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Kinetics of *p*-Cresol Degradation by an Immobilized *Pseudomonas* sp.†

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A *p*-cresol (PCR)-degrading *Pseudomonas* sp. was isolated from creosote-contaminated soil and shown to degrade PCR by conversion to protocatechuate via *p*-hydroxybenzaldehyde (PBA) and *p*-hydroxybenzoate (PHB). Cells of the *Pseudomonas* sp. were immobilized in calcium alginate beads and in polyurethane foam. The relationship between the PCR concentration and the PCR transformation rate was investigated in batch and continuous culture bioreactors. The biodegradation kinetics of PBA and PHB also were investigated. In batch culture reactors, the maximum PCR degradation rate (V_{max}) for the alginate-immobilized *Pseudomonas* sp. cells was 1.5 mg of PCR g of bead^{-1} h^{-1} while the saturation constant (K_s) was 0.22 mM. For PHB degradation, the V_{max} was 0.62 mg of PHB g of bead^{-1} h^{-1} while the K_s was 0.31 mM. For polyurethane-immobilized *Pseudomonas* sp. cells, the V_{max} of PCR degradation was 0.80 mg of PCR g of foam^{-1} h^{-1} while the K_s was 0.28 mM. For PHB degradation, the V_{max} was 0.21 mg of PHB g of foam^{-1} h^{-1} and the K_s was 0.22 mM. In a continuous column alginate bead reactor, the V_{max} for PCR transformation was 2.6 mg g of bead^{-1} h^{-1} while the K_s was 0.20 mM. The V_{max} and K_s for PBA transformation in the presence of PCR were 0.93 mg g of bead^{-1} h^{-1} and 0.063 mM, respectively. When PHB alone was added to a reactor, the V_{max} was 1.48 mg g of bead^{-1} h^{-1} and the K_s was 0.32 mM. The effects of various aeration methods on degradation rate were investigated. Oxygen limitation was suggested in the alginate-immobilized-cell system. The rates of degradation of both PCR and PHB increased when pure oxygen was supplied to the reactors. Competition for oxygen between the PCR and PHB transformation enzymes was suggested. Oxygen limitation was not observed in the polyurethane-immobilized-cell systems.

Bacteria can degrade and thereby detoxify a wide range of hazardous synthetic compounds. Our goal is to harness these abilities to remove toxic chemicals from polluted water. A promising method to accomplish this goal lies in the use of immobilized bacterial cells. Here we report results of investigations of the ability of immobilized *Pseudomonas* cells to degrade the toxic phenol *p*-cresol (PCR). A variety of immobilization methods and bioreactor designs were examined.

Immobilized cells have been defined as cells that are entrapped within or associated with an insoluble matrix. Mattiasson (15) discussed six general methods of immobilization: covalent coupling, adsorption, biospecific affinity, entrapment in a three-dimensional polymer network, confinement in a liquid-liquid emulsion, and entrapment within a semipermeable membrane. Under many conditions, immobilized cells have advantages over either free cells or immobilized enzymes. By preventing washout, immobilization allows a high cell density to be maintained in a bioreactor at any flow rate. Catalytic stability can be greater for immobilized cells, and some immobilized microorganisms tolerate higher concentrations of toxic compounds than do their nonimmobilized counterparts (7, 14, 18).

One potential disadvantage of immobilization is the increased resistance of substrates and products to diffusion through immobilization matrices. Owing to the low solubility of oxygen in water and the high local cell density, oxygen transfer often becomes the rate-limiting factor in the performance of aerobic, immobilized cell systems (1). Thus, when aerobic cells are used, aeration techniques become very important considerations in bioreactor design.

Although cell immobilization is commonly used in processes for microbial production of specialty chemicals (4, 13, 20), few studies have addressed the use of immobilized cells to degrade toxic compounds (2, 3, 14, 18). We recently presented data on the degradation of pentachlorophenol by an alginate-immobilized *Flavobacterium* sp. (K. O'Reilly, R. Kadakia, J. Steiert, R. Korus, and R. Crawford, *in* J. Glasser (ed.), *Chemical and Biochemical Detoxification of Hazardous Waste*, in press). Complete mineralization of the biocide was accomplished by the immobilized cells in both batch and continuous culture reactors.

