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Biodegradation of Endosulfan by *Klebsiella oxytoca* KE-8 Immobilized on Activated Carbon

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Endosulfan degrading ability of *Klebsiella oxytoca* KE-8 immobilized by entrapment with activated carbon was examined. Endosulfan degradation by the immobilized bacterial strains on several different activated carbon based support materials was investigated. Based on results, activated carbon (8×30 mesh) was chosen as a support material. The immobilized *Klebsiella oxytoca* KE-8 with the cell density of 4 mg g^{-1} (dry weight) degraded 22.18 ug ml^{-1} endosulfan within 5 days at pH 7.0, 30℃ in batch shake flask cultures. Also, we an experimented recycle packed bed column mode and continuous packed bed column mode for endosulfan degradation. Under optimum operation condition, the immobilized cells in a laboratory scale pack bed column with support beads were able to degrade endosulfan completely in defined minimal salt medium at a maximum rate of 129.6 ug ml^{-1} per day. Moreover, the endosulfan degradation activity could be demonstrated at 4℃ for one month without significant decrease in activity. Results of this study suggest that immobilized cells of *Klebsiella oxytoca* KE-8 might be applicable to endosulfan contaminated site.

Key Words: Biodegradation, Detoxification, Endosulfan, Immobilization, *Klebsiella oxytoca*

INTRODUCTION

Endosulfan has been widely used as a broad-spectrum cyclodiene insecticide on a wide range of crops including vegetable, fruits, cotton and rice system. However, the intensive use of endosulfan resulted to serious environmental problems because they are either recalcitrant or biodegraded very slowly. Endosulfan could persist in soil and water environments for 3 to 6 months or more(Kullman and Matsumura., 1996; Awasthi et al., 2000; Sethunathan et al., 2002; Lee et al., 2006).

Detoxification of pesticides through biological means is receiving serious attention as an alternative to existing methods, such as incineration and landfill. A preliminary step in the investigation of enzymatic technologies for endosulfan detoxification is the definitive identification of a biological source, capable to degrade endosulfan. Microorganisms have increasingly been investigated as a source of xenobiotic-degrading enzymes (Sutherland et al., 2002; Siddique et al., 2003a). Several studies have reported that the isolation of bacterial co-culture(Awasthi et al., 1997; 2003) and mixed cultures(Miles and Moy., 1979; Sutherland et al., 2000) capable of degrading endosulfan. Our group has

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reported that the endosulfan and endosulfan sulfate degread bacteria, *Klebsiella oxytoca* KE-8(Kwon et al., 2005). However, these detoxifying processed do not appear to be entirely satisfactory because these are because of toxic effects to fish and aquatic invertebrates and these have been implicated in mammalian gonadal toxicity, genotoxicity and neurotoxicity(Siddique et al., 2003a; Sutherland et al., 2004). Moreover, the use of pure cultures of microorganisms, specially adapted to metabolize the contaminant, can be envisaged as an attractive alternative(Prieto et al., 2002). The microbial purification of wastewater has been reported to be efficient, preferably in immobilized system, using synthetic and natural carriers.

Immobilization of microorganisms on inert supports shows an increasing interest because of the benefits that can be obtained from the process. The main advantages in the use of immobilized cells in comparison with suspended ones include the retention in the reactor of higher concentrations of microorganisms, protection of cells against toxic substances and prevention of suspended bacterial biomass in the effluent(Jianlong et al., 2002; Mordocco et al., 1999; Kok et al., 1999). Moreover, immobilization of microbial cells provides, in general, high degradation efficiency and good operational stability(Prieto et al., 2002; Dhar et al., 1998; Suzuki et al., 1998). Many kinds of materials have been selected as carrier for immobilization of microorganisms to treat wastewater; network polymers, alginate, polyacylamide hydrazide, calcium alginate, activated pumice and activated carbon(Pai et al., 1995; Murakami et al., 2003; Pazarlioglu and Telefoncu., 2005; Rahman et al., 2006; Karigar et al., 2006).

The objective of this study was to develop a simple and efficient bioprocess suitable for the removal of endosulfan contaminants wastewater.

