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Application of solid-phase microextraction–gas chromatography–mass spectrometry to characterize intermediates in a joint solar–microbial process for total mineralization of Aroclor 1254

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Abstract

A combined solid-phase microextraction–GC–MS analytical technique was used to monitor the formation of metabolites in the biodegradation of biphenyl, which were originally obtained from the solar photodechlorination of Aroclor 1254 by *Pseudomonas pseudoalcaligenes* KF707 and *Burkholderia* sp LB400. In both cases, the following metabolites were detected: 2-hydroxybiphenyl (2-OH-BP), 2,3-dihydroxybiphenyl (2,3-di-OH-BP), and benzoic acid, which was detected as its benzoate derivative 1-methylethylbenzoate. A time course study for the formation and disappearance of these metabolites was used to construct a degradation pathway, which in both cases, involved the formation of 2-OH-BP and 2,3-di-OH-BP.

Keywords: Mineralization; Aroclor 1254; Biphenyl; Hydroxybiphenyls; Benzoic acid

1. Introduction

Solid-phase microextraction (SPME) is a solventless and rapid extraction technique that uses polymer-coated fibers for the extraction of organic compounds from an aqueous or gaseous phase sample followed by thermal desorption in the injection port of a gas chromatograph for subsequent detection and quantification. The technique is known for its speed and sensitivity which enables detection in the $\mu g\ l^{-1}$ range [1–5].

Although SPME has been widely used for the trace analysis of organic compounds in several aqueous based matrices, little is known on the applicability of the technique for monitoring organic biotransformations in biological matrices [6]. Until recently, exhaustive sample preparation and separation techniques (e.g. liquid–liquid extraction (LLE) followed by chromatographic clean-up procedures) were required to isolate and identify intermediates from biotransformation processes [5,7]. When such intermediates are formed in trace amounts, LLE may not be as practical as SPME for the extraction of such metabolites particularly from a fermentation broth where emulsion formation, due to the presence of surfactant-like products, constitutes an extraction and phase separation problem. Additionally, relatively large volumes of organic solvents are needed during LLE. These problems added together may render LLE unsuitable for the detection of small quantities of (bio)degradation intermediates thus leading to the loss of valuable information on the metabolic pathway.

The main objective of this study was to apply

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SPME in combination with GC–MS to identify the metabolites formed during the biodegradation by *Pseudomonas pseudoalcaligenes* KF707 and *Burkholderia* sp LB400 of biphenyl (BP) originally produced from the reductive photodechlorination of polychlorinated biphenyls (PCBs) [8]. The unique thermal and chemical stability that makes PCBs, e.g., Aroclor 1254, industrially useful has also made them a threat to the environment. They are resistant toward direct solar photodegradation and microbial biodegradation and, therefore, tend to persist indefinitely [9]. Solar photodegradation is one of the most natural and most economical degradation routes for environmental pollution. Unfortunately, most PCB congeners do not absorb strongly above 300 nm (<4% of sun radiation reaching us is below 300 nm) that their direct photolysis by sunlight often proceeds with very low quantum efficiency [8,10,11]. Previously, we were able to completely dechlorinate Aroclor 1254 to BP by sunlight using phenothiazine as a sensitizer in alkaline 2-propanol [8]. The BP that is generated was then mineralized with two aerobic bacteria *Pseudomonas pseudoalcaligenes* KF707 and *Burkholderia* sp LB400.

Several studies have described products that are generated from the microbial degradation of PCBs and its reduced product BP under different microbial conditions. For example, through extensive GC–MS analysis Massé et al. [12] reported the formation of several key metabolites including monohydroxylated and dihydroxylated derivatives (2-OH-4′-Cl-BP, 4-OH-4′-Cl-BP, 2,3-di-OH-4′-Cl-BP, and 3,4-di-OH-BP) and 4-chlorobenzoic acid during the degradation of 4-chlorobiphenyl (3-Cl-BP) by a Gram-negative strain-206. Whereas Williams and May [13] reported the formation of benzoic acid derivatives as metabolites from the aerobic degradation of PCBs in sediments.

The present work describes the utility of SPME–GC–MS in the identification of key metabolites formed during the degradation of BP obtained from the photodegradation of Aroclor 1254 by solar radiation in alkaline 2-propanol. A time profile of the appearance and disappearance of the detected metabolites was used to elucidate the degradation pathway of BP (Scheme 1) by *Pseudomonas pseudoalcaligenes* KF707 and *Burkholderia* sp LB400.

