Biodegradation **12:** 455–463, 2001.
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The roles of intermediates in biodegradation of benzene, toluene, and *p*-xylene by *Pseudomonas putida* F1

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Accepted 21 December 2001

Key words: BTX, intermediates, oxygen effects, *Pseudomonas putida* F1

Abstract

Several types of biodegradation experiments with benzene, toluene, or *p*-xylene show accumulation of intermediates by *Pseudomonas putida* F1. Under aerobic conditions, the major intermediates identified for benzene, toluene, and *p*-xylene are catechol, 3-methylcatechol, and 3,6-dimethylcatechol, respectively. Oxidations of catechol and 3-methylcatechol are linked to biomass synthesis. When oxygen is limited in the system, phenol (from benzene) and *m*-cresol and *o*-cresol (from toluene) accumulate.

Introduction

Because of the frequent presence of benzene, toluene, and *p*-xylene (BTX) at sites contaminated by petroleum products, biodegradation of BTX has been studied extensively. Depending on the bacterial culture and community, degradation of BTX can proceed along drastically different pathways. For example, Figure 1 summarizes the reported initial steps of toluene degradation by different *Pseudomonas* strains (Duetz et al. 1994). None of the reactions shown in Figure 1 releases net electrons (shown as H) or carbon-containing components that can be used directly in synthesis. Therefore, the pathways must proceed beyond the last compounds shown if synthesis of new biomass is to occur. Because of the differences in chemical structures and the enzymes needed, benzene or *p*-xylene may or may not be biodegraded using enzymes of the pathways shown in Figure 1.

Degradation of toluene by *Pseudomonas putida* mt-2 follows the *tol* pathway, in which the side-chain methyl group is first oxidized in a monooxygenase-catalyzed reaction (Assinder & Williams 1990). Because xylenes have two methyl groups, they can also be degraded via the *tol* pathway. Benzene, on the other hand, cannot be used by *P. putida* mt-2 as sole carbon source. Several other bacterial strains, such as *Pseudomonas aeruginosa* and several *Nocardia* sp., also degrade alkylbenzenes similarly (Gibson & Subramanian 1984).

Pseudomonas cepacia G4 and *Pseudomonas mendocina* KR1 first attack the aromatic ring of toluene with monooxygenase enzymes. Neither *P. cepacia* G4 nor *P. mendocina* KR1 can grow with benzene as sole carbon source (Shields et al. 1989; Whited & Gibson 1991). The former, however, shows very weak growth on *p*-xylene, while the latter shows no growth on *p*-xylene.

Toluene degradation by *P. putida* F1 follows another typical pathway, the *tod* pathway, in which the aromatic ring is dioxygenated first and produces 3-methylcatechol (Gibson et al. 1970a; Spain & Gibson 1988). Benzene can be degraded by *P. putida* F1 via the same pathway and produces catechol. Catechol and 3-methylcatechol can be further degraded by *P. putida* F1. However, the product from dioxygenation of *p*-xylene, 3,6-dimethylcatechol, is a dead-end product of the *tod* pathway (Gibson et al. 1974). Therefore, *P. putida* F1 cannot use *p*-xylene as sole carbon source. Similar pathways are observed with other bacterial strains, such as *Pseudomonas mildenbergii*, an *Achromobacter* sp., and *Nocardia coral-*

