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# Effect of Dual Substrates on Aniline Mineralization by *Pseudomonas testosteroni* 6Fl

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# *Pseudomonas testosteroni* 6F1의 아닐린 분해에 미치는 이차기질의 영향

조경연•전효곤•배경숙•고영희*

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**The simultaneous mineralization of aniline and other secondary carbon sources by *Pseudomonas testosteroni* 6Fl were evaluated by the lag time and the enzyme induction level. The lag time for aniline mineralization by *P. testosteroni* 6Fl was 7 hours, whereas the lag time for aniline and readily utilizable secondary substrates were 1-3 hours. This stimulated degradation resulted from the simultaneous use of secondary substrates and aniline, the increased rate of enzyme induction, and the increased rate of the cell growth. The enzyme induction level of *P. testosteron* 6Fl were varied according to the kinds of secondary substrate.**

In natural ecosystem, organic pollutants frequently occur in mixtures with other synthetic as well as natural organic compounds. Therefore, it is important to understand how the biodegradation of a polluting compound is affected by the presence of other compounds. Recent works had shown that the degradation of low concentrations of organic compounds could be stimulated by the addition of readily degraded organic substrate (1-3). Such findings may have both practical and ecological significances. In a practical sense, it would be beneficial if these findings could be applied to the operation of waste treatment systems to stimulate the breakdown of synthetic compounds. In an ecological context, many natural environments are carbon-limited, and therefore it would be advantageous for an organism to metabolize a variety of organic compounds that are present at low concentrations.

In addition to enhancing the mineralization of synthetic organic compounds, supplementary substrates may affect the kinetics of biodegradation of organic compounds. Schmidt *et al.* showed that the kinetics of biodegradation of organic compounds were altered by the presence of compounds that the organism could use simultaneously with the test substrate. When the two substrates were metabolized simultaneously, the degradation of the compound that was present at low concentration was enhanced if the growth of the population is increased by the presence of the other substrate(3). This enhancement of biodegradation has been termed secondary substrate utilization(4).

In this paper we demonstrated the effects of secondary substrates on shortening the lag time for cell growth, and expression and induction level of aniline oxidizing enzymes of *P. testosteroni* 6F1.

## Materials and Methods

### Media

The minimal salt medium contained 3.2g of $K_2HPO_4$, 4.9g of $NaH_2PO_4$, 1.0g of $NH_4Cl$, 0.48g of

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$MgSO_4 \cdot 7H_2O$, 0.03g of $CaCl_2$, 0.01g of $FeSO_4 \cdot 7H_2O$, 0.01g of $MnCl_2 \cdot 4H\ O$, 0.001g of $CoCl_2 \cdot 6H_2$, and 0.001g of $Na_2MoO_4 \cdot 2H_2O$ per liter of distiled water. Individual salt solution was sterilized separately and mixed(5). The final pH of the medium was 6.5. Single substrate media was composed of minimal salt medium and aniline as a sole carbon source. Dual substrate media was composed of single substrate media containing other secondary substrate.

**Bacterial strain**

*Pseudomonas testosteroni* strain 6F1 was isolated from water sample of the Han River previously(6).

**Cultivation in dual substrate medium**

*P. testosteroni* 6F1 was precultured in 3 m*l* of Luria broth for 10 hours at 30°C with shaking. After washing twice with saline, 0.5 m*l* of cell suspension was transfered to 50 m*l* of minimal salts medium containing 3 mM of aniline and 2 or 3 mM of each test compounds. The bacterium was cultured at 30°C on a rotary shaker at 250 rpm. At an intervals of hour, the turbidity of culture broth was measured at 660 nm, and the concentration of aniline was determined by diazo coupling reaction (7).

**Induction of aniline-oxidizing enzyme systems**

*P. testosteroni* 6F1 was allowed to grow on single or dual substrate media to late exponential phase. Cells were then washed and placed on an ice bath to stop further enzyme synthesis.

**Enzyme assay**

The activity of aniline dioxygenase was assayed by measuring the aniline uptake rate in resting cells(6). The activity of catechol 1,2-dioxygenase was assayed in cell-free extracts by the procedure of Hegeman(8) with the molar extinction coefficients reported by Dorn and Knackmuss(9).

## Results

**Induction of aniline dioxygenase**

The strain 6F1 could utilize aniline, catechol and benzoate. In these strain, all these three aromatic compounds induced catechol 1,2-dioxygenase. However, only aniline induced aniline dioxygenase (Table 1). This strain neither utilized other aniline derivatives nor accumulated catechols from these compounds(6).

**Table 1. Induction of aniline oxygenase and catechol-1,2-dioxygenase by aniline and metabolites.**

| Compounds | Aniline uptake[a] rate | Catechol-1,2 dioxygenase |
|---|---|---|
| Aniline | 0.11 | 0.067 |
| Benzoate | 0.007 | 0.145 |
| Catechol | 0.005 | 0.013 |
| Succinate | 0.002 | 0.001 |

a unit: μmoles/min, mg of protein of cells.

**Table 2. Effects of dual substrate media containing aniline and various additional carbon or carbon and nitrogen sources on growth of *P. testosteroni* 6F1.**

| Substrate | Lag time(hour) Substrate[a] alone | Lag time(hour) Aniline and[b] substrate | Growth type* |
|---|---|---|---|
| Aniline | 7 | | |
| Acetate | 3 | 5 | a |
| Citrate | 4 | 2 | c |
| Glucose | -[c] | 7 | d |
| Glycerol | - | 7 | d |
| Lactate | 1 | 1 | b |
| Succinate | C[d] | 3 | c |
| Alanine | 3 | 3 | b |
| Arginine | 20 | 7 | d |
| Glutamate | 2 | 2 | b |
| Proline | 13 | 6 | c |
| Tryptophan | 20 | 7 | d |

[a]2 or 3 mM of substrate.
[b]2mM of aniline and 2 or 3mM of substrate.
[c]Not be assimilated.
[d]Non-inducible.
*Refered to Fig. 1.

**Effect of secondary substrate on the aniline utilization**

Studies about the stimulative effect of secondary substrate on the cell growth and the rate of mineralization of aniline were maded in dual substrate media containing aniline and various secondary substrates. Table 2 showed that the addition of several substrates, such as major metabolites of cell, to the minimal aniline medium reduced the lag time and promoted the cell growth of the strain 6Fl. As shown in Fig. 1, there were three types of growth

on dual substrate media. When acetate was added to aniline minimal medium, it was preferentially utilized to aniline. Though dioxy-curve was observed, mineralization of aniline was promoted by addition of acetate (Fig. 1-a). In dual substrate media containing aniline, and lactate, citrate, or glutarate the growth of *P. testosteroni* 6F1 and mineralization of aniline was rapidly promoted. So, it need only one or two hours of lag time as if it grow on this additional substrate alone (Fig. 1-b). The most extraordinary phenomena were observed when suc-

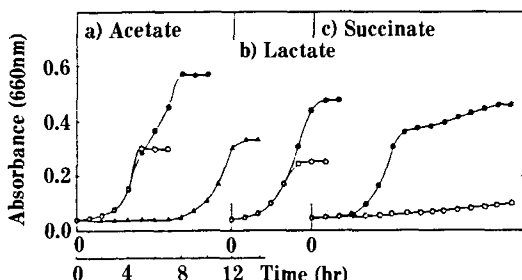


**Fig. 1. Growth of *P. testosteroni* strain 6F1 in dual substrate media containing aniline and various additional carbon or carbon and nitrogen sources. Control media contained either aniline or the additional substrate alone.**

▲ - ▲ growth on aniline alone.
○ - ○ growth on the additional substrate alone.
● - ● growth on aniline and the additional substrate.

**Table 3. Induction level of aniline uptake rate and catechol 1,2-dioxygenase by several compounds.**

| Compounds* | Aniline[a] uptake rate | Catechol 1,2-[a] dioxygenase |
|---|---|---|
| Aniline | 0.11(100) | 0.067(100) |
| Acetate | 0.063(57) | 0.036( 54) |
| Fumarate | 0.053(48) | 0.082(122) |
| Lactate | 0.15(136) | 0.074(110) |
| Pyruvate | 0.13(118) | 0.070(104) |
| Peptone | 0.18(164) | 0.091(136) |
| Succinate | 0.079(72) | 0.058( 87) |
| Yeast extract | 0.13(118) | 0.060( 90) |
| Non-induced[b] | 0.001 | 0.0001 |

*Containing 5mM of aniline and 5mM or 0.15% of substrate.
[a]unit: $\mu$moles/min, mg of protein of cells.
[b]grown on LB medium.

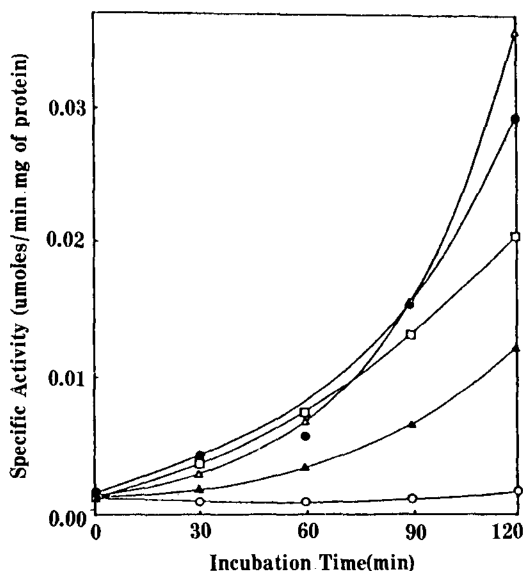


**Fig. 2. Induction of catechol 1,2-dioxygenase in dual substrate media containing aniline and various additional substrates.**

○ - ○ aniline.
▲ - ▲ aniline and acetate.
△ - △ aniline and fumarate.
□ - □ aniline and succinate.
■ - ■ aniline and yeast extract.

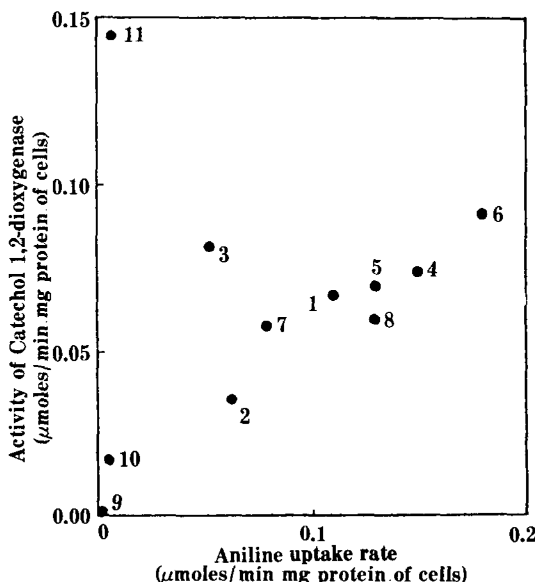


**Fig. 3. Relationship between the activity of catechol 1,2-dioxygenase and aniline uptake rate.**

Each cells grown on; 1. aniline, 2. aniline and acetate, 3. aniline and fumarate, 4. aniline and lactate, 5. aniline and pyruvate, 6. aniline and peptone, 7. aniline and succinate, 8. aniline and yeast extract, 9. non-induced, 10. catechol, 11. benzoate.

cinate was added. The strain 6F1 grew very slowly and with no exponential phase on succinate alone. But, when succinate was added to minimal aniline medium, it promoted the growth of the strain 6F1 and the lag time was shortened (Fig. 1-c). Fig. 2 showed that catechol 1,2-dioxygenase was more rapidly induced in succinate added aniline minimal salts media than aniline minimal media. There was no effect by additional substrate if it was not utilized by this strain(d-type).

**Induction of aniline metabolisms**

To see the induction levels of aniline dioxygenase and catechol 1,2-dioxygenase on dual substrate media containing aniline and various additional substrates, the strain 6F1 was cultured, harvested at late exponential phase, and the enzyme activity of this cells was measured (Table 3). When cells were grown on aniline and peptone, it showed the highest enzyme activities. And, when the additional substrate was acetate it showed the lowest enzyme activities. Fig. 3 showed that the level of enzyme induction and the ratio of aniline dioxygenase to catechol 1,2-dioxygenase activity depended on the additional substrates. So, it was appeared that induction level of these two enzymes might be slightly related to each other upto a certain point but not be completely coordinated.

On the basis of these results, it was concluded that the induction of enzyme systems of aniline metabolism might be promoted at early stage of growth (Fig. 2) rather than repressed by the additional substrates, but the induction level of enzymes varied with respect to the additional substrates.

Uptake of aniline by non-induced cell, aniline induced cell, and aniline and peptone induced cell were shown in Fig. 4. Aniline and peptone induced cells uptake aniline more efficiently than aniline induced cells.

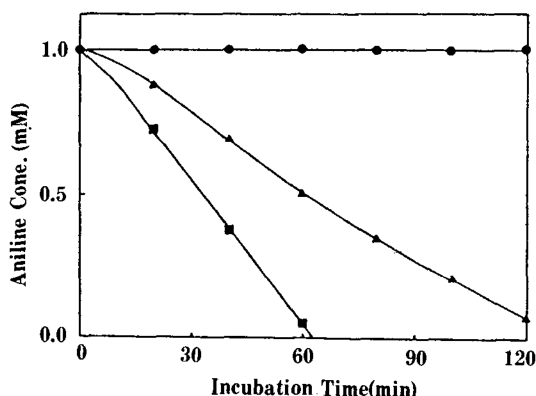


**Fig. 4. Uptake of aniline by induced cells of *P. testosteroni* strain 6F1.**

●-● non-induced.
▲-▲ induced by 5 mM of aniline.
■-■ induced by 5mM of aniline and 0.15% of peptone.

## Discussion

There were several reports on promotion or repression of aromatic compound mineralization by secondary substrate. Aoki *et al.* reported that *Frateuria* sp. ANA-18 preferentially consumed glucose rather than aniline and utilization of aniline was repressed by the addition of glucose(10). However, they also reported that *R. erythropolis* AN13 used aniline in preference to glucose in the dual substrate media and aniline mineralization was promoted by glucose with an increase of the cell growth (11). Steven *et al.* demonstated the effect of a stimulatory substrate (glucose) on the kinetics of *p*-nitrophenol mineralization by *Pseudomonas* sp.(12). In that study, they showed that the shapes of the curves describing biodegradation of substrate could change markedly in response to a second substrate, and the stimulatory effect of glucose on *p*-nitrophenol mineralization resulted from the increased rate of growth of the *p*-nitrophenol mineralizing population when it is also metabolizing glucose.

When *P. testosteroni* 6F1 was allowed to grow on minimal media containing aniline, it needs 7 hours of lag time to utilize aniline. But, on the dual substrate media supplemented with aniline and other substate, they utilized aniline with shortened lag time. And, induction of the aniline oxidizing activity in this strain was rapidly promoted by incubation with aniline and other utilizable substate in both cases either this substate was utilized rapidly or slowly than aniline. These results implied that the rapidly utilizable substrates increase expression level of aniline oxidizing enzymes by providing energy and intermediates. Therefore, inducers, intermediates of aniline metabolism, should rapidly accumulated and aniline metabolism might be rapidly induced by these compounds. In case of succinate, it was utilized slowly than aniline. But, the cells grew with reduced lag time on dual media containing aniline and succinate, because succinate might supply energy and intermediate to the cells in

lag phase though it was not enough to supply all the energy and intermediate for the cell growth.

Another effect of second substrate on aniline utilization of *P. testosteroni* 6F1 was the variation of the induction level of aniline oxidizing enzymes. In minimal salts medium containing 5 mM of aniline and 0.15% of peptone, aniline oxidizing enzymes of *P. testosteroni* 6F1 was induced higher than in that containing only aniline.

This study demonstated the stimulaneous effects of second substrates on the lag time for enzyme expression and induction level of aniline oxidizing enzyme of *P. testosteroni* 6F1. The lag time which was need for enzyme expression could decrease markedly and the induction level was varied in response to second substrate. Knowledge gained from such studies should aid in finding means to enhance the degradation of organic toxicants at waste disposal sites.

## 요 약

*Pseudomonas testosteroni* 6F1의 아닐린 분해에 미치는 이차기질의 첨가효과를 보기 위하여 균생장에 필요한 유도기간과 효소활성유도 정도를 조사하였다. 아닐린만 존재하는 배지에서 *P. testosteroni* 6F1은 7시간의 유도기간을 필요로한데 비하여 쉽게 이용가능한 이차기질을 첨가해준 경우 아닐린 분해에 필요한 유도기간이 1-3시간으로 줄어들었다. 이러한 아닐린 분해 촉진효과는 이차기질과 아닐린이 서로 빨리 이용되면서 균체의 생장과 분해효소의 활성유도시기를 앞당겼기 때문이었으며, 이때 아닐린 분해효소의 최종활성 정도는 첨가해준 이차기질에 따라 다르게 나타났다.

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