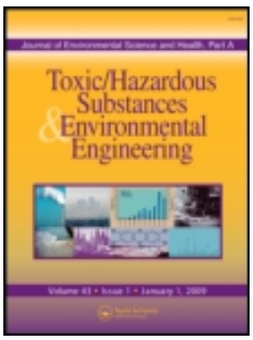

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DEGRADATION OF DIMETHYLPHTHALATE BY CELLS OF *BACILLUS* SP. IMMOBILIZED IN CALCIUM ALGINATE AND POLYURETHANE FOAM

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ABSTRACT

A *Bacillus* sp. which is capable of degrading dimethylphthalate (DMP) was immobilized in calcium alginate and polyurethane foam for efficient and long term degradation of DMP. Freely suspended cells (10^{12} cfu ml^{-1}) degraded a maximum of 20 mM DMP. Whereas, alginate-(10^{12} cfu g^{-1} beads) and polyurethane foam-entrapped ($0.34\times10^{6-9}$ cfu g^{-1} foam cubes) cells degraded a maximum of 40 mM DMP within 12–15 days of incubation. Polyurethane foam-entrapped cells degraded 30 mM of DMP at 4 days and alginate-entrapped cells degraded within 10 to 12 days of incubation irrespective of the cell population. When the initial concentration of DMP increased to 50 mM, the DMP degrading ability of the immobilized cells was not increased even after 20 days. Repeated batch cultures by alginate-entrapped cells with initial 35 mM DMP loading could be reused for a maximum of 20 cycles. However, the degradation rate was gradually decreased when the beads were reused for more than 15 cycles. On the other hand, the foam-entrapped cells, with the same initial DMP loading there was no decrease in DMP degrading ability and could be reused for more than 20 cycles. The packed bed reactor with alginate-entrapped cells ($1\times10^{10-12}$ cfu g^{-1} bead) could be continuously operated for 7–8 days with an initial 25 mM DMP at a flow

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rate of 50 ml h^{-1}. Whereas, the polyurethane foam-entrapped cells ($1\times10^{6-9}$ cfu g^{-1} foam cubes) could be operated continuously for more than 90 days with the same initial DMP loading at a flow rate of 100 ml h^{-1}. Thus the enhanced degradation of DMP could be achieved by immobilizing the cells of *Bacillus* sp. in calcium alginate and polyurethane foam as compared to that of freely suspended cells.

Key Words: Alginate; *Bacillus* sp.; Dimethylphthalate; Polyurethane foam.

INTRODUCTION

Phthalate esters, which can make up 40% of the weight of some plastic materials, are produced in excess for the use in plastic industries. The toxic nature of phthalate esters has been investigated and are considered to be teratogenic to man and animals (1). These phthalate esters are often detected at relatively high levels in the ecosystem, hence contribute to environmental pollution. US-Environmental Protection Agency (EPA) has placed them in a priority list of pollutants (2). The biodegradation of phthalate esters has been extensively studied by using free microbial cells. Immobilization of cells is one of the advantageous procedures to avoid the problem associated with the use of free bacterial cells (3,4). The main advantages in using whole cell-immobilized microorganisms are being the high density, enhanced activity of immobilized viable cells on reuse, protection of cells from the external environmental perturbations and their use in continuous reactors (5). Immobilized cells are extensively used for the production of useful chemicals and degradation of toxic products from industrial waste (6–12).

In the present study, the rate of degradation of dimethylphthalate by cells of *Bacillus* sp. immobilized in calcium alginate and polyurethane foam were compared with those of freely suspended cells. The DMP degrading ability, longevity and operational stability of immobilized cells of *Bacillus* sp. were compared by cultivating the cells in batch, semicontinuous cultures and continuous packed bed reactor. Further, the efficiency of polyurethane foam and calcium alginate-immobilized cells to degrade DMP is also discussed.