In work summarized here, we examined the degradation kinetics of PCR (4-methylphenol). PCR was chosen as a target compound because it, like pentachlorophenol, is common in wood treatment wastewaters. Two immobilization matrices, calcium alginate and polyurethane foam, were examined.

(A preliminary report of part of this work was presented at the International Conference on Water and Wastewater Microbiology, Newport Beach, Calif., 8 February 1988.)

MATERIALS AND METHODS

Isolation of the bacterium. The bacterial strain used in these experiments was isolated from creosote-contaminated soil. Soil was placed on the surface of mineral salts agar plates containing a defined medium (see below) plus 100 mg of PCR liter^{-1} as the sole carbon source. Halos of growth appeared around the soil particles. Successive streaking of these halos onto PCR plates allowed the isolation of a pure culture that could utilize PCR as its sole source of carbon and energy. The bacterium, a motile rod, is gram negative, fluorescent, catalase positive, and oxidase positive and has

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tentatively been identified as a *Pseudomonas* sp. (strain PCR).

Cell growth. The bacterium was grown in a minimal salts medium (K_2HPO_4, 3.6 mM; KH_2PO_4, 1.4 mM; $NaNO_3$, 5.9 mM; $MgSO_4$, 0.4 mM; $FeSO_4$, 50 μM [pH 7.3]). Sodium glutamate (4 g liter^{-1}) and 200 mg of PCR liter^{-1} were supplied as simultaneous carbon sources. Cell growth was monitored by using a spectrophotometer by monitoring culture turbidity at 560 nm. When the A_{560} reached 1.5, cells were collected into a paste by centrifugation.

Immobilization in alginate. Cell paste was mixed 1:1 (wt/wt) with cold (5°C), sterile 4% sodium alginate (type VII; Sigma Chemical Co., St. Louis, Mo.). Cold 2% sodium alginate was added to bring the mixture to the final desired volume. Five grams (wet weight) of cells was used for each 100 ml (final volume) of alginate solution. The alginate-cell mixture was added dropwise to cold 50 mM $CaCl_2$. Each drop hardened into a bead containing entrapped cells. The beads were hardened further by being stirred in $CaCl_2$ for 30 min and then were collected by filtration. They were stored at 5°C in HEPES immobilization buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 50 mM; $CaCl_2$, 1 mM; $MgSO_4$, 1 mM; $FeSO_4$, 20 μM; sodium glutamate, 0.5 g liter^{-1} [pH 7.3]).

Immobilization in polyurethane. A cell suspension was prepared by mixing 10 g of cell paste with 20 ml of buffer (carbon-free growth medium). Polyurethane prepolymer (HYPOL FHP2000; W. R. Grace Co., Lexington, Mass.) was cooled on ice, and an equal part (weight/weight) of buffer was added. The mixture was stirred well for about 1 min. Two parts of the cell suspension then were added, and the mixture was stirred for an additional 1 min. The reaction vessel was kept on ice for 2 h while the polyurethane foam hardened. The foam was removed from the vessel, rinsed with buffer to remove free cells, and stored in buffer at 0°C.

Chemical determinations. Concentrations of PCR and its degradation intermediates were monitored by high-performance liquid chromatography (Hewlett Packard 1090 liquid chromatograph) by using a reverse-phase, C18 column (250 by 0.2 mm) (PP/6474A; Phenomenex, Rancho Palos Verdes, Calif.). The solvent used was a 1:1 mixture of acetonitrile and 50 mM sodium acetate (pH 4.5). PCR biotransformation products were identified on the basis of comparison of their UV-visible spectra (by using the chromatograph's diode array detector) and retention times with those of known standards.

