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Development of Versatile Strains of *Pseudomonas* Degrading Various Persistent Aromatic Hydrocarbons*

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다양한 난분해성 방향족 탄화수소를 분해하는 *Pseudomonas*의 균주개발

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ABSTRACT: To develop the new strains of microorganisms having the degradative ability for various aromatic hydrocarbons, the hybrid plasmid pKG2 having the 2,4-Dichlorophenoxyacetic acid (2,4-D) degradative genes, the hybrid plasmid pKG3 containg the naphthalene degradative genes and TOL plasmid were introduced into *Pseudomonas putida* KUD12 and *P. putida* KUP10 by transformation or conjugation which originally have the degradative ability of the synthetic surfactants and phthalate esters, respectively. From *P. putida* KUD12, the new strains of *P. putida* KUD101(pKG2), KUD102(pKG3), KUD103(TOL) and KUD202(pKG3, TOL) were obtained, and KUD106(pKG2), KUD107(pKG3), KUD108(TOL) were originated from the *P. putida* KUP10. The degradative abilities in *P. putida* KUD101, KUD102 and KUD107 were similar with those of the original strains. The *P. putida* KUD103, KUD106 and KUD202 had a little lower and *P. putida* KUD108 had a better degradative abilitie than those of the original ones. In the case of mixed cultures, the mixed culture of KUD107 and KUD108 had a better degradative abilities than those of the other mixed cultures.

KEY WORDS □ ***Pseudomonas putida*, hybrid plasmid, degradative ability**

Soil microorganisms are able to transform and degrade various persistent aromatic hydrocarbons (Franklin *et al.*, 1981). Several genera of soil bacteria, particularly *Pseudomonas* spp, are known to degrade and utilize a variety of these compounds *via* the presence of partial or complete catabolic pathways encoded by either chromosome or plasmid (Wheelis, 1975: Chakrabarty, 1976). Some of these degradative pathways are related with large plasmids such as TOL, NAH, SAL and CAM and most of the plasmids are transmissible from a donor to a recipient cell by conjugation (Dunn and Gunsalus, 1973). Because most of the microorganisms, however, can degrade a specific substrate, several microorganisms must be added and co-cultured to treat the wastewater containing various aromatic hydrocarbons. Otherwise, multifunction microorganisms must be constructed.

In this study, to develop the new strains having degradative abilities for various persistent aromatic hydrocarbons, *Pseudomonas putida* KUD12 (Choi and Lee, 1989) and *P. putida* KUP10 (Song and Lee, 1988) which has a degradative ability of akylbenzene sulfonate (ABS) and phthalate esters, respectively, were used as a host cell. Hybrid plasmids pKG2, pKG3 and TOL plasmid having 2,4-Dichlorophenoxyacetic acid (2,4-D), naphthalene and toluene degradative genes, respectively, were introduced into the host cell by conjugation or transformation, and their degradative abilities for aromatic hydrocarbons were measured and compared with that of the original strains.

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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)	This study
KUD102 (pKG3)	/pKG3 (ABS$^+$ Nah$^+$ Km^r Ap^r Sm^r)	This study
KUD103 (pWWO)	/pWWO (ABS$^+$ Tol$^+$)	This study
KUD106(pKG2)	/pKG2 (DEHP$^+$, 2.40$^+$, Tc^r)	This study
KU107 (pKG3)	/pKG3 (DEHP$^+$ Nah$^+$ Km^r Ap^{r}Sm^r)	This study
KUD108 (pWWO)	/pWWO (DEHP$^+$ Tol$^+$)	This study
KUD202 (pKG3, pWWO)	/pKG3, pWWO (ABS$^+$ Nah$^+$ Tol$^+$ Ap^r Km^r Sm^r)	This study
KUD12	Wild strain (ABS$^+$)	Choi and Lee (1989)
KUP10	Wild strain (DEHP$^+$)	Song and Lee (1988)
AC812 (pKG2)	*recA, trpB*/pKG2 (2,4-D$^+$ Tc^r)	Lée and Lee (1989)
AC812 (pKG3)	*recA, trpB*/pKG3 (Nah$^+$ Km^r Ap^r Sm^r)	Lee and Lee (1989)
PpG1901 (NAH7)	*met*$^-$*/NAH7 (Nah*$^+$ Sal$^+$ Tra$^+$)	Yen and Gunsalus (1985)
mt-2 (pWWO)	/pWWO (Tol$^+$)	Worsey and Williams (1975)
Pseudomonas aeruginosa		
PAO303(Rms 148)	*arg*$^-$*/Rms148 (Sm^r)*	Jacoby (1977)
Escherichia coli		
C600 (RP4)	/RP4 (Ap^r Tc^r Km^r)	Barth and Grinter (1977)
Alcaligenes eutrophus		
JMP134 (pJP4)	/pJP4 (2,4-D$^+$ 3CB$^+$ Hg$^+$)	Don and Pemberton (1981)

