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Effects of Salicylate and Glucose on Biodegradation of Phenanthrene by *Burkholderia cepacia* PM07

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Abstract The stimulatory effects of exogenous salicylate as a pathway inducer on phenanthrene biodegradation were investigated using *Burkholderia cepacia* PM07. The phenanthrene degradation rate was greatly enhanced by increasing the salicylate additions, and the maximum rate was 19.6 mg l^{-1} d^{-1} with the addition of 200 mg l^{-1} of salicylate, 3.5 times higher than that (5.6 mg l^{-1} d^{-1}) without the addition of salicylate. The degradation rate was decreased at higher concentrations of salicylate (above 500 mg l^{-1}), and cell growth was significantly inhibited. The phenanthrene degradation was not affected by increasing glucose up to 2 g l^{-1}, although dramatic microbial growth was obtained. The stimulatory effect of exogenous salicylate decreased in the presence of glucose. After the addition of 200 mg l^{-1} of salicylate, approximately 60% of the initial phenanthrene (50 mg l^{-1}) was degraded after 96 h. However, with extra addition of 200 mg l^{-1} of glucose, the phenanthrene degradation rate decreased, and only 18.5% of the initial phenanthrene was degraded.

Key words: Biodegradation, bioremediation, induction, glucose, phenanthrene, salicylate

Polycyclic aromatic hydrocarbons (PAHs) are released to the environment as a result of the incomplete combustion of fossil fuels or by accidental discharge during the transport, use, and disposal of petroleum products [15]. Remediation of sites contaminated with these compounds is a major environmental concern because of their toxic and carcinogenic properties [6, 15, 21, 22]. Bioremediation is the most desirable method because of its use of a natural degradation process, which uniquely offers the potential for complete eradication [1, 13]. Because of the stability of the condensed ring structure in PAHs, the intrinsic biodegradation is quite slow in the natural system. Furthermore, their hydrophobicity and tendency to sorb to organic matters greatly decrease the fraction of PAH available to microorganisms [9]. Therefore, the natural degradation process of PAHs is very slow, resulting in its persistence in the environment for a long period of time.

Recently, various strategies in the addition of supplements have been attempted to enhance the biodegradation rate of target compounds by stimulating the growth and metabolism of microorganisms. The most common supplements are general nutrient sources, such as carbon and nitrogen. A primary carbon source (e.g., glucose) can not only become building materials for cell synthesis while causing an increase in the microbial population, but also provide energy needed for the degradation of target compounds. In a recent example, it was reported that phenanthrene degradation was enhanced by the individual addition of yeast extract, acetate, glucose, or pyruvate [23].

One of the strategies proposed to enhance the degradation of specific PAH is to offer bacteria with one or more known pathway inducers to stimulate both the selective growth of PAH degraders and the induction of PAH metabolism. The potential pathway inducers, which are produced as intermediates during PAH degradation, include salicylate, salicylaldehyde, 1-hydroxy-2-naphthoate, catechol, phthalate, gentisate, and cinnamate [4]. Previous studies have shown that some of the pathway inducers could stimulate biodegradation of PAHs [4, 11, 12]. For example, salicylate was one of the most effective inducers for the biodegradation of various molecular weight PAHs: naphthalene [12, 16], benz[a]anthracene [7], phenanthrene, fluoranthene, pyrene, benz[a]pyrene, benz[a]anthracene, and chrysene [4]. However, in some cases, it has been reported that

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there is no effect or a negative effect on degradation of naphthalene and phenanthrene by the addition of salicylate [3, 5, 8]. In most of this research, kinetic study has not been conducted intensively by using various initial concentrations of an inducer and a carbon source using single- and co-additions. The objectives of this study were (1) to investigate the stimulating effects of metabolic inducers with salicylate and additional carbon source with glucose on phenanthrene biodegradation, (2) to investigate the stimulatory and inhibitory effects of various initial salicylate concentrations, and (3) to compare kinetic variations with cell growth phase.