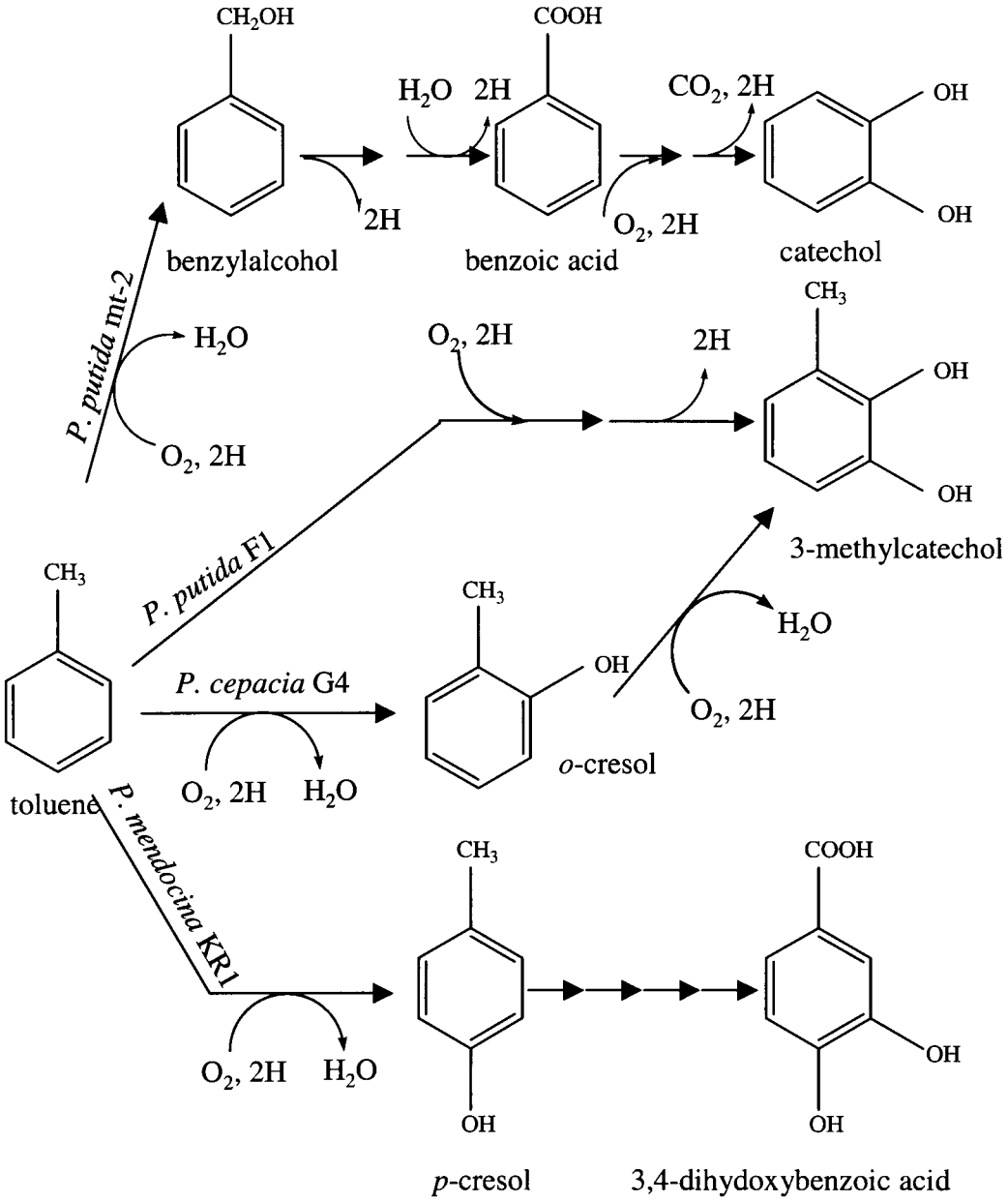

Figure 1. Different toluene-degradation pathways for *Pseudomonas* strains (after Duetz et al. 1994).

lina (Rittmann et al. 1994), a microbial consortium and a *Pseudomonas* strain (PPO1) (Oh et al. 1994), and *Pseudomonas fragi* and *Pseudomonas fluorescens* (Chang et al. 1993).

Further degradation of catechol or 3-methylcatechol occurs by ring cleavage. The reaction products are or can be easily converted to tricarboxylic acid cycle (TCA cycle) intermediates, which are further broken down into CO_2 and water (Madigan et al. 2000; Rittmann & McCarty 2001; Rittmann et al. 1994). Because none of the reaction pathways shown in Figure 1 results in a net release of electrons (H), it must be the subsequent oxidation of the catechol intermediates that supports biomass synthesis. This phenomenon is similar to the fungal growth on sucrose, which happens primarily after the conversion of sucrose to intermediates such as glucose and fructose (Crueger & Crueger 1990).

Each reaction is modulated by a number of factors, such as concentrations of the enzymes, substrates, and cosubstrates (such as oxygen and or an electron carrier). Consequently, each reaction in the pathway proceeds at its own rate, and substrates for the slowest reaction step can accumulate. The overall rate of reaction will be determined by how fast this slowest-reacting substrate can be transformed. For example, the overall rate of toluene mineralization may be controlled by the toluene itself or by one of its intermediate(s). When an intermediate controls the overall mineralization kinetics, the growth rate of the cells does not depend directly on the transformation rate of toluene.

Results from prior studies suggest that intermediates, rather than the original substrates, control the bacterial growth of cells during BTX degradation. In their study of biodegradation of BTX by two *Pseudomonas* isolates, Chang et al. (1993) observed that the biomass production lagged behind the removal of the single growth substrate (toluene or benzene). They also reported that biomass continued to grow even after all the growth substrates had been completely removed. They recognized that the phenomenon might be the result of slow degradation of intermediates. In order to fit the experimental data of substrate removal and biomass growth to model output, they numerically incorporated a time lag into the Monod model. The model with a time lag in biomass growth fit the experimental data better than the model without the lag. The fates of the intermediates were disregarded in the model.