MATERIALS AND METHODS

Microorganism and Growth Conditions

A DMP degrading *Bacillus* sp. was isolated from the samples collected from the biological waste-water treatment plant in our laboratory. The bacterium was maintained on agar-slants of DMP-mineral salts medium (MM1).

Medium

Two different media were used in this study as described previously (13). The medium used for precultivation (MM1) of the bacterium contained ($g l^{-1}$) K_2HPO_4, 6.3; KH_2HPO_4, 1.83; $(NH_4)_2NO_3$, 1.0; $MgSO_4$, 0.1; $FeSO_4$, 0.1; $MnSO_4$, 0.1; $NaMoO_3$, 0.005. The pH was adjusted to 7.0 and the medium was supplemented with 6 mM DMP.

For the degradation studies, the mineral salts medium (MM2) contained ($g l^{-1}$) K_2HPO_4, 0.15; $(NH_4)_2NO_3$, 1.0; $MgSO_4$, 0.2; $FeCl_3$, 0.1 and $CaCl_2$, 0.2. The pH was adjusted to 7.0 and DMP at various concentrations (20–50 mM) was used as sole carbon source.

Mode of DMP Supplementation

DMP was initially dissolved in minimum amount of N, N′-dimethylformamide and forced into the mineral salts medium containing Tween-80 ($80 \times$ cmc) with the help of syringe. The medium was then subjected to ultrasonication thrice for one min each time and finally, the medium appeared milky.

Immobilization

Bacillus sp. was grown on MM1 containing DMP as sole carbon source. The cells were harvested during mid-logarithmic growth phase by centrifugation at 5000 *g* for 10 min at 15°C and used for immobilization.

Immobilization of Cells in Alginate

The alginate entrapment of cells was performed as described previously (13). Sodium alginate (4% w/v) was sterilized and mixed with bacterial cells (15 g wet weight/50 ml alginate solution). This alginate-cell mixture was extruded drop wise through a burette fixed with tappered pipette tip into cold, sterile 0.2 M calcium chloride solution. The entrapped cells were left to harden in a fresh $CaCl_2$ solution for 2 h with gentle agitation. Finally the beads were washed with distilled water and transferred into an appropriate medium containing various DMP concentrations.

Immobilization of Cells in Polyurethane Foam

Immobilization of cells was performed in polyurethane foam cubes (9). The polyurethane foam sheet was obtained from local supplier, Mukesh

Foam Products, Bangalore, India. The foam was elastic, with low density (15 kg m^3) and provided large surface area. The foam was cut into approx. 5 mm cubes, washed twice with distilled water and dried. Approximately 200 foam cubes (4 g) in 500 ml conical flasks containing 100 ml distilled water were autoclaved. The water was decanted and the foam cubes were dried under vacuum. The bacterial suspension was added into the flasks containing foam cubes and the contents were mixed by stirring on a magnetic stirrer for 2 h. The flasks were then allowed to stand undisturbed for additional 2 h. The medium was then removed and the immobilized foam cubes were washed with saline and were used for degradation studies. The bacterial cells in the culture were counted using standard plate count method by crushing 1 g cell-immobilized-foam cubes under aseptic conditions.

Batch and Semicontinuous Degradation of Dimethylphthalate

The batch degradation of DMP was carried out for both freely suspended cells and cells immobilized in alginate and polyurethane foam. The exponentially growing cells were harvested by centrifugation as described earlier and the cells were suspended in 1/10th volume of 0.05 M potassium phosphate buffer (pH 7.0). This cell suspension was made into 3 aliquots for the degradation studies of DMP by free cells and cells immobilized in calcium alginate and polyurethane foam. For freely suspended cell studies, one aliquot of cell-suspension was added to 500 ml Erlenmeyer flasks containing DMP-mineral salts medium with different concentrations of DMP (30, 40 and 50 mM) and with suitable controls. The second and third aliquots of cell-suspension was used for the degradation studies of DMP at the above concentrations by cells immobilized in calcium alginate and polyurethane foam.