2. Materials and methods

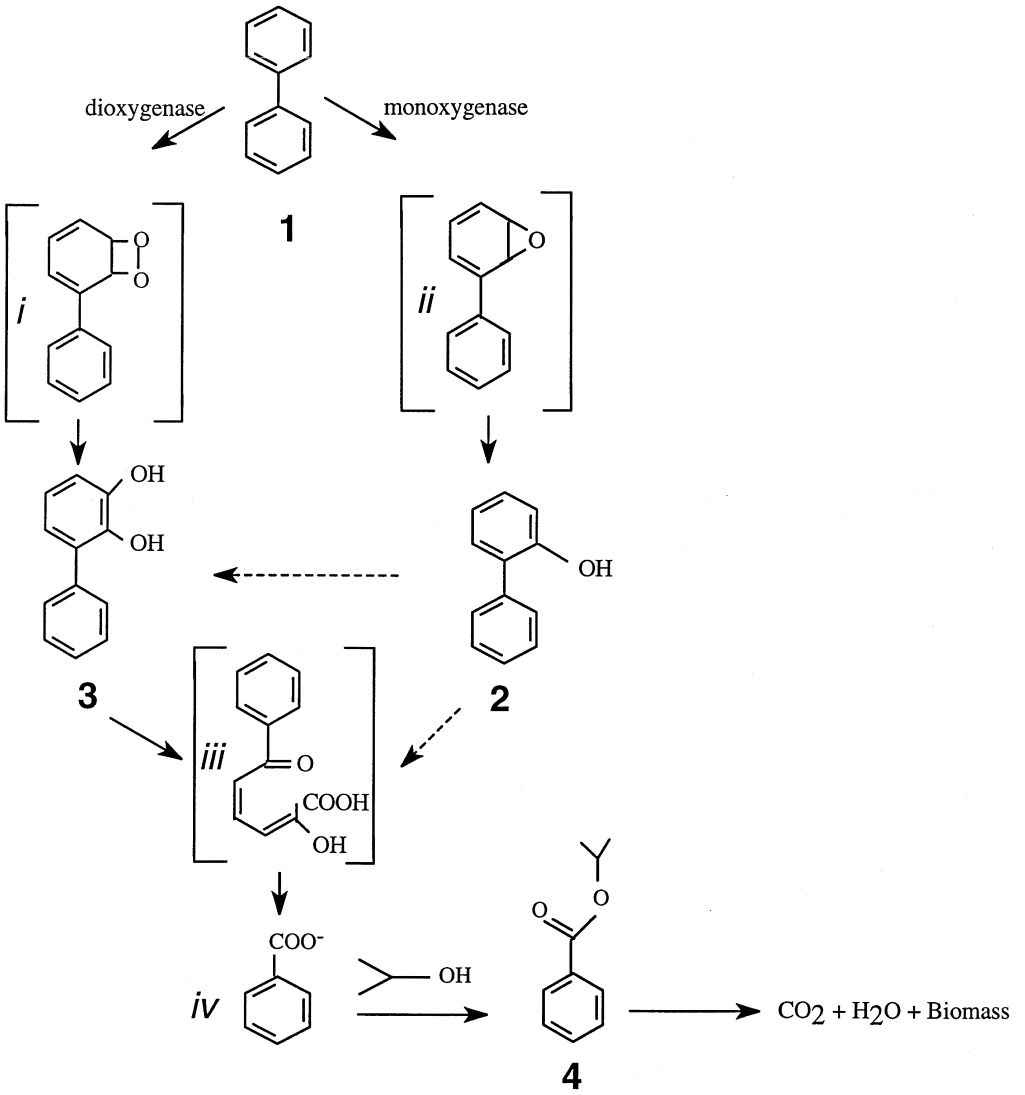
Biphenyl, 3-chlorobiphenyl, 2,2′-dihydroxybiphenyl (2,2′-di-OH-BP), benzoic acid (99% purity) and phenothiazine all with purity ≥99% were obtained from Aldrich, Milwaukee, WI, USA. Aroclor 1254 was obtained from Chem Service, West Chester, PA, USA and analar grade KOH from Anachemia, Rouses Point, NY, USA. Uniformly labelled BP [U-^{14}C] BP (purity >98%), 2-hydroxybiphenyl (2-OH-BP) (99% purity), and 4-hydroxybiphenyl (4-OH-BP) (99% purity) were obtained from Sigma Chemicals Co., St. Louis, MO, USA. All solvents were of pesticide grade and obtained from Fisher Scientific, Montréal, Canada.

2.1. Photolysis and photoproducts of Aroclor 1254

Aroclor 1254 (1105 ppm) was photolyzed in alkaline 2-propanol (pH 8) using solar radiation in the presence of the sensitizer phenothiazine (PT) (0.1%, w/v) following the procedure described in Ref. [8]. After 12 h of photolysis sample aliquots (100 μl) of the photolyzed mixture were diluted with water and their pH adjusted to between 3 and 5 by treatment with dilute nitric acid (0.5 N) for subsequent analysis by SPME–GC–MS. For the analysis of released chloride ions a sample from the photolyzed mixture was extracted with hexane. The remaining aqueous layer was analyzed for Cl^- with an SP 8100 HPLC connected to a 25×0.46 cm PRP-X 100 Hamilton column and a Waters 431 conductivity detector as described in Ref. [8].

