: 493-499.
9. Elizabeth MH, Jonathan JD, Murray RG, Julia MF., Identification and Characterization of the emhABC Efflux System for Polycyclic Aromatic Hydrocarbons in *Pseudomonas fluorescens* cLP6a. J. *Bacteriol.* 2003; **185**: 6233-6240.
10. Foght JM, Westlake DS., Transposon and spontaneous deletion mutants of plasmid-borne genes encoding polycyclic aromatic hydrocarbon degradation by a strain of *Pseudomonas fluorescens*. *Biodegradation*, 1996; **7**: 353-366.
11. Fumiyasu F, Hisako H, Hideto T., Isolation and transposon mutagenesis of a *Pseudomonas putida* KT2442 toluene-resistant variant:involvement of an efflux system in solvent resistance. *Extremophiles*, 2008; **2**: 395-400.
12. Gyorgy B, Gyorgy V, Istvan M S, Andras P. Sandor D, Janos S, Janos M, Tadashi H, Anita M, Istvan S., n-Alkane uptake and utilisation by *Streptomyces* strains. *Antonie van Leeuwenhock*, 2001; **79**: 269-276.
13. Hua F, Wang H., Uptake modes of octadecane by *Pseudomonas* sp. DG17 and synthesis of biosurfactant. *J. Appl. Microbiol*. 2012; **112**: 25–37.
14. Isken S, De Bont JAM., Active efûux of toluene in a solvent-resistant bacterium. *J. Bacteriol*. 1996; **178**: 6056–6058.
15. Kim IS, Foght JM, GrayMR., Selective transport and accumulation of alkanes by *Rhodococcus erythropolis* S+14He. *Biotechnology and Bioengineering.* 2002; **80**: 650-659.
16. Kieboom J, Dennis JJ, Zylstra GJ, de Bont JAM., Active efflux of organic solvents by *Pseudomonas putida* S12 is induced by solvents. *Bacteriol*, 1998; **180**: 67-72.
17. Laila D, Venkateshwarlu K, Anthony Kar LL, Krishna K., Fluorinated Lipid Constructs Permit Facile Passage of Molecular Cargo into Living Cells. *J. Am. Chem. Soc*. 2009; **131**: 12091-12093.
18. Miyata N, Iwahori K, Foght JM,Gray MR., Saturable, energy-dependent uptake of phenanthrene in aqueous phase by *Mycobacterium* sp. strain RJGII-135.*Appl. Environ. Microbiol*. 2004; **70**: 363-369.
19. Murray R, Gray MR, Bugg T., Selective biocatalysis in bacteria controlled by active membrane transport. *Ind. Eng. Chem. Res*. 2001; **40**: 5126-5131.
20. Nikaido H., Multidrug efflux pumps of Gram-negative bacteria. *J. Bacteriol*. 1996; **178**: 5853-5859
21. Puntus IF, Sakharovsky VG, Filonov AE, Boronin AM., Surface activity and metabolismof hydrocarbon-degrading microorganisms growing on hexadecane and naphthalene. *Process. Biochem*. 2005; **40**: 2643-2648.
22. Sharma A, Kachroo D, Kumar R., Time dependent influx and efflux of phenol by immobilized microbial consortium. *Environmental Monitoring and Assessment*, 2002; **76**: 195-211.
23. Shishido M, Toda M., Apparent zero-order kinetics of phenol biodegradation by substrate-inhibited microbes at low substrate concerntrations. *Biotechnol. Bioeng.* 1996; **50**: 709-717.
24. Sonja I, Jan AM, de Bont., Bacteria tolerant to organic solvents .*Extremophiles*, 1998; **2**: 229-238.
25. Stelmack PL, Gray MR, Pickard M A., Bacterial adhesion to soil contaminants in the presence of surfactants.*Appl. Environ. Microbiol*. 1999; **65**: 163-168.
26. Swaranjit SC, Pooja S., Synthesis of rhamnolipid biosurfactant and mode of hexadecaneuptake by *Pseudomonas species*. *Microbial Cell Factories*, 2009; **8**: 1-7.
27. Tamura K,Dudley J,Nei M,Kumar S., MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution.* 2007; **24**(8).

28. Tecon R, van der Meer JR., Effect of two types of biosurfactants on phenanthrene availability to the bacterial bioreporter *Burkholderia sartisoli* strain RP037. *Appl. Microbiol. Biotechnol*.2010; **85**: 1131-1139.
29. Wentzel A, Ellingsen TE, Kristian KH, Zotchev SB, Throne HM., Bacterial metabolism of long-chain n-alkanes. *Appl. Microbiol. Biotechnol.* 2007; **76**: 1209-1221.
30. Whitman BE, Lueking DR, MihelcicJR., Naphthalene uptake by a *Pseudomonas fluorescens* isolate. *Can. J. Microbiol.* 1998; **44**: 1086-1093.