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Biodegradation of Toluene using Biofilms in a Bubble Column Bioreactor

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Biodegradation of toluene in liquid effluent stream was carried out using biofilms of *Pseudomonas putida* formed on celite particles in the bubble column bioreactor. Silicon rubber tubing was installed at the bottom of the bioreactor and liquid toluene was circulated within the tubing. Toluene diffused out of the tube wall and was transferred into the culture broth where degradation by biofilms occurred. The operating variables affecting the formation of biofilms on celite particles were investigated in the bubble column bioreactor, and it was found that formation of bifilm is favored by high dilution rate and supply rate of carbon source which stimulate the growth of initially attached cells. Continuous biodegradation of toluene using biofilms was stablely conducted in the bioreactor for more than one month without any significant fluctuation, showing a removal efficiency higher than 95% at the toluene transfer rate of 1.2 g/L/h.

Aromatic solvents such as benzene, toluene and xylenes (BTX) are widely used in chemical and process industries such as refineries, paint, and pharmaceutical manufacturing. As one of the major pollutants, these solvents have been causing detrimental effects on the environment (8, 12, 21, 22, 26, 27).

Much effort has been directed to the biodegradation of these compounds in the waste stream. Since these solvents are highly volatile and slightly soluble in water, they are present in both liquid and gaseous effluent streams. A few processes have been reported for the biodegradation of BTX in gaseous effluents : biofilters, trickling filters, and bioscrubbers. However, the main disadvantages of these processes are low degradation capacity, less than 0.04 g/L/h, and difficulty of operation (21,26,27). On the other hand, little attention has been paid to the development of biodegradation process for aromatic solvents in the liquid effluent streams even though significant quantities of these solvents are known to be discharged in liquid effluents by the chemical industries.

In our previous papers, we developed a novel bioreactor for the microbial degradation of toxic aromatic solvents such as benzene, toluene and xylenes in liquid effluent stream (1, 17). Silicon rubber tubing was immersed in a completely mixed and aerated bioreactor, and liquid solvent was circulated within the tubing from a solvent reservoir using a diaphragm pump. In this system, liquid solvent diffused out of the tube wall and was transferred into the culture broth where the liquid solvent was aerobically degraded by microorganisms. The degradation rate of liquid solvent in the bioreactor was found to be considerably higher than that of the conventional processes.

However, biodegradation process using freely suspended cells are generally known to have some shortcomings compared with those using immobilized microorganisms in the treatment of industrial wastewaters. There are two main advantages of the processes using immobilized microorganisms. Higher biomass concentration can be maintained even at high dilution rate which allows higher volumetric degradation rate. A stable operation is possible because immobilized cells are often found to be more resistant against shock loading of inhibitory compounds than freely suspended cells (10, 11, 13, 15, 16, 18, 28, 29).

In this paper, we examined the biodegradation of toluene as a model solvent using biofilms of *Pseudomonas putida* developed on celite particles in a bubble column bioreactor. Recently, bubble column bioreactor has been gaining increasing importance in the area of biotechnology, particularly in wastewater treatment because this bioreactor provides a favorable mixing and a mass transfer property combined with low shear stress of the biological materials (7, 9, 15, 19, 23). For the efficient supply of liquid toluene into the bioreactor, silicon rubber tubing was immersed in the bubble column

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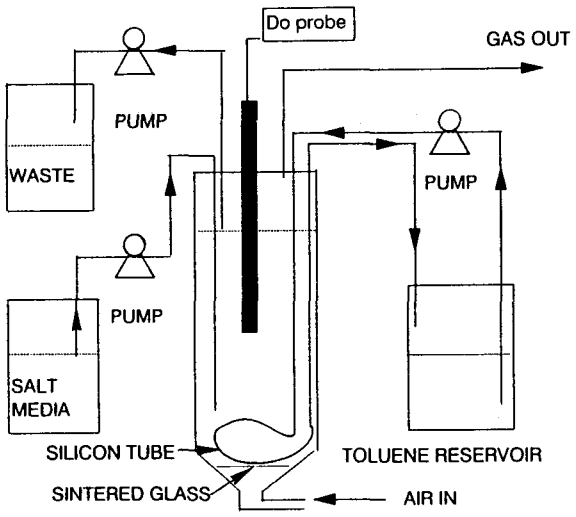