Acting as a cosubstrate for oxygenation reactions (e.g., Figure 1) and a terminal electron acceptor, oxygen is important during aerobic degradation of BTX. By affecting the regeneration of electron carrier NAD^+ from NADH + H^+, oxygen also affects the availability of the reduced electron carrier NADH + H^+, thus indirectly affecting any reaction using NAD^+ or NADH + H^+ as a cosubstrate (Bae & Rittmann 1996a,b). As a result, the oxygen concentration has significant and complex impact on the overall rate of BTX degradation. For example, Malmstead et al. (1995) found that, during biodegradation of quinoline, the oxygen concentration affected dioxygenase reactions and respiration reactions, but to markedly different extents. Dioxygenation was slowed much more easily by a low oxygen concentration than was respiration. This means that the overall degradation rate can be slowed significantly by low dissolved oxygen concentrations, even when respiratory use of oxygen is not significantly affected.

Because intermediates and oxygen play very active roles during BTX degradation, this study investigates their interacting effects on the mineralization of BTX by *P. putida* F1 and the resulting growth of *P. putida* F1.

Experimental setup and analytical methods

Three types of reactors were used in the study: open-system batch reactors, closed-system batch reactors, and a closed-system chemostat. The temperature of all experiments was 22 °C. The minimal mineral medium for all experiments was composed of KH_2PO_4 (2040 mg l^{-1}), Na_2HPO_4 (2130 mg l^{-1}), $(NH_4)_2SO_4$ (1000 mg l^{-1}), $CaCl_2{\cdot}2H_2O$ (11 mg l^{-1}), $MgSO_4{\cdot}7H_2O$ (200 mg l^{-1}), $FeSO_4{\cdot}7H_2O$ (7 mg l^{-1}), $ZnSO{\cdot}7H_2O$ (2 mg l^{-1}), $MnSO_4{\cdot}H_2O$ (1.54 mg l^{-1}), $CuCl_2$ (0.21 mg l^{-1}), $CoCl_2{\cdot}6H_2O$ (0.404 mg l^{-1}), H_3BO_3 (0.11 mg l^{-1}), and $Na_2MoO_4{\cdot}2H_2O$ (0.25 mg l^{-1}). The *P. putida* F1 culture used in this study was maintained in petri dishes on agar prepared with the same minimum mineral medium. Toluene was the sole carbon source and was supplied by dripping 4–8 drops of toluene on a piece of filter paper and sticking it onto the inside of the top of the petri dish. Every month, the pure culture was transferred to a new set of dishes.

The open batch system included a 1-liter flask with a side-port and a J-shaped glass tube supplying benzene, toluene, or *p*-xylene from the vapor phase. During operation, 500 ml of minimum mineral medium were autoclaved and then added to the flask; then one of the substrates (pure liquid form) was loaded into the J-shaped tube, which was suspended in the reactor headspace, and a cotton plug was used to cover the flask opening. The reactor was inoculated with *P. putida* F1 and placed on a stirrer plate. Toluene vapor (from the J-tube) and oxygen (through the cotton plug) filled the headspace and diffused into the medium to support the growth of bacteria. Sampling could be done through the sampling port without interrupting the supply of toluene or oxygen. This system provided a high concentration of the BTX substrate at all times, despite its biodegradation and volatilization.

Closed-system batch reactors were used when the volatile substrates were fed via the liquid phase only and uncontrolled volatilization losses were unacceptable. The closed-system batch reactors were 1-liter glass bottles with Teflon-faced septa at the center of

the sealing caps. Unless specified otherwise, 800 ml of autoclaved medium was inoculated with *P. putida* F1 and saturated with oxygen. Then, the 200-ml headspace was purged with oxygen. Finally, predetermined amounts of saturated solutions of toluene, benzene, or *p*-xylene (solubility of 519 mg l^{-1}, 1827 mg l^{-1}, or 207 mg l^{-1}at 21 °C, respectively) were added to yield initial concentrations of 10–30 mg l^{-1}, and the bottles were sealed immediately. The medium was stirred vigorously for the substrates to reach equilibrium between the gas and liquid phases. Samples were taken through the septum using a gas-tight syringe equipped with a 16-gauge needle.