For the calcium alginate-entrapped cell studies (1×10^{12} cfu g^{-1} beads), 25 g wet weight (approx. 1500 Nos) of beads were added to each 500 ml Erlenmeyer flask containing 100 ml DMP-mineral salts medium with the above mentioned DMP concentrations with appropriate controls without cell entrapped beads. For foam-entrapped cell studies ($0.34 \times 10^{6-9}$ cfu g^{-1} foam cubes), 4 g of foam cubes (approx. 120 Nos) were added to each 500 ml Erlenmeyer flask containing 100 ml DMP-MM2 with the above-indicated DMP concentrations and with suitable controls. The freely suspended cells and the entrapped cells in both matrices were incubated at room temp. ($30 \pm 2^{\circ}C$) on a rotary shaker under identical culture conditions for 4, 8 and 12 days, respectively. The control experiments for the adsorbed DMP (3–4 mM) to alginate/foam were carried out in sterile medium at the above-mentioned DMP loadings. The adsorbed DMP from the sterile conditions were considered as undegraded DMP, which was used to correct the degradation of DMP by this bacterium.

For long term and efficient degradation of DMP by immobilized cells in calcium alginate and polyurethane foam, the repeated batch culture studies were carried out. After every incubation period (5 days), the spent medium was decanted and beads/foam cubes were washed with water and transferred into a fresh DMP-mineral salts medium (35 mM). The degradation process was carried out under identical culture conditions.

Continuous Degradation of Dimethylphthalate

The continuous degradation of DMP was carried out in a packed bed reactor. The reactor used was a long cylindrical glass column (4×50 cms with a working volume of 650 ml) with outlet facility at every 5 cm. The bottom of the column was packed with a circular foam pad followed by a porous glass frit. The reactor was packed with respective cell-entrapped matrices, 250 g cell-entrapped alginate beads and 25 g cell-entrapped polyurethane foam cubes to a height of 25 cms for alginate and 30 cms for foam with a working volume of 60 and 120 ml, respectively. The aeration was adjusted to 0.5 bar, which was passed from the bottom of the packed column, and also provided good mixing of culture medium with DMP. The DMP-MM2 was continuously supplied from an inlet at the bottom with different initial DMP loadings at different flow rates (25, 50, 75 and 100 ml h^{-1}). The effluent was continuously removed from the outlet at the top and residual DMP was estimated for each set of experiments (13).

Analysis of DMP

The spent medium was extracted with ether, dried over anhydrous Na_2SO_4, concentrated and analyzed spectrophotometrically at 274 nm by using Shimadzu UV/vis light spectrophotometer (model 160 A) with appropriate dilutions and with suitable controls.

RESULTS

Degradation of Dimethylphthalate by Freely Suspended and Immobilized Cells

The degradation of DMP was carried out by both freely suspended cells and cells entrapped in calcium alginate and polyurethane foam in batch cultures. Freely suspended cells (1×10^{12}) degraded 20 mM DMP at 4 days of incubation from an initial 30 mM DMP. With the same cell population, only 15 mM DMP was degraded after 4 days of incubation from an initial 40 and 50 mM of DMP concentrations (Fig.1). No further degradation of DMP

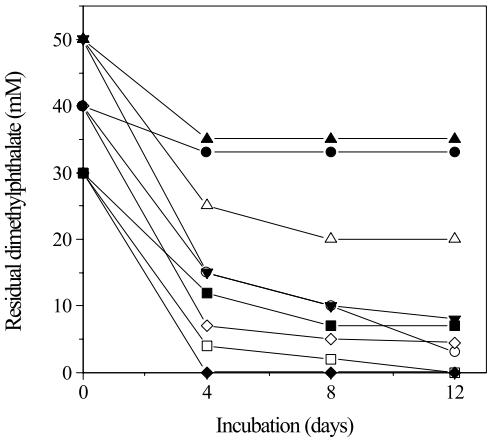

Figure 1. Degradation of dimethylphthalate by freely suspended cells (10^{12} cfu ml^{-1}); alginate-immobilized cells (10^{12} cfu g^{-1}) and polyurethane foam-immobilized cells ($0.34 \times 10^{6-9}$ cfu g^{-1} foam cubes) in shaken cultures. ▲ ● ■ represent degradation by freely suspended cells; △ ○ □ represent degradation by alginate-immobilized cells and ▼ ◇ ◆ represent degradation by polyurethane foam-immobilized cells at different concentrations.

was occurred even after 20 days of incubation. Further, when the cell population was doubled, no significant change was observed in the degradation rate of DMP (data not shown).