Fig. 1. Schematic diagram of the bubble column bioreactor

bioreactor as mentioned above. A schematic diagram of the bubble column bioreactor is shown in Fig. 1. The formation of a stable biofilm of high density is crucial for a successful startup and operation of the process. The operating variables affecting the formation of biofilms on celite particles and biodegradation of toluene using biofilms in the bubble column bioreactor were investigated.

THEORETICAL CONSIDERATION

Toluene Transfer Rate

Toluene transfer rate through the silicon rubber tubing in the bioreactor was experimentally determined in the absence of microorganism based on the material balance for toluene as follows :

$$R_t \cdot V = F_g \cdot C_g + (Q_t \cdot X) \cdot V + \frac{dC_t}{dt} \cdot V \quad (1)$$

By taking a steady-state for toluene in the culture broth, Eq. (1) becomes

$$R_t \cdot V = F_g \cdot C_g + (Q_t \cdot X) \cdot V \quad (2)$$

In the absence of microbial growth, the toluene transfer rate per culture volume is obtained by measuring a steady-state concentration of toluene in the exit gas as follows :

$$R_t = \frac{(F_g \cdot C_g)}{V} \quad (3)$$

Toluene Degradation Rate

Toluene degradation rate per unit culture volume is determined from equation (1) :

$$Q \cdot X = R_t - \frac{F_g \cdot C_g}{V} - \frac{dC_t}{dt} \quad (4)$$

In batch operation, the toluene degradation rate can be estimated by measuring both the toluene concentration in the exit gas and the change in dissolved toluene concentration with time at a fixed toluene transfer rate. The toluene degradation rate under steady-state condition is obtained by measuring the toluene concentration in the exit gas under the same condition.

Removal efficiency of toluene

Removal efficiency of toluene is calculated by dividing the total degradation rate by total transfer rate :

$$RE(\%) = \frac{Q_t \cdot X}{R_t} \cdot 100 \quad (5)$$

MATERIALS AND METODS

Materials

Toluene was purchased from Sigma Chemical Company (St.Louis, MO). Celite (250~300 μm diameter) was obtained from Manville Company (Denver. CO). All other chemicals were of reagent grade. Silicon tubing (0.157 cm ID, 0.318 cm OD) was obtained from Dow Corning (Midland, MI).

Microorganism and Cultivation

Pseudomonas putida ATCC23973 was used. When degradation experiments were conducted, the follwing medium was used : 5.8 g/L KH_2PO_4, 4.5 g/L K_2HPO_4, 2.0 g/L $(NH_4)_2SO_4$, 0.34 g/L $MgCl_2 \cdot 6H_2O$, 0.02 g/L $CaCl_2$, 0.002 g/L $FeSO_4 \cdot 7H_2O$, 0.0016 g/L $MnCl_2 \cdot 4H_2O$.

Bioreactor

The bioreactor was a bubble column type with a water jacket and the diameter and height of the bioreactor were 5.5 and 28.5 cm, respectively. A predetermined length of silicon rubber tubing was installed at the bottom of the bioreactor, and liquid toluene was circulated at the flow rate of 30 ml/min within the tubing from a reservoir using a diaphragm pump (Fig. 1). Toluene transfer rate in the bioreactor was varied by changing the length of the silicon rubber tubing. Water-saturated air was supplied through a sintered glass placed at the bottom of the bioreactor. Dissolved oxygen was monitored using a oxygen probe (Ingold, Switzerland). Unless stated otherwise, working volume of the bioreactor was fixed at 300 ml, and temperature was controlled at 30°C.