The closed chemostat is illustrated in Figure 2. The reactor had a liquid volume of 1.45 liter. In order to minimize precipitation during autoclaving, the chemicals for the minimal mineral medium were separated into two groups. Group A included the pH buffers and nitrogen and phosphorus sources (KH_2PO_4, Na_2HPO_4, and $(NH_4)_2SO_4$). Group B included the rest of chemicals that provided metals and trace elements critical for bacterial growth. Ten-times concentrated solutions of group A and B (10X-A and 10X-B) were diluted by reverse osmosis (RO) water. The saturated substrate stock was injected into the bacterial culture directly. A Teflon gas-sampling bag was attached to the top of bottle containing the substrate stock to maintain atmospheric pressure in the bottle and prevent toxic vapor from releasing into the air. The reactor was closed to the atmosphere and maintained at a small positive pressure (∼1 cm of H_2O) by a regulator connected to an oxygen tank. When oxygen was consumed, the pressure in the headspace decreased slightly, and more oxygen was supplied. This oxygen system provided a fully aerobic environment in the reactor (DO > 12 mg l^{-1}).

Analyses performed in this study include determining the concentrations of suspended biomass, BTX in gas and liquid phases, and BTX intermediates. The suspended biomass was measured according to its optical density (OD) at 540 nm with a spectrophotometer, which was calibrated to biomass dry weight per liquid volume (mg l^{-1}) after correcting for salts in the medium. Biomass dry weights used in the calibration were measured on a balance after the sample standards were filtered through a pre-weighed filter paper (47-mm, 0.2-mm) and then dried at 104 °C (Yu 1998). An HP 5890 gas chromatograph (GC) equipped with a flame-ionization detector (FID) with a fused silica column (Model DB-5 0.53mm ID, J&W Scientific) was used to analyze the concentrations of

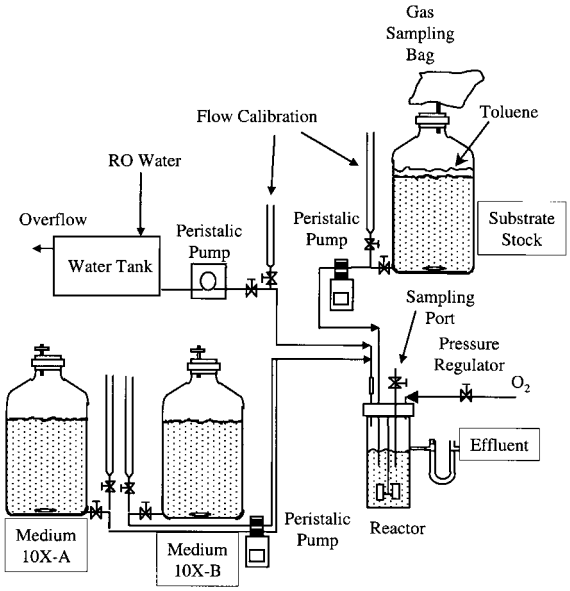

Figure 2. Closed chemostat setup.

toluene, benzene, or *p*-xylene in the gas or liquid phase. For each sample, 1 ml of liquid sample was first filtered (0.2-μm pore size) to remove the biomass. Then the BTX compound in the sample was extracted into methylene chloride (DCM), which was injected using a HP automated injector (Model 6890). The GC/FID output signals were analyzed by an HP integrator (Model 3396). The concentrations of BTX in the samples were calculated from their peak areas on the GC/FID chromatographs based on standard calibration curves (Yu 1998).

Chemical oxygen demand (COD) of intermediates was measured with Standard COD vials from Hach Co. (Loveland, CO). During a COD test, 2 ml of filtered liquid sample were added to the vial containing the chemical oxidizing agent and measured for absorbance using a spectrophotometer following Hach's standard method. The COD value of the sample was obtained from a standard calibration curve, which was prepared following the protocol provided by Hach Co.

A Hitachi HPLC system equipped with a diode-array detector was used to identify intermediates of BTX degradation. A HP-Hypersil column (C-18, 5 μm, 2.1-mm ID, and 10-cm length) was used to separate the compounds. The mobile phase included acetonitrile and 10 mM KH_2PO_4 buffer (pH 2.88) at a volume-to-volume ratio of 20-to-80. The flowrate used was 0.2 ml/min or, when a guard column was installed in front of the HP-Hypersil column, 0.5 ml/min.

The chromatograms were collected at 207 nm. The HPLC analysis in this study was mostly qualitative. Therefore, no calibration curves were made.