Batch reactor experiments. Batch reactor experiments were performed in 125-ml flasks with either 50 or 100 ml of 50 mM MOPS (3-[morpholino]propanesulfonic acid, pH 7.3) buffer with a ratio of grams of matrix per milliliter of medium of 0.1. To determine the effect of the aeration method on degradation rates and extents, three reactor designs were tested. Two were air-lift-type reactors, in which the beads were circulated by the addition of either air or gaseous oxygen (1 liter min^{-1}). The third reactor was a flask, which was shaken at 200 rpm.

Kinetics experiments were performed in 125-ml flasks containing 100 ml of 50 mM MOPS plus the compound of interest. Initial concentrations of PCR were varied between 10 and 100 mg liter^{-1}; those of *p*-hydroxybenzoate (PHB) were varied between 25 and 200 mg liter^{-1}. Oxygen gas was added at a rate sufficient (1 liter min^{-1}) to maintain circulation of the beads. Transformation rates of the PCR degradation intermediate PHB also were investigated. To determine initial degradation rates, samples were removed eight times over a period of 25 min for analysis by high-performance liquid chromatography. Degradation rates were calculated by using the linear regression of time versus concentration. Kinetic parameters were estimated by the linear regression method described by Cornish-Bowden (5).

Continuous column experiments. A column reactor (60 by 4 cm) (760 ml) with sampling ports every 5 cm was constructed from polycarbonate. Input ports were located at the bottom of the column. An oxygen electrode (IL530; Instrumentation Laboratory, Inc., Lexington, Mass.) was installed 10 cm below the top of the column. Fifty grams of calcium alginate-immobilized *Pseudomonas* cell beads was added with enough MOPS buffer (10.4 g of MOPS liter^{-1}, 0.24 g of $MgSO_4$ liter^{-1}, 0.22 g of $CaCl_2$ liter^{-1} [pH 7.3]) to fill the column. A flow rate through the reactor of the same buffer was maintained at 2.5 ml min^{-1}. Oxygen gas was provided at 650 ml min^{-1}. After 4 h, the input medium was changed to MOPS plus 200 mg of PCR liter^{-1}. The column was then run for 4 days to allow for equilibration.

By adjusting the flow rate and the input PCR concentrations, the column was operated at a series of input rates between 15 and 178 mg of PCR h^{-1}. The effluent concentrations of PCR and of its degradation intermediates were monitored by high-performance liquid chromatography. After steady state had been reached for a particular set of conditions, the rate of PCR transformation was determined by a material balance equation: $V_t = F(C_i - C_e)$, where V_t was the transformation rate, F was the flow rate, C_i was the influent PCR concentration, and C_e was the effluent PCR concentration. To determine the transformation rates of biotransformation intermediates, the following equation was used: $V_t = V_p - FC_e$, where V_p was the rate of production of the compound within the reactor, i.e., the V_t of the previous compound in the pathway.

Similar experiments were run with PHB in place of PCR. Here, the input rate was varied between 34 and 170 mg of PHB h^{-1}.

Enzyme assays. Enzyme assays were performed to verify the predicted degradative pathway and to determine relationships between oxygen utilization and enzyme activity. The *Pseudomonas* sp. was grown in minimal salts medium with 4 g of sodium glutamate liter^{-1}. To compare induced and uninduced activities, cells were grown with or without 200 mg of PCR liter^{-1}. The cultures were grown overnight, and the cells were collected by centrifugation when the A_{560} reached 2.8. The cell paste was suspended in 20 ml of 50 mM Tris buffer (pH 8.0) and centrifuged again. The cells were suspended in 15 ml of cold enzyme buffer (8) (50 mM Tris, 1 mM EDTA, 1 mM dithiothreitol, 1 μM flavin adenine dinucleotide [pH 8.0]). Cell extracts were prepared by passing the cell suspensions through a French press at 10,000 lb in^{-2}. The extracts were centrifuged at 20,000 × *g* for 15 min and then at 140,000 × *g* for 2.5 h. The extracts were kept on ice. The protein contents of extracts were measured by using the Bio-Rad protein assay system (Bio-Rad Laboratories, Richmond, Calif.) standardized to known concentrations of bovine serum albumin.

