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Effect of additional carbon source on naphthalene biodegradation by *Pseudomonas putida* G7

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Abstract

Addition of a carbon source as a nutrient into soil is believed to enhance in situ bioremediation by stimulating the growth of microorganisms that are indigenous to the subsurface and are capable of degrading contaminants. However, it may inhibit the biodegradation of organic contaminants and result in diauxic growth. The objective of this work is to study the effect of pyruvate as another carbon source on the biodegradation of polynuclear aromatic hydrocarbons (PAHs). In this study, naphthalene was used as a model PAH, ammonium sulfate as a nitrogen source, and oxygen as an electron acceptor. *Pseudomonas putida* G7 was used as a model naphthalene-degrading microorganism. From a chemostat culture, the growth kinetics of *P. putida* G7 on pyruvate was determined. At concentrations of naphthalene and pyruvate giving similar growth rates of *P. putida* G7, diauxic growth of *P. putida* G7 was not observed. It is suggested that pyruvate does not inhibit naphthalene biodegradation and can be used as an additional carbon source to stimulate the growth of *P. putida* G7 that can degrade polynuclear aromatic hydrocarbons.
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Keywords: Inhibition; Diauxic growth; Pyruvate; Naphthalene; Bioremediation

1. Introduction

Polynuclear aromatic hydrocarbon (PAH) compounds are common groundwater contaminants and highly carcinogenic chemicals [1]. Bioremediation has been studied extensively in the last two decades as a means removing PAHs, especially from contaminated soils [2,3]. Numerous strains of soil microorganisms that are capable of degrading PAHs have been isolated and characterized. The following strains are known to degrade naphthalene, which is one of the most studied PAHs: *Pseudomonas putida* G7, *Rhodococcus* sp. strain B4, *Oscillatoria* sp. strain JCM, and *Alcaligenes* sp. strain NP-Alk [2–6].

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Various technologies and procedures of bioremediation are currently being used, and a number of promising approaches have been suggested. Some of these technologies are for in situ bioremediation, in which subsurface pollution is degraded without the need to capture and to deliver contaminants to an above-ground treatment system [7]. In situ bioremediation is based on stimulating the growth of microorganisms, primarily bacteria that are indigenous to the subsurface and that can degrade contaminants [7,8]. When supplying oxygen (or other electron acceptors) and nutrients, microorganisms can degrade a number of soil and groundwater contaminants including PAHs, to carbon dioxide and water [9]. The added nutrients are often nitrogen and phosphorus when the contaminants are composed mainly of hydrocarbons. Addition of a carbon source as a nutrient will increase the biomass of soil microorganisms, but may inhibit the biodegradation of contaminants and result in diauxic growth. This has been observed in the culture of *Eschericia coli* in a medium containing two carbon sources, one of which is more preferable to *E. coli* metabolism [9].

The objective of this work is to study the effect of a carbon source added as a nutrient on the biodegradation of polynuclear aromatic hydrocarbons (PAHs). Pyruvate and naphthalene were used as a model carbon source and a model PAH, respectively. Pyruvic acid, which is named as 2-oxopropanoic acid by IUPAC system of nomenclature, is quite soluble in water. Its melting point and boiling point under 1 atm are 11.8 and 165 °C, respectively [10]. *P. putida* G7 was used as a model naphthalene-degrading microorganism. Ammonium sulfate and oxygen were added into a culture medium as a nitrogen source and an electron acceptor, respectively. If pyruvate were a prefereable carbon source and inhibited the biodegradation of naphthalene until the depletion of pyruvate, diauxic growth would be observed [11]. Batch culture experiments were performed to see if diauxic growth was present. A preculture of *P. putida* G7 on pyruvate or naphthalene was transferred to a new medium containing both pyruvate and naphthalene. Concentrations of pyruvate and naphthalene in the new medium should be those allowing *P. putida* G7 similar growth rates when either of them is the sole carbon source. Growth kinetics of *P. putida* G7 on naphthalene was available from the work of Ahn et al. [12]. In this study, growth kinetics of *P. putida* G7 on pyruvate was determined from a chemostat culture of *P. putida* G7 using pyruvate as a carbon and energy source. Yield coefficients for pyruvate, ammonium sulfate, and oxygen were also estimated from batch culture experiments to determine the increase of biomass from the consumption of these nutrients. It is important to add the optimal amount of nutrients for in situ bioremediation from the knowledge on the growth kinetics and yield coefficients [8]. For instance, the addition of nutrients less than the optimal amounts would result in a slow biodegradation, and the addition of nutrients more than the optimal amounts might clog the pores in the soil due to the excessive increase of biomass, thereby causing cessation of the bioremediation.

2. Materials and methods

2.1. Bacterial strain and culture medium

P. putida G7 was used in all experiments [13,14]. The minimal medium for the culture of *P. putida* G7 [15] contained 3.0 g of K_2HPO_4, 1.5 g of KH_2PO_4, 0.01 g of NaCl, 0.1 g of $MgSO_4$, 0.001 g of $FeSO_4{\cdot}7H_2O$ and 1.0 g of $(NH_4)_2SO_4$ per liter of solution. The pH

of the medium was 7.0. For batch growth experiments using naphthalene, naphthalene was added 1 day prior to addition of the seed culture. To prepare minimal medium plates, noble agar (Difco, Detroit, MI, USA) was added at the level of 1.5% (w/v). All chemicals used for the culture media were of analytical grade.

2.2. Analytical methods

Cell growth was monitored by absorbance measurements of bacterial cultures at 600 nm optical density (OD_{600}), which were obtained using a Shimadzu UV160U Spectrophotometer and referenced to distilled water. To correct for the absorbance produced by dissolved extracellular material, samples were filtered through a 0.2 μm PTFE filter (Nalge Co., Rochester, NY, USA) to remove cells, and the filtrate's absorbance at 600 nm was subtracted from that for the unfiltered sample.

Pyruvate in the culture medium was analyzed using a pyruvate assay kit (Sigma Chemical Co., St. Louis, MO, USA). Concentrations of ammonia and oxygen were measured by an ammonia specific ion electrode (Orion Research Inc., Boston, MA, USA) and an oxygen electrode (Microelectrodes Inc., Bedford, NH, USA), respectively. Naphthalene in the culture medium was analyzed by high-performance liquid chromatography (Hewlett-Packard series 1050) using an ultraviolet detector (Hewlett-Packard Co.). Separations were performed on an Hypersil Reversed-Phase HPLC column (Hewlett-Packard Co. [2.1 by 100 mm]). Samples were eluted under isocratic conditions with 50% acetonitrile (HPLC grade) and 50% water (HPLC grade) at a constant flow rate of 1 ml/min. Naphthalene was detected by setting the ultraviolet wavelength at 254 nm. The naphthalene detection limit was 0.3 mg/l.

2.3. Preculture

Two seed cultures were prepared on minimal medium plates. One seed culture was grown on plates saturated with naphthalene vapor in a desiccator and the other was grown on plates containing 0.1% (w/v) sodium pyruvate, which corresponded to 0.78 g of pyruvate per liter. The seed cultures were stored at 5 °C. Fresh inoculum was prepared by placing colonies from the seed culture plates into a 250 ml flask with 50 ml of fresh minimal medium containing 0.1 g of solid naphthalene or 0.1% (w/v) sodium pyruvate. Cells were pregrown aerobically to an optical density (at 600 nm) of 0.4. The cells were then harvested and washed twice with fresh medium without a carbon source before being used as an inoculum. Levels of the inocula were always lower than 1% and carry-over of carbon source from seed cultures should be negligible.

2.4. Batch culture

Cells were cultured with shaking (New Brunswick G24 Environmental Incubator Shaker) at 175 rpm in 250 ml baffled flasks containing 50 ml minimal medium. The incubator temperature was maintained at 24 °C.

The presence of diauxic growth was determined by the presence of the lag period during the batch culture in the medium containing both pyruvate and naphthalene (Table 1, con-

Table 1
Batch culture conditions for determination of diauxic growth

Condition	Carbon source of inoculum	Carbon source of batch culture
I	Pyruvate	Pyruvate
II	Pyruvate	Naphthalene
III	Pyruvate	Pyruvate + naphthalene
IV	Naphthalene	Pyruvate
V	Naphthalene	Naphthalene
VI	Naphthalene	Pyruvate + naphthalene

ditions III and VI). As controls, culture media containing single carbon source were used (Table 1, conditions I, II, IV, and V).

In the experiments designed to estimate yield coefficients for pyruvate and ammonia, the same amount of inoculum (0.2 %) was added to culture flasks, and the initial cell concentrations were measured. At a certain interval, 2 ml samples were taken. After measuring the absorbance at 600 nm, filtered samples were analyzed to determine concentrations of pyruvate and ammonia. To estimate a yield coefficient for oxygen, the culture media containing 1 g/l pyruvate and 1 g/l ammonium sulfate were added to culture flasks. Immediately after the same amount of inoculum (0.2%) was added to culture flasks, and the initial cell concentrations were measured, the culture flasks were sealed with a rubber stopper containing an oxygen electrode. Oxygen partial pressure in the head space was measured and the amount of O_2 consumed was calculated by assuming equilibrium between the head space and the medium.

2.5. Chemostat cultures

The minimal medium with sodium pyruvate was prepared for the chemostat culture. With a magnetic stirring bar inside the medium reservoir, the medium was introduced into a bioreactor for the chemostat culture Fig. 1. Flow rates of the minimal medium were controlled by a variable-speed pump equipped with a multi-channel mini-catridge (Cole-Parmer Instrument Co., Vernon Hills, IL, USA), and ranged from 0.5 to 5.0 ml/min. A 0.6 l jacketed

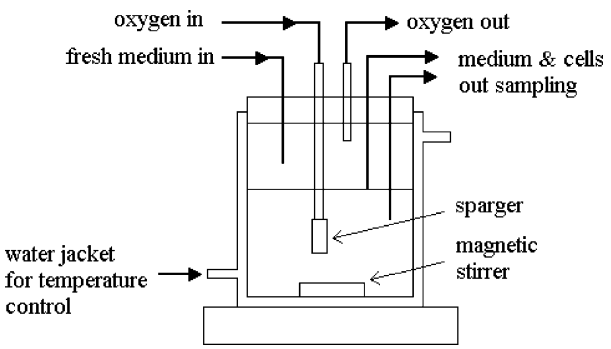

Fig. 1. Schematic diagram of continuous stirred tank bioreactor for chemostat experiments.

glass beaker with a rubber stopper (Fig. 1) was used as a fermentor with a working volume of about 0.3 l. The culture was maintained at 24 ± 1 °C by a water jacket connected to a circulating constant-temperature water bath (Fig. 1). The culture medium was continuously stirred and the pH was measured regularly with a pH meter outside the fermentor on discrete samples; pH remained constant at 7.0. For a sufficient supply of oxygen, pure oxygen gas was introduced into the fermentor through a sparger. The fermentor was run batchwise after inoculation until the onset of growth was clearly visible. The medium supply was then started and a specific dilution rate was set by adjusting the flow rate. A culture sample was taken regularly, and its optical density and pyruvate concentration were measured. Steady state was considered to be achieved when these parameters varied by <2% over consecutive residence times. O_2 concentration was also measured regularly by carefully inserting an oxygen electrode into the culture medium. Ammonia concentrations were measured on samples withdrawn from the fermentor.

3. Results and discussions

3.1. Yield coefficients for pyruvate, oxygen and ammonia

The yield coefficient for pyruvate (Y_P) was determined by plotting the optical density versus pyruvate concentration in the culture medium (Fig. 2). Data points fitted to each line shown in Fig. 2 were from the same culture experiment. The yield coefficient for pyruvate was determined as an average of the absolute values of slopes of the lines in Fig. 2. In the same manner, the yield coefficient for ammonia (Y_A) was determined from a plot of the optical density versus ammonia concentration in the culture medium Fig. 3. The relationship

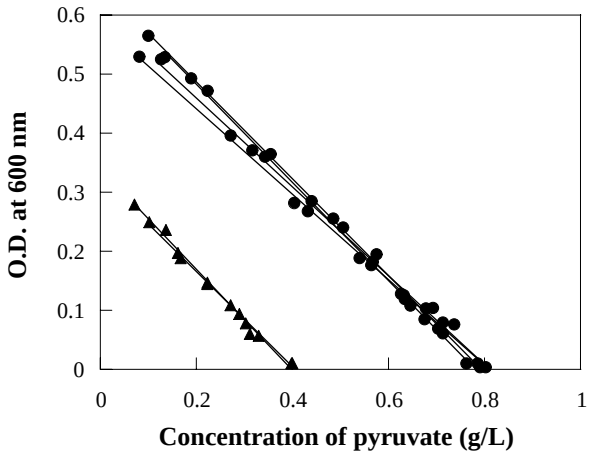

Fig. 2. Yield coefficient of *P. putida* G7 for pyruvate (Y_P). Data points fitted to each line were from the same culture experiment. The initial concentrations of pyruvate were 0.785 ± 0.014 g/l (●) and 0.399 ± 0.001 g/l (▲). $Y_P = 0.80 \pm 0.04$ OD_{600}/pyruvate (g/l) = 0.30 ± 0.02 g cells/g pyruvate.

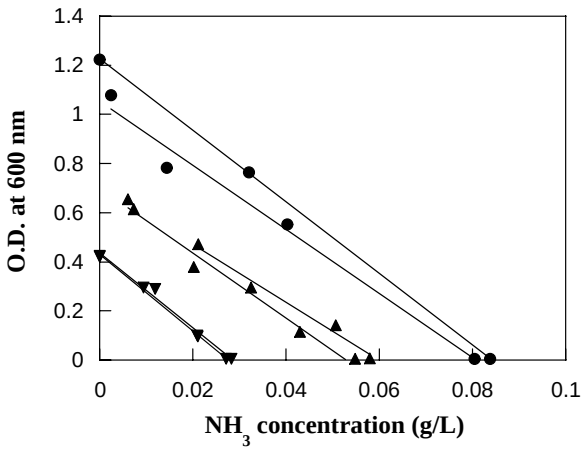

Fig. 3. Yield coefficient of *P. putida* G7 for ammonia (Y_A) with pyruvate as a carbon source under O_2 sufficient conditions. Data points fitted to each line were from the same culture experiment. The initial concentrations of ammonia were 0.082 ± 0.002 g/l (●), 0.056 ± 0.002 g/l (▲), and 0.028 ± 0.001 g/l (▼). Y_A = 14.0 ± 1.35 OD_{600}/ammonia (g/l) = 5.17 ± 0.50 g cells/g ammonia.

between the amount of oxygen consumed and the increase in the optical density of *P. putida* G7 culture is plotted in Fig. 4. The yield coefficient for oxygen (Y_O) was determined as an average of the slopes of the lines connecting each datum point and the origin. The yield coefficients in the unit of gram cell formed per gram of substrate consumed were calculated by applying the experimentally-determined conversion factor, 0.37 g cell/(OD_{600} l) [12], and summarized with those in the unit of OD_{600} per substrate (g/l) in Table 2. Based on the ratios of these yield coefficients, 0.57 g of ammonia (3.82 g of ammonium sulfate) and

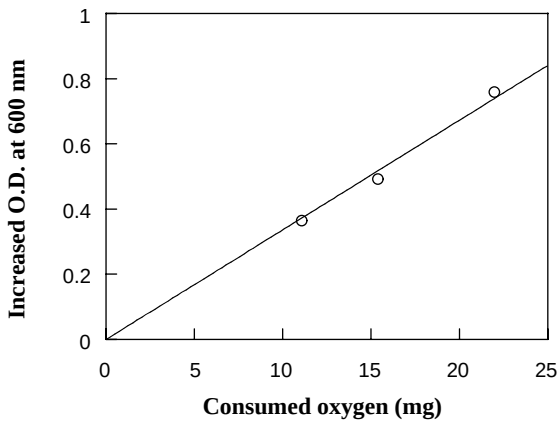

Fig. 4. Oxygen yield coefficient (Y_O) with pyruvate and ammonium sulfate as a carbon source and a nitrogen source, respectively, under O_2 sufficient conditions. Y_O = 1.68 ± 0.04 OD_{600}/oxygen (g/l) = 0.62 ± 0.02 g cell/g oxygen.

Table 2
Yield coefficients of *P. putida* G7 for pyruvate, ammonium and oxygen when pyruvate is a carbon source

Substrate	Yield coefficient in OD at 600 (nm)/substrate (g/l)	Yield coefficient in g cell/g substrate[a]
Pyruvate	0.80 ± 0.04	0.30 ± 0.02
Ammonium	14.0 ± 1.35	5.17 ± 0.50
Oxygen	1.68 ± 0.04	0.62 ± 0.02

[a] Calculated by applying the conversion factor of 0.37 g dry cell/(OD_{600} l) [12].

0.476 g of oxygen are additionally required for the consumption of 1 g of added pyruvate by *P. putida* G7.

3.2. Determination of kinetic parameters in chemostat cultures

The steady-state pyruvate concentrations at different dilution rates in chemostat cultures are shown in Fig. 5. At steady state in a chemostat, the dilution rate is numerically equal to a growth rate. Less than 20% of added ammonia was consumed and the concentration of dissolved oxygen in the chemostat was maintained at a level higher than that of air-saturated water. Therefore, the culture in the chemostat must have been limited solely by pyruvate. The growth kinetics of *P. putida* G7 on pyruvate could be adequately described by the well-known Monod kinetics [11].

$$\mu = \frac{\mu_m S}{K_s + S} \tag{1}$$

where μ_m and K_s are maximum specific growth rate constant and Monod constant, respectively. μ_m and K_s were determined by non-linear regression as $0.80 \pm 0.01\ h^{-1}$ and 0.082 ± 0.01 g/l, respectively. The fitted equation is shown as the dashed line in Fig. 5.

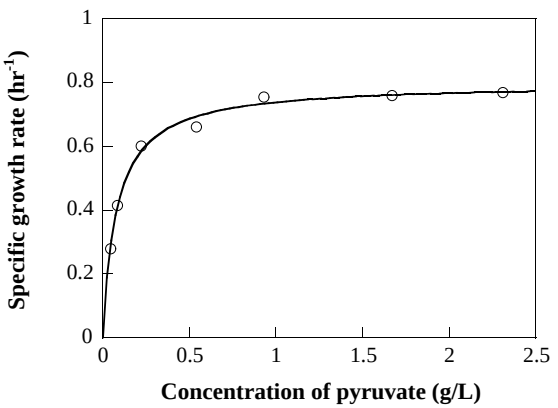

Fig. 5. Growth rates of *P. putida* G7 on pyruvate, the sole carbon source. The experimental data are from a chemostat culture conducted with a sufficient supply of ammonium sulfate and oxygen. μ_m (maximum specific growth rate constant) $= 0.80 \pm 0.01\ h^{-1}$ and K_s (Monod constant) $= 0.082 \pm 0.01$ g/l from non-linear regression.

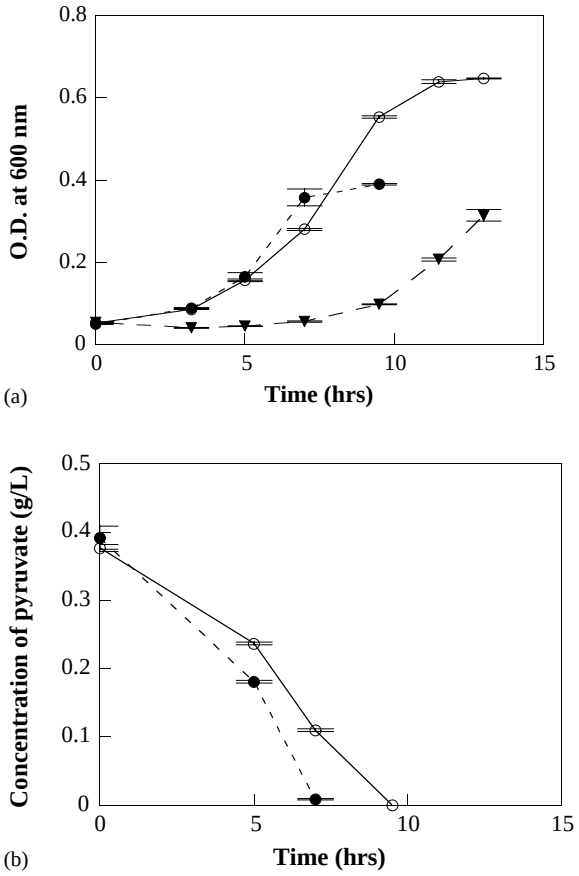

Fig. 6. Culture of pyruvate-bred preculture of *P. putida* G7 in the medium with (●) pyruvate only as a C-source (condition I in Table 1), in the medium with (▼) naphthalene only (condition II in Table 1), and in the medium with (○) pyruvate and naphthalene (condition III in Table 1); (a) cell growth depicted by optical density (OD) at 600 nm, (b) pyruvate concentrations (g/l). The initial concentrations of naphthalene in cases II and III were 24.1 ± 0.2 ppm. The experimental data were obtained from batch experiments.

3.3. *Effect of pyruvate on naphthalene biodegradation*

Results of batch culture experiments with the pyruvate-bred preculture of *P. putida* G7 under the culture conditions listed in Table 1 (conditions I, II, and III) are shown in Fig. 6a and b. When transferred to the minimal medium containing pyruvate, which was the same carbon source as in preculture, there was no lag as shown in Fig. 6a (condition I). But in the medium containing naphthalene as the sole carbon source (condition II), cell growth did not occur for the first 7 h. During this lag, cells must have prepared themselves for naphthalene metabolism. In the medium containing both pyruvate and naphthalene, there was no pattern of diauxic growth (Fig. 6a), condition III). Cell densities depicted by OD_{600} were virtually

the same for conditions I and III, and one could speculate that the consumption of pyruvate was followed by the consumption of naphthalene without a lag. But pyruvate concentrations in condition I and III were not the same (Fig. 6b). The concentration of pyruvate in condition III was always higher than that in condition I at the same culture time. After ca. 7 h, pyruvate was depleted in condition I, but still remained in the medium in condition III. Note that cell growth did not occur at this time in condition II (Fig. 6a). For the culture in condition III, the concentration of naphthalene in the medium was about 24 ppm in the beginning, but it was only about 2 ppm after 7 h. These results imply that cell growth in condition III occurred consuming both pyruvate and naphthalene before pyruvate was depleted, and that the addition of pyruvate accelerated the adaptation of *P. putida* G7 to naphthalene.

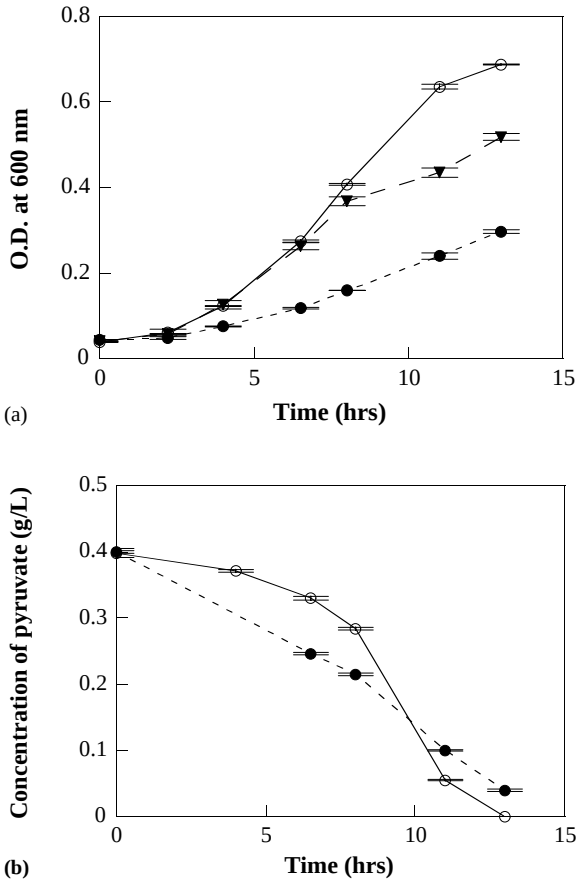

Fig. 7. Culture of naphthalene-bred preculture of *P. putida* G7 in the medium with (●) pyruvate only as a C-source (condition IV in Table 1), in the medium with (▼) naphthalene only (condition V in Table 1), and in the medium with (○) pyruvate and naphthalene (condition VI in Table 1); (a) cell growth depicted by OD at 600 nm, (b) pyruvate concentrations (g/l). The initial concentrations of naphthalene in cases II and III were 23.8 ± 0.3 ppm. The experimental data were obtained from batch experiments.

Naphthalene-bred preculture of *P. putida* G7 was transferred into the minimal media with various carbon sources (Table 1 conditions IV, V, and VI), and the results are shown in Fig. 7a and b. When transferred to the minimal medium containing naphthalene only (condition V), which was the same carbon source as in the preculture, no lag was observed as shown in Fig. 7a. In the medium containing pyruvate only (condition IV), *P. putida* G7 did not show any lag (Fig. 7a), and the consumption of pyruvate occurred at the beginning of the culture experiment (Fig. 7b). In the medium containing both pyruvate and naphthalene, pyruvate consumption also occurred at the beginning of the culture, even though the rate of consumption was lower than that in condition IV. Enzymes involved in a metabolic pathway of pyruvate consumption are believed to be constitutively synthesized in *P. putida* G7. As shown in Fig. 7a, a pattern of diauxic growth was not observed in the medium containing both pyruvate and naphthalene (condition VI), and the cell growth depicted by OD_{600} was larger than that in the medium containing pyruvate only (condition IV), even though pyruvate consumption in condition VI was smaller than that in condition IV (Fig. 7b). These results also imply that *P. putida* G7 does not have a preference for either pyruvate or naphthalene, and that pyruvate can be supplied as a carbon source to stimulate the degradation of naphthalene by *P. putida* G7.

4. Conclusions

The inhibitory effect of another carbon source on the biodegradation of polynuclear aromatic hydrocarbons (PAHs) was studied with the batch culture experiments of a PAH-degrading microorganism in a medium containing both the carbon source and PAH. Pyruvate and naphthalene were used as a model carbon source and a model PAH, respectively. *P. putida* G7 was used as a model naphthalene-degrading microorganism. From a chemostat culture of *P. putida* G7 using pyruvate as a carbon and energy source, the growth kinetics of *P. putida* G7 was determined. At concentrations of naphthalene and pyruvate giving similar growth rates of *P. putida* G7, batch culture experiments were performed. As diauxic growth was not observed in the cultures with both pyruvate and naphthalene, it is believed that pyruvate does not inhibit biodegradation of naphthalene. Pyruvate rather accelerated the adaptation of *P. putida* G7 to naphthalene. Therefore, pyruvate is believed to be a candidate for carbon sources that can be added to PAH-contaminated soil to stimulate the growth of PAH-degrading microorganisms indigenous to the subsurface, and to enhance in situ bioremediation.

Acknowledgements

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