MATERIALS AND METHODS

Microorganism

Bacterial cultures were used in the experiments. Experiments were performed with pure cultures of *K. oxytoca* KE-8, isolated from agriculture soil by repeated transfers on a minimal salt medium with endosulfan as the sole source of carbon and energy (Kwon et al., 2005).

Medium and culture conditions

K. oxytoca KE-8 were grown and maintained at 30℃ on minimal salt medium (MSM) with endosulfan as the sole carbon source. The Minimal salt medium (MSM) contained (L^{-1}) 4.35 g K_2HPO_4, 1.7 g KH_2PO_4, 1 g NH_4NO_3, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.05 g $MnSO_4 \cdot 5H_2O$ and 0.01 g $FeSO_4 \cdot 7H_2O$. The pH of MSM was adjusted at pH 6.8~7.0. Endosulfan was added after autoclaving.

Cell immobilization

Prior to immobilization, *K. oxytoca* KE-8 were obtained from separate pure cultures using the MSM which contained 20 ug ml^{-1} endosulfan (incubated at 30℃, 130 rpm in 100 ml MSM). After which, different two cultures were centrifuged (13000 rpm for 10 min) and washed three times with 0.1 M Phosphate buffer. After complete consumption of endosulfan, these cells with a cell titer and MSM were placed into a flask which contained the activated carbon.

The activated carbons used was type JG-20 (Jeil Activated Carbon IND Co. LTD, Kroea) with a size of 8×30 mesh and with the internal surface areas specified as 900~1100 MIN (m^2 g^{-1}). Morphological and physical properties of activated carbons are given in Table 1. Prior to immobilization, carriers were treated with methanol and 1 M acetic acid and finally washed with distilled water in ultrasonic cleaner at room temperature.

The immobilization lasted about 24 h in flasks at 30℃, 130 rpm shaking incubator. Control cells were inactivated by autoclaving at 121℃ for 15 min before adding to the flask(Ehrhardt and Rehm., 1985).

Table 1. Morphological and physical properties of activated carbons (Test Report)

Test item	Unit	SPECIFICATIONS
Total moisture	%	3.5
Iodine number	ml g^{-1}	952
Hardness	%	96.4
Bulk density	g ml^{-1}	0.48
Average particle diameter	mm	2~3
Specific surface area	m^2 g^{-1}	900~1100
Mesh size	mesh	8×30

Endosulfan determination

Reversed-phase liquid chromatography (HPLC) was used to analyze the residual concentration of endosulfan in liquid culture broths or effluents. HPLC comprised a Sykam system controller-S3210 UV-vis detector (Sykam, Germany). The analytical column was Mightysil RP-18 column (Kanto Chemical Co., Japan) and mobile phase was acetonitrile/water (70:30 v/v) at a flow rate of 1 ml min^{-1}. The column oven temperature was maintained at 35℃ and solute was detected at 214 nm(Lee et al., 2006).

Endosulfan biodegradation : Batch cultures (Degradation of endosulfan by free cells)

Batch culture of free cells (*K. oxytoca* KE-8: 9×10^7 cells) were performed in a 50 ml Erlenmeyer flask containing 20 ml of MSM with 24 ug ml^{-1} of endosulfan. Incubation of samples was carried out at 30℃ on shaking incubator at 130 rpm for the desired 3 days period. Endosulfan concentration and cell growth were monitored at certain time intervals. Control cells were inactivated by autoclaving at 121℃ for 15 min before adding to the flask.

Batch cultures (Degradation of endosulfan by immobilized cells)

Equal quantity of immobilized cells was added into a 50 ml Erlenmeyer flask containing 20 ml sterile MSM, the other conditions was the same as above for free cells. Samples of the culture broths (Inclusive of immobilized cells: activated carbon) were taken each day for residual endosulfan analysis.

Recycled packed bed column and Continuous packed bed column

Immobilized cells were suspended in 50 ml MSM and percolated through a bed comprised of 78 g (dry weight) of support material (immobilized matrix) in a glass column. The temperature was kept at 30℃ by circulating water through the reactor jacket. Minimal salt medium containing endosulfan was introduced to the packed bed column with a peristaltic pump (Tokyo Rikakikai Co., Japan) through the lower inlet port. Packed bed column operating conditions are given in Table 2.