MATERIALS AND METHODS

Materials and Microbial Cultures

Burkholderia cepacia PM07 was isolated from a PAH-contaminated soil by enrichment on phenanthrene as the sole carbon source [20]. Cultures of *B. cepacia* PM07 were preserved in cryostat vials with 50% glycerol at −70°C. To recover a frozen culture, a sterilized toothpick was inserted into the cryostat vial and inoculated into 5 ml of NB (nutrient broth, Difco, U.S.A.). For the inoculation, the cultures grown in NB for 24 h were cultivated in mineral salt medium (MSM) containing 200 mg l^{-1} of salicylate for 2 days. The cultures were harvested by centrifugation and washed twice with MSM. It was finally resuspended in MSM and used as inoculum for all biodegradation experiments.

The mineral salt medium for biodegradation experiments contained 50 mM NH_4Cl, 5 mM NaH_2PO_4, 11.3 mM Na_2HPO_4, 10 mM KCl, 2 mM Na_2SO_4, 1.25 mM $MgSO_4 \cdot 7H_2O$, 0.02 mM $CaCl_2$, 0.1 mM $Na_2MoO_4 \cdot 2H_2O$, 1 mM EDTA, and 5 ml per 1 l of a trace solution containing 0.12 mM HCl, 5 mM $ZnSO_4 \cdot 7H_2O$, 20 mM $FeSO_4 \cdot 7H_2O$, 10 mM $MnSO_4 \cdot H_2O$, 1 mM $CuSO_4 \cdot 5H_2O$, 2 mM $CoCl_2 \cdot 6H_2O$, and 0.8 mM H_3BO_3. The final pH was 7.0.

Biodegradation Experiments

The biodegradation experiments used completely soluble phenanthrene in MSM containing Triton X-100 at a final concentration of 1 wt%. Phenanthrene crystals were solubilized to 50 mg l^{-1} in the surfactant medium, and the medium was then autoclaved at 121°C for 20 min. Into a 250-ml Erlenmeyer flask were added 100 ml of the surfactant medium containing phenanthrene and microbial inoculum at a level of 3.2×10^5 CFU (colony forming unit) ml^{-1}. The flasks were shaken at 150 rpm in a rotary shaker at 25°C for 10 days.

To examine the effect of an additional carbon source on biodegradation of phenanthrene, glucose was added to the medium at concentrations of 200, 500, and 2,000 mg l^{-1}. Glucose was sterilized by filtering (0.2-μm pores) and added to the autoclaved medium. Salicylate as a metabolic inducer was examined at various concentrations ranging from 20 to 2,000 mg l^{-1}. The effect of the addition of both glucose and salicylate was tested at 200 mg l^{-1}. Three control tests were also performed, including (1) a test done with phenanthrene as a single carbon source, (2) a test for the growth of microorganisms in a control medium without phenanthrene, and (3) a test to assess the abiotic loss of phenanthrene. Duplicate runs were performed for all the experiments. Liquid samples were taken from the flasks at appropriate times and used for measurements of CFU, phenanthrene, salicylate, and glucose.

Analytical Methods

Phenanthrene was analyzed by HPLC (high performance liquid chromatography, Dionex, U.S.A.) using a UV detector at 254 nm. The analytical column was a reversed-phase Supelcosil™ LC-PAH column (150 mm×4.6 mm) [10, 21]. The column was eluted with mobile phase (74.5% acetonitrile, 24.5% deionized water, and 1% acetate) at a flow rate of 1.5 ml min^{-1}. The minimum detectable concentration was approximately 0.01 mg l^{-1} for phenanthrene and 1 mg l^{-1} for salicylate. For the analysis of phenanthrene and salicylate, approximately 1 ml of liquid was withdrawn with a disposable glass Pasteur pipette and placed into a 1.5-ml borosilicate glass vial. This was immediately centrifuged at 6,000 ×*g* for 10 min. The supernatant was injected directly into the HPLC system. The CFUs were counted after a 7-day incubation period on nutrient agar (Difco, U.S.A.) at 25°C.

