# Application of the Genetically Engineered Strains of *Pseudomonas* Degrading Various Persistent Aromatic Hydrocarbons to Wastewater*

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# 다양한 난분해성 방향족 탄화수소를 분해하는 *Pseudomonas* 개발균주의 폐수에의 적용

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**ABSTRACT: Genetically engineered strains of *Pseudomonas,* which could degrade the various aromatic hydrocarbons, were acclimated to the synthetic wastewater with increasing substrate concentration. All of the tested strains except KUD101 showed 70-90% of the COD removal efficiencies. Acclimated strains, *Pseudomonas putida* KUD106, KUD107 and KUD108 were inoculated into the ordinary activated sludge and these sludges were used in the dyeing or alkylbenzene wastewater treatment system. In the case of the wastewater containing alkylbenzene compounds, COD removal efficiency was 12-14% higher than that of the ordinary activated sludge, while it was not effective for dyeing wastewater. On the other hand, the floc formation of the activated sludge inoculated with genetically engineered strains was more rapid in both wastewaters tested than that of the activated sludge by the ordinary natural strains.**

**KEY WORDS □ *Pseudomonas putida,* aromatic hydrocarbons, activated sludge**

Water pollutions have been generally caused by urban sewage and factory wastewater. In case of the sewage, 80-90% organic compounds could be removed by activated sludge, but most of recalcitrants, toxic compounds, nitrate, and phosphate were not removed and released in the steam, river and ocean. So main water pollutants are recalcitrants and dyes (Grady, 1986). These compounds have showed several toxicities, and can cause cancer and mutation for the organisms (Alexander, 1981).

But various microorganisms, especially, *Pseudomonas* spp. in soil, freshwater and seawater has degradative capacity for these recalcitrants, and plasmids harbored in these microorganisms are related to the degradative capacity (Wheelis, 1975: Chakrabarty, 1976).

In the previous paper, to construct multifunction strains having degradative ability for recalcitrants, some studies have been proceded: *Pseudomonas putida* KUD12 and *P. putida* KUP10 which could degrade alkylbenzen sulfonate (ABS) and di-2-ethyl hexyl phthalate (DEHP), respectively, were isolated and used as host cells. By congugation and transformation, multifunction microorganisms harbouring hybrid plasmid pKG2, pKG3 and TOL plasmid were constructed.

In this paper, first of all, these genetically engineered strains of microorganisms were acclimated to artifical wastewater containing recalcitrants. Thereafter the acclimated microorganisms were inoculated into the activated sludge of the wastewater treatment factory. We used these activated sludge for the treatment of dyeing wastewater and alkylbenzene wastewater.

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**Table 1.** *Bacterial strains and plasmids*

| Strain/Plasmid | Relevant characteristics | Source or Reference |
|---|---|---|
| *Pseudomonas putida* | | |
| KUD101(pKG2) | /pKG2(ABS$^+$ 2,4-D$^+$ $Tc^r$) | Lee *et al.* (1990) |
| KUD102(pKG3) | /pKG3(ABS$^+$ Nah$^+$ $Km^r$ $Ap^r$ $Sm^r$) | Lee *et al.* (1990) |
| KUD103(pWWO) | /pWWO(ABS$^+$ Tol$^+$) | Lee *et al.* (1990) |
| KUD106(pKG2) | /pKG2(DEHP$^+$ 2,4-D$^+$ $Tc^r$) | Lee *et al.* (1990) |
| KUD107(pKG3) | /pKG3(DEHP$^+$ Nah$^+$ $Km^r$ $Ap^r$ $Sm^r$) | Lee *et al.* (1990) |
| KUD108(pWWO) | /pWWO(DEHP$^+$ Tol$^+$) | Lee *et al.* (1990) |
| KUD202(pKG3, pWWO) | /pKG3, pWWO(ABS$^+$ Nah$^+$ Tol$^+$ $Km^r$ $Ap^r$ $Sm^r$) | Lee *et al.* (1990) |
| KUD12 | Wild strain(ABS$^+$) | Choi and Lee (1989) |
| KUP10 | Wild strain(DEHP$^+$) | Song and Lee (1988) |
| PpG1901(NAH7) | *met*$^-$/NAH7(Nah$^+$ Sal$^+$ Tra$^+$) | Yen and Gunsalus (1985) |
| mt-2(pWWO) | /pWWO(Tol$^+$) | Worsey and Williams (1975) |
| *Alcaligenes eutrophus* | | |
| JMP134(pJP4) | /pJP4(2,4-D$^+$ 3CB$^+$ Hg$^+$) | Don and Permberton (1981) |

## MATERIALS AND METHODS

**Bacterial strains and plasmids**

Bacterial strains and plasmids used in this study are listed in Table 1.

**Medium and synthetic wastewater**

PAS salt medium (Chatterjee *et al.*, 1981) was used, and as a single carbon source, di-2-ethyl hexyl phthalate, toluene, 2,4-Dichlorophenoxyacetic acid (2,4-D), naphthalene or alkyl benzen sulfonate were added and the final concentrations were adjusted to 1 mg/m*l* respectively. For acclimation of the artificial strains, synthetic wastewater was prepared as follows: $Na_2HPO_4$ (1 g/*l*) and $KH_2PO_4$ (1 g/*l*) as a buffer; $(NH_4)_2SO_4$ or $NaNO_3$ (1 g/*l*) as a nitrogen source. The concentration of the carbon sources added was the following: in cases of *P. putida* KUD101, KUD102, KUD103 and KUD202 the concentration of ABS was adjusted to 100 ppm in the early stage and increased to 200 ppm in the later stage; in cases of *P. putida* KUD106, KUD107 and KUD108 the concentration of DEHP was adjusted to 500 ppm in the early stage and increased to 700 ppm in the later stage. Other carbon sources were adjusted to 200 ppm and increased to 400 ppm, respectively.

**Acclimation of the strains to synthetic wastewater**

Constructed microorganisms were activated in PAS minimal medium and these were inoculated and grown in LB liquid medium (OD., 0.8-1.2, 600 nm). Cultured medium was centrifuged at 4°C for 10 minutes in 5,000 rpm. The pellet was washed in PAS minimal medium in order to remove organic substances and inoculated into the synthetic wastewater containing various substrates.

This acclimation experiment was operated by semi-continuous cultivation. That is, the effluent sample (50 m*l*) was withdrawn from the reactor and was exchanged by influent (50 m*l*) containing fresh medium. To measure the several factors for wastewater treatment efficiency, effluent sample was centrifuged and microorganisms were removed. Thereafter supernatant was used for measurement of chemical oxygen demand (COD) and pH. This measurement was carried out at an interval of 3 days.

**Efficiencies of the strains for the wastewater treatment**

Acclimated strains, *P. putida* KUD107 and KUD108 were introduced into the two kinds of activated sludges, at dyeing and alkylbenzene wastewaters. To obtain activated sludges inoculated with constructed microorganisms, a modified method of Kurane *et al.* (1979) was employed. That is, the mixed liquor suspended solid (MLSS) of the introduced strains and the ordinary activated sludge was adjusted to the same rate. Thereafter MLSS was adjusted to 1000 mg/*l* for dyeing wastewater and 5000 mg/*l* for alkylbenzene wastewater approximately. Ordinary activated sludge and activated sludge inoculated with mixed all original microorganisms were used as a control.

At first, pH and dissolved oxygen (DO) were measured at an interval of 24 hours. If reactors were

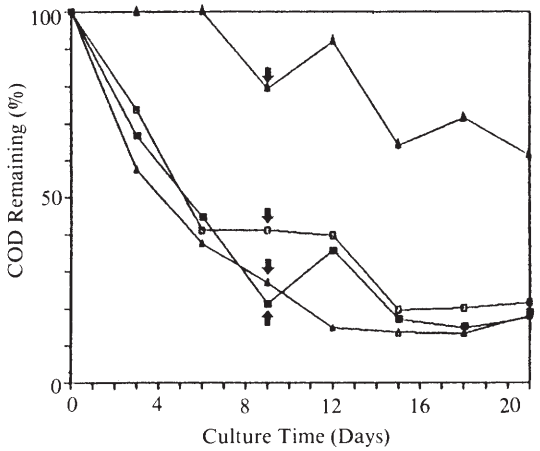


**Fig. 1.** *COD removal efficiencies of various detergent degrading strains during their acclimation.*
Bacterial strains were cultured at 30°C for 21 days in synthetic wastewater. Aliquotes of the samples were picked out, centrifuged and checked for COD at an interval 3 days. The arrow indicates the point of adding the increased substrate concentration. Symbols: □: *P. putida* KUD102(pKG3), ■: *P. putida* KUD103(pWWO), ▲: *P. putida* KUD101 (pKG2), △: *P. putida* KUD202(pKG3, pWWO).

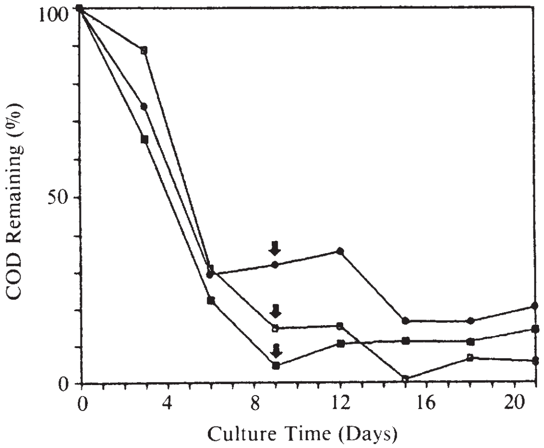


**Fig. 2.** *COD removal efficiencies of various phthalate degrading strains during their acclimation.*
Bacterial strains were cultured at 30°C for 21 days in synthetic wastewater. Aliquotes of the samples were picked out, centrifuged and checked for COD at an interval 3 days. The arrow indicates the point of adding the increased substrate concentration. Symbols: □: *P. putida* KUD107(pKG3), ■: *P. putida* KUD108(pWWO), ●: *P. putida* KUD106 (pKG2).

allowed to stand for 1 hour, activated sludge was sedimented. Then supernatant 100 m*l* was withdrawn and undisposed wastewater 100 m*l* was added. Subsequently COD, MLSS, sludge volume (SV), sludge volume index (SVI) were checked on for the supernatant by Standard Methods (APHA *et al.*, 1985).

## RESULTS AND DISCUSSION

### Acclimation in synthetic wastewater

In all cultures, pH of the influent showed average 6.7 and that of the effluent was 6.9-7.7. Temperature was kept up 30°C. COD removal rate for synthetic wastewater treatment of the constructed strains, *P. putida* KUD101, KUD102, KUD103 and KUD202 represented 19.0%, 63.3%, 68.9% and 74%, respectively (Fig. 1). And the COD removal rates of *P. putida* KUD106, KUD107 and KUD108 for synthetic wastewater treatment were 68%, 76.8%, and 81.2%, respectively. These value except *P. putida* KUD101 showed the possibility that the constructed microorganisms could be introduced into the ordinary wastewater treatment. Especially COD removal rate of *P. putida* KUD202 was higher than that of any other strains. These phenomenon indicated that wastewater treatment efficiency could be improved by the use of the genetically engineered multi-functional microorganisms. *P. putida* KUD101 harvouring plasmid pKG2 was lower COD removal rate than that of other constructed strains.

When the substrate concentration was changed at 9th day, COD removal rates of *P. putida* KUD102, KUD107, KUD202 were not influenced and showed gradual improvement (Fig. 1 and 2). These results represented that hybrid plasmid pKG3 have a large effect for the wastewater treatment and genetically engineered microorganisms could be acclimated in the new circumstance with increasing substrate concentration.

### Dyeing wastewater treatment

*P. putida* KUD106, KUD107, and KUD108 which were competent to treat synthetic wastewater during the acclimation were introduced to the activated sludge of the dyeing wastewater in single or mixed. As a control, ordinary activated sludge and activated sludge inoculated with mixed all original microorganisms, that is, *P. putida* PpG1901(NAH7), mt-2 (pWWO), KUP10 and *Alcaligenes eutrophus* JMP-134 were used. During the period of treatment, pH of all reactors was changed from 8.0 to 8.5-8.7, and temperature and DO were kept up 30°C, 3-4 mg/*l*, respectively. MLSS showed 1,000-2,000 mg/*l*. The SV of the ordinary activated sludge showed 15 m*l*/100 m*l* and that of the activated sludge inocu-

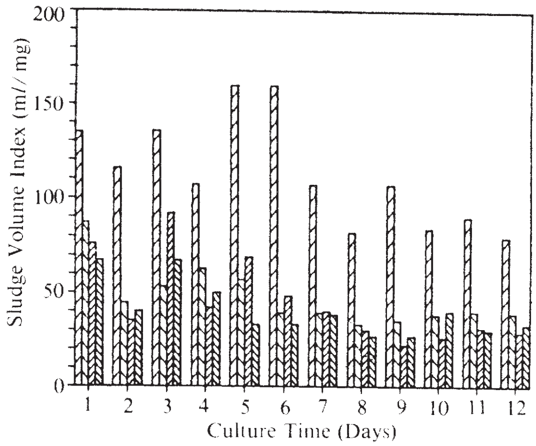


**Fig. 3.** *Comparison for the SVI values of four types of activated sludge during dyeing wastewater treatment procedure.*

SV and MLSS were measured by Standard Methods. SVI was calculated by the following formula: SVI(ml/mg) = SV(ml/l) × 1000/MLSS(mg/l)
Symbols: ▨: Ordinary activated sludge, ▧: Average value of the activated sludge inoculated with single constructed strain, ▨: Average value of the activated sludge inoculated with mixed strains, ▧: The activated sludge inoculated with mixed original strains.

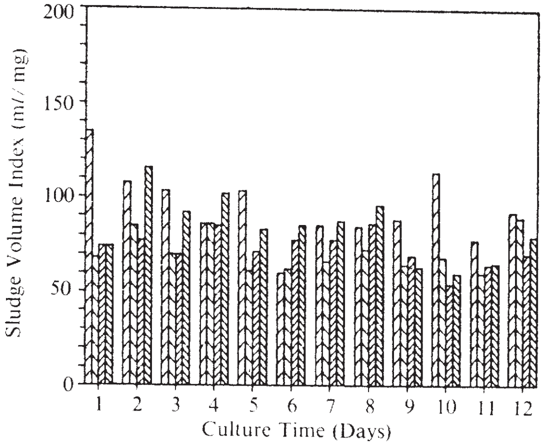


**Fig. 4.** *Comparison for the SVI values of four types of activated sludge during alkylbenzene wastewater treatment procedure.*

SV and MLSS were measured by Standard Methods. SVI was calculated by the following formula:
SVI(ml/mg) = SV(ml/l) × 1000/MLSS(mg/l)
Symbols: ▨: Ordinary activated sludge, ▧: Average value of the activated sludge inoculated with single constructed strain, ▨: Average value of the activated sludge inoculated with mixed strains, ▧: The activated sludge inoculated with mixed original strains.

lated with constructed strains were 5 ml/100mg. The SVI of the ordinary activated sludge and activated sludge inoculated with single constructed strain represented 90 ml/mg, 35 ml/mg respectively (Fig. 3). In contrast, the SVI of the activated sludge inoculated with the mixed strains was 25 ml/mg (Fig. 3). In a microscopic examination of the activated sludge inoculated with constructed microorganisms, filamentous microorganisms observed in the early stage were disappeared in the late stage. These result was caused by the capacity that the constructed microorganisms could occupy the space of filamentous microorganisms. Because the capacity of the floc formation was improved, SVI value was decreased naturally. But the acclimation velocity of the ordinary activated sludge was faster than that of the activated sludge inoculated with constructed microorganisms and the COD removal efficiencies of the two activated sludge showed similar value, average 67~68% (Fig. 5). Therefore, it is considered that in dyeing wastewater treatment, the constructed strains couldn't support any other good effects except floc formation capacity.

**Alkylbenzene wastewater treatment**

During the period of treatment, pHs of all reac-

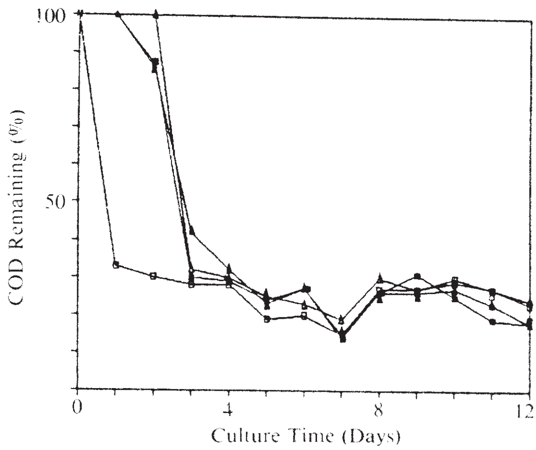


**Fig. 5.** *COD removal efficiencies of four types of activated sludge during dyeing wastewater treatment by semi-continuous cultivation.*

The reactors were allowed to stand for 1 hour and the supernatant (100 ml) was withdrawn. Then COD was measured by the Standard Methods.
Symbols: □: Ordinary activated sludge, ■: Average value of the activated sludge inoculated with single constructed strain, ▲: Average value of the activated sludge inoculated with mixed strains, Δ: The activated sludge inoculated with mixed original strain.

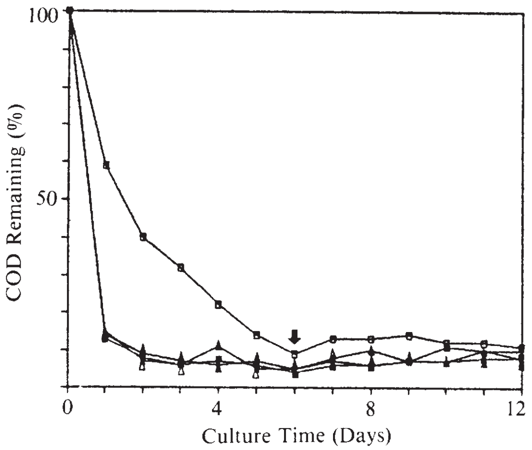


**Fig. 6.** *COD removal efficiencies of four types of activated sludge during alkylbenzene wastewater treatment by semi-continuous cultivation.*

The reactors were allowed to stand for 1 hour and the supernatant (100 ml) was withdrawn. Then COD was measured by the Standard Methods. The arrow indicates the point of adding the increased wastewater concentration.

Symbols: □: Ordinary activated sludge, ■: Average value of the activated sludge inoculated with single constructed strain, ▲: Average value of the activated sludge inoculated with mixed strains, △: The activated sludge inoculated with mixed original strains.

tors were changed from 7.1 to 5.2-6.0. These gradual change from neutrality to acidity was caused by sulfuric acid contained in alkylbenzene wastewater. DO, MLSS and temperature were kept up 2-3 mg/*l*, 5,000 mg/*l* and 28.5 °C. SVI of the activated sludge inoculated with constructed strains showed 70-80 m*l*/mg approximately, but that of the ordinary activated sludge showed 103 m*l*/mg (Fig. 4). Among these values, SVI of the activated sludge inoculated with *P. putida* KUD106, KUD107 and KUD108 together was 61.5 m*l*/mg, value having good sedimentation capacity. COD removal rate of the activated sludge inoculated with constructed strains was stabilized in one day, but that of ordinary activated sludge was stabilized in 5-6 days. COD removal rate of the former was 12-14% higher on the average than that of the latter and average removal rate of the former was kept up 92-93% after stabilization (Fig. 6).

From this point of view, remaining recalcitrants, which ordinary activated sludge cannot remove, can be disposed by the activated sludge inoculated with the constructed strains. Also we concluded that the deflocculation by the filamentous microorganisms can be prevented by the genetically engineered strains.


## 적 요

다양한 난분해성 방향족 화합물을 분해하는 개발된 *Pseudomonas* 균주들을 기질농도를 높여가며 인공폐수에서 적응시킨 결과, *Pseudomonas. putida* KUD101 균주를 제외한 모든 공시균주가 70-90%의 COD 제거효율을 나타내었다. 또 인공폐수에서 적응시킨 개발균주 *P. putida* KUD106, KUD107, 그리고 KUD108을 원슬러지에 첨가한 후, 이 새로운 슬러지의 염색폐수와 알킬벤젠이 주성분인 공장폐수의 COD 제거율을 비교한 결과, 염색폐수에서는 효과가 없었으나 공장폐수에서는 원슬러지보다 평균 12-14% 향상된 COD 제거효율을 나타내었다. 반면에 플록형성에 있어서는 염색폐수와 공장폐수 모두에서 원슬러지보다 개발균주를 첨가한 슬러지에서 플록이 빨리 형성되었다.


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