Continuous packed bed column cultivated, operation conditions were similar in continuous and batch-recirculation mode experiments. The entire closed system consisting of the packed column, feed vessel, waste vessel, and connecting tubing and filter vents were sterilized by autoclaving. Endosulfan was then added to the feed reservoir.

Table 2. Experimental conditions of batch-recirculation, continuous packed bed system

Parameter	Value					Unit
Diameter			2.5			cm
Length			30			cm
Inner volume			140			ml
Use volume			90			ml
Flow rate	0.16	0.25	0.5	1	2	ml min^{-1}
HRT	25	50	100	200	300	min

RESULTS

Immobilization of microorganism

The immobilization capacity of activated carbon particles with different materials (Granular activated carbon and Pellet activated carbon) was compared. As a result, the granular activated carbon 8×30 mesh (Type JG-20) was the best attachable to microorganisms (results not shown). Thus, it was chosen and used as the supporting material to immobilized *K. oxytoca* KE-8.

The quantity of immobilized microorganism cells attached to the surface of the activated carbon 8×30 mesh is dependent on immobilization time. After about 12 h an adsorption balance was reached in immobilized cells. To get a high cell density on the activated carbon surface, further investigation was conducted by increasing the immobilization time to 24h. The experimented 1 g of activated carbon was achieved with 4 mg (9×10^7 cells) of *K. oxytoca* KE-8 (Fig. 1). The immobilization of *K. oxytoca* KE-8 could be demonstrated by scanning electron micrographs (Hitachi Type S-2500, Japan) (Fig. 2). The bacteria were able to penetrate into the activated carbon macropores. Many of the activated carbon particles were interspersed by deep macropores (Fig. 2A), which may permit the cells to enter the inner portion of the activated carbon. But, the surface structure of the activated carbon can be observed here in contrast to Fig. 2(B). Figure 2B shows both many cells. Specifically macropores, crevices and the entrances of the macro-

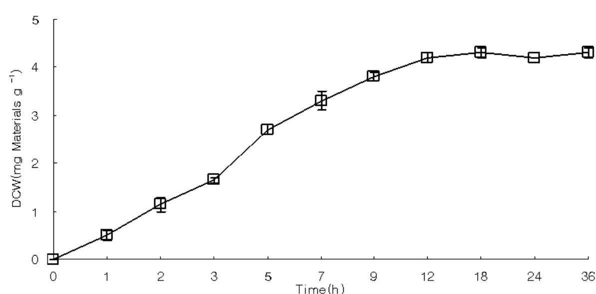
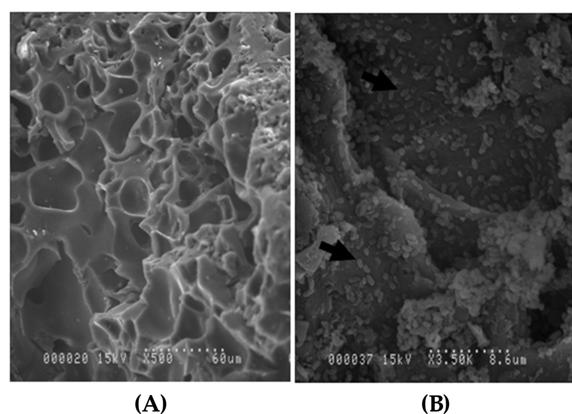


Fig. 1. Time-dependence of immobilization on activated carbon column with 8×30 mesh by *K. oxytoca* KE-8 (□).



(A) (B)

Fig. 2. SEM micrographs of the internal surface of an active carbon column with 8×30 mesh. Without whole cell (A), and with whole cell-*K. oxytoca* KE-8 (B) (Model : Hitachi, S-2500C, Japan).

pores were colonized by the bacteria.