Biodegradation of Toluene in the Bioreactor

Toluene was aerobically degraded using biofilm in the bubble column bioreactor. In continuous operation, culture medium was fed at the predetermined flow rate into the bioreactor and the culture broth was withdrawn using a peristaltic pump to maintain a constant liquid volume in the bioreactor. Culture broth and exit gas were

periodically analyzed to determine the concentrations of toluene and biomass.

Biomass loading on celite particles

The biomass loading (defined as the dry cell mass per unit mass of celite particles) was determined as follows : a portion of biofilms-formed celite particles (2~4 ml) was withdrawn from the bioreactor and washed with 100 ml of distilled water to remove the free and loosely attached cells. The washed celite particles were incubated in an oven at 105°C for 24 h, and dry weight was measured. Biofilms developed on celite particles were removed by successively treating with 6 N HCl and 10 N NaOH, and celite particles were thoroughly washed with 1000 ml of distilled water. Biomass-free particles were dried in an oven at 105°C for 24 h and weighed. The biomass loading on celite particles was obtained by calculating the weight difference. Duplicate samples were taken for each determination.

Scanning electron microscopy

Biofilm-formed celite particles withdrawn from the bioreactor were washed with 50 mM phosphate buffer (pH 7.2) and treated with 2.5% glutaraldehyde solution for fixation. After they were extensively washed with distilled water, celite particles were then vacuum dried, coated with gold in a spurter coating unit, and scanned using a scanning electron microscope (535M Model, Philips Co. Netherlands) at an accelerating voltage of 20 kV.

Analysis

The concentration of toluene was determined using a gas chromatograph (HP5890 Model, Hewlett-Packard Co., Palo Alto, CA.) equipped with a flame ionization detector. A stainless steel column (6 ft × 1/8 in. ID) packed with Chromosorb HWP (100~120 mesh) was used. Temperature of both injector and detector were maintained at 250°C, and the column temperature was maintained at 170°C. Flow rate of nitrogen as a carrier gas was 30 ml/min. When determining the concentration of toluene in the exit gas of the bioreactor, the exit gas was collected using a home-made gas collector equipped with a Teflon-coated rubber stopper, and 100 μl of collected gas was injected into the gas chromatograph using a microsyringe. The concentration of toluene in the culture broth was determined after filtering the culture broth through a polycarbonated filter (0.45 μm pore size, Millipore). Dry cell weight was determined by weighing the filtered bacterium after drying at 105°C for 4 h.

RESUTS AND DISCUSSION

Toluene transfer rate in the bubble column bioreactor

Toluene is transferred in the liquid phase through the tubing into the culture broth via tube walls. Thus, the toluene transfer rate is thought to be affected by the operation parameters such as aeration rate and circulation rate within the tubing. The toluene transfer rate varied significantly with the aeration rate as shown in Fig. 2. Toluene transfer rate was almost independent of circulation rate of liquid toluene within the tubing (data not shown). In this work, the aeration rate was fixed at 1 L/min to prevent the formation of excess foam and flooding, and circulation rate of liquid toluene within the tubing was fixed at 30 ml/min. The toluene transfer rate into the bioreactor was controlled by changing the length of the silicon tubing immersed in the bioreactor

Effect of celite particles on oxygen transfer

In order to investigate the effect of celite particles on the oxygen transfer, the oxygen transfer coefficient was determined using a dissolved oxygen probe at different amounts of biomass-free celite particles in the bioreactor. As shown in Fig. 3, the oxygen transfer coefficient was

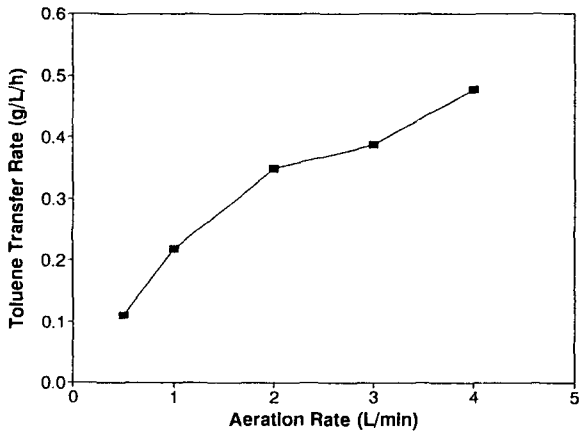

Fig. 2. Effect of aeration rate on the toluene transfer rate

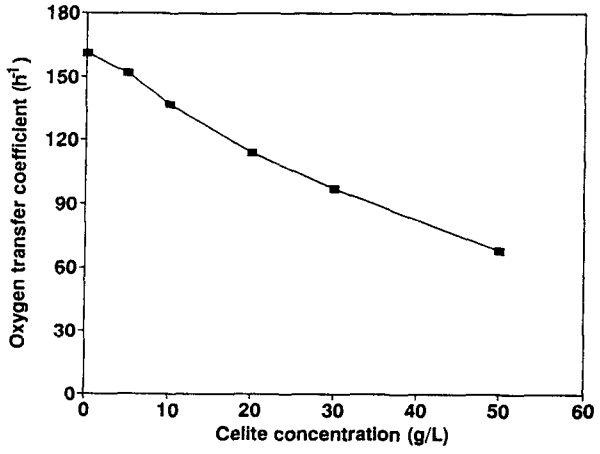

Fig. 3. Effect of celite concentration on the oxygen transfer coefficient

found to decrease with increasing celite concentration. It was also reported that the oxygen transfer coefficient was significantly decreased from 144 to 72 h^{-1} with increasing concentration of sand particles (25). Another factor to be considered in determining the proper concentraton of celite particles in the bioreactor is the mixing of celite particles. We tested the mixing at different amounts of celite particles under fixed aeration rate, and found that celite particles were well circulated at the concentration lower than 20 g/L in the bioreactor. Accordingly, the concentration of celite was fixed at 20 g/L by taking into consideration the oxygen transfer rate required for the complete biodegradation and mixing.

Development of biofilms on celite particles

It has been generally known that a requisite event in the formation of biofilms is the adsorption of bacterial cells on solid substratum. This indicates the colonization of microorganisms on the solid support. When environmental conditions for cell growth are satisfied, the formation of biofilms on solid support will occur through successive process of continuous adsorption, growth of adsorbed cells and detachment of attached cells. Most of the researches concerning formation of biofilms have been dealing only with the effect of physico-chemical and biological interactions occurring between the microorganisms and surface of the solid support (4, 5, 10, 13, 26, 28, 29). And although several reports on the biofilm formation process have been appearing recently, their conclusions have not supported each other (10, 13, 18). In addition, studies regarding the operation variables affecting the formation of biofilms have not been systematically conducted.

We conducted the formation of biofilms in the bubble column bioreactor as described in the experimental section and investigated the effect of operating variables, such as dilution rate and supply rate of carbon source (i.e. toluene), which directly affect the growth of microorganisms in continuous cultivation.

Fig. 4 shows the electron microscopic photos of the biofilms developed on the celite particles after 5 days of continuous cultivation at different dilution rates when toluene transfer rate was fixed at 0.395 g/L/h. Concentration of freely suspended and attached cells were also determined at each dilution rate and are shown in Fig. 5. As can be seen from these results, the formation of biofilms on the surface of celite particles was insignificant at low dilution rate even though the concentration of freely suspended cells was very high (Fig. 4a). On the contrary, entire portion of pores was found to be covered with microorganisms at higher dilution rate (Fig. 4b). In this case, the concentration of freely suspended cells was lower than that observed at lower dilution rate (Fig. 5). The maximum specific growth rate of microrganism used in this work was determined to be about 0.43 h^{-1} on toluene. The presence of freely suspended cells at a dilution rate much higher than the maximum specific growth rate implies that the detachment of biofilms is

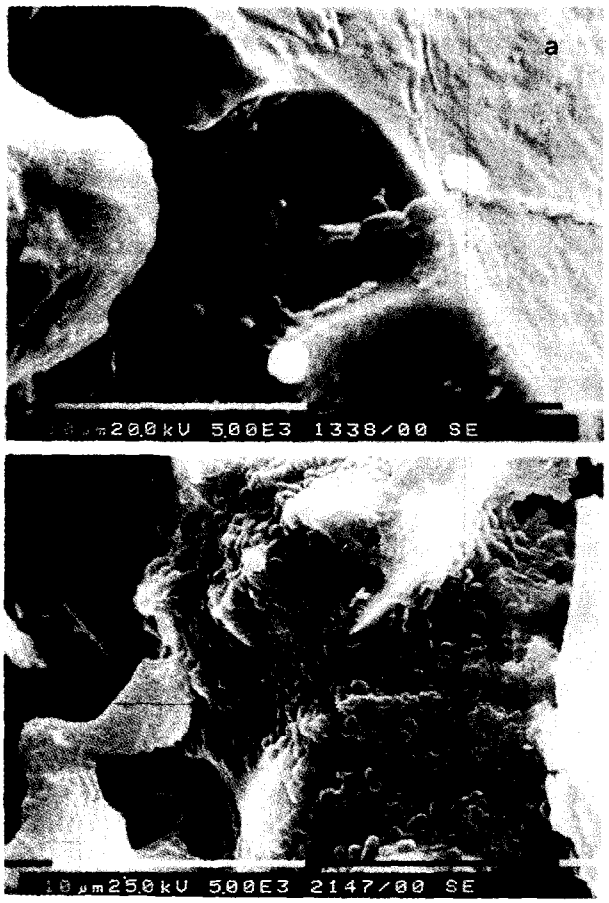

Fig. 4. Electron microscopic photos of the attached cells at different dilution rates when the touene transfer rate was fixed at 0.395 g/L/h.
Dilution rate: (a) 0.1 h^{-1}; (b) 1.15 h^{-1}. Bar: 10 μm

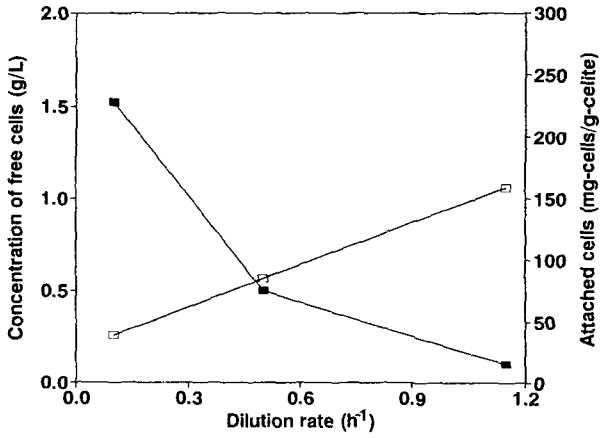

Fig. 5. Concentrations of free and biofilm-formed cells at various dilution rates when the toluene transfer rate was fixed at 0.395 g/L/h.
Symbols are: (■) Free cells; (□) Attached cells.

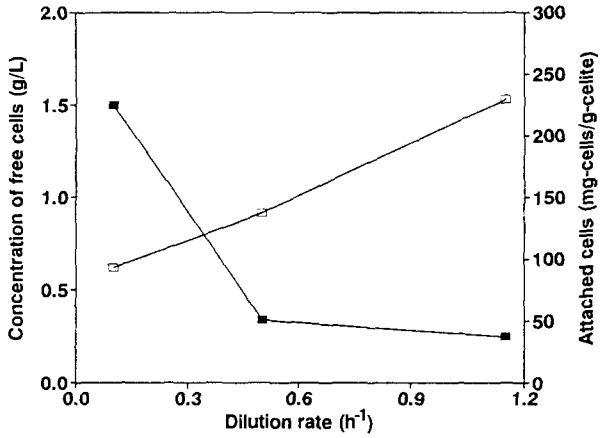

Fig. 6 Concentration of free and biofilm-formed cells at various dilution rates when the toluene transfer rate was enhanced to 1.2 g/L/h.
Symbols are: (■) Free cells; (□) Attached cells.

also considerable due to hydraulic shear.

As the dilution rate increases, the supply rate of nutrients becomes higher, while the concentration of freely suspended cells adversely decreases. Thus, more nutrients are available for the growth of initially colonized cells on the surfaces, which causes the biofilms to develop and become thicker. In other words, the growth of microorganism after initial colonization induced by the physico-chemical and biological interactions between the freely suspended cells and the surface of solid support plays a crucial role in the development of biofilms.

Based on the observation made, the effect of toluene transfer rate on the formation of biofilms was investigated. Fig. 6 shows the concentration of freely suspended and attached cells after 5 days of continuous cultivation when the toluene transfer rate was enhanced to 1.2 g/L. The amount of attached cells on celite particles was increased compared with that obtained at the toluene transfer rate of 0.395 g/L/h over the whole range of dilution rate, while no significant variation in the concentration of freely suspended cells was observed. Fig. 7 shows the electron microscopic photos of biofilms formed at elevated toluene transfer rate. The surface of celite particles was observed to be more significantly covered with biofilms at higher dilution rate, and in addition, microorganisms became lengthened and outward growth was considerable when compared with those observed at the toluene transfer rate of 0.395 g/L/h (Fig. 7(b)). It seems that the uptake rate of carbon source by attached microorganism increased with increasing toluene transfer rate, and consequently the growth of attached cells became enhanced, resulting in the increased biomass loading on the celite particles. These results also imply that the formation of biofilms is favored when the growth of initially attached cells is stimulated.

Fig. 7 Electron microscopic photos of the attached cells at different dilution rates when the toluene transfer rate was enhanced to 1.2 g/L/h.
Dilution rate: (a) 0.1 h^{-1}; (b) 1.15 h^{-1}. Bar: 10 μm

Continuous biodegradation using biofilm

Degradation of toluene was continuosly performed using biofilms at different dilution rates and toluene transfer rates in the bubble column bioreactor. Average concentration of freely suspended and attached cells, dissolved oxygen, and removal efficiency at steady state are shown in Table 1. The removal efficiency was almost similar even though the toluene transfer rate was enhanced by threefold, which indicates that the specific degradation rate was increased significantly with increasing toluene transfer rate.

Although the concentration of attached cells was decreased with decreasing dilution rate, the removal efficiency was maintained at similar level, and this seems to be due to the fact that the concentration of freely suspended cells was increased with decreasing dilution rate, and consequently a portion of degradation by free cells was increased. As the biofilms grow outward from the surface of the celite particles, the diffusion of organic carbon or oxygen becomes impeded and one or both of these substances will not penetrate to the basal layer of the biofilms, resulting in the limitation of toluene

Table 1. Continuous biodegradtion of toluene in the bubble column bioreactor at different dilution rates and toluene transfer rates

Toluene transfer rate (g/L/h)	Dilution rate (h-1)	Free cells (g/L)	Adsorbed cells (g/L)	Dissolved oxygen concentration (%saturation)	Removal Efficiency (%)
0.395	0.1	1.52	0.78	72	96.7
	0.5	0.56	1.7	65	96.2
	1.15	0.1	3.18	48	95.6
1.2	0.1	1.5	1.83	61	94.8
	0.5	0.34	2.74	48	94.5
	1.15	0.25	4.6	30	95.3

degradation by mass transfer. This implies that the thickness of biofilms should be properly controlled to improve the biodegradation efficiency.

Degradation of toluene was stablely conducted without any significant fluctuation for more than one month in the bubble column bioreactor. It is expected that bubble column bioreactor will be succesfully applied to the biodegradation of inhibitory aromatic solvents using biofilms.

NOMENCLATURE

C_g : Concentration of toluene in gas phase (g/L)
C_l : Concentration of toluene in liquid phase (g/L)
F_g : Gas flow rate (L/h)
Q_t : Specific degradation rate of toluene (g toluene/g cell/h)
R_t : Volumetric toluene transfer rate (g/L/h)
X : Biomass concentration (g/L)
V : Working volume of the bioreactor (L)

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