2.2. Conditions for the biodegradation of BP

The two bacterial strains used in the study were *Burkholderia* sp LB400, obtained from Herman L. Research and Development Center of the General Electric Company, New York State, USA and *Pseudomonas pseudoalcaligenes* KF707 obtained from Kensuke Furukawa, Department of Agricultural Chemistry, Kyushu University, Fukuoka, Japan. Flasks (serum bottles) were each charged with a minimal salt medium (40 ml), 1 ml aliquots (8×10^8 spores/ml) of the microorganism, and BP (100 mg/l). Further details on microcosm preparation can be



Scheme 1. A constructed metabolic pathway of BP (**1**) biodegradation by *Pseudomonas pseudoalcaligenes* KF707.

found in Ref. [14]. Some serum bottles (microcosms) were supplemented with uniformly labeled [U^{14}C]-BP (100 000 dpm) and then fitted with a small test tube containing 1.0 ml of 0.5 *M* KOH to trap liberated carbon dioxide ($^{14}CO_2$). The head-space in each microcosm was flushed with oxygen gas to maintain aerobic conditions and then sealed with Teflon coated serum caps for incubation at 31°C in a rotary shaker (Brunswick, NJ, USA) at 135 rpm. Control microcosms were prepared using the bacterial culture medium without BP and a second contained an autoclaved bacterial medium followed by the addition of BP with no microorganisms being added. Microcosms with [U^{14}C]-BP were routinely sampled (daily or every two days) for the determination of $^{14}CO_2$ in the KOH trap using a Packard, Tri-Carb 4530 liquid scintillation counter (Model 2100 TR, Packard Instrument Company, Meriden, CT, USA). Microcosms that did not receive [U^{14}C]-BP were reserved for SPME–GC–MS analysis of

residual BP and its metabolites in the aqueous phase after filtration. The cultures were sampled at intervals by removing an aliquot (2 ml) for SPME–GC–MS analysis.

2.3. Solid-phase microextraction followed by GC–MS

A fused-silica fiber coated with an 85-μm polyacrylate polymer (Supelco, Bellefonte, PA, USA) 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, 2-ml aliquots of the cell suspension were acidified with H_3PO_4 (pH 2) and filtered with a Millex-HV 0.45-μm filter to remove cells and suspended material. Analytes were absorbed directly from the MSM filtrate onto the fiber and then thermally desorbed inside the GC injector for analysis by GC–MS. Thermodynamic equilibrium for the partitioning of BP and its intermediate metabolite 2-OH-BP between the SPME sorbent and the aqueous phase was achieved in roughly 40 min as shown in Fig. 1. Stirring was found to be critically important to obtain the thermodynamic equilibrium for the analyte distribution between the aqueous phase and the sorbent coating. Eventually a 40 min adsorption followed by 10 min desorption into the GC injector (250°C) were found to be typical conditions for reproducible analysis. Recovery was determined using 4-OH-BP (99%) as an internal standard.

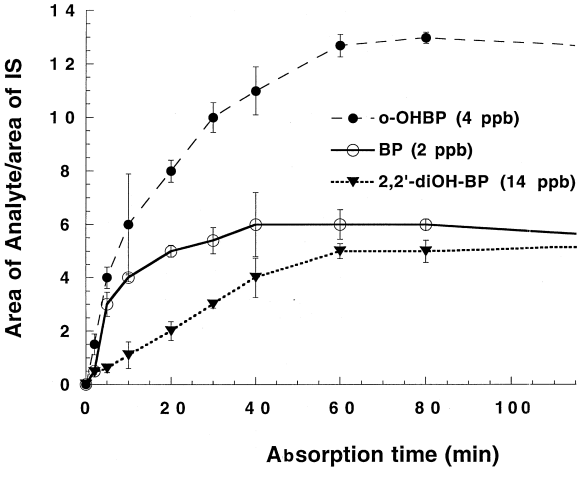


Fig. 1. Representative absorption isotherms of biphenyl, BP: 2-hydroxbiphenyl (2-OH-BP) and 2,2′-dihydroxybiphenyl (2,2′-di-OH-BP) using polyacrylate coated SPME with stirring. I.S.: 4-OH-BP (14 ppb).

A Varian GC–MS equipped with a Saturn II ion trap detector (transfer line temperature 220°C) was connected to a DB-5 capillary column (30 m×0.25 mm I.D.×0.25 μm film). A splitless injection was used for the first 6 min, followed by split injection (ratio 1/10) for the remainder of the GC program. The carrier gas was helium, and the temperature of the injection port was 250°C. The initial oven temperature (90°C) was increased at a rate of 15°C/min to 210°C, followed by 7°C/min to a final temperature of 280°C. The mass spectrum was obtained using an electron impact of 70 eV with a filament emission current of 30 mA, a mass range of 20–400 amu and a scan rate of 2 scans/s. Based on signal-to-noise ratio of 3 the detection limit (DL) was lower than 1 ppb.

Metabolites were identified by comparison with authentic standards and the profile of their formation was followed by their area counts. A time study, to monitor the formation and disappearance of metabolites during degradation, was carried out by analyzing the BP treated culture medium at different time intervals ranging from $t=0$ h, 4 h, 6 h, 24 h, 3 weeks, and 5 weeks [15].

3. Results and discussion

3.1. Metabolites from the degradation of BP by Pseudomonas pseudoalcaligenes KF707 and Burkholderia sp LB400

Fig. 2 shows the photosensitized transformation of Aroclor 1254 to BP by solