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Detection of intermediate metabolites of benzene biodegradation under microaerophilic conditions

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Abstract

The intermediate metabolites of benzene transformation by a microaerophilic bacterial consortium, adapted to degrade gasoline and benzene at low concentrations of dissolved oxygen (<1 mg l^{-1}), were identified. The examined range of initial DO concentration, 0.05 to 1 mg l^{-1}, was considerably lower than the previously reported values believed to be necessary to initiate benzene biodegradation. An extensive transformation of benzene, higher than the theoretical predictions for its aerobic oxidation, was observed. Phenol was identified as the most stable and the major intermediate metabolite which was subsequently transformed into catechol and benzoate. The use of ^{13}C-labeled compounds identified benzene as the source of phenol, and phenol as the source of catechol and benzoate, suggesting the involvement of a monooxygenase enzymatic system in biodegradation of benzene at low DO concentrations. A metabolic sequence was proposed to describe the simultaneous detection of catechol and benzoate during the microaerophilic transformation of benzene. The results of this work demonstrate that it is possible to transform benzene, a highly carcinogenic hydrocarbon and a major contaminant of groundwater, to more easily biodegradable compounds in the presence of very small amounts of oxygen.

Introduction

The biodegradation of benzene under fully aerobic or strict anaerobic conditions has been extensively studied and many intermediate metabolites of these processes have been identified (Axcell & Geary 1975; Berry et al. 1987; Ribbons & Eaton 1982; Kuhn et al. 1988; Grbic-Galic 1989; Harwood and Gibson 1997; Kazumi et al. 1997). Benzene is easily degraded under aerobic conditions by pure cultures or by consortia. Its biodegradation under strict anaerobic conditions has also been reported under nitrate-, manganese-, iron- or sulfate-reducing as well as under methanogenic conditions (Goud et al. 1985; Lee et al. 1988; Hutchins 1991; Lodaya et al. 1991; Lovley et al. 1995; Coates et al. 2001). However, there is little information concerning benzene biodegradation at low concentrations of dissolved oxygen (DO), particularly about its degradation pathway and the type of metabolites produced under these conditions. Biodegradation of benzene at low DO concentrations has significant environmental implications because of the toxic and carcinogenic effects of this highly soluble monoaromatic hydrocarbon combined with low DO concentrations usually found under in situ conditions and downgradient of gasoline spills. As emphasized by Caldwell and Suflita (2000), identification of intermediate metabolites of benzene biodegradation under in situ conditions is of utmost importance because of the higher polarity and water-solubility of these compounds which facilitates their migration in the environment.

There are several reports on the requirement for a minimum concentration of dissolved oxygen in the range of 1.0–1.5 mg l^{-1} to initiate the biodegradation of monoaromatic hydrocarbons and in particular benzene which has been shown to be the most recalcitrant hydrocarbon at low DO concentrations (Chiang et al. 1989; Wilson & Bouwer 1997). According to

Viliesid and Lilly (1992), dissolved oxygen concentration regulates the synthesis and activity of the enzymes responsible for the initial attack on aromatic ring of hydrocarbons. Consequently, a minimum dissolved oxygen concentration is required in order to ensure a complete expression of the aerobic enzymes. The requirement for a minimum (threshold) concentration of dissolved oxygen (2 ppm) to sustain the biodegradation of BTX by soil indigenous microorganisms was reported by Chiang et al. (1989). These authors observed little or no biodegradation of hydrocarbons even at low concentrations of 120–200 ppb, with DO concentrations below 0.5 mg l^{-1}. Olsen et al. (1995) showed that eight different strains of bacteria isolated for growth on benzene under aerobic conditions were completely unable to degrade benzene under oxygen-limited conditions. It appears that a critical oxygen concentration is needed to induce aerobic hydrocarbon biotransformation. This concentration depends on the type of hydrocarbon(s) and the specific microbial population present in the system, and was reported to be in the range of 1.0–1.5 mg O_2 l^{-1} (Wilson and Bower 1997).

The objectives of the present study were to investigate the biological transformation of benzene at low DO concentrations previously believed to be limiting, and to identify the type of intermediate metabolites produced under these conditions. The detection of intermediate metabolites of hydrocarbon biodegradation has proven to be difficult because of the inherent chemical properties of these compounds including their volatility and polar structure, the high reactivity and fast rate of their transformation, as well as limited capabilities of the employed analytical instruments. Furthermore, the production and subsequent transformation of many metabolites take place in a very short time, causing the lack of their detection due to infrequent sampling procedure.

In this work, an extensive methodology was employed for the detection and identification of intermediate metabolites of benzene biotransformation under microaerophilic conditions. The methodology included a combination of analytical techniques using gas chromatography/mass spectrometry (GC/MS), liquid chromatography/mass spectrometry (LC/MS) and solid phase microextraction-gas chromatography/mass spectrometry (SPME-GC/MS). The SPME technique is known for its speed and sensitivity and enabled the detection of unstable products of benzene metabolism at trace amounts. This effort resulted in identification of several intermediate metabolites, not previously associated with degradation of benzene in the presence of molecular oxygen at the concentrations examined in this work.

Dissolved oxygen concentrations ranging from 0.05 to 1 mg l^{-1} were used in the experimental system, establishing a condition known as microaerophilic. The microbial culture used in this study had been adapted to degrade gasoline and pure benzene at low availability of dissolved oxygen in packed-bed biobarriers (Yerushalmi et al. 1999; Yerushalmi & Guiot 2001).

Materials and methods

Microbial biomass

The microbial biomass used in the present study was obtained from three laboratory-scale, packed-bed bioreactors which were employed in the biodegradation of gasoline and benzene for over two years. The microbial culture used as the inoculum of the bioreactors, also known as biobarriers, was an aerobic culture isolated by enrichment techniques from the top layers of a gasoline-contaminated soil sample from Montreal, Canada. The details of enrichment procedure are described elsewhere (Yerushalmi & Guiot 1998). The supplied oxygen to the biobarriers was far less than the requirements for complete aerobic biodegradation of hydrocarbons, implying that the microbial consortium was adapted to degrading petroleum hydrocarbons at low concentrations of oxygen. The removal efficiencies of benzene in the biobarriers ranged from 63.9% to 99.9% with inlet benzene concentrations of 0.4 to 35.1 mg l^{-1}. The removal efficiencies of gasoline with inlet concentrations of 3.7 to 74 mg l^{-1}, ranged from 86.6% to 99.9%. The operation of biobarriers was previously reported (Yerushalmi et al. 1999; Yerushalmi & Guiot 2001). Only the free-floating cell suspensions (not the attached microbial biofilm) were withdrawn from the biobarriers. The biomass samples were mixed together under an atmosphere of $N_2 : CO_2$ (90% : 10%) and were distributed in the experimental bottles under anaerobic conditions. A 10% inoculum was used throughout this study.