Oxygen utilization was monitored with a Clark-type oxygen electrode (YSI 5350; Yellow Springs Instrument Co., Yellow Springs, Ohio). The volume of the reaction vessel was 3 ml, and the temperature was maintained at 30°C. The system was calibrated by monitoring oxygen utilization upon the oxidation of known concentrations of NADH by phenazine methosulfate (PMS) (16). The oxidation of PCR was monitored by the method of Hopper and Taylor (11). In this assay, uptake of oxygen results from the reoxidation of PMS which had been reduced by the PCR-oxidizing enzyme. Fifty

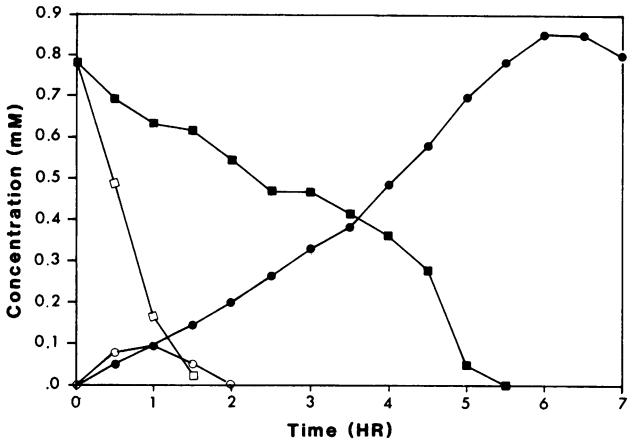

FIG. 1. Effect of aeration method on the degradation of PCR by alginate-immobilized *Pseudomonas* cells. The concentrations of PCR and PHB in shake flask (■, PCR; ●, PHB) and oxygen gas-lift (□, PCR; ○, PHB) reactors are shown.

microliters of 10 mM PCR was added to 50 mM glycine buffer (pH 9.0) with 0.1 ml of cell extract and 1 μg of PMS. The oxidation of PHB was tested in the Tris-dithiothreitol-flavin adenine dinucleotide buffer described above (8). A 0.1-ml portion of cell extract was added, endogenous oxygen uptake was monitored for about 1 min, and then 50 μl of either 10 mM NADH or 10 mM NADPH was added and the oxygen concentration was measured for an additional 1 min. Finally, 50 μl of 10 mM PHB was added and the oxygen uptake rate was again determined. The oxidation of protocatechuate (3,4-dihydroxybenzoate) was monitored in 50 mM Tris (19).

RESULTS

Immobilization. The typical yield following immobilization of cells within alginate was about 0.6 g of beads ml of cell-alginate suspension^{-1}. Individual beads had a diameter of 3 mm and a wet and dry weight of 15 and 0.8 mg, respectively. At the time of immobilization, each bead contained 1×10^8 to 2×10^8 viable cells, as determined by plate counts of disrupted beads.

The polyurethane immobilization method produced 40 g of wet foam having a volume of about 100 ml. The foam was cut into 1-cm^3 pieces for use in experiments. The exact number of cells immobilized per unit volume of foam was not determined.

Degradative pathway. Two PCR biotransformation products, *p*-hydroxybenzaldehyde (PBA) and PHB, were identified in the culture media. Lower concentrations of PHB were detected in reactors supplied with oxygen gas than were detected in reactors supplied with air (Fig. 1).

Batch reactor experiments. Immobilized *Pseudomonas* cells completely degraded PCR in flask reactors having initial PCR concentrations of less than 250 μg ml^{-1}. Partial degradation was observed in reactors with PCR concentrations as high as 1 mg ml^{-1}. In the alginate system, the rate of PCR degradation and the concentration of PHB detected were influenced dramatically by the method of aeration. In the presence of O_2, very little PHB was detected and that amount was present only transiently during the first 2 h of operation. In the shake flask, PCR was converted stoichiometrically to PHB; the PHB was subsequently degraded (Fig. 1). Also, the rate of PHB degradation was lower in the shake flask (0.29 mg of PHB g of bead^{-1} h^{-1}) than in the oxygen-sparged reactor (0.54 mg of PHB g of bead^{-1} h^{-1}). In the polyurethane system, the PCR and PHB degradation rates were not affected by the aeration method (Table 1). Volumetric reaction rates can be calculated by dividing the degradation rates given by the density of the cell immobilization matrices ($d_{alginate}$ = 1.07 g cm^{-3}; $d_{polyurethane}$ = 0.40 g cm^{-3}).

