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Addition of *Rhodococcus fascians* AC6 to prevent inhibition of the toluene degradation from ethyl acetate in biofiltration of VOCs-contaminated air stream

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***Abstract*—In treatment of both ethyl acetate and toluene-contaminated air, selecting an adequate mixed microbial consortium as an inoculum is essential for effective toluene degradation with no serious inhibitory effect from ethyl acetate. *Rhodococcus sp.* B5 has the ability for uptake of both ethyl acetate and toluene but it encounters readily the problem of substrate-competitive inhibition from high load of ethyl acetate. Our present work indicates that adding a fast ethyl-acetate-degrading strain (not capable of degrading toluene), *Rhodococcus fascians* AC6, together with strain B5 to biofilters can make a steady progress in removal of toluene, and alleviate the afore-mentioned substrate competition inhibition by ethyl acetate. The results were confirmed by using two different filter media at various conditions. Under the optimal media moisture content, commercial grade of peat moss is preferred to be a filter media for treatment of relatively hydrophobic toluene, and scraped sugarcane stem could alleviate the effect of the toxic intermediates on biodegradation of toluene. The inoculation concentration of microbial consortium in biofiltration must be high enough to maintain the high removal efficiency, and to prevent the substrate competition inhibition from decomposed compounds.**

Keywords- biofiltration*, Rhodococcus fascians, Rhodococcus sp.*; *ethyl acetate; toluene; biodegradtion

I. INTRODUCTION

Biofiltration is an old biotechnology used to treat odorous gases. Now it has been applied to industrial waste gases containing volatile organic compounds (VOCs). A considerable quantity of VOCs has been produced each year from many industrial point sources, e.g., printing and coating facilities, foundries and opto-electronics industries, and paint-manufacturing plants [1,2]. According to our previous report in a field study (data not shown), a comparable concentration of ethyl acetate (30-100 ppmv) was inhibitory for toluene or xylene degradation (10-30 ppmv). In addition, Deshusses *et al.* [3] reported that even a 2 g m^{-3} of ethyl acetate could apparently inhibit the removal of concurrent toluene.

An adaptation limitation, competitive inhibition, non-competitive inhibition and reaction product inhibition can be used to explain the biological inhibition in biofiltration. The acidic intermediates might lower down the pH of media and then reduced the removal efficiency of biofiltration [4]. Deshusses *et al.* [3] found that a large amount of ethanol was measured in treating 6.9 and 12.4 g m^{-3} of ethyl acetate and toluene, respectively, and no removal of toluene occurred. After their microbiological test, those facultative toluene degraders were far outnumbered by microorganisms capable of degrading ethyl acetate. Hubert *et al*. [5] and Mars *et al.* [6] have also reported the results regarding the microbial community competition in removal of toluene. However, little research was proposed to provide a total solution for the common inhibition phenomena; in this case, the bacterium capable of degrading both ethyl acetate and toluene will first degrade the most readily biodegradable ethyl acetate rather than toluene. Thus, the goal of this study was to demonstrate an adequate microbial consortium, which could utilize both ethyl acetate and toluene with a great and stable removal efficiency under various environmental factors or operating conditions.

II. MATERIALS AND METHODS

A. Chemicals

All the chemicals used were from Merck and of analytical grade.

B. microorganisms

Only the strain with the ability for uptake of both ethyl acetate and toluene as well as the strain capable of degrading only ethyl acetate were chosen. The former is *Rhodococcus sp.* B5 *CCRC 17223*, and the latter is *Rhodococcus fascians* AC6 *CCRC 17224*.

C. Shake-flask culture

The standard Hydrocarbon Minimal Medium (HCMM2, as seen in Table 1) was used as a basic culture medium [7]. In the beginning, an aliquot (0.1-1 ml samples) of supernatant fluid from the isolated stock cultures was evenly spread on a NB medium, and then incubated for 24-36 hours at 30 ^{o}C. The colony on the surface was transferred to an autoclaved modified HCMM2 mineral salt solution. The optical density (OD_{600nm}) was adjusted to 0.1. An acid-rinsed serum bottle with a volume of 163 ml was filled with 25 ml of the modified HCMM2 solution. The bottle was sealed with Teflon/Silicon septum. Using a syringe, 2, 4, 20, 40-μl ethyl acetate and 2, 4-μl toluene were injected together into each bottle. The volumetric ratio of toluene to ethyl acetate (T/E ratio) was maintained in proportion of 1:1 to 1:10. The

gaseous concentrations of ethyl acetate and toluene were measured regularly according to a headspace method [8]. The dry cell weight was determined by drying the cells for 24 h at 105 ^{o}C. All assays were conducted in triplicates with uninoculated controls.