RESULTS AND DISCUSSION

Biodegradation of Phenanthrene

The results of the primary biodegradation tests during 2 days of culture with various conditions are summarized in Table 1. No appreciable removal of phenanthrene (only 1.8%) occurred for the abiotic control (Test B1). In Test B2, although a carbon source was not added, the cell number increased from 3.2×10^5 ml^{-1} to 4.2×10^6 ml^{-1}, corresponding to 1.8 mg cells l^{-1}. The microorganisms could not use the surfactant Triton X-100 in the basal medium as a carbon source. The small increase of cell number was attributed to cell growth on organic impurities in the medium containing high surfactant concentration (10 g Triton X-100 l^{-1}). Also, this result indicated that the strain was not significantly inhibited by the surfactant. Using phenanthrene as a single carbon source (Test B3), however, 13.2% of the initial phenanthrene was degraded with a degradation rate of 3.3 mg l^{-1} d^{-1} and a specific degradation rate of 0.35 d^{-1}. The microorganisms grew from 3.2×10^5 ml^{-1} to 2.2×10^7 ml^{-1} after 2 days of culture.

When glucose was supplemented as an additional carbon source, the final number of microorganisms increased 30 times for 500 mg l^{-1} of glucose, and 150 times for

Table 1. Cell growth and biodegradation of phenanthrene with the addition of glucose or salicylate after 2 days.

	Glu (0d) [mg l^{-1}]	Sal (0d) [mg l^{-1}]	Phn (2d) [mg l^{-1}]	CFU (2d) [ml^{-1}]	Phn degraded (%)
B1*	0	0	49.1±1.3	-	1.8±2.5
B2**	0	0	-	$(4.2\pm0.3)\times10^6$	-
B3	0	0	43.4±3.2	$(2.2\pm0.02)\times10^7$	13.2±6.4
G1	500	0	46.0±4.4	$(6.5\pm0.7)\times10^8$	14.2±8.7
G2	2,000	0	43.3±2.3	$(3.4\pm0.1)\times10^9$	16.7±4.7
S1	0	500	35.8±1.5	$(1.8\pm0.1)\times10^8$	30.5±2.9
S2	0	2,000	48.7±1.8	$(2.0\pm0.3)\times10^3$	2.6±3.6

Glu: Glucose; Sal: Salicylate; Phn: phenanthrene.
Initial phenanthrene concentration was 50 mg l^{-1} and initial CFU was 3.2×10^5 ml^{-1}.
Data presented are means of duplicate samples±standard deviations.
*Performed without inoculation. **Performed without carbon addition.

2,000 mg l^{-1} of glucose over that of Test B3, as shown in Table 1. However, the percentage of phenanthrene degradation did not significantly increase. In contrast, using salicylate at a concentration of 500 mg l^{-1}, a greatly enhanced degradation was observed, and 30.5% of the initial phenanthrene was degraded. The phenanthrene degradation rate was 7.1 mg l^{-1} d^{-1}, 2.2 times faster than that of the control test B3. Compared with the control, the higher cell number, 1.8×10^8 ml^{-1}, was obtained with the salicylate addition. At a high concentration of salicylate (2,000 mg l^{-1}), significant cell death below inoculum size was observed, and phenanthrene was not degraded.

Effect of Salicylate Addition

Figures 1 and 2 show the effects of phenanthrene degradation with various salicylate concentrations, ranging from 0 to 700 mg l^{-1}. The phenanthrene degradation rate and the number of microorganisms increased with the increase of the salicylate concentration up to 200 mg l^{-1}. Figure 1 shows that the trends of cell growth and phenanthrene degradation were consistently observed in all salicylate additions at concentrations lower than 200 mg l^{-1}. The pattern of cell growth had two sequential growth periods: the first showed rapid growth mainly on salicylate, and the second showed slow growth and a stationary state on phenanthrene after a short decrease of cell numbers. Phenanthrene was degraded most rapidly between 8 to 24 h, the period corresponding to the exponential growth phase. Phenanthrene degradation rates during the stationary phase (after 24 h) gradually decreased. Phenanthrene was not completely degraded in all cases. For example, the remaining phenanthrene was 36.9 mg l^{-1} at 576 h without salicylate addition, and 5.8 mg l^{-1} with the addition of 200 mg l^{-1} of salicylate.