TABLE 1. Effect of aeration on the transformation rates of PCR and PHB by a polyurethane-immobilized *Pseudomonas* sp.

Compound/initial concn (μg ml^{-1})	Transformation rate[a]	
	Shake flask	Oxygen gas
PCR		
100	0.46	0.42
150	0.44	0.48
PHB		
25	0.11	0.10
100	0.33	0.30

[a] Milligrams of compound transformed per gram of foam per hour.

In oxygen gas-lift reactors, the relationship between PCR transformation rate (V_t) and concentration (S) fits a saturation kinetic model (Fig. 2) (17): $V_t = (V_{max} \cdot S)/(K_s + S)$. For the alginate-immobilized *Pseudomonas* cells, the maximum degradation rate (V_{max}) was 1.5 mg of PCR g of bead^{-1} h^{-1} (±0.01) while the saturation constant (K_s) was 0.22 mM (±0.03). For PHB degradation (Fig. 3), the V_{max} was 0.62 mg of PHB g of bead^{-1} h^{-1} (±0.03) while the K_s was 0.31 mM (±0.04).

For the polyurethane-immobilized *Pseudomonas* cells, the V_{max} was 0.80 mg of PCR g of foam^{-1} h^{-1} (±0.04) while the K_s was 0.28 mM (±0.05). For PHB degradation, the V_{max} was 0.21 mg of PHB g of foam^{-1} h^{-1} (±0.04) while the K_s was 0.22 mM (±0.01).

Continuous column experiments. The transformation rates for PCR and PBA in continuous column alginate bead reactors also followed saturation kinetics. For PCR, the V_{max} was 2.6 mg g of bead^{-1} h^{-1} (±0.004) while the K_s was 0.20 mM (±0.04). The V_{max} and K_s for PBA transformation

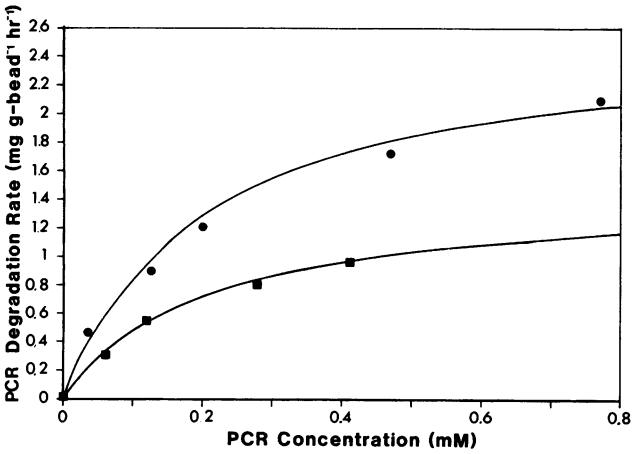

FIG. 2. Effect of PCR concentration on the rate of PCR degradation by alginate-immobilized *Pseudomonas* cells in batch (■) and continuous culture (●) oxygen gas-lift reactors. The lines are the predicted values calculated by using the kinetic parameters discussed in the text.

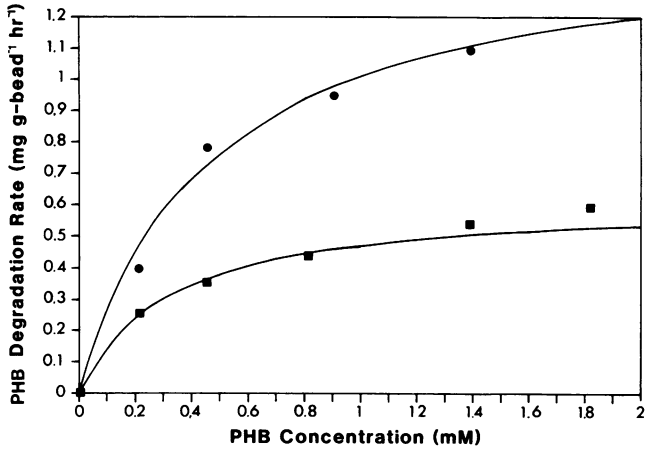

FIG. 3. Effect of PHB concentration on the rate of PHB degradation by alginate-immobilized *Pseudomonas* cells in batch (■) and continuous culture (●) oxygen gas-lift reactors.