TABLE I. THE STANDARD HCMM2 MEDIUM [12]

Contents	Concentrations (mg/L)
KH_2PO_4	1.36×10^3
Na_2HPO_4	1.42×10^3
KNO_3	0.50×10^3
$(NH_4)_2SO_4$	2.38×10^3
$MgSO_4$-$7H_2O$	0.05×10^3
$CaCl_2$	0.01×10^3
H_3BO_3	2.86
$MnSO_4$-H_2O	1.54
$Fe(NH_4)_2(SO_4)_2$-$6H_2O$	3.53
$CuSO_4$-$5H_2O$	0.039
$ZnCl_2$	0.021
$CoCl_2$-$6H_2O$	0.041
Na_2MoO_4-$2H_2O$	0.025

D. Biofiltration

Three biofilter columns made of acrylic fiber were all 30-cm long and 8-cm ID (Figure 1). The details of the associated systems could be obtained according to our previous work [9]. The nutrient-containing water used for medium moisture control consisted of 1.33 g l^{-1} NH_4Cl, 0.6 g l^{-1} KH_2PO_4, and 2.272 g l^{-1} Na_2HPO_4 (Merck, Taiwan). A commercial grade of peat moss (Klasmann-Deilmann, Germany) or laboratory-prepared scraped sugarcane stem was selected as media packing. They were scraped or sieved out at the size of 1.19-2.00 mm and then dried at 105 ^{o}C for 24 hours. An approximate amount of water that contained 19.58 g l^{-1} $NaHPO_4$, 9 g l^{-1} KH_2PO_4, and 5 g l^{-1} NH_4Cl (Merck, Taiwan) was added as neutralizing agents to buffer the media and to adjust its moisture content to the specified values of 5 (for peat moss) and 10 (for scraped sugarcane stem) water per dry solid (g g^{-1}), or otherwise mentioned. The wetted media were placed in a sealed plastic box and stayed 24 h for moisture equilibrium. Then, only 20-cm in height of the reactor was filled with the media. The biofilter with mixed microbial consortium was originally inoculated with 10 ml of each of above-mentioned bacterial strains at an OD_{600nm} value of 0.4, but the ones using a pure strain were inoculated with full 20-ml. The VOCs-contaminated air streams (ethyl acetate: conc., 100 ppmv, loading rate per media volume, 36.0 g m^{-3} h^{-1}; toluene: 10 ppmv, 3.8 g m^{-3} h^{-1}; space velocity for biofiltration, 100 h^{-1}) were made using two gaseous mass flow controllers (SIERRA, Mass-Trak series 810, Monterey, US) rated at 100 ml min^{-1} and 1,000 ml min^{-1} for ethyl acetate and toluene, respectively. The room temperature was maintained between 24-26 ^{o}C for all experiments.

E. Gas phase analysis

VOCs were analyzed using an automatic gas chromatograph system (China Series 8700F, Taipei County, Taiwan) equipped with a flame ionization detector and a capillary column (15 m; 0.53 mm ID) (Suppelco, SPB-5, Bellefonte, US). Nitrogen (99.95% purity) was used as a carrier gas at a flow rate of 10-ml N_2 min^{-2}. The GC oven temperature, injector temperature, and detector temperature were maintained at 150 ^{o}C, 200 ^{o}C, and 200 ^{o}C, respectively.

III. RESULTS AND DISCUSSION

A. Microbial Characteristics and Ethyl Acetate Inhibition

The optimal initial pH was controlled at 7.0 for biodegradation of ethyl acetate or toluene in the shake flask culture or biofiltration

Strain B5 is able to use both ethyl acetate and toluene as carbon sources. Their time course profile under various T/E ratios is depicted in Figure 2. Obviously, feeding 4-μl toluene (T/E ratio=1/0) alone into the 163-ml flask containing 25-ml modified HCMM2 medium gave the fast degradation rate in removal of toluene. The degradation rate became affected by the initial addition of 4-μl ethyl acetate (T/E ratio=1/1) and leveling off when the T/E ratio was 1/10. Since ethyl acetate concentration at the T/E ratio of 1:10 became leveling off before complete degradation and the final associated residual dissolved oxygen and pH were 1.0-1.5 mg/L and below 5.0, respectively, it is believed that toluene was not even degraded or oxidized after the acidic intermediate had produced. Subsequently, the other experiment with much more buffer intensity in the medium to maintain the pH was carried out, and the final complete degradation of ethyl acetate with barely improved toluene degradation efficiency (data not shown) implied that the strain still encountered the problem of substrate competitive inhibition from ethyl acetate.