Results and discussion

Observations for the different biodegradation experiments

Three open-batch reactors, loaded with toluene, benzene, or *p*-xylene, were inoculated with a small colony of *P. putida* F1 from the petri dish. Within one day, flasks fed with toluene and benzene showed growth of biomass with concentrations up to 70 mg l^{-1}. In the flask fed with *p*-xylene, OD measurement showed no biomass growth one week after inoculation. A flask reactor containing 50 mg l^{-1} biomass (grown on toluene) was then setup and supplied with *p*-xylene alone from the headspace. No increase of biomass was observed after a week. The color of the medium, on the other hand, turned slightly brown a few hours after inoculation and stayed that way. The brown color suggested that *p*-xylene was being incompletely degraded as a cometabolic substrate. HPLC analysis of the liquid showed that 3,6-dimethylcatechol accumulated in the flask. The batch degradation of benzene also showed the transient buildup of an intermediate, as a light yellow color appeared rapidly during the experiments. The color in this case disappeared during the later stage of experiments, indicating the intermediate was subsequently removed from the medium. The batch experiments showed that toluene and benzene were readily transformed by *P. putida* F1. Toluene and benzene were able to support the growth of *P. putida*, but *p*-xylene was not able to support biomass growth and was degraded incompletely.

The chemostat was fed continuously with only one growth substrate, either toluene or benzene. Biomass, substrate, and COD concentrations in the liquid were measured daily. During the chemostat experiments, the analysis of liquid samples supported the presumption that intermediates, rather than toluene or benzene, were supporting the biomass growth. Throughout the chemostat experiments, neither toluene nor benzene was detected in either the gas or the liquid phase of the reactor by GC/FID (detection limit of 0.1 μg l^{-1} for gas and 0.1 mg l^{-1} for liquid samples). However, analysis of the effluent showed soluble COD in the samples. The fact that biomass was synthesized and maintained in the reactor without the presence of detectable toluene or benzene suggests that the reaction from toluene or benzene to the intermediates was faster than the degradation of intermediates.

One interesting phenomenon took place when the substrate was switched from toluene to benzene when the biomass concentration was relatively high (53 mg l^{-1}) and dilution rate was quite high (7.3 day^{-1}). Each substrate concentration was 70 mg l^{-1} in the influent. Within 20 minutes of the switch to benzene, the color of the reactor contents became bright yellow. The color gradually intensified to greenish yellow. After about 3 hours, the color was darker, and the biomass appeared to be washing out of the reactor. Five and half-hours after the switch, the reactor medium was a light yellow color, and the liquid phase was almost clear of biomass. About 20 hours after the switch, bacterial growth became obvious again in the chemostat. After another three hours, the biomass concentration in the chemostat was dense again, nearly returning the biomass concentration prior to the switch.

Several conclusions can be drawn from the observations after the switch from toluene to benzene. First, toluene-degrading *P. putida* was able to transform benzene to its intermediate (catechol, identity demonstrated below) readily without any adaptation period. This suggests that either the same dioxygenase enzyme attacked toluene and benzene, or a benzene-specific dioxygenase was co-induced with toluene. Second, the subsequent steps for catechol transformation likely involved different enzymes from those transforming 3-methylcatechol (identity of the toluene intermediate as 3-methylcatechol also is shown below). If the same enzyme was used to degrade catechol and 3-methylcatechol, degradation should be immediate and could generate electrons and carbon for biomass growth. In reality, new biomass synthesis took place only after induction of a dioxygenase for catachol. Thus, the chemostat experiments suggest that degradation of catechol or 3-methylcatechol was responsible for biomass synthesis, and each catechol intermediate seems to have its own dioxygenase. Furthermore, no utilizable carbon or electrons are released from the transformation from toluene and benzene to their intermediates.

The presumption that degradation of the intermediates supported biomass growth, while the transformation of toluene or benzene to the intermediate did not support growth, was further proved by benzene and toluene degradation in closed-system batch reactors. If only toluene or benzene supported biomass

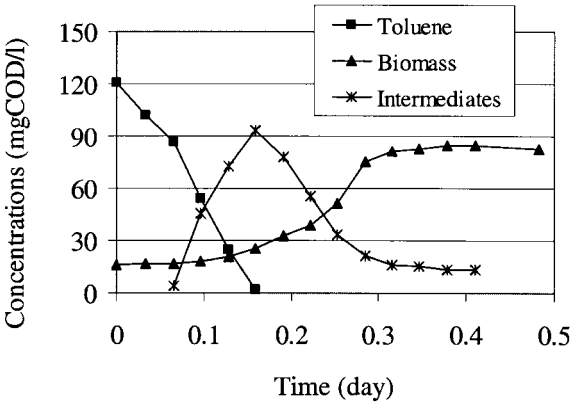

Figure 3. Closed-system batch experiment of aerobic toluene degradation.

growth, the biomass growth would stop once toluene or benzene was depleted from the closed-system batch reactors. Instead, in the closed-system batch reactors, the biomass concentration continued to increase for several hours after depletion of the benzene or toluene. For example, Figure 3 shows the loss of toluene, the delayed growth of biomass, and the COD values of the filtered liquid phase (intermediates), which increased at the beginning of the experiment and then decreased after toluene was depleted. Once the COD value of intermediates stabilized to a small, but non-zero value, biomass growth also stopped. These results clearly show that biomass growth was more closely associated with the change in the intermediates than to removal of toluene. Similar trends were observed during benzene degradation in closed-system batch reactors.