Biodegradation of endosulfan from defined mineral salt medium in shaken flasks

The degradation of endosulfan was first studied at optimum temperature and pH of immobilized cells. Degradation of 25 ug ml^{-1} endosulfan by immobilized cells in MSM was studied at 20, 25, 30, 35 and 40℃ (results not shown). A temperature of 30℃ was found to be the optimum temperature for degradation of endosulfan with 55% being degraded by the immobilized cells. Degradation of 25 ug ml^{-1} endosulfan by immobilized cells in MSM was studied at pH 3.0, 5.0, 7.0, 9.0 and 11.0 (results not shown). Endosulfan degradation was optimum at pH 7.0. Thus, endosulfan degradation was studied in batch experiments at pH 7.0, temperature 30℃ and concentration of 25 ug ml^{-1} by free cells and immobilized cells. In order to distinguish between endosulfan adsorption and endosulfan degradation by microorganism, the activated

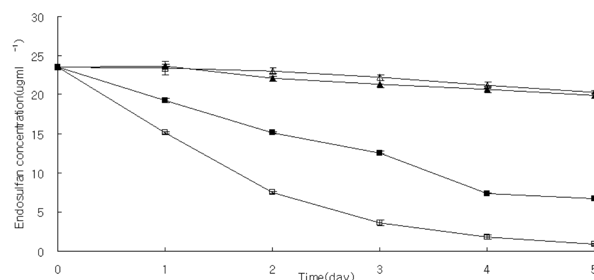


Fig. 3. Degradation in batch culture of endosulfan in defined mineral medium by free cells (closed symbols) and immobilized cells (open symbols) at 30℃, pH 7.0, (△)Autoclaved-*K. oxytoca* KE-8; (□)*K. oxytoca* KE-8.

carbon was treated with endosulfan and its levels in MSM was monitored for 5 days (Fig. 3). Results show that the endosulfan concentration in MSM was the same in the channels of autoclaved free cells and autoclaved immobilized cells.

On the other hand, the endosulfan concentration in the medium decreased clearly with the non-autoclaved free cells and non-autoclaved immobilized cells until 5days (Fig. 3). The free cells *K. oxytoca* KE-8 could degrade 16.88 ug ml^{-1} (71%) endosulfan in MSM containing 23.54 ug ml^{-1} endosulfan for 5 days. Moreover, rapid degradation of endosulfan by *K. oxytoca* KE-8 was observed in MSM with initial concentration of 23.54 ug ml^{-1} endosulfan. *K. oxytoca* KE-8 degraded 96% of endosulfan in 5 days of incubation. The concentration of endosulfan in MSM decreased to 0.90 ug ml^{-1} in 5 days (Fig. 3).

The immobilized *K. oxytoca* KE-8 adsorbed on activated carbon was able to degrade endosulfan faster than the free cells *K. oxytoca* KE-8.

Recycled packed bed column

The experimental packed bed column was designed to facilitate continuous measurement of the biodegradation rate following a perturbation from steady state. Bioreactor operating conditions are given in Table 2. The effect of the hydraulic retention time (from 25 to 300 min) on the performance of bioreactor was studied by varying the influent flow-rate. In the *K. oxytoca* KE-8 immobilized cells column, a gradual decrease in degradation rate, up to complete inhibition, was observed with the increase of hydraulic retention time (Fig. 4). At hydraulic retention time of 25 and 50 min, endosulfan was consumed without a lag phase. At 100, 200, 300 min, a marker lag phase of approximately 18

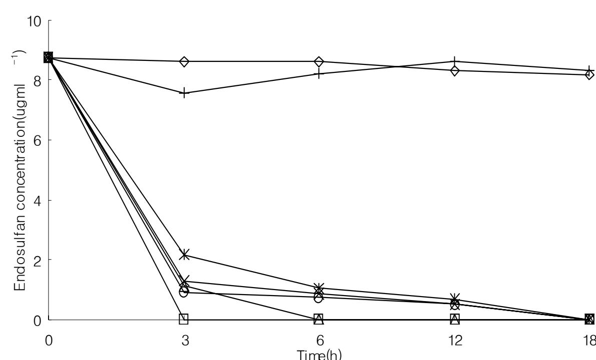


Fig. 4. Degradation of endosulfan in the recycled packed bed bioreactor (*K. oxytoca* KE-8) (◇); Influent, (+); Autoclave immobilized cells, (□); HRT-25 min, (△); HRT-50 min, (○); HRT-100 min, (X); HRT-200 min, (※); HRT-300 min.

h was already observed. But then degradation began at a rate approximately parallel to that observed at the previous concentration on influent and autoclave immobilized cells (Fig. 4). When almost complete endosulfan degradation was attained, at the end of the log phase, the recycled packed bed column was switched to recycled condition by fixing an initial hydraulic retention time, for 25 min. As a result, no significant difference was observed between the degradation times of each run until the tenth run (data not shown).