On the other hand, the removal efficiency in degradation of pure ethyl acetate (4 or 40 μl; the T/E ratio = 0/1 or 0/10) by strain B5 was about the same as that in the presence of toluene (4 μl; the T/E ratio = 1:1 or 1:10). It is noted that the degradation of ethyl acetate was not affected by the initial addition of toluene. In addition, the fast removal rate in lower concentration of ethyl acetate but incomplete degradation in higher concentration implies that strain B5 is only able to metabolize the intermediates at a low concentration level and becomes inhibited by the accumulated concentration of intermediates (acetic acid). Cha *et al.* (1999) reported that the accumulated acid intermediate caused further decomposed slowly during the reaction. Thus, ethyl acetate is degraded slowly right after the pH value below 5.0.

Strain AC6 is able to use ethyl acetate but toluene as carbon sources. The time course of their degradation under various T/E ratios is depicted in Figure 3. Obviously, toluene was not degraded at all, and the complete degradation of 40-μl ethyl acetate (for both T/E ratios of 0/10 and 1/10) occurred soon (within 12 h) compared with that by strain B5 in Figure 2. In the meantime, the resulting pH value for microbial growth at the T/E ratio of 1:10 was maintained above the minimum value, 5.5 (data not shown), below which no bacteria could survive. This indicates that strain AC6 is a fast ethyl-acetate-degrading bacterium that can be used to treat a high concentration of ethyl acetate and its

intermediates as well. Moreover, the removal efficiency in degradation of pure ethyl acetate (4 or 40 μl; the T/E ratio = 0/1 or 0/10) by strain AC6 was about the same as that in the presence of toluene (4 μl; the T/E ratio = 1:1 or 1:10). Apparently, no inhibitory effect occurs at that concentration of toluene.

B. Improvement of toluene degradation demonstrated in an AC6-added shake-flask culture

To demonstrate the microbial consortium can alleviate the substrate-competition inhibition problem, the mixed-VOCs degradation experiments inoculated with strain B5 only, and the consortium of strain B5 and strain AC6, were conducted under various T/E ratios. The results of their initial degradation rates are listed in Table 2.

TABLE II. EFFECT OF MICROBIAL CONSORTIUM ON THE BIODEGRADATION OF BOTH ETHYL ACETATE AND TOLUENE IN SHAKE FLASK CULTURES UNDER VARIOUS T/E RATIOS AT 30 ^{o}C.

T/E ratios	Initial degradation rate (g m^{-3} h^{-1})			
	Strain B5 only		*Strain B5 + strain AC6*	
	Ethyl acetate	*Toluene*	*Ethyl acetate*	*Toluene*
1/0	-	1.064	-	0.574 (1.148*)
1/2	0.181	0.712	0.192	0.521 (1.042*)
1/5	0.641	0.673	0.660	0.359 (0.718*)
1/10	1.008	0.709	1.402	0.408 (0.816*)

*: The mixed cultures had equal amount of strain B5 and strain AC6 in it. Since the strain AC6 was not able to degrade toluene, the mixed culture had only half equivalent amount of bacteria that can degrade toluene. Thus, it is reasonable to double the initial degradation rate of toluene for the consortium with strain B5 and strain AC6 so as to compare the rate made by pure strain B5.

The initial degradation rate of toluene by strain B5 decreased with the decrease of T/E ratio, indicating that the substrate-competition inhibition occurred in biodegradation. However, the initial degradation rate of toluene was significantly improved by seeding the consortium of strain B5 and strain AC6. Especially for the T/E ratio equal to 1/2, the ability of strain B5 to degrade initially toluene was completely recovered while strain B5 was being associated with strain AC6. For the rest of T/E ratios, it is expected that adding more amount of strain AC6 to the system could significantly recover the initial degradation rate of toluene. The whole idea is demonstrated in the following biofiltration test result. In addition, the initial degradation rate of ethyl acetate by strain B5 only was slower than that by the consortium of strain B5 and strain AC6. This result is in agreement with the above-mentioned characteristics of strain B5 that is not a fast ethyl-acetate-degrading bacterium.