Medium composition

A minimal salts medium (MSM) with the following composition was used as the culture medium; in (g/L): $KH_2\ PO_4$, 0.87; $K_2H\ PO_4$, 2.26; $(NH_4)_2\ SO_4$, 1.1; and $Mg\ SO_4.7H_2O$, 0.097. To this solution was

added 1 ml (per liter) of a trace metals solution composed of, in (g l^{-1}): Co $(NO_3)_2.6H_2O$, 0.291; AlK $(SO_4)_2.12H_2O$, 0.474; Cu SO_4, 0.16; Zn $SO_4.7H_2O$, 0.288; Fe $SO_4.7H_2O$, 2.78; Mn SO_4. H_2O, 1.69; Na_2 MoO_4. $2H_2O$, 0.482; and Ca $(NO_3)_2$. $4H_2O$, 2.36. The final pH of the medium was 6.9–7.1. The medium was sterilized by autoclaving at 120 °C for 20 minutes. Distilled water was used in the preparation of medium throughout this study. A 0.5 ml of a 0.2% resazurin solution was added per liter of culture medium to the anaerobic and microaerophilic bottles as an indicator of oxygen.

Chemicals

The chemical compounds used in the preparation of culture medium were of reagent grade from BDH (Montreal, Canada). Non-radioactive benzene, as well as the uniformly labeled (UL) ^{14}C-benzene and ^{13}C-benzene were obtained from Sigma (Oakville, Ontario, Canada). Benzoic acid, cathecol, resorcinol, phenol and UL-^{13}C phenol, acetic and propionic acids were purchased from Aldrich Chemicals (Danvers, MA., USA). Butyric, valeric and hexanoic acid were purchased from Aldrich-Chemie (Steinheim, Germany). All solvents were of pesticide grade from Fisher Scientific (Montreal, Quebec). The reagents used for derivatization of metabolites were N,O-bis-trimethylsilyl trifluoroacetamide (BSTFA) (Pierce Chemical Co., Rockford, USA) and acetic anhydride from Anachemia (Rouses Point, NY, USA).

^{14}C-Benzene mineralization

The extent of mineralization of ^{14}C-labeled benzene by the microbial culture in the presence of various levels of dissolved oxygen was measured by microcosm tests. The tests were carried out in 120-ml serum bottles equipped with a KOH vial that trapped the produced carbon dioxide. The CO_2 produced by transformation of the labeled substrate was subsequently measured by scintillation counting. Values in disintegrations per minute (dpm) were then converted to mg or % of substrate mineralized over a given period of time. All experiments were performed in closed environment in order to prevent evaporation of benzene and the volatile intermediate metabolites. Negative controls with benzene but without biomass, as well as controls with killed biomass and without benzene were present to correct for the interferences from biomass or the volatilization of benzene.

Experimental condition

The experiments were performed at room temperature (20-25 °C) in 120 or 160-ml serum bottles with working liquid volumes of 40 or 57 ml, respectively. The liquid in the bottles was sparged with a mixture of $N2 : CO_2$ (90% : 10%) for 10 minutes before the bottles were closed with stoppers and were autoclaved at 120 °C for 20 minutes. After autoclaving and while the bottles were still hot (∼95 °C), the liquid was sparged again with the $N_2 : CO_2$ mixture until it cooled down to room temperature (∼25 °C). The liquid turned colorless following the addition of 30 μl of a $Na_2S.9H_2O$ solution (0.1 g/40 ml) to each bottle, indicating the complete removal of traces of oxygen and the presence of anaerobic condition. The microaerophilic conditions were established by the addition of air at predetermined amounts to the anaerobic bottles in order to obtain the desired concentrations of dissolved oxygen ranging from 0.05 to 1 mg l^{-1}. The liquid turned pink in all of the bottles and at all levels of the initial DO concentration as soon as air was introduced in the bottles. Parallel control experiments showed the presence of oxygen, indicated by the pink color of liquid, in the sterile non-inoculated bottles for the entire duration of experiments. The volume of air added to each bottle was determined by using the Henry's constant of oxygen at 25 °C (756.7 atm.L $mole^{-1}$) and by assuming equilibrium between the gas and liquid phase. Benzene was added aseptically to each bottle, yielding initial concentrations of 10.9 ± 1.4 mg/l or 24.9 ± 0.2 mg l^{-1} during the different series of experiments. The examined initial dissolved oxygen concentrations were 0.05, 0.1, 0.2, 0.5 and1.0 mg l^{-1}.

The initial DO concentration in the bottles was measured in parallel experimental bottles to ensure the establishment of the desired oxygen level in the experimental system. Measurements were made by a glass polarographic DO probe Model E05643 (Cole Parmer, Chicago, Ill, USA) and monitored on a DO controller Model 01972 (Cole Parmer, Chicago, Ill, USA). The accuracy of DO measurements was ±1%.

Aerobic and anaerobic controls were present during every set of experiments. The aerobic control was prepared by continuous sparging of the liquid phase with air for 10 minutes resulting in an initial DO concentration of 8.5 mg l^{-1}, while the anaerobic control did not receive any air and remained colorless during the entire course of the experiments. The negative control had 0.5 or 1.0 mg l^{-1} dissolved oxygen

concentration and did not receive any benzene in order to identify the potential metabolites that might have originated from biomass. The reported values are averages of three to five replicate measurements.

The stability of microbial consortium was verified by repeating benzene biodegradation experiments under similar operating conditions over a period of 15 months. The variability of the obtained results was within 10%.

Analytical techniques

The concentrations of non-radioactive benzene and phenol in the liquid phase were determined by HPLC analysis. A Thermo Separation Products HPLC equipped with a ternary pump (model TSP SP-8800), an autosampler (model SP-8880) and a UV detector was used in these analyses. The samples (20 μl) were injected into a C-18 column (25 cm $\times$ 4.6 mm ID, 3 μm particle size, Sphereclone, Phenomenex, CA, USA) using a mobile phase of acetic acid/methanol (70/30) at a flow rate of 1 ml min^{-1}. Detection was done at 210 nm. Liquid samples (1 ml) were withdrawn from the experimental bottles at the desired intervals and were filtered through a 0.22 μm millex-GP (Millipore, MA, USA) filter paper before analysis. The concentration of compounds was estimated from their corresponding chromatographic peaks using the appropriate calibration data. The gas phase concentrations of benzene and phenol were determined by using their corresponding Henry's constant and by assuming equilibrium between the gas and liquid phase.