MATERIALS AND METHODS

Bacterial strains and plasmids

Bacterial strains and plasmids used in this work are listed Table 1. Hybrid plasmid pKG2 was constructed by using the vector pRK290 and EcoRI B fragment of pJP4 plasmid having 2,4-D degradative genes (Lee and Lee, 1989). Hybrid plasmid pKG3 was constructed by using the vector pKT240 and EcoRI A fragment of NAH7 plasmid having naphthalene degradative genes (Lee and Lee, 1989).

Media

L-broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% glucose) was used as a complete medium, and PAS (Chatterjee *et al.*, 1981) was used as a minimal medium and ABS, naphthalene, 2,4-Dichlorophenol, Di-2-ethyl hexyl phthalate (DEHP) and benzylalcohol was added to final concentration of 1 mg/ml, respectively.

Isolation of plasmid

Plasmid DNAs in conjugants or transformants were isolated by the procedure of Hansen and Olsen (1978) and then purified with ethidium bromide-CsCl density gradient. Plasmids were checked by agarose gel electrophoresis.

Conjugation

The conjugation procedure was same as described by De Graaf *et al.* (1973). Donor and recipient cells were grown in liquid media to the late expotential phase respectively, and then mixed in the 1:1 ratio relative to their optical density. 1.0 ml of the mixture of cells was filtrated and this filtrate was incubated overnight at 30°C and then washed with 0.7% NaCl solution. The cell suspension was diluted and spreaded onto selective media.

Transformation

The transformation of *Pseudomonas* was carried out according to the procedure of Nakazawa (1983).

Analytical method

The degradative abilities for aromatic hydrocarbons were measured by Gas Chromatography and acetonitrile was used as a solvent for naphthalene, 2,4-Dichlorophenol, and benzylalcohol.

RESULT AND DISCUSSION

Construction of 2,4-D degrading strains and their degradative abilities

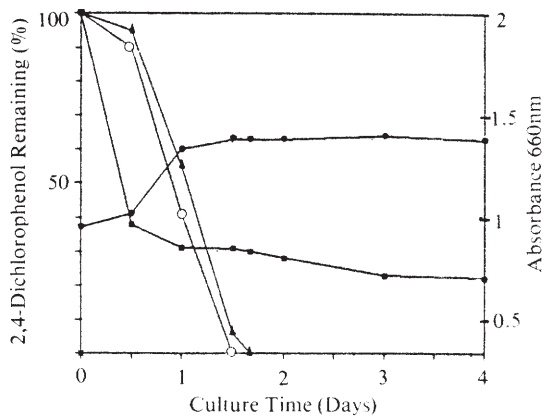

Fig. 1. *2,4-Dichlorophenol degradation by various bacterial strains and growth curve of KUD106.*
Bacterial strains were cultured at 30 °C for 4 days in PAS minimal medium containing 2,4-Dichlorophenol. Aliquotes of the samples were picked out and dissolved in acetonitrile.
Symbols: ○ : *A. eutrophus* JMP134 (pJP4), ▲ : *P. putida* KUD101 (pKG2), ■ : *P. putida* KUD106 (pKG2), ● : Growth curve of KUD106.

In order to develop the new strains which degrade 2,4-D and ABS or 2,4-D and phthalate esters, hybrid plasmid pKG2 (Lee and Lee, 1989) was introduced into *P. putida* KUD12 and KUP10 by transformation, respectively. From *P. putida* KUD12, the new strain of *P. putida* KUD101 was obtained, and KUD106 was originated from the KUP10. The transformants, *P. putida* KUD101 and KUD106, were resisted to tetracycline and could be grown on mininal media containing 2,4-D and ABS or 2,4-D and phthalate esters, respectively.

Degradative ability of 2,4-Dichlorophol in *P. putida* KUD101 strain was similar to the original strain, *Alcaligenes entrophus* JMP134 (Fig. 1). But, *P. putida* KUD106 strain had a little lower ability than that of original one, though, it showed rapid degradative ability in early stage.

Agarose gel electrophoresis of plasmid DNA from KUD101 and KUD106 were shown in Fig. 2.

Construction of toluene degrading strains and their degradative abilities

By conjugation of *P. putida* KUD12 and *P. putida* mt-2(TOL) or *P. putida* KUP10 and mt-2, *P. putida* KUD103 and KUD108 strains were obtained, respectively. These conjugants, harboring TOL plasmid, KUD103 and KUD108 were selected by growth on minimal media containing benzylalcohol and ABS or benzylalcohol and phthalate esters, respectively. Also, hybrid plasmid pKG3 which has the naph-

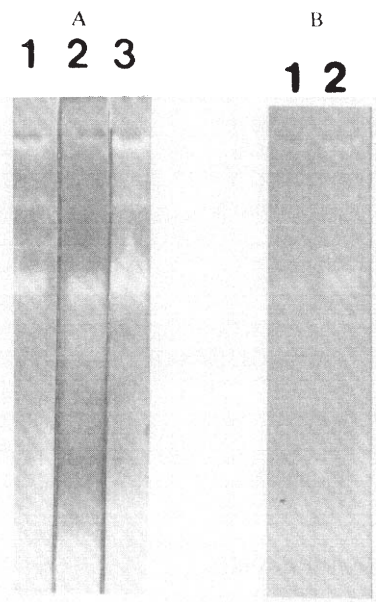

Fig. 2. *Agarose gel electrophoresis of plasmid DNA from the KUD101 (A) and KUD106 (B) strain.*
Plasmid DNA was electrophoresed for 2 hours at 100 V on 0.7% agarose gel.
A: 1. Size marker: *Pseudomonas aeruginosa* (Rms 148), 2. *Pseudomonas putida* KUD12, 3. *Pseudomonas putida* KUD101 (pKG2).
B: 1. *Pseudomonas putida* KUP10, 2. *Pseudomonas putida* KUD106 (pKG2).

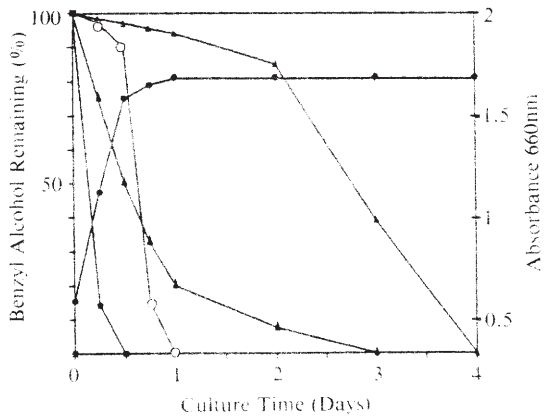

Fig. 3. *Benzylalcohol degradation by various bacterial strains and growth curve of KUD108.*
Bacterial strains were cultured at 30 °C for 4 days in PAS minimal medium containing benzylalcohol. Aliquotes of the samples were picked out and dissolved in acetonitrile.
Symbols: ○ : *P. putida* mt-2(pWWO), ▲ : *P. putida* KUD103(pWWO), ■ : *P. putida* KUD108 (pWWO), △ : *P. putida* KUD202(pKG3, pWWO), ● : Growth curve of KUD108.

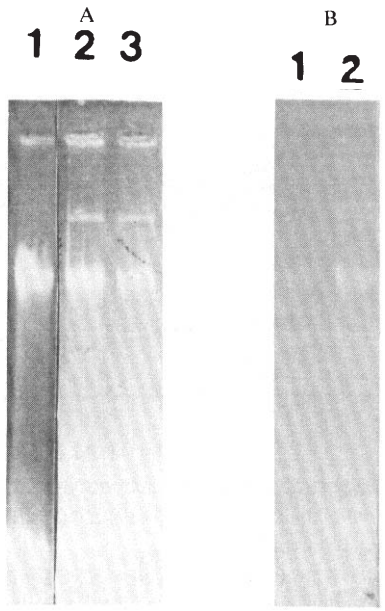

Fig. 4. *Agarose gel electrophoresis of plasmid DNA from the KUD103 (A) and KUD108 (B) strain.*
Plasmid DNA was electrophoresed for 2 hours at 100 V on 0.7% agarose gel.
A: 1. *Pseudomonas putida* KUD12, 2. *Pseudomonas putida* KUD103(pWWO), 3. *Pseudomonas putida* mt-2(pWWO).
B: 1. *Pseudomonas putida* KUP10, 2. *Pseudomonas putida* KUD108(pWWO).

thalene degradative genes was introduced into *P. putida* KUD103, and the new strain of *P. putida* KUD202 which degrades toluene, naphthalene and ABS was obtained.

Degradative abilities of benzylalcohol in *P. putida* KUD108 had a better, and *P. putida* KUD103 and KUD202 had a little lower abilities than that of the original strain (Fig. 3). *P. putida* KUD108 showed a similarity in degrading pattern with original strain. In comparison, KUD103 and KUD202 which originated from KUD12 were different pattern with the original one and each other.

Fig. 4, showed the agarose gel electrophoresis of plasmid DNA of KUD103 and KUD108 strains.

Construction of naphthalene degrading strains and their degradative abilities

To develop the new strains which degrade naphthalene and ABS or naphthalene and phathalate esters, hybrid plasmid pKG3 (Lee and Lee, 1989) was introduced into *P. putida* KUD12 and KUP10 by transformation, respectively. From *P. putida* KUD12, the new strains of *P. putida* KUD102 was obtained and KUD107 originated from KUP10. The

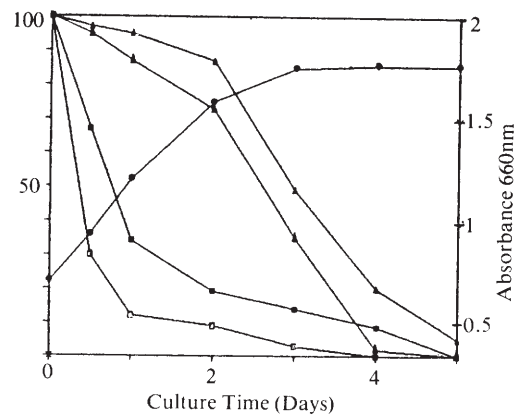

Fig. 5. *Naphthalene degradation by various bacterial strains and growth curve of KUD107.*
Bacterial strains were cultured at 30°C for 5 days in PAS minimal medium containing naphthalene. Aliquotes of the samples were picked out and dissolved in acetonitrile.
Symbols: □: *P. putida* PpG1901(NAH7), ▲: *P. putida* KUD102(pKG3), ■: *P. putida* KUD107 (pKG3), △: *P. putida* KUD202(pKG3, pWWO), ●: Growth curve of KUD107.

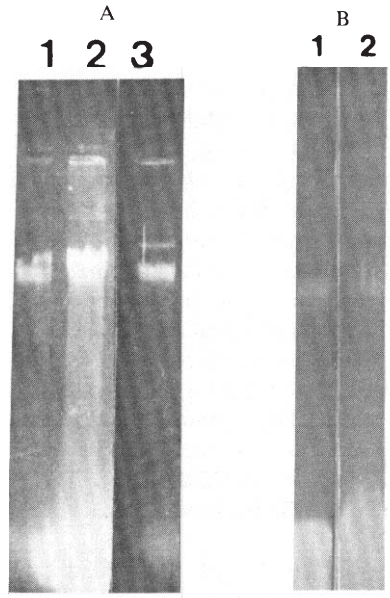

Fig. 6. *Agarose gel electrophoresis of plasmid DNA from the KUD102 (A) and KUD107 (B) strain.*
Plasmid DNA was electrophoresed for 2 hours at 100V on 0.7% agarose gel.
A: 1. *Pseudomonas putida* KUD12, 2. *Pseudomonas putida* KUD102(pKG3), 3. Size marker: *Escherichia coli* C600(RP4).
B: 1. *Pseudomonas putida* KUP10, 2. *Pseudomonas putida* KUD107 (pKG3).

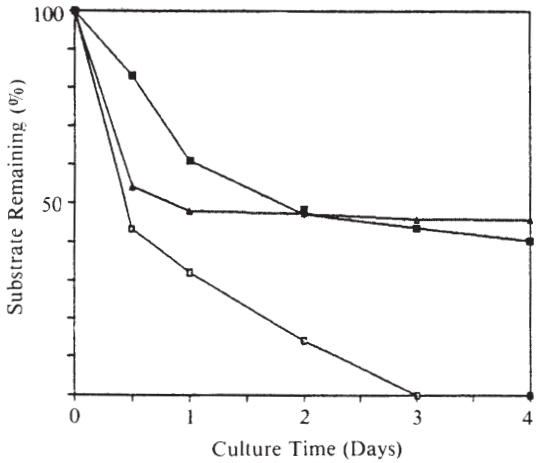

Fig. 7. *2,4-Dichlorophenol, naphthalene and DEHP degradation by mixed culture of KUD106(pKG2) and KUD107(pKG3) strains.*
Bacterial strains were cultured at 30 °C for 4 days in PAS minimal medium containing 2,4-Dichlorophenol, naphthalene and DEHP. Aliquotes of the samples were picked out and dissolved in acetonitrile.
Symbols: □ : Naphthalene, ■ : 2,4-Dichlorophenol, △ : DEHP.

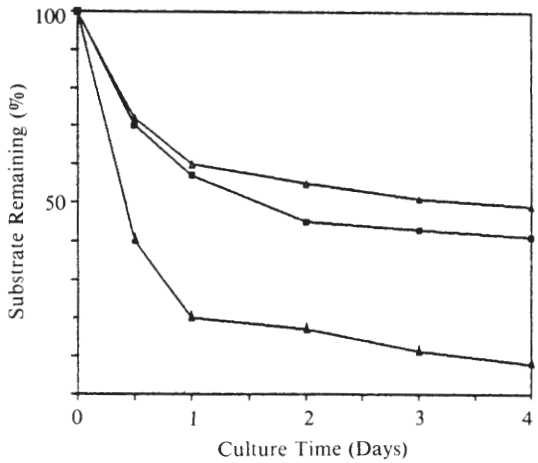

Fig. 8. *2,4-Dichlorophenol, benzylalcohol and DEHP degradation by mixed culture of KUD106(pKG2) and KUD108(pWWO) strains.*
Bacterial strains were cultured at 30 °C for 4 days in PAS minimal medium containing 2,4-Dichlorophenol, benzylalcohol and DEHP. Aliquotes of the samples were picked out and dissolved in acetonitrile.
Symbols: ▲ : 2,4-Dichlorophenol, ■ : Benzyl alcohol, △ : DEHP.

transformants, *P. putida* KUD102 and KUD107 were restisted to kanamycin and could be grown on minimal media containing naphthalene and ABS or naphthalene and phthalate esters, respectively.

Generally, degradative abilities of naphthalene in constructed strains had a little lower abilities than that of original strain, *P. putida* PpG1901 (Fig. 5). *P. putida* KUD107 showed a similarity in degrading pattern with original strain. In comparison, KUD102 and KUD202 which originated from KUD12 were different from the original one.

Agarose gel electrophoresis of plasmid DNA from KUD102 and KUD107 strain were shown in Fig. 6. Also, Fig. 11 showed the agarose gel electrophoresis of plasmid DNA of KUD202 strain.

Degradative abilities in mixed cultures of constructed strains

The mixed culture of KUD106 and KUD107 showed 15% higher degradative ability of 2,4-Dichlorophenol than that of single culture of KUD106 strain (Fig. 7). But in case of the mixed culture of KUD106 and KUD108 (Fig. 8), degradative ability of 2,4-Dichlorophenol had lower ability than that of single culture of KUD106 strain.

Degradative ability of benzylalcohol in mixed culture of *P. putida* KUD107 and KUD108 was similar

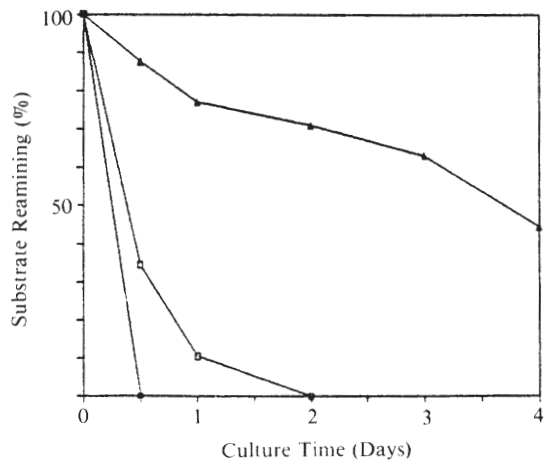

Fig. 9. *Naphthalene, benzylalcohol and DEHP degradation by mixed culture of KUD107(pKG3) and KUD 108(pWWO) strains.*
Bacterial strains were cultured at 30 °C for 4 days in PAS minimal medium containing naphthalene, benzylalcohol and DEHP. Aliquotes of the samples were picked out and dissolved in acetonitrile.
Symbols: □ : Naphthalene, ■ : Benzyl alcohol, △ : DEHP.