Continuous packed bed column

Cultivation, operation conditions were similar in continuous and batch-recirculation mode experiments. Bioreactor operating conditions are given in Table 2. The effect of the Hydraulic retention time (from 25 to 300 min) on the performance of bioreactor was studied by varying the influent flow-rate. As a result, when almost complete endosulfan degradation was attained, at the end of the log phase, the recycled packed bed column was switched to recycled condition by fixing an initial hydraulic retention time, at 100 min. In both the *K. oxytoca* KE-8 immobilized cells column, endosulfan degradation rate was at the highest level at 100 min. Endosulfan degradation rations were 95% or over for the *K. oxytoca* KE-8 immobilized cells column (Table 3).

Finally, long-term continuous endosulfan biodegradation using a defined MSM containing 9 ug ml^{-1} endosulfan fed at a constant hydraulic retention time of 100 min was assessed during 2 days. Bioreactor

Table 3. Degradation of endosulfan in the continuous packed bed bioreactor

HRT (min)	Flushing effluent (ug ml^{-1})	Effluent reservoir (ug ml^{-1}) KE-8	Degradation ration (%) KE-8
25	8.73	1.21	86.14
50	8.73	0.77	91.18
100	8.73	<0.1	>95
200	8.73	<0.1	>95
300	8.73	<0.1	>95

Table 4. Experimental conditions of continuous system and storage stability

Parameter	Value	Unit
Diameter	2.5	cm
Length	30	cm
Inner volume	140	ml
Use volume	90	ml
Flow rate	0.5	ml min^{-1}
HRT	100	min

Storage Stability Temperature 4℃

operating conditions are given in Table 4. Based on results, the calculated average rate of biodegradation was 129.6 ug endosulfan ml^{-1} per day. The endosulfan concentration in the effluent was always negligible and lower concentrations due to the time spent for changing and connecting the new pack bed column, were observed (Fig. 5).

Storage stability of endosulfan degradation activity

If endosulfan degradation activity was stably maintained in the immobilized cells during storage, we can use the stored cells as the need arises. Bioreactor operating conditions are given in Table 4. Activated carbon with immobilized cells *K. oxytoca* KE-8 that had been used for one degradation activity stored for 1, 15 and 30 days at 4℃. Endosulfan degradation activity after one month had duration of 81%, compared to 98% before storage (Fig. 6).

DISCUSSION

This study demonstrates that the bacterial strains, *K. oxytoca* KE-8 were able to degrade endosulfan in free cells, as well as in immobilized cells. The immo-

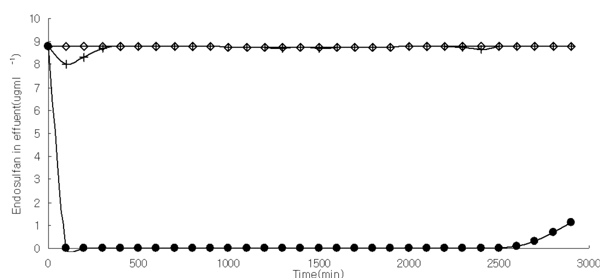


Fig. 5. Continuous biodegradation of endosulfan by *K. oxytoca* KE-8 cells immobilized in pack bed column (◇);Influent (+); Autoclave immobilized *K. oxytoca* KE-8 cells; (•)*K.oxytoca* KE-8 immobilized cells.

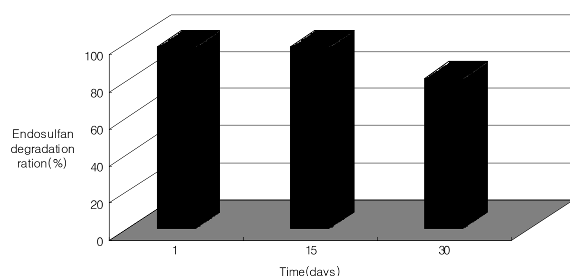


Fig. 6. Storage stability of endosulfan degradation activity. After the storage at 4℃. Endosulfan degradation was performed at 30℃, immobilized cells *K. oxytoca* KE-8.

bilization of *K. oxytoca* KE-8 adsorbed on activated carbon was able to degrade endosulfan faster than the free cells *K. oxytoca* KE-8 (Fig. 3). Since whole cell immobilization is a useful technique that would allow for recycling of the biocatalyst, it may have other beneficial effects such as increasing the resistance of the entrapped cells to the toxicity of xenobiotic compounds. Immobilized cells bioreactor (packed bed column) studies showed that endosulfan- degrading microorganism could be applied into an immobile degradation site. Complete removal of the endosulfan could be achieved; thus, immobilized bacteria could be applied to the soil to decrease the environmental contamination caused by endosulfan.

Immobilization of microbial cells by adsorption on activated carbon offers the advantage of making the process easy to handle under favorable physiological conditions. SEM observation of the external surface of an adsorption activated carbon is shown in Fig. 2. The outer pores of the activated carbon have a diameter of >5×10^{-8} m(Ehrhardt and Rehm., 1985). Therefore the cells will attach on the external surface of the activated carbon and can allow only the entry of some big macropores. A similar phenomenon was reported by Ehrhardt and Rehm (1985), Morsen and Rehm (1987), Ehrhardt and Rehm (1989), Morsen and Rehm (1990) and Khaled et al. (1996) on activated carbon. The degradation of toxic compounds by bacteria culture immobilized on activated carbon has been reported as a combination of physical adsorption and biological degradation(Ehrhardt and Rehm., 1985). This combined effect assumes an intrinsic relationship between the activated carbon and bacteria in the same environment, thus regenerating the surface of the adsorbent carrier as diffusion and degradation proceed. The adsorbed material desorbs, diffuses out of the carbon and can then be metabolized. This can be described as a mechanism of the following nature: bulk ⇒ adsorption ⇒ desorption ⇒ diffusion ⇒ biodegradation; in which the main part of the organic material is adsorbed at the begining of the process, followed by slow diffusion and biodegradation(Khaled et al., 1996).

The use of exponentially growing cells to inoculate the packed bed column resulted in a faster adsorption of cells on activated carbon as well as in a higher initial rate of endosulfan degradation. Under optimized conditions using a defined MSM containing 9 ug ml^{-1} endosulfan and fed at 0.16 ml min^{-1} (HRT; 25 min: Recycled packed bed column bioreactor operating conditions are given in Table 2), immobilized cells of *K. oxytoca* KE-8 were able to degrade endosulfan at a maximum rate of 3 ug endosulfan ml^{-1} per hour (Fig. 4). Moreover, in long-term continuous experiment (2 days; 2880 min), using an influent endosulfan concentration of 9 ug ml^{-1} (2 days; 261 ug ml^{-1}) fed at 0.5 ml min^{-1} (HRT; 100 min; long-term continuous bioreactor operating conditions are given in Table 2) flow rate, average rate of endosulfan biodegradation of about 129.6 ug endosulfan ml^{-1} per day were calculated (Fig. 5).

Most of the data reported on endosulfan biodegradation were obtained with defined mineral medium. These data are difficult to be compared among them because they are usually expressed in different units and experimental, procedures were performed under different experimental conditions using distinct microbial stains. However, the rate of endosulfan biodegradation reported here for immobilized cells of *K. oxytoca*

KE-8 are higher than the best values reported by other authors: 23.5 ug endosulfan ml^{-1} per 7 days for Pseudomonas sp. KS-2P(Lee et al., 2006) and 100 ug and 95 ug endosulfan ml^{-1} per 12 days and 18 days for *Fusarium ventricosum* and *Pandoraea* sp., respectively(Siddique et al., 2003b).

It is remarkable that in this new case, cells were entrapped in activated carbon and were held stable for one month. The excellent stability during storage of the biocatalyst shows that the practical application of this method is highly promising. Because of the results described above, activated carbon seems to be better suited to serve as a carrier for the immobilization of *K. oxytoca* KE-8 and in the treatment of endosulfan site were high concentrations of pollutants are expected. Furthermore, the immobilized cells of *K. oxytoca* KE-8 might be applicable to endosulfan contaminated site.

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