were 0.93 mg g of bead^{-1} h^{-1} (±0.001) and 0.063 mM (±0.007), respectively. The oxygen concentration of the bulk solution was about 95% of saturation during the degradation of PCR.

When PCR alone was added to a continuous culture reactor, the ability of the reactor to transform PHB initially increased as the concentration of PHB (formed from PCR) increased, but the ability to transform decreased at higher PCR input rates (Fig. 4). The decreased PHB degradation rate at the higher PCR input rate appears to be due to the competition of the two systems for oxygen. When PHB alone was added to a reactor, its transformation followed saturation kinetics, with a V_{max} of 1.48 mg g of bead^{-1} h^{-1} (±0.14) and a K_s of 0.32 mM (±0.6) (Fig. 3).

Enzyme assays. The specific activities of the assayed enzymes are listed in Table 2. There was greater activity in the extracts of cells that had been exposed to PCR during growth. The ratio of activity between induced and uninduced extracts differ for the three enzymes. The oxidation of PCR required the presence of the electron acceptor PMS. The addition of PHB led to oxygen utilization only if NADPH or NADH was also added to the reaction. The order of addition did not appear to be important. The reaction rate (118 nmol of O_2 mg of protein^{-1} min^{-1}) in the presence of NADPH was 26 times higher than the rate (4.5 nM O_2 mg of protein^{-1} min^{-1}) in the presence of NADH. The results indicate that an NADPH-specific monooxygenase was responsible for PHB oxidation (12). Protocatechuate oxidation did not require the presence of PMS, NADPH, or NADH.

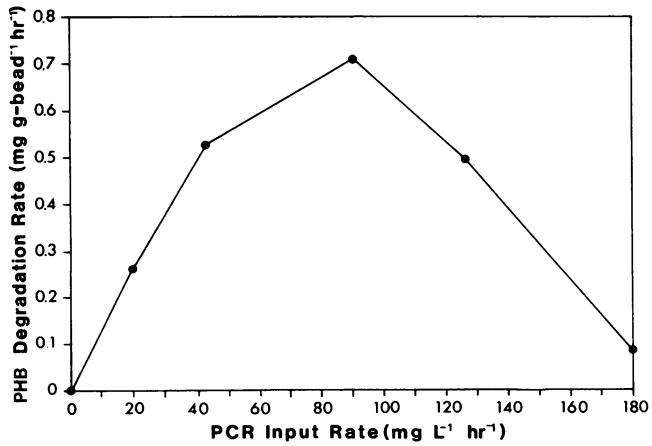

FIG. 4. Degradation rate of endogenously formed PHB at different PCR input rates in a continuous column reactor.

TABLE 2. Oxidation of aromatic substrates by cell extracts of a PCR-degrading *Pseudomonas* sp.

Substrate	Oxidation[a] by:	
	Uninduced extract	Induced extract
PCR	ND	140
PHB[b]	4.9	118
Protocatechuate	12.3	1,500

[a] Nanomolar of substrate utilized per milligram of protein per minute. ND, No activity detected.
[b] With NADPH as cosubstrate.