Identification of the intermediates

HPLC chromatograms of samples taken at data points 3, 4, 7, and 10 in Figure 3 show the accumulation and subsequent consumption of 3-methylcatechol (RT 3.07 min at 0.5 ml min^{-1} with guard column) in the experiment. The peak areas at these data points are 202860, 337080, 423480, and 11840, respectively. Accumulation of 3-methylcatechol during toluene biodegradation confirms that *P. putida* F1 used the *tod* pathway (Figure 1), which involves an initial dioxygenase enzyme that requires two cosubstrates: oxygen and the reduced electron carrier $NADH + H^+$.

Because the biodegradation pathway for benzene begins with hydroxyl substitution at vicinal carbons, catechol should buildup in the batch experiment with benzene. We identified the intermediate of aerobic benzene degradation in an open-system batch reactor. First, *P. putida* F1 was grown to a high density (200 mg l^{-1}) with toluene vapor fed from the headspace. Then, toluene was removed for three hours to let the bacteria consume all the intermediates accumulated in the flask. Next, benzene vapor was supplied from the headspace for one hour before a sample was taken for HPLC analysis. Finally, benzene was replaced by toluene for two hours, and another sample was collected for HPLC analysis. Figure 4 shows the HPLC chromatograms of catechol and 3-methylcatechol standards (a) together with the two samples (b and c). As expected, catechol (RT 3.9 min at 0.2 ml min^{-1}) was found in the first HPLC sample. However, a second peak (RT 7.7 min) also was observed in Figure 4(b). The compound was later identified as phenol using HPLC. In Figure 4(c), the majority of catechol had been consumed, while 3-methylcatechol (RT 8.6 min) accumulated when benzene was replaced by toluene. The removal of phenol must have been slower than catechol, as phenol persisted. In addition, other compounds (RT 17.0 and 18.2 min) were detected. They were later identified as *m*-cresol and *o*-cresol by comparing with standard HPLC chromatographs.

When *p*-xylene was fed in the open-batch reactor, the accumulated intermediate was 3,6-dimethylcatechol (RT 4.4 min), which is consistent with the dioxygenation reaction forming catechol and 3-methylcatechol.

Effects of oxygen

The presence of phenol and cresols in the open-batch experiments probably was due to oxygen limitation in the reactor, which was aerated only through mixing with a stir bar. Phenol or cresols were never detected in the chemostat experiments, where the oxygen concentration was always maintained above 12 mg l^{-1}. To examine the effects of oxygen during toluene degradation, two 165-ml closed-system batch reactors were seeded with 10 mg $l^{-1}$of *P. putida* F1. Toluene was added to 50 mg l^{-1}, the bottles were sealed, and the headspace of one bottle was flushed with pure oxygen and the other with pure nitrogen for about 30 seconds. The cultures were then stirred vigorously with stir bars. HPLC samples were collected every several hours from each bottle.

The chromatograms of samples collected three hours after the start show the presence of different intermediates. A significant amount of 3-methylcatechol (RT 3.07 min) accumulated in the oxygen bottle (peak area 1282365). Several small peaks also were present