C. Adding strain AC6 to biofilter to improve the toluene removal efficiency

In order to know the role of strain AC6 playing in the mixed culture, the experiments inoculated with strain B5 and strain AC6, or both of them were conducted twice by using either the commercial grade of peat or the bagasse as filter media, based on the predetermined optimal conditions (pH 7, NH_4Cl as nitrogen source, MC=4 & 8 for peat and scraped sugarcane stem, respectively). Obviously, the removal of toluene is much improved and in a steady progress with the inoculants of both strain B5 and strain AC6 rather than their individuals (Figure 4). As aforementioned in shake-flask cultures, the biodegradation of toluene was inhibited by the presence of ethyl acetate because the substrate competitive inhibition or the acidic intermediate products would damage the activity of strain B5. Thus, adding a fast-ethyl-acetate degrading bacterium, strain AC6, can degrade the ethyl acetate in time, to prevent the removal efficiency of toluene from dropping. Consequently, adding these two strains of bacteria as a consortium can be a good inoculation strategy in biofiltration of mixed VOCs that cause the substrate-competition inhibition problem.

Compared with Figure 4A, Figure 4B shows a better performance in biofiltration of toluene. Since the commercial grade of peat moss, compared with bagasse, has the lower optimal MC level, it is more favorable for hydrophobic toluene to adsorb. In other words, the mass transfer rate of toluene in peat moss is higher than that in bagsse. Moreover, the lower optimal MC level in peat moss could not dilute efficiently the decomposed toxic-intermediate compounds. Therefore, the removal efficiency of toluene in peat moss became worse after it reached the maximum, but that in bagasse became leveling off.

According to Figure 4B and 4C, the toluene removal efficiency couldn't reach 100 % although the volume ratios of inoculants to the media free water in biofiltration and in shake-flask cultures were kept the same (10%). Moreover, initially adding twice amount of inoculants (Figure 4C) gives a much more improvement in the degradation of toluene. As aforementioned, the produced biomass and the decomposed compounds from the media would compete with toluene for cell metabolism. Thus, the concentration of inoculants, i.e. strain B5 and strain AC6, must be high enough to increase the removal efficiency and to prevent the substrate competition inhibition and the microbial community competition caused by the indigenous microorganisms.

IV. CONCLUSIONS

Adding an enough fast-ethyl-acetate degrading strain AC6 can degrade ethyl acetate in time so as to enhance the degradation of toluene. Under the optimal media moisture content, 4 and 8 g-H_2O/g-solid for commercial grade of peat moss and bagsse, respectively, commercial grade of peat moss is preferred to be a filter media for treatment of relatively hydrophobic toluene, and bagasse could alleviate the effect of the toxic intermediates on biodegradation of toluene. The inoculation concentration of microbial consortium in biofiltration must be high enough to maintain the high removal efficiency, and to prevent the substrate competition inhibition.

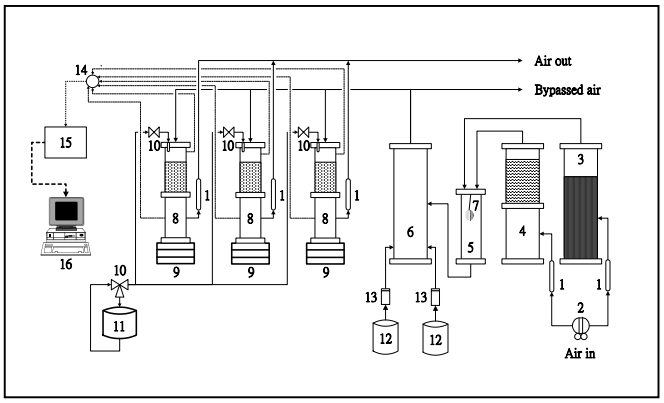

Figure 1. Schematic diagram of laboratory-scale biofilter. (1) rotameter; (2) air blower; (3) humidifier; (4) air dryer with silica gel; (5) equalization chamber for temperature and humidity; (6) equalization chamber for VOCs; (7) 100-Watt electric bulb; (8) column with neutralized-peat or otherwise mentioned; (9) electronic balances; (10) electric solenoid; (11) nutrient solution; (12) pure VOC liquids; (13) mass flow controllers; (14) sixteen-way stream selector; (15) gas chromatograph; (16) computer with two data acquisition cards