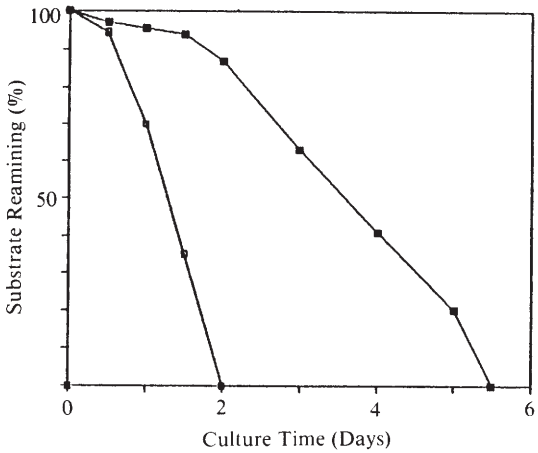

Fig. 10. *Naphthalene and benzylalcohol degradation by mixed culture of KUD102(pKG3) and KUD(103) (pWWO) strains.*
Bacterial strains were cultured at 30 °C for 5.5 days in PAS minimal medium containing naphthalene and benzylalcohol. Aliquotes of the samples were picket out and dissolved in acetonitrile.
Symbols: □ : Benzyl alcohol, ■ : Naphthalene.

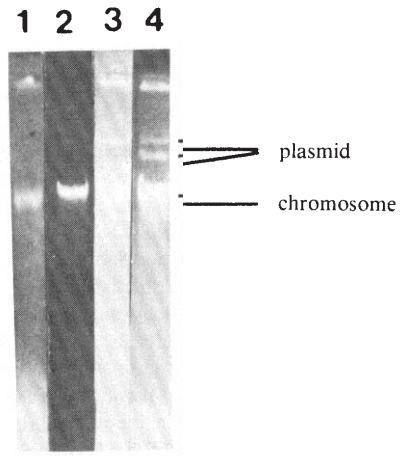

Fig. 11. *Agarose gel electrophoresis of plasmid DNA from the KUD202 strain.*
Plasmid DNA was electrophoresed for 2 hours at 100V on 0.7% agarose gel.
1. *Pseudomonas putida* KUD12. 2. *Pseudomonas putida* KUD102(pKG3), 3. *Pseudomonas putida* mt-2(pWWO), 4. *Pseudomonas putida* KUD202 (pKG3, pWWO).

to those of the single culture of KUD108 (Fig. 3 and 9). In the case of mixed cultures of KUD102 and KUD103 or KUD106 and KUD108, the mixed culture of KUD102 and KUD103 had a better (Fig. 3 and 10) and the mixed culture of KUD106 and KUD108 had a little lower degradative abilities of benzylalcohol than those of single culture of KUD103 or KUD108 (Fig. 3 and 8).

The mixed culture of KUD102 and KUD103 had a little lower (Fig. 5 and 10), and mixed cultures of KUD107 and KUD106 or KUD107 and KUD-108 had a better degradative abilities of naphthalene than those of single culture of KUD107 or original strain (Fig. 5, 7 and 9).

적 요

여러 가지 난분해성 방향족 화합물을 분해하는 균주들을 개발하기 위해 2,4-D 분해 유전자를 갖는 재조합 플라스미드 pKG2, 나프탈렌 분해 유전자를 갖는 재조합 플라스미드 pKG3 그리고 TOL 플라스미드를 합성세제 분해능을 갖는 *P. putida* KUD12와 프탈산에스테르를 분해하는 *P. putida* KUP10에 각각 형질전환 또는 접합시켰다. *P. putida* KUD12로부터 KUD101(pKG2), KUD102(pKG3), KUD103(TOL), KUD202(pKG3, TOL) 균주를, 또 *P. putida* KUP10으로부터 KUD 106(pKG2), KUD107(pKG3), KUD108(TOL) 균주를 각각 개발하였다.
개발균주의 분해능은 KUD101과 KUD102 그리고 KUD107이 원분해 균주와 비슷하였고, KUD103과 KUD106 그리고 KUD202는 원분해 균주보다 분해능이 떨어졌고 KUD108의 분해능은 원균주보다 우수하였다. 개발균주들의 혼합배양에서는 KUD107과 KUD108의 혼합배양이 다른 혼합배양들보다 분해능이 우수한 것으로 나타났다.

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