DISCUSSION

The intermediates detected during degradation of PCR by our *Pseudomonas* sp. and the results of the enzyme assays indicate that the PCR degradative pathway of this bacterium (Fig. 5) is similar to that determined by Dagley and Patel (6). The initial step is the hydroxylation of the methyl group to an alcohol (9, 10). Water is the source of the oxygen atom, and the excess electrons are passed directly to the electron transport chain through flavin and *c*-type cytochrome subunits (10). The *p*-hydroxybenzyl alcohol is further oxidized

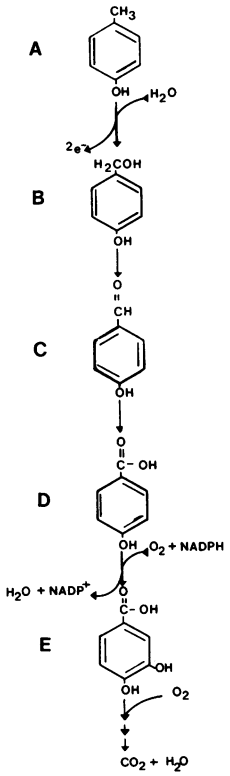

FIG. 5. Probable initial steps of the pathway of PCR degradation by *Pseudomonas* strain PCR. Steps: A, PCR; B, *p*-hydroxybenzyl alcohol; C, PBA; D, PHB; E, protocatechuate.

to an aldehyde and then to a carboxyl. The next step is further hydroxylation of the benzyl ring by a monooxygenase to protocatechuate. The oxidation of protocatechuate by the extract of induced cells indicates that this compound is the substrate for a ring-cleaving dioxygenase.

Oxygen limitation of the entrapped cells was suggested in the alginate immobilization system. The degradation rates for both PCR and PHB increased when pure oxygen was supplied to the reactors. Oxygen limitation was not obvious for the polyurethane-immobilized cells, as the transformation rate of neither PCR nor PHB was influenced by the aeration method. One explanation for the differences between the two systems may be the structures of the matrices. The alginate forms solid, spherical beads; thus, oxygen must diffuse through the outer cell matrix layers to reach cells deeper within the beads. The polyurethane foam has relatively large pores caused by the production of CO_2 during polymerization. These pores allow the interior of the foam to be in closer contact with the bulk solution as compared with the interior of the alginate beads. The density of cells within the matrix also may be involved. Although the number of cells originally added per final gram of matrix was the same in the polyurethane and alginate immobilization systems, the large pores in the polyurethane contained free cells that were removed from the foam before it was used. The lower rate of transformation per gram of matrix (1.5 mg of PCR g of matrix^{-1} h^{-1} for alginate versus 0.8 mg of PCR g of matrix^{-1} h^{-1} for polyurethane) indicates a lower cell density.

The PCR and PHB transformation enzymes may compete for oxygen. In batch reactors with alginate-immobilized cells (Fig. 1), only traces of PHB were detected when oxygen gas was supplied; in the shake flasks, however, all of the PCR was converted to PHB before PHB was further transformed. In the continuous culture reactor (Fig. 4), PHB transformation was inhibited when PCR was being transformed at high rates. Under limited availability of oxygen, early steps in the PCR pathway, as well as endogenous cellular respiration, apparently deplete the oxygen concentration within the alginate matrix, thus inhibiting the activity of the PHB-transforming monooxygenase. This is the first enzyme in the degradative pathway that utilizes oxygen directly as a co-substrate (6, 10). PHB accumulates until all the PCR has been converted to PHB, and the oxygen can be used for further transformation of PHB.

Although reports have been published on the use of immobilized microorganisms to degrade aromatic compounds (2, 3, 7, 14, 18), we believe that this is the first instance in which immobilized-cell degradation kinetics both of a parent compound and of two of its degradative intermediates have been investigated simultaneously. Understanding the kinetics of transformation of catabolic intermediates was important in the development of the polyurethane technique and in optimizing bioreactor design. The degradation kinetics of pathway intermediates could be important in the environmental application of immobilized-cell technology, since the intermediates formed during degradation of some xenobiotic molecules can be more toxic than the original contaminant (21).

In summary, PCR was degraded effectively by a *Pseudomonas* sp. immobilized in either calcium alginate or polyurethane. Saturation kinetics were observed for the degradation of both the cresol and its degradation intermediates, PBA and PHB. Transformation of PCR and PHB was limited by oxygen in the alginate system. Oxygen limitation was not observed in the polyurethane system.

ACKNOWLEDGMENTS

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