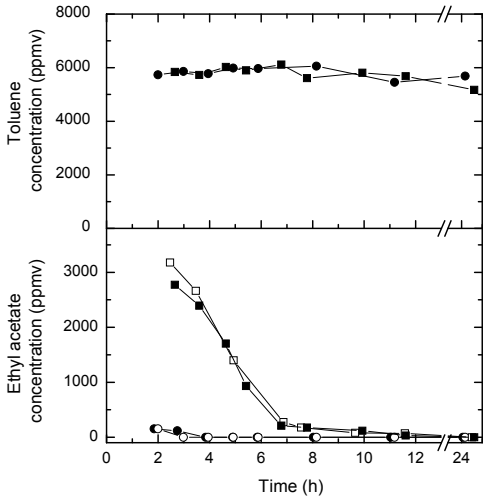

Figure 3. Biodegradation of ethyl acetate and toluene in shake flask cultures by strain AC6 feeding different amount of VOCs. For ethyl acetate only (open); mixed VOCs (close).
—■— 4-μL toluene and 40-μL ethyl acetate (T/E=1/10)
—●— 4-μL toluene and 4-μL ethyl acetate (T/E=1/1)

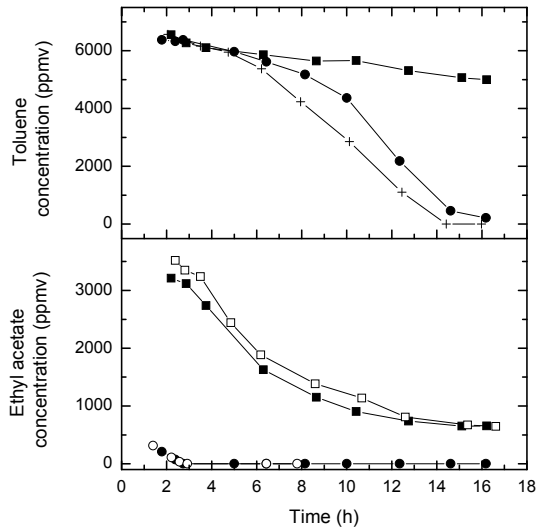

Figure 2. Biodegradation of ethyl acetate and toluene in shake flask cultures by strain B5 feeding different amount of VOCs. For ethyl acetate only (open); mixed VOCs (close).
—■— 4-μL toluene and 40-μL ethyl acetate (T/E=1/10)
—●— 4-μL toluene and 4-μL ethyl acetate (T/E=1/1)
—+— 4-μL toluene (T/E=1/0)

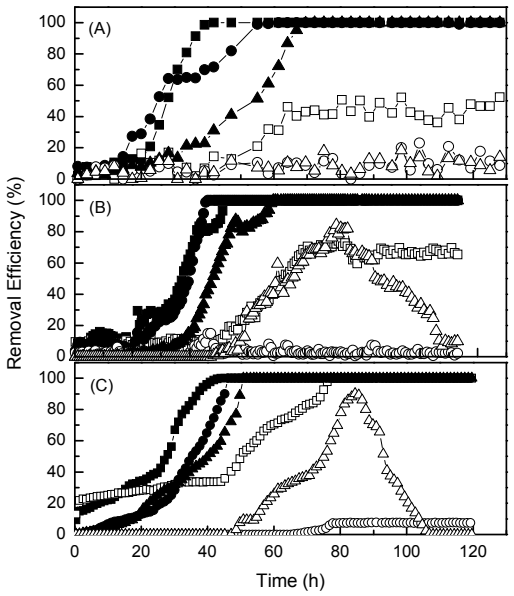

Figure 4. Biofiltration of ethyl acetate (close) and toluene (open) with various microbial consortium using NH_4Cl and scraped sugarcane stem (MC=8) (A); using peat (MC=4) (B); and under the same condition as (B) except doubling the inoculant concentration (MC=4) (C).
—■— strain B5 and strain AC6
—●— strain AC6
—▲— strain B5

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