Figure 2 shows that the phenanthrene degradation rate decreased with increasing salicylate addition to above 200 mg l^{-1}. The time for adaptation before active cell growth also increased with a corresponding increase in the addition of salicylate. In particular, cell numbers initially

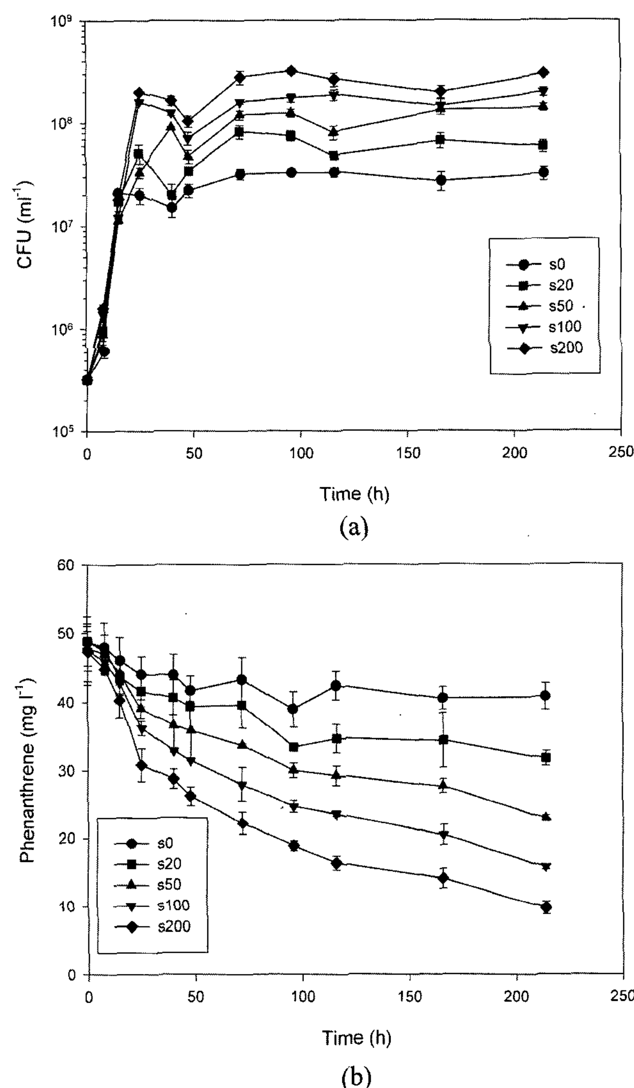

Fig. 1. Enhanced phenanthrene biodegradation by the addition of salicylate at concentrations lower than 200 mg l^{-1}.
The character 's' and the number in legends represent salicylate and the concentrations added, respectively. (a) CFU, (b) phenanthrene. The error bars indicate the distribution of duplicates.

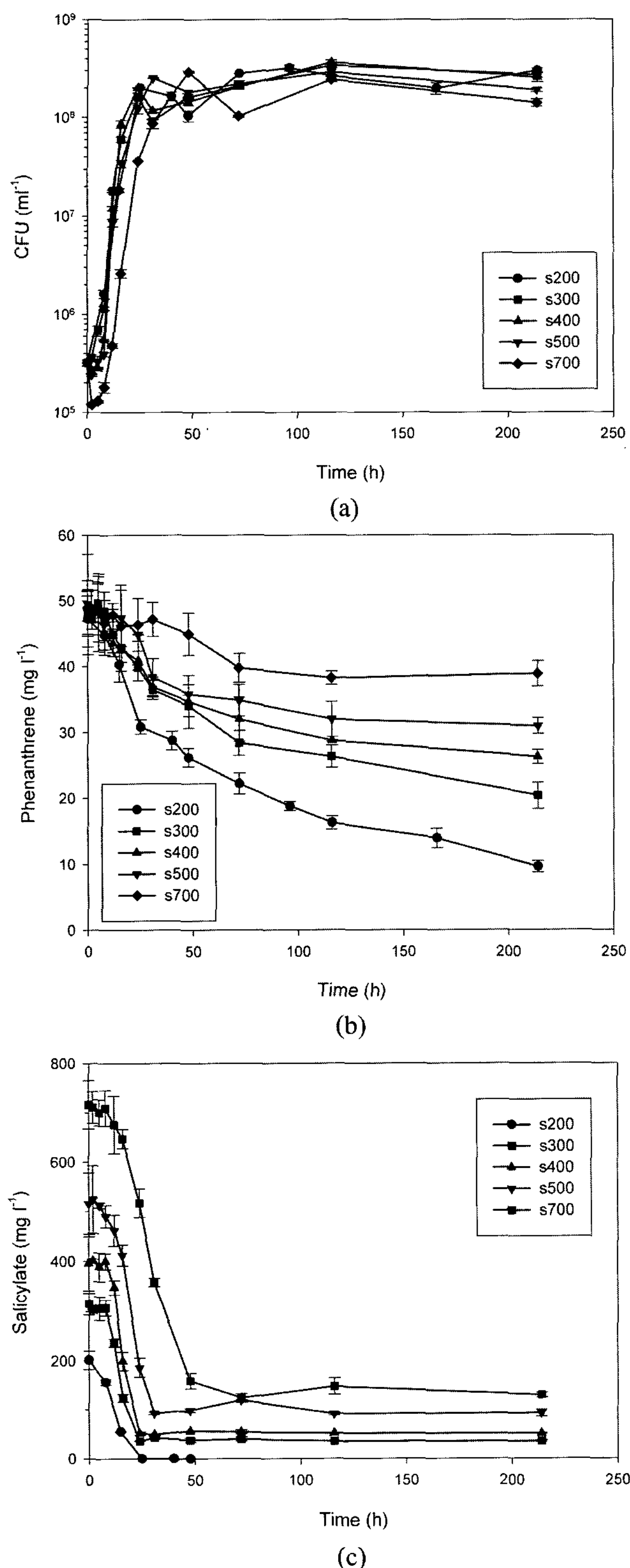

Fig. 2. Decrease of stimulatory effect on phenanthrene degradation by the addition of salicylate at concentrations higher than 200 mg l^{-1}. The character 's' and the number in legends represent salicylate and concentrations added, respectively. (a) CFU. (b) Phenanthrene. (c) Salicylate. The error bars indicate the distribution of duplicates.

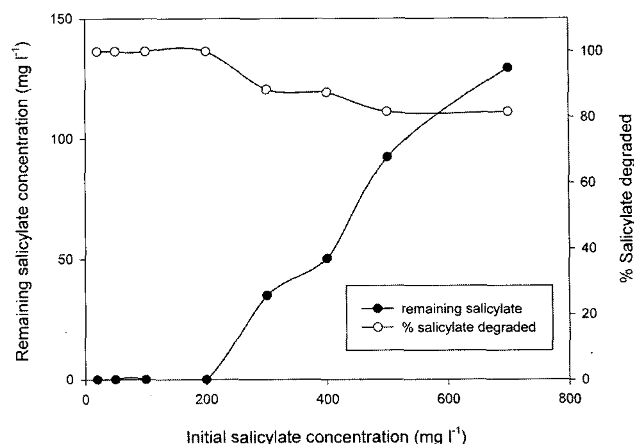

Fig. 3. Remaining salicylate and percentage of salicylate degraded with various concentrations of salicylate addition.

decreased when the salicylate added was above 400 mg l^{-1}, and it drastically dropped from 3.2×10^5 ml^{-1} to 1.2×10^5 ml^{-1} with 700 mg l^{-1} of salicylate. The cell numbers at the stationary phase did not reach a high level parallel to the addition of salicylate.

Salicylate was completely degraded in 24 h with the addition of 200 mg l^{-1} of salicylate (Fig. 2c) or below (data not shown). In contrast, although the degradation rates of salicylate in the growth phase were quite similar in all cases, the initial salicylate remained for a long time without further degradation with the addition of 300 mg l^{-1} of salicylate. The amount of remaining salicylate increased with the increased addition of salicylate. As shown in Fig. 3, the total amount of salicylate degraded was not constant; however, the percentage of salicylate degraded was almost constant at around 80%, as shown in Fig. 3. In separate tests, cell growth was completely inhibited at above 1,000 mg l^{-1} of salicylate, whereas phenanthrene and salicylate were not degraded at all.

The stimulatory effect of the salicylate addition on the degradation of various PAHs has been elucidated in several previous studies. The transformation of benz[a]anthracene has been shown to be induced by salicylate in *Sphingomonas yanoikuyae* [7]. It was observed that the transformation of naphthalene, phenanthrene, fluoranthene, and pyrene by *Pseudomonas saccharophila* P15 was stimulated by salicylate [16]. Using the same strain, salicylate was found to greatly enhance initial rates of removal of high molecular weight PAHs: fluoranthene, pyrene, benz[a]anthracene, chrysene, and benzo[a]pyrene, which are not used for growth of the strain [4]. It was also reported that preincubation of the strain P15 with other potential inducers such as naphthalene, phenanthrene, 1-hydroxy-2-naphthoate, or salicylaldehyde greatly increased the initial rate of phenanthrene removal [4].

However, induction by the pathway inducers was not always effective in some cases and was dependent on

environmental conditions. It has been reported that salicylate or other pathway inducers (phthalate, gentisate, and cinnamate) decreased the mineralization of phenanthrene or pyrene as well as the number of degrading bacteria in the study using various natural soil samples [3]. There was no effect of the salicylate addition on the phenanthrene degradation [8] or on naphthalene dioxygenase activity [5]. In our study, the addition of salicylate greatly enhanced the phenanthrene degradation rate (e.g., 3.5 times higher at 200 mg l^{-1} of salicylate than without salicylate). However, the rate of phenanthrene degradation and initial cell growth rate were inhibited by the salicylate at concentrations higher than 200 mg l^{-1} (Fig. 2). In such high range of concentrations, the salicylate was not completely degraded, and the remaining amount increased was proportional to the amount initially added (Fig. 3). The reason that most of the salicylate was degraded but some portion (about 10–20%) still remained without degradation at all is not clear. It is likely that the intracellular concentration of salicylate became so high that downstream metabolism could not keep up.

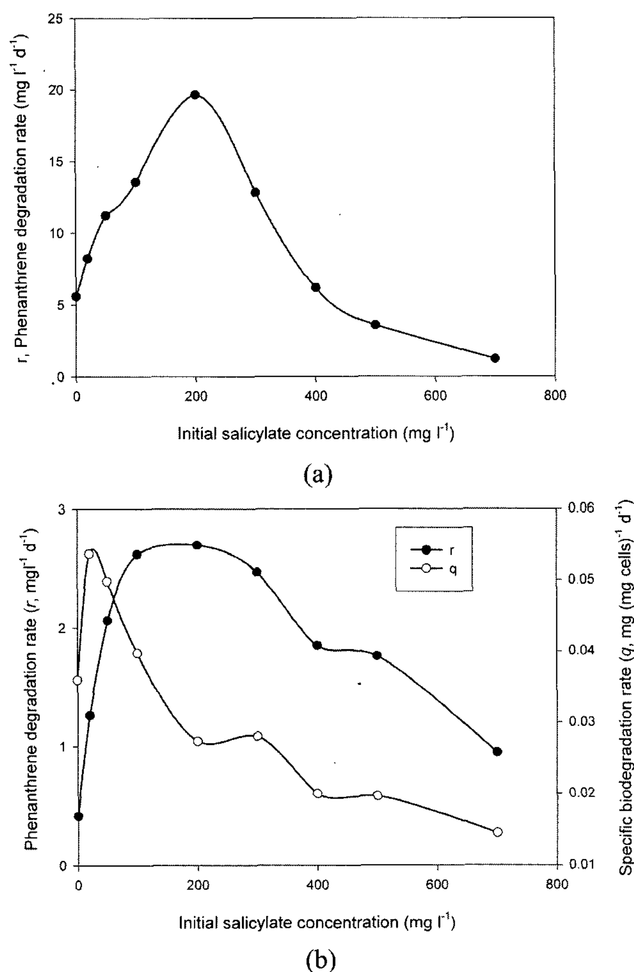

Fig. 4. Phenanthrene degradation rate per reactor volume (r) and per cell mass (q) at different cell phases.
(a) Growth phase (8 to 24 h). (b) Stationary phase (24 to 214 h).

As shown in Fig. 4, the pattern of phenanthrene biodegradation was distinctive according to the growth phase and the stationary phase. The calculation for the biodegradation rates was performed using the data from 8 to 24 h for the growth phase and from 24 to 214 h for the stationary phase. The phenanthrene degradation rate in the growth phase was the maximum with 200 mg l^{-1} of salicylate and declined with the addition of more salicylate (Fig. 4a). The degradation rate with 200 mg l^{-1} of salicylate was 19.6 mg l^{-1} d^{-1}, 3.5 times higher than the 5.6 mg l^{-1} d^{-1} value obtained in the test without the addition of salicylate. The phenanthrene degradation rate in the stationary phase in all cases was significantly lower than that in the growth phase, although much of the phenanthrene still remained in the medium. The degradation rate in the stationary phase was 1.3–13.5 times lower than that in the growth phase, and the difference became larger by increasing the salicylate addition. Similar to the growth phase, the maximum degradation rate in the stationary phase was observed at around 200 mg l^{-1} of salicylate and at 2.7 mg l^{-1} d^{-1} of phenanthrene. However, the maximum value of specific degradation rate was observed with the much smaller addition of 20 mg l^{-1} of salicylate. With the addition of more than 20 mg l^{-1} of salicylate, the specific biodegradation rate decreased as more salicylate was added. In particular, with more than 200 mg l^{-1} of salicylate added, the specific biodegradation rate was lower than the control test with no salicylate addition.

The overall degradation rate of phenanthrene was much higher in the growth phase than in the stationary phase (Fig. 4). The slope of phenanthrene degradation was changed at the time (after 24 h) when salicylate degradation ceased, which was observed in the whole range of salicylate addition (Fig. 1). These results imply that the existence of salicylate in the medium might support an enzyme activity to degrade phenanthrene. On the other hand, the maximum value of the specific degradation rate was found at the lowest concentration of salicylate addition (Fig. 4b). This suggests that the degradation activity of the unit cell can be decreased to some extent during cell growth on an easily degradable inducer (salicylate), as well as glucose. The rapid cell differentiation during active cell growth can cause plasmid loss mainly by segregation, which is the division of the cell into two daughter cells when only one plasmid is present [14]. Such plasmid loss would be prominent during the growth, using a general carbon source such as glucose. Therefore, the addition of supplements for enhancing the bioremediation rate of contaminated soils should be focused on the possibility of increasing the cell activity directly related with biodegradation rather than on cell mass itself.

Effect of Glucose Addition

The effects of glucose as a primary carbon source on the degradation of phenanthrene or salicylate were examined

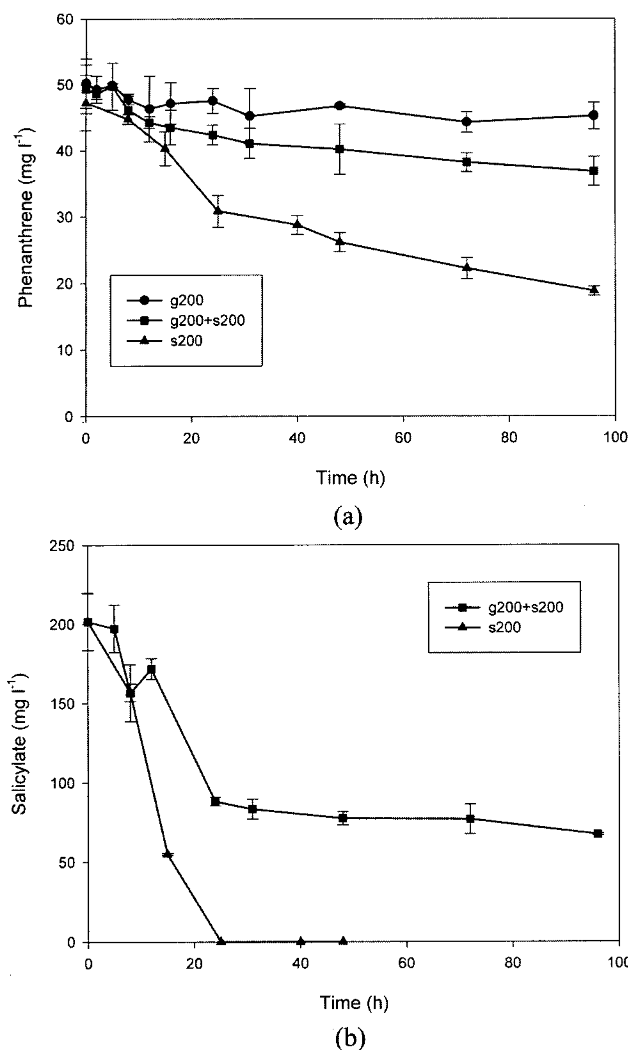

Fig. 5. The effect of glucose and salicylate with single addition or co-addition on phenanthrene degradation and salicylate uptake. The characters 'g,' 's,' and the number in the legends represent glucose, salicylate, and the concentrations added, respectively. (a) Phenanthrene. (b) Salicylate. The error bars indicate the distribution of duplicates.

over time, as shown in Fig. 5. After the addition of 200 mg l^{-1} of salicylate, approximately 60% of the initial phenanthrene was degraded after 96 h. However, with extra addition of glucose, the phenanthrene degradation rate decreased, and only 18.5% of the initial phenanthrene was degraded. This degradation by the addition of both glucose and salicylate was higher than that (7%) of the case where only glucose was added without the addition of salicylate. Salicylate was completely degraded within 24 h with the addition of only salicylate. In contrast, salicylate was partially degraded in the presence of glucose with the remaining approximately 35% of the initial salicylate without further degradation after 24 h.

Reports on the effect of the addition of a primary carbon source on the PAHs biodegradation differ as to whether it is stimulatory [2, 23] or not [16–18, 23]. It has been shown that the presence of cosubstrates enhances PAH degradation [2]. Phenanthrene degradation has been enhanced by the individual addition of yeast extract, acetate, glucose, and pyruvate at a concentration of 50 mg l^{-1} [23]. On the contrary, supplementation of the media with high glucose levels diminished specific fluoranthene degradation [18]. Although increasing glucose concentrations from 0.45 to 3 g l^{-1} resulted in a better bacterial growth, it has been reported that the degradation of phenanthrene was reduced significantly [19]. Sometimes, the stimulatory effect depends on the salinity: glucose addition significantly increased bacterial growth and phenanthrene degradation at high salinity, but caused a slight decrease in biodegradation at low salinity [17]. It has been reported that the use of readily available carbon source (glucose) did not enhance degradation of phenanthrene in the Guayanilla Bay water, as carbon was not a limiting factor [24]. In the present study, the extent of phenanthrene degradation seems to be unaffected by increasing glucose up to 2 g l^{-1}, although dramatic microbial growth was obtained, thus decreasing the specific biodegradation rates (per cell mass) significantly (B3, G1, G2 in Table 1). Such an inhibition of phenanthrene degradation by glucose was likely due to catabolite repression and/or plasmid loss during rapid cell growth on glucose. The inhibition becomes much more dramatic when induced by salicylate (Fig. 5). In this test, some of the salicylate remained without further degradation when glucose was added. This implies that the degrading ability may not be recovered once it has been lost during its rapid growth on glucose.

The stimulatory effects of exogenous salicylate as a pathway inducer on phenanthrene biodegradation were investigated using *Burkholderia cepacia* PM07. The phenanthrene degradation rate was greatly enhanced by increasing the salicylate concentrations, and the maximum rate was obtained with the addition of 200 mg l^{-1} of salicylate. The higher concentrations of salicylate significantly inhibited the cell growth. The results demonstrate that salicylate can be a good stimulant to enhance the biodegradation rate of polycyclic aromatic hydrocarbons. The stimulatory effect of exogenous salicylate decreased in the presence of glucose as a primary carbon source. The results of glucose tests suggest that the inherent presence of easily degradable carbon sources in natural soils could alter the biodegradation rate of contaminants. Therefore, the addition of supplements to enhance the natural biodegradation rate should be designed carefully according to various carbon substrates, including inducers, for a successful bioremediation.

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