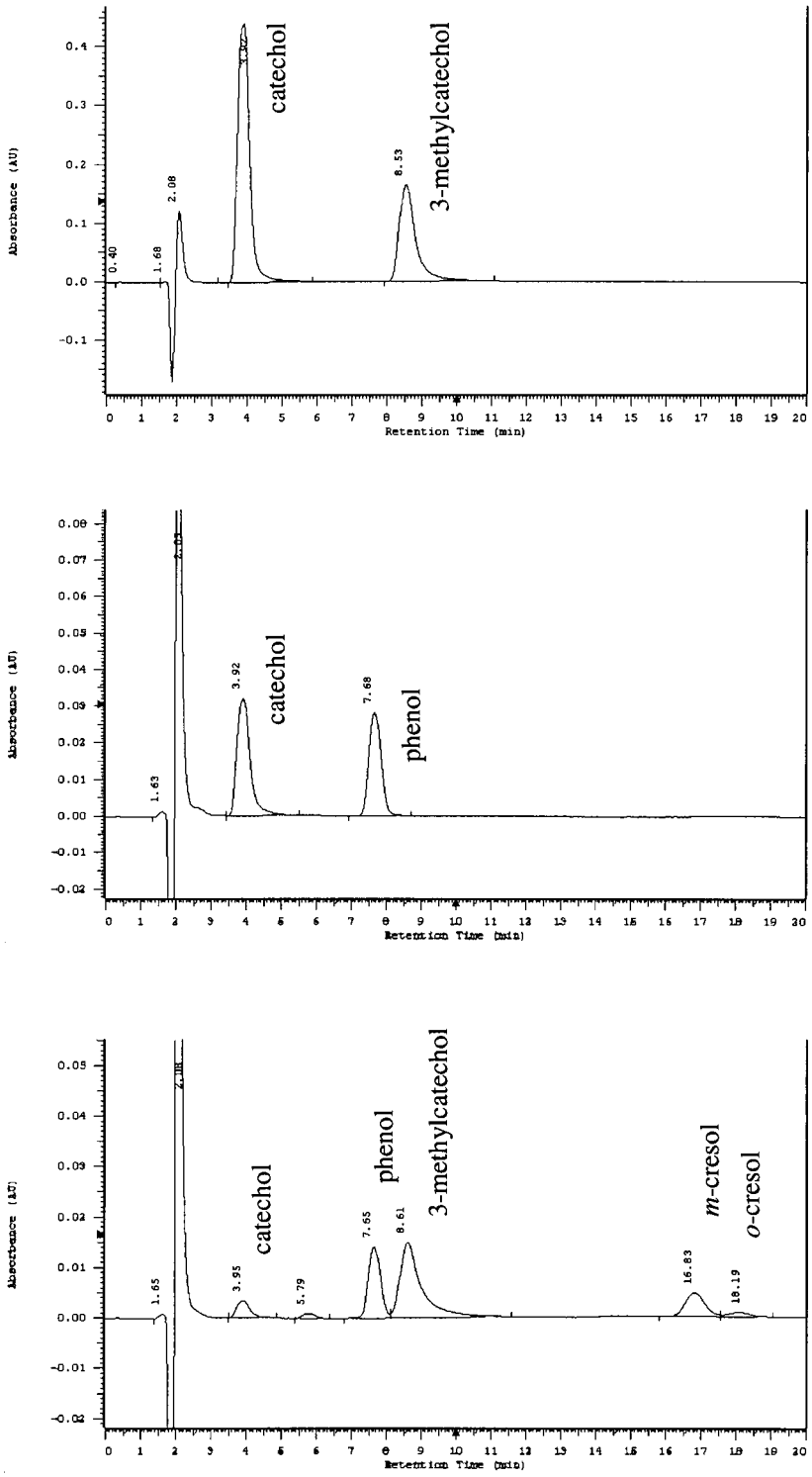

Figure 4. Intermediates of aerobic degradation of benzene and toluene. Graph (a): standards of catechol and 3-methylcatechol, (b): benzene intermediates, (c): toluene intermediates and residues of benzene intermediates.

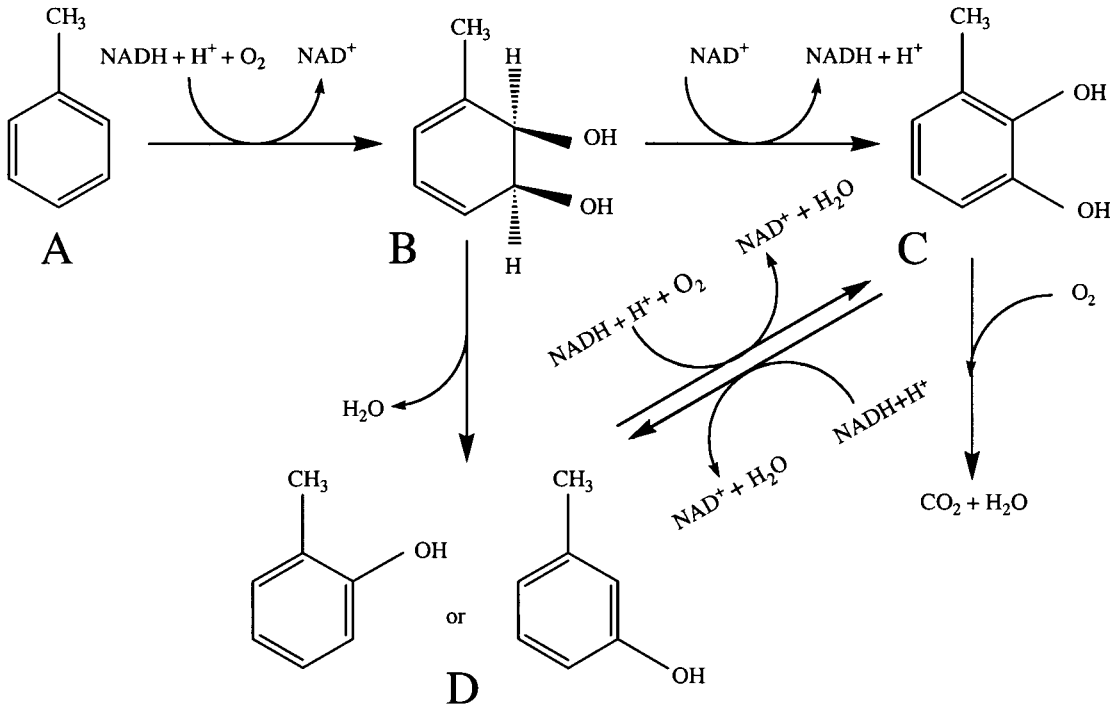

Figure 5. Possible toluene-degradation pathway by *P. putida* F1 for the formation of cresols from toluene. Similar pathway produces phenol from benzene. A = toluene, B = cis-toluene dihydrodiol, C = 3-methylcatechol, D = cresols.

(peak area 113564 at RT 2.24 min and peak area 42274 at RT 5.79 min), but not identified. Toluene was identified a RT of 30.9 min (peak area 580680). On the other hand, 3-methylcatechol clearly was absent in the nitrogen bottle, while the only non-toluene compounds accumulated in significant quantity were the *m*-cresol and *o*-cresol (RT 5.79 and 6.3 min) with a total peak area of 136920. The toluene peak of 1605540 was much larger than that of the oxygen bottle. The larger toluene peak in the nitrogen bottle indicates slower transformation of toluene when oxygen was severely limiting.

Figure 5 shows the possible reactions that led the formation cresols from toluene when oxygen was limited as a cosubstrate for dioxygenation. The reaction pathway from toluene to *cis*-toluene dihydrodiol and then to 3-methylcatechol is well known. When oxygen is not limiting, 3-methylcatechol is further degraded to CO_2 and H_2O. However, when oxygen is limiting, most oxygen could be consumed during transformation from toluene to *cis*-toluene dihydrodiol, because this step is faster than the transformation of 3-methylcatechol. Thus, a bottleneck develops in the path of electron flow from the donor to the acceptor. The electrons are "trapped" in the electron carriers ($NADH + H^+$) inside the cells, thus causing the concentration of NAD^+ to decrease relative to $NADH + H^+$ (Bae & Rittmann 1996a,b). As shown in Figure 5, NAD^+ facilitates the reaction from *cis*-toluene dihydrodiol to 3-methylcatechol. When NAD^+ is low in the cells and $NADH + H^+$ high, it becomes more likely for *cis*-toluene dihydrodiol to lose a water molecule to form *m*-cresol or *o*-cresol.

Implications of the observations

The formation and accumulation of intermediates during the biodegradation of BTX has important implication for the treatment of BTX-contaminated waters and gases. First, all the observed intermediates – phenol, cresols, and the catechols – are toxic to humans and other organisms in the environment. Second, these intermediates contain most of the initial chemical oxygen demand (COD) and all of the dissolved organic carbon (DOC) of the original BTX. These two factors show that successful treatment of the BTX contamination is not effected until the intermediates are removed. Third, oxidation of the catechols allows biomass synthesis. Hence, the viability of any microbiological process demands that the catechols be

mineralized so that their electrons and carbon can be utilized to synthesize and maintain the biomass that transforms the BTX.

Conclusions

When oxygen was available to *P. putida* F1, toluene, benzene, and *p*-xylene were transformed by a dioxygenase reaction that produced a catechol intermediate, but did not support biomass growth. Instead, mineralization of the intermediates – catechol for benzene and 3-methylcatechol for toluene – supported biomass growth. The 3,5-methyl catechol produced from *p*-xylene was a dead-end product, making transformation of *p*-xylene strictly cometabolic for *P. putida* F1. Being a cosubstrate in oxygenation reactions and a terminal electron acceptor, oxygen affected the biodegradation rates and the intermediates formed. When oxygen was severely limiting, phenol (from benzene) and *m*-cresol and *o*-cresol (from toluene) were formed via an alternative pathway that involved only the first step of the initial dioxygenation. It appears that phenols and the cresols persisted when oxygen was absent. In order to have a viable microbiological process that achieves treatment goals (removal of toxicity, COD, and DOC), the catechol intermediates must be mineralized.

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