Benzene and metabolites were also analyzed by the GC/MS in a selective ion mode (6890 HP GC connected to an HP 5773 MSD). The metabolites in liquid samples were extracted with ethyl acetate at pH 2.0 using concentrated HCl. Ethyl acetate extract obtained from incubation mixtures was evaporated once under a gentle stream of nitrogen gas and dissolved in a small amount of ethyl acetate and dried over $NaSO_4$. N,O-bis-trimethylsilyl trifluoroacetamide (BSTFA) was added to form trimethylsilyl (TMS) derivatives of benzene metabolites, and the sample was heated at 70 °C for 20 min. The extracts (1 μl) were injected into a DB-5 capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 μm) using an HP 7683 automatic sampler. The carrier gas was helium, and the injector temperature and the temperature at the detector interface were kept at 265 °C and 280 °C respectively. The oven temperature was initially held at 50 °C for 2 min, then increased to 280 °C at a rate 10 °C min^{-1} and held for 5 minute.

Phenol and metabolites of benzene biotransformation were analyzed a second time with a Varian 3400 gas chromatograph GC/SPME (Solid-Phase Microextraction) connected to a Varian Saturn II ion trap mass spectrometer and fitted to an autosampler (Varian 8200 cx). Separation was done using a SPB-5, 0.25 μm film thickness, 30 m $\times$ 0.25 mm i.d., Supelco Canada (Oakville, Ontario). The column was held at 50 °C for 2 min, increased at a rate of 10 °C min^{-1} to 280 °C and held for 5 min. The transfer line was held at 280 °C. The mass spectrometers were operated in the EI mode and were tuned to decaflurotriphenylphosphine (DFTPP) in accordance with U.S. EPA method 525. The mass spectrum was obtained using an electron impact of 70 eV with a filament emission current of 30 mA, a mass range of 20 to 300 amu and a scan rate of 2 scans/second. A fused silica fiber coated with an 85 μm polyacrylate polymer (Supelco, Bellefonte, PA) was conditioned by placing it inside the injection port of a GC/MS at 300 °C until a blank background was produced (about 2 h). At each sampling time, 1 ml aliquots of the cell suspension were acidified with 1N HCl (pH3) and filtered with a Millex-HV 0.45-μm-pore-size filter to remove suspended material including biomass. A 10 min adsorption time from the aqueous solution followed by a 10 min desorption inside the GC injector (265 °C) were found appropriate for reproducible analyses. Acetylation in aqueous solution was done to detect metabolites hydroxyl groups. Acetic anhydride (1 %) was added to 1 ml of incubation mixture and was agitated for 5 minutes before adsorption with SPME fiber. Metabolites were identified by comparison with authentic standards and the profile of their formation was followed by their area counts.

The analysis of ^{13}C-compounds by LC-MS, to identify and further verify benzene metabolites, used a HPLC (Hewlett Packard, model 1100) equipped with a photodiode array detector and coupled to a Micromass PlatformII benchtop single quadrupole mass detector. The samples (100 μl) were injected into a Supelco C-18 column (25 cm $\times$ 4.6 mm, 5 μm particle size) held at 35 °C. The mobile phase was a methanol/water (30 : 70) mixture. Water was acidified with acetic acid (pH = 4) and was delivered at a flow rate of 1 ml/min with a post column split ratio of 5 : 95. Analyte ionization was done in negative atmospheric pressure chemical ionization mode (APCL -), leading to mass spectra containing [M-H] ions. The electro-

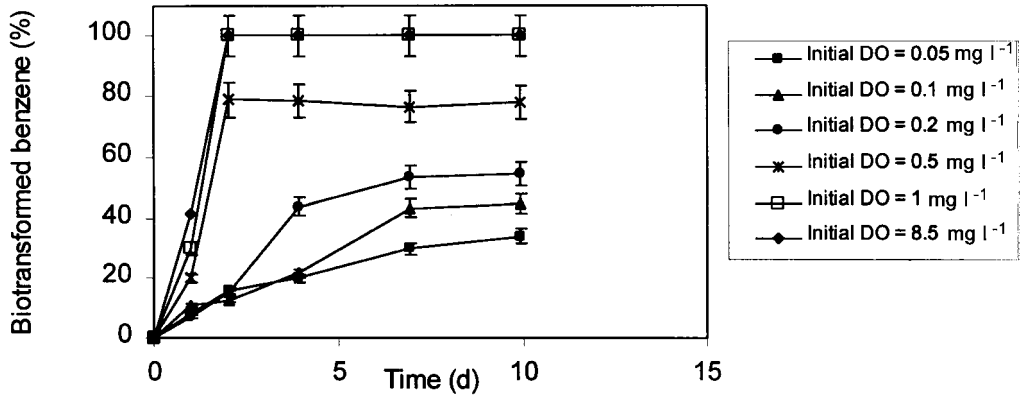

Figure 1. Dynamics of benzene biotransformation (10.9 mg l^{-1}) under microaerophilic condition.

Table 1. Comparison of benzene biotransformation with its expected theoretical mineralization under aerobic and microaerophilic conditions

Initial DO (mg l^{-1})	% Benzene biotransformation		% Theoretical mineralization expected[1]		% Benzene mineralized[2]
	B_i[3] = 10.9 ± 1.4 (mg l^{-1})	B_i = 24.9 ± 0.2 (mg l^{-1})	B_i = 10.9 ± 1.4 (mg l^{-1})	B_i = 24.9 ± 0.2 (mg l^{-1})	B_i = 24.9 ± 0.2 (mg l^{-1})
0.05	34.0 ± 1.7	NA	8.4	3.8	0.8
0.1	44.6 ± 2.2	65.1 ± 2.9	20.5	7.5	0.9
0.2	54.4 ± 2.1	NA	41.2	14.8	13.2
0.5	77.8 ± 2.3	84.7 ± 2.3	72.9	37.1	27.7
1.0	100	98.6 ± 0.4	100	73.4	50.8
8.5[4]	100	100	100	100	98.4

[1] Based on the available oxygen in the system and the requirement of 3.08 mg oxygen per mg benzene mineralized.
[2] Measured by $^{14}CO_2$ recovery.
[3] B_i = Initial benzene concentration.
[4] Aerobic system.
NA = data not available.

spray probe tip potential was set at 3.5 KV with a skimmer voltage of 30 V. The temperatures of the APCL probe and the source were 250 °C and 150 °C, respectively. Dry nitrogen gas at atmospheric pressure was used to evaporate electrospray droplets. Single ion monitoring technique (SIM) was used in order to improve the detection limit of compounds. The signals were corrected for the background noise.