It is evident from the immobilized cell studies that alginate-entrapped cells (1×10^{12} cfu g^{-1} beads) degraded 25 mM DMP at 4 days and the complete degradation of DMP at 10–12 days of incubation was observed (Fig. 1). On the other hand, the complete degradation of DMP at 4 days of incubation was observed with foam-entrapped cells ($0.34 \times 10^{6-9}$ cfu g^{-1} foam cubes) (Fig. 1). When the initial concentration of DMP was raised to 40 mM, no significant change in degradation rate of DMP was noticed in both alginate- and foam-entrapped cells. Whereas, for initial 50 mM DMP, alginate entrapped cells degraded 30 mM and foam-entrapped cells degraded 40 mM DMP at 12 days of incubation. Furthermore, when the incubation period was extended upto 20 days, there was no increase in the degradation rate of DMP.

Semicontinuous Degradation of Dimethylphthalate by Immobilized Cells

The repetitive degradation of DMP by immobilized cells in both alginate and polyurethane foam (Fig. 2) was carried out with initial 35 mM DMP for 5 days. It was observed that the alginate-entrapped cells (10^{7-12} cfu g^{-1} beads) could be reused for 20 cycles and foam-entrapped

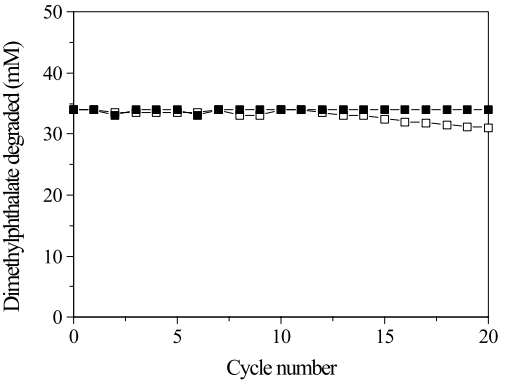

Figure 2. Repetitive batch fermentations for semicontinuous degradation of dimethylphthalate by alginate- (□) and polyurethane foam-immobilized cells (■).

cells (10^{6-9} cfu g^{-1} foam cubes) for more than 20 cycles without loosing their DMP degrading capacity. However, the alginate-entrapped cells (10^{2-3} cfu ml^{-1}) were gradually leaked out due to its mechanical instability and thus, the decreased degradation rate with increased cycle number was observed. Whereas, the polyurethane foam-entrapped cells showed higher degradation efficiency even though there was cell leakage (10^{3} cfu ml^{-1}).

Continuous Degradation of Dimethylphthalate by Immobilized Cells

The continuous degradation of DMP by immobilized cells in a packed bed reactor was carried out by allowing DMP (25 and 50 mM) to pass through the packed bed reactor at different flow rates (25, 50, 75 and 100 ml^{-1}). The initial 25 mM DMP was completely degraded with a flow rate of 25 ml h^{-1} in both alginate- (10^{10-12} cfu g^{-1}) and foam-entrapped ($1 \times 10^{6-9}$ cfu g^{-1}) cells (Table 1). Whereas, a slight difference in the degradation of DMP at flow rate of 50 ml h^{-1} was observed. However, at flow rates of 75 and 100 ml h^{-1}, the foam-entrapped cells showed no change in the degradation rate as compared to alginate-entrapped cells in which the degradation rate reduces to about 40%. When the initial DMP loading was increased to 50 mM, alginate-entrapped cells degraded 28, 20, 18 and 10 mM DMP and foam-entrapped cells degraded 43, 33, 30 and 27 mM DMP at the flow rates of 25, 50, 75 and 100 ml h^{-1}, respectively. It was observed that for an initial 25 mM DMP loading with a flow rate of 50 ml h^{-1}, the packed bed reactor was efficiently operated continuously for 15 days with alginate and more than 60 days with foam-entrapped cells.

Table 1. Degradation of Dimethylphthalate by Alginate- and Polyurethane Foam-Immobilized *Bacillus* sp. in a Packed Bed Reactor*

Flow rate (ml h^{-1})	Residence time (h)	Initial conc. of DMP (mM)	DMP degraded (mM)		Degradation efficiency (%)	
			Alginate-entrapped	Foam-entrapped	Alginate-entrapped	Foam-entrapped
025	4.0	25	24	24	91	91
	4.0	50	28	43	56	86
050	2.0	25	22	22	88	88
	2.0	50	20	33	40	66
075	1.7	25	15	22	60	88
	1.7	50	18	30	36	66
100	1.0	25	15	22	60	88
	1.0	50	10	27	20	54

*Cell numbers were in the range 1×10^{10} cfu g^{-1} beads and 1×10^{12} cfu g^{-1} beads in alginate and 1×10^{6} and 1×10^{9} cfu g^{-1} foam cubes in polyurethane foam.

DISCUSSION

The *Bacillus* sp. capable of degrading DMP was used to compare the degradation rate by free cells with that of cells immobilized in calcium alginate and polyurethane foam. The freely suspended cells degraded a maximum of 20 mM DMP at 4 days of incubation and there was no significant change in degradation activity when the initial DMP concentration was increased as high as 50 mM. Whereas, the cells immobilized in alginate and foam, a maximum of 40 mM DMP was degraded at the same incubation period, which is comparatively much higher than those of freely suspended cells (Fig. 1). Furthermore, the polyurethane foam-entrapped cells retained the DMP degrading ability for long term in reusing for more than 20 cycles with initial 35 mM of DMP. Whereas, the alginate-entrapped cells showed the lower degradability of DMP with increased cycle numbers. This may be due to the mechanical instability of alginate beads.

The results obtained from the continuous degradation of DMP in a packed bed reactor suggest that the rate of degradation of DMP with foam-entrapped cells was effective when compared to alginate-entrapped cells. However, at 50 mM DMP the alginate-entrapped cells degraded a maximum of 28 mM and foam-entrapped cells degraded 43 mM of DMP at a flow rate of 25 ml h^{-1}, Furthermore, when the flow rate was increased to 100 ml h^{-1}, the alginate-entrapped cells showed only 20% and foam entrapped cells showed 54% of DMP degrading activity. Thus it is evident from the above results that the complete degradation of DMP was observed only at a lower initial concentration (25 mM DMP) in both matrices (Table 1). As the initial concentration of DMP increases, the degradation

rate substantially decreases. This may be due to the high polar nature of DMP resulting in low solubility in aqueous medium.

The use of free cells for the degradation of various toxic compounds for industrial applications has number of disadvantages. It is mostly because of low mechanical strength, low density of cell-population and difficulty in biomass-effluent separation. Immobilization techniques have now been well established in overcoming these problems (8,13,14,15). Calcium alginate, agar and polyurethane foam have also been extensively used as matrices for immobilization of whole microbial cells. Moreover, enhancement in the activity of viable immobilized cells on reuse have also been reported (16). Immobilized bacterial cells have been shown to have altered metabolism, enhanced enzyme induction and reduced specific cell growth.

Thus the present study reveals that the immobilized *Bacillus* sp. showed more efficient degradation of DMP in both the calcium alginate and polyurethane foam when compared to that of the freely suspended cells. The bacterium used in this study have an ability to degrade the other phthalate esters like, diethylphthalate, dibutylphthalate and dioctylphthalate.

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