Results

Benzene biotransformation under microaerophilic conditions

Benzene was transformed at all the examined initial dissolved oxygen (DO) concentrations of 0.05 to 1 mg/l. As shown in Figure 1, the overall biotransformation of benzene ranged from 34.0% to 100% with the increase of DO concentration from 0.05 to 1 mg l^{-1}. The extent of benzene biotransformation was equal to or higher than the theoretical predictions for its aerobic mineralization based on the total availability of oxygen in the system and the requirement of 3.08 mg oxygen per mg benzene (negligible cell growth) (Table 1). The total available oxygen in the experimental system included the dissolved oxygen as well as the amount present in the head space of the bottles. This observation suggested that benzene was either partially transformed into intermediate metabolites, or alternatively, it was degraded via anaerobic pathways following the depletion of oxygen in the system. Experiments under strict anaerobic condition showed the lack of benzene biotransformation. The anaerobic bottles were incubated for an extended period of almost six months. No sign of benzene biodegradation or accumulation of by-products was observed under anaerobic conditions after extensive analysis.

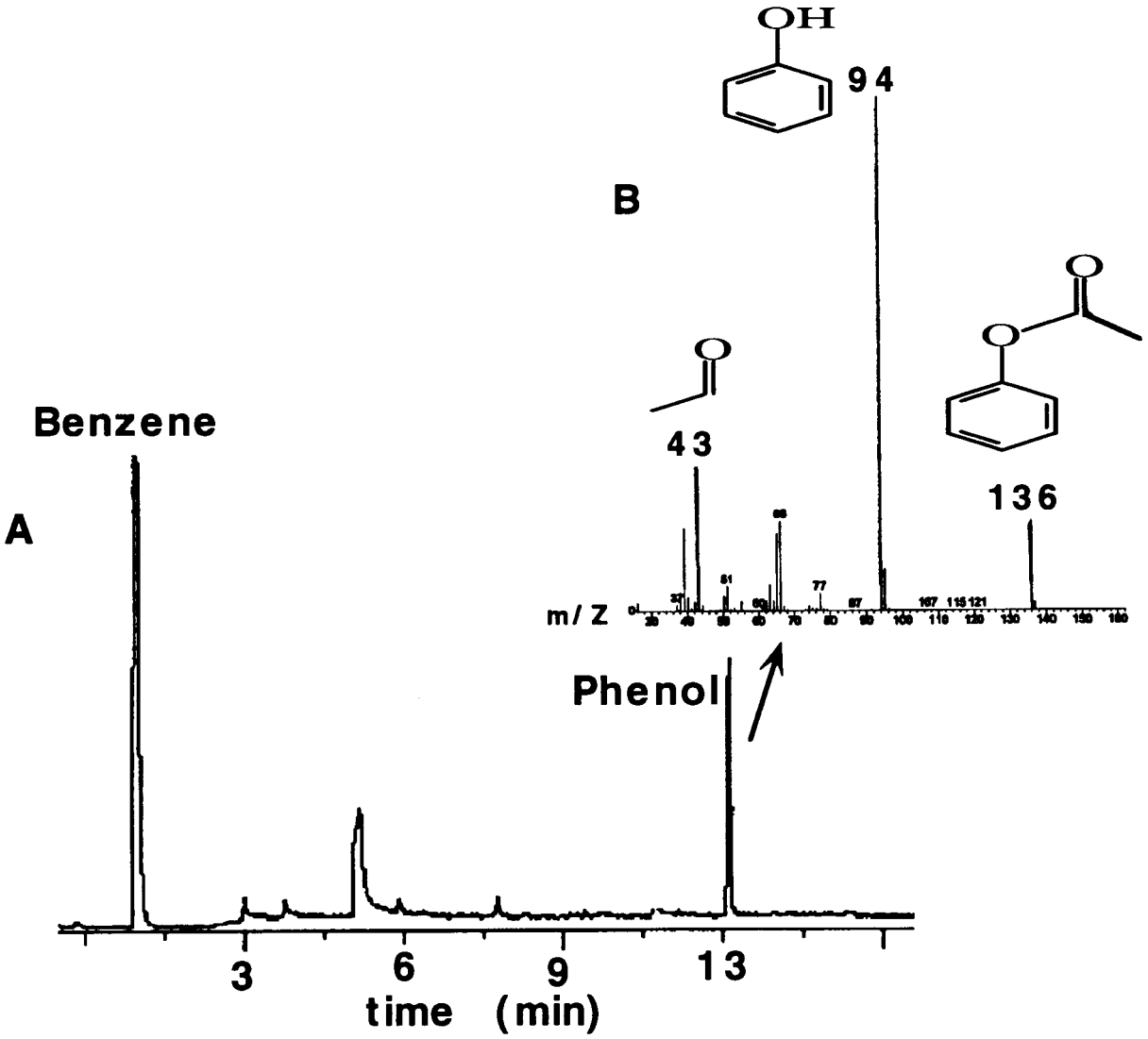

Figure 2. GC-MS-SPME total ion chromatogram of benzene and phenol during [^{12}C]benzene biotransformation under microaerophilic condition at initial DO = 0.5 mg l^{-1} (A), and a typical mass spectral profile of acetylated derivative of [^{12}C]phenol (B).

Furthermore, mineralization experiments at initial DO concentrations of 0.05 to 1 mg l^{-1} revealed that benzene was not completely mineralized in the absence of adequate amount of oxygen (Table 1). These findings suggested that benzene was partially transformed into other compounds during its metabolic degradation.

Identification of intermediate metabolites

The intermediate metabolites of benzene biotransformation were identified from their molecular (*m/z*) mass ions and by comparison of their retention times (rt, min) with authentic standards.

Analysis of liquid samples by HPLC identified phenol as an intermediate metabolite of benzene biotransformation under microaerophilic conditions. The production of phenol was also confirmed by SPME-GC/MS method. A typical SPME-GC/MS total ion chromatogram of unlabeled benzene biotransformation under microaerophilic condition is presented in Figure 2 (initial DO = 0.5 mg l^{-1}). The mass ions occurring at *m/z* 43, 94 and 136 correspond to acetyl, phenol and the acetyl derivative of phenol, respectively.

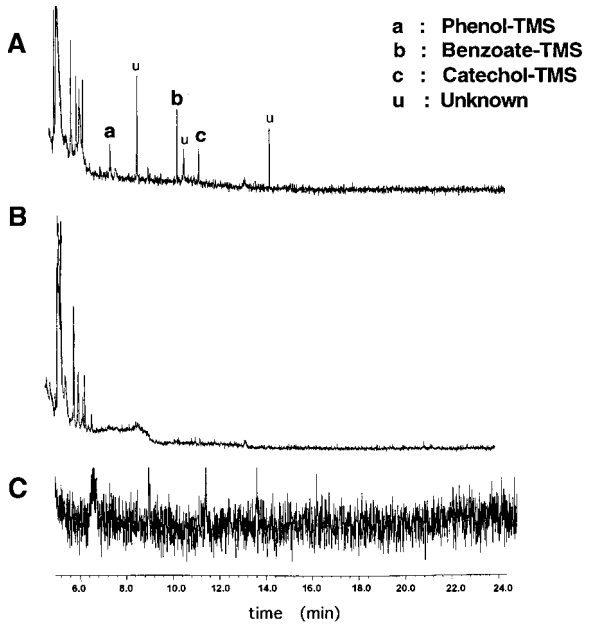

Figure 3. GC-MS total ion chromatogram of TMS-derivatized metabolites of benzene biotransformation under microaerophilic condition (A), chromatogram of the derivatized control without benzene (B), and chromatogram of extracts without derivatization (C).

Experiments with [^{13}C-UL]benzene were performed in order to establish that phenol did indeed originate from benzene. The results of analyses by LC/MS and SPME-GC/MS positively identified benzene as the origin of phenol under microaerophilic conditions. The mass spectrum of [^{13}C]phenol showed mass ions at *m/z* 43, 100 and 142, representing acetyl, [^{13}C]phenol and the acetyl derivative of [^{13}C]phenol. The results also indicated that phenol completely originated from benzene (100% ^{13}C). The recovery of transformed benzene carbon in phenol ranged from 6.7% $\pm$ 0.5% to 27.4% $\pm$ 0.7% under the examined range of initial DO concentration.

With increasing time of incubation and following the derivatization procedure, several new peaks appeared on the chromatograms. Figure 3 shows a typical GC/MS chromatogram of a liquid sample withdrawn from an experimental bottle with an initial DO = 0.5 mg l^{-1} after 24 hours of incubation. The chromatograms showed the simultaneous presence of benzoate and catechol identified by their retention times of 10.8 and 11.7 min, respectively, and by their mass spectral profiles (Figure 4A and B). The mass ions at *m/z* 194 and 254 correspond to the silylated derivatives of benzoate and catechol, respectively. Many unknown peaks were also present on the chromatograms (Figure 3). The detection of catechol and benzoate became possible only through frequent sampling of the liquid culture followed by their rapid analysis. A derivatization step was required in order to stabilize these polar metabolites. Further sampling in time and subsequent analysis of liquid samples by GC/MS-SPME revealed the presence of a catechol isomer, resorcinol (1,3 dihydroxybenzene, rt = 13.89 min, *m/z* 194) among the metabolites of benzene biotransformation under microaerophilic conditions (Figure 5). The formation of resorcinol from phenolic compounds has been reported under anaerobic condition (Schink et al., 1992). No fatty acids were detected under microaerophilic conditions. Also, no metabolites were found in the sterile chemical or biological controls.

The appearance of phenol before catechol and benzoate during the biotransformation of benzene, and the simultaneous detection of the latter two metabolites suggested that phenol was the precursor of catechol and benzoate. In order to verify this hypothesis, experimental bottles (initial DO = 0.2 or 0.5 mg l^{-1}) were amended with [^{13}C-UL]phenol after the oxidation of benzene. GC/MS analysis of liquid samples, after extraction with ethyl acetate and subsequent derivatization with BSTFA and acetic anhydride, showed the presence of benzoate and catechol, identified by their respective mass spectral profiles. An example of the mass spectral profiles of derivatized [^{13}C]catechol and [^{13}C]benzoate is presented in Figure 6 (A and B). The mass ions at *m/z* 200, 158 and 116 (Figure 6A) correspond to [^{13}C]catechol diacetate, [^{13}C]catechol-acetyl (after losing one acetyl group) and [^{13}C]catechol (after losing two acetyl groups). The mass spectral profile of derivatized benzoate (Figure 6B) included the mass ion at *m/z* 201 representing the silylated derivative of [^{13}C]benzoate. These findings established phenol as a possible origin of catechol and benzoate during microaerophilic degradation of benzene. However, it is not possible to determine if during benzene oxidation, these metabolites were also produced by an alternative pathway, by-passing phenol. The occurrence of a mass ion at *m/z* 201, representing silylated [^{13}C]benzoate (Figure 6B) demonstrates the incorporation of ^{13}C in all carbon positions including the carboxyl group.

Discussion

Biological degradation of aromatic compounds proceeds by their transformation into a few central inter-

mediate metabolites. Catechol has been identified as the characteristic intermediate metabolite during aerobic aromatic metabolism (Gibson et al. 1968; 1970), while the production of benzoic acid has been associated with anaerobic transformation of aromatic compounds (Evans & Fuchs 1988; Grbic-Galic 1989). The simultaneous detection of catechol and benzoate in the presence of low concentrations of dissolved oxygen suggested that the microbial consortium used in this study had the ability of benzene biotransformation via alternative metabolic pathways.

Based on the detection of [^{13}C]phenol following amendments with [^{13}C]benzene, and further detection of [^{13}C]catechol and [^{13}C]benzoate following amendments with [^{13}C]phenol, a tentative sequence for the transformation of benzene under microaerophilic conditions is proposed, representing phenol as a central metabolite and the product of ring oxidation of benzene, further transformed into catechol and benzoic acid (Figure 7).

Phenol can be produced by the transformation of benzene under anaerobic conditions (Vogel and Grbic-Galic 1986; Grbic-Galic & Vogel 1987; Caldwell & Suflita 2000). However, in the present work strict anaerobic conditions prevented the transformation of benzene. It should be noted that sulfate, previously shown to serve as a terminal electron acceptor (TEA) in anaerobic biodegradation of benzene (Lovley et al. 1995; Phelps et al. 1996), was supplied in experimental bottles at abundant concentrations. This indicates that the microbial consortium could not degrade benzene via anaerobic routes using sulfate as the TEA. Hence, the proposed pathway proceeds through an obligate first step which is oxygenase dependent and controlled by the oxygen concentration. The regulatory effects of oxygen concentration or its partial pressure on the synthesis and activity of the aerobic enzymes are reported before (Viliesid & Lilly 1992). There are fundamental enzymological differences between the group of bacteria active under hypoxic conditions and those that are active under fully aerobic conditions (Olsen et al. 1994a). Under aerobic conditions, monooxygenases or dioxygenases catalyze aromatic compounds by introducing hydroxyl groups into the ring structures (Kitayama et al. 1996; Krooneman et al. 1996; Olsen et al. 1997). It is conceivable that the low concentrations of oxygen under microaerophilic condition suppressed the activity of dioxygenase enzymatic systems as observed by several investigators (Viliesid & Lilly 1992; Krooneman et al. 1996). Krooneman et al. (1996) showed that dioxygenases, normally active under aerobic conditions during the degradation of 3-chlorobenzoate (3CBA), were suppressed when 3CBA-degrading bacteria were isolated under reduced O_2 partial pressures. Under these conditions, the bacterial isolates metabolized 3CBA via an alternative pathway not requiring the dioxygenase activity. They concluded that the availability of oxygen played a significant role in the early steps of substrate degradation. Hack et al. (1994), in a study of toluene transformation to toluene dihydrodiol showed that low DO concentrations reduced the amount and the activity of toluene dioxygenase in *Pseudomonas putida*. Similarly, Viliesid and Lilly (1992) reported that the induction of catechol 1,2-dioxygenase of *P. putida* was strongly reduced at lower partial pressures of oxygen. With the suppressed activity of the dioxygenase system, the formation of phenol from benzene and its subsequent transformation into catechol could be catalyzed by a monooxygenase enzymatic system, incorporating one oxygen atom into the aromatic ring. The involvement of monooxygenases during metabolic degradation of aromatic structures is known. Zhou et al. (1999) reported the biotransformation of benzene to phenol and its further oxidation to catechol by a monooxygenase enzymatic system. Olsen et al. (1994b) showed that toluene-3-monooxygenase incorporated one atom of oxygen into toluene, producing *meta*-cresol that was hydroxylated into 3-methyl catechol and further degraded via *meta*- ring cleavage. Literature has other examples of benzene transformation into phenol through monooxygenase systems. They include: the production of phenol by a mutant of *Pseudomonas aeruginosa* growing on benzene (Yarmoff et al. 1988; Kitayama et al. 1996); by a *Xanthobacter* Py2 strain through the action of alkane monooxygenase (Zhou et al. 1999), and through the induction of toluene-3-monooxygenase by benzene, converting it to phenol (Olsen et al. 1994b).

As an alternative to its further oxidation to catechol, phenol, the central intermediate, can also be transformed into benzoic acid as a result of oxygen deficiency. The sequence of phenol transformation into benzoate in the absence of molecular oxygen starts by its carboxylation, to produce *para*-hydroxybenzoic acid, followed by dehydroxylation to give benzoate (Knoll & Winter 1987; Lack & Fuchs 1994; Li et al. 1996). Caldwell and Suflita (2000) recently demonstrated that phenol produced from benzene, was further transformed into benzoate under sulfate-reducing conditions. Similar to the findings of the present study, these authors also observed that the mass ions asso-

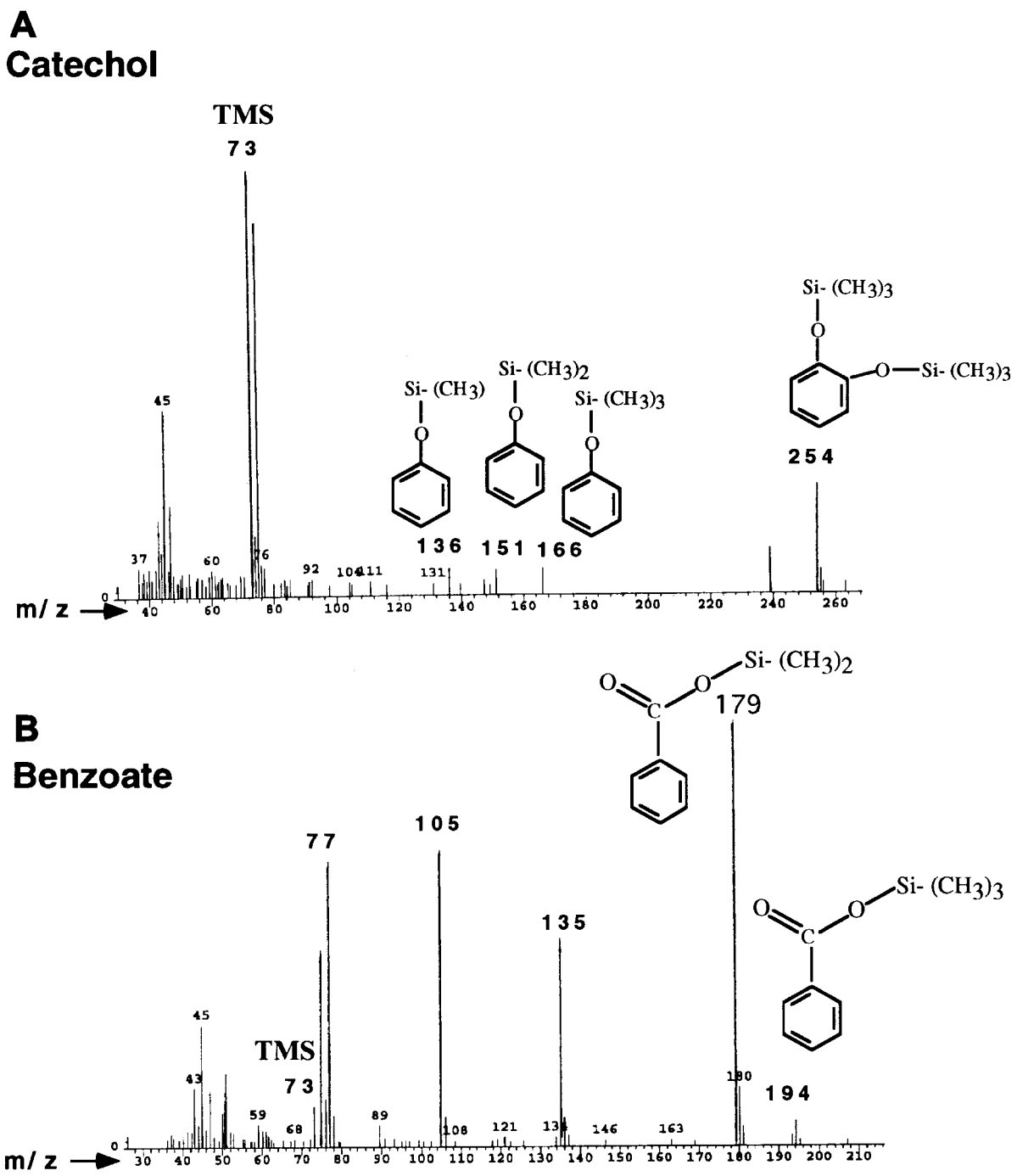

Figure 4. Mass spectral profiles of sylilated derivative of [^{12}C]catechol (A), and sylilated derivative of [^{12}C]benzoate (B) produced during biotransformation of [^{12}C]benzene under microaerophilic condition.

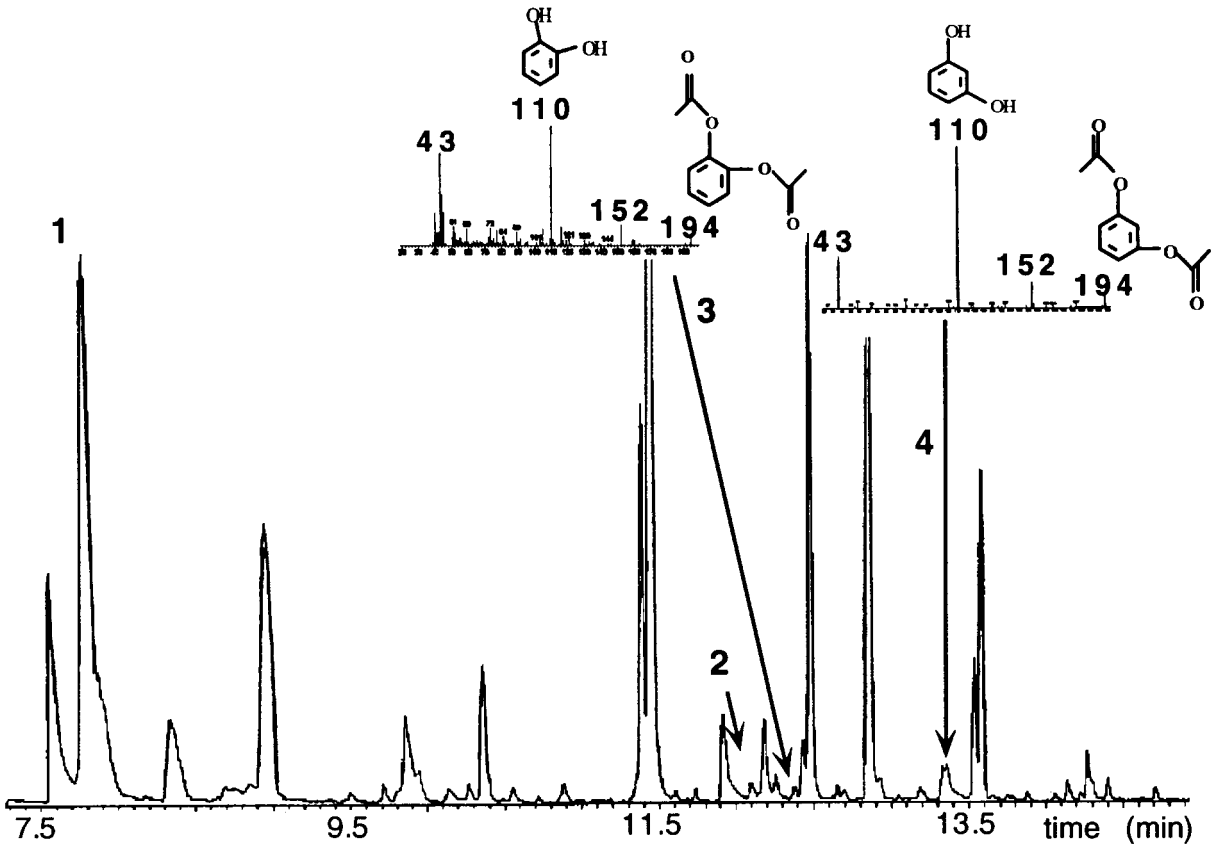

Figure 5. GC-MS-SPME total ion chromatogram of acetylated derivatives of metabolites including phenol (1), benzoate (2), catechol (3) and resorcinol (4) produced during biotransformation of benzene under microaerophilic condition at initial DO = 0.5 mg l^{-1}.

ciated with the formation of benzoate exhibited an increase of 7 mass units during the degradation of ^{13}C-benzene. They concluded that another atom of ^{13}C was incorporated into the metabolite during carboxylation of the ring by a ^{13}C-labeled fragment liberated during the metabolism of benzene. In the present work, the lack of detection of known metabolites of ring reduction (e.g., cyclohexanol, cyclohexanone, cyclohexane) (Evans & Fuchs 1988; Caldwell & Suflita 2000) at any time at the low DO concentrations employed, and the lack of benzene biotransformation under strict anaerobic conditions suggest that the enzymes responsible for phenol transformation into benzoic acid were activated after benzene was processed through an oxidative step.

The activation of anaerobic degradation at low residual oxygen concentrations were previously reported by Krooneman et al. (1999) where 3-chlorobenzoate (3CBA) was anaerobically degraded at residual oxygen concentrations of 3 μM or less. Their data showed that anoxic phototroph *Rhodopseudomona palustris* was able to metabolize 3CBA via anaerobic pathways when there were high oxygen input levels, as long as there was a second substrate that could be aerobically metabolized, leaving low oxygen concentrations in the system.

The results of this work show that in the presence of extremely low DO concentrations, usually recognized as insufficient to trigger the activity of aerobic enzymes, or in the absence of microorganisms capable of anaerobic degradation of benzene, it is still possible to degrade benzene, a potentially recalcitrant hydrocarbon. Under microaerophilic condition, the microbial consortium transformed benzene into metabolites that are less recalcitrant. These metabolites may further be degraded via aerobic pathways if adequate amounts of oxygen become available (Figure 7, Route 1). Alternatively, the products of anaerobic metabolism could be further degraded anaerobically following the complete depletion of oxygen, upon the

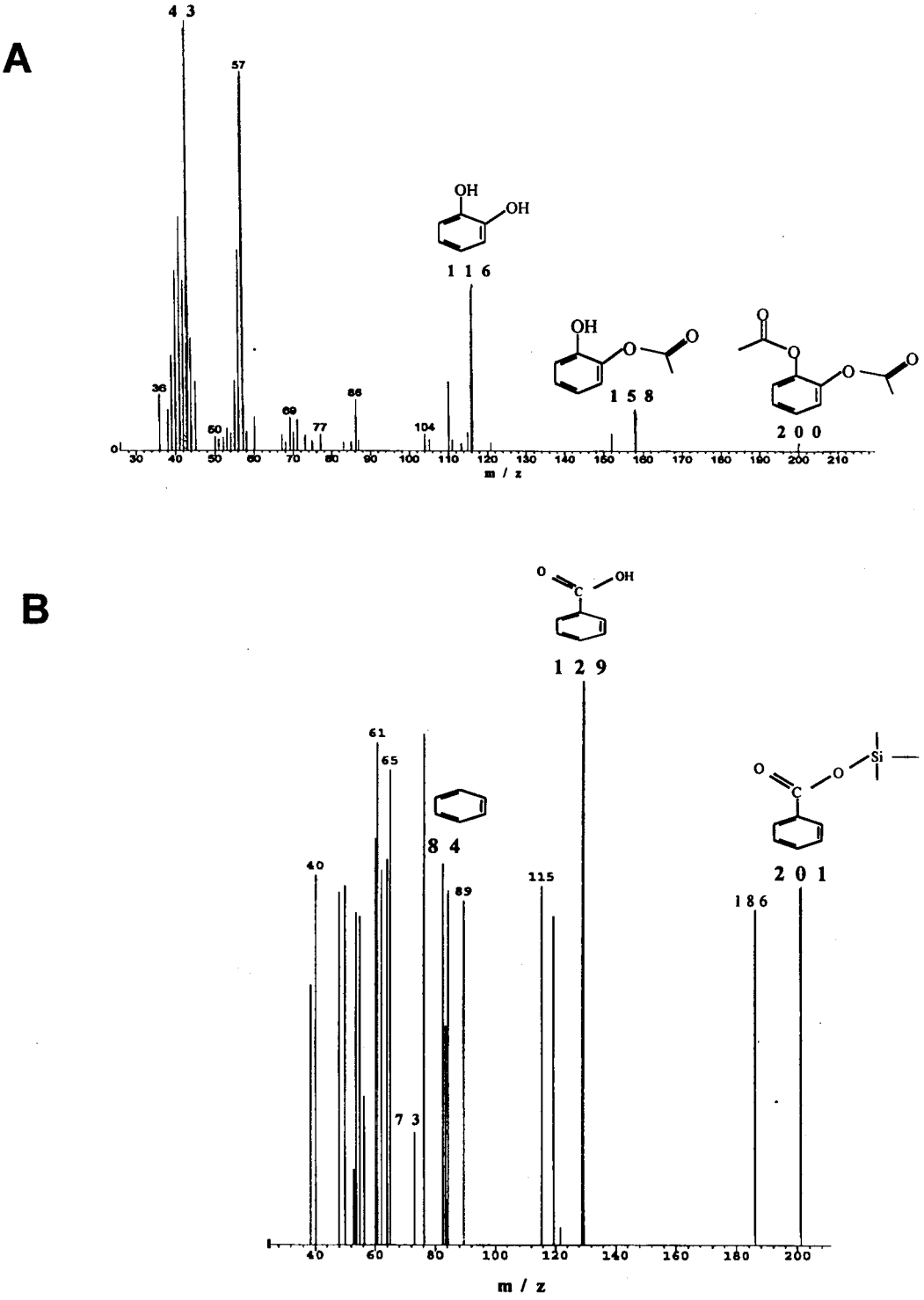

Figure 6. Mass spectral profile of acetylated derivative of [^{13}C]catechol produced from [^{13}C]phenol (A), and mass spectral profile of TMS derivative of [^{13}C]benzoate produced from [^{13}C]phenol (B).

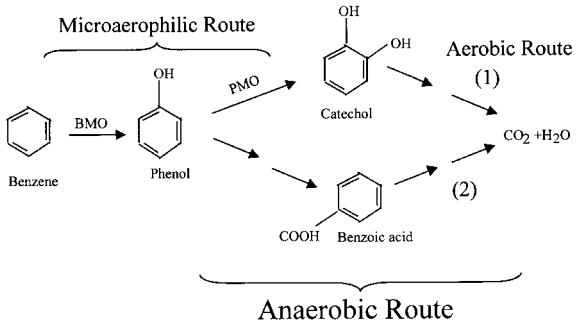

Figure 7. Proposed pathway of benzene biotransformation under microaerophilic condition. The complete mineralization of benzene would be conditional to the presence of adequate amount of oxygen (1), or the presence of appropriate microorganisms (2). BMO = benzene monooxygenase, PMO = phenol monooxygenase.

presence of appropriate anaerobic microorganisms in the environment (Figure 7, Route 2) (Tschech & Fuchs 1987; Gorny & Schink 1994; Heider & Fuchs 1997; Harwood et al. 1999). The proposed sequence presents an alternative route for remediation of groundwater contaminated with toxic and recalcitrant compounds.

Conclusions

The results of this work demonstrate that it is possible to transform benzene, a potentially toxic and recalcitrant contaminant of groundwater, at very low DO concentrations of 0.05 to 1 mg l^{-1}, previously believed to limit its biodegradation. The high biotransformation of benzene compared to the theoretical aerobic mineralization indicated its partial and incomplete transformation at low concentrations and low availability of oxygen. The use of elaborate methodology and sophisticated analytical instruments enabled the detection of unstable metabolites produced at trace amounts during benzene biotransformation. Under conditions known as microaerophilic, the microbial consortium transformed benzene via an alternative metabolic pathway, forming phenol as the major and the most stable intermediate metabolite which was subsequently transformed to catechol and benzoic acid. The formation of phenol from benzene and its further transformation into catechol and benzoic acid were confirmed by the use of ^{13}C-labeled compounds, suggesting the suppressed activity of dioxygenases and the involvement of monooxygenase enzymatic system in the metabolic sequence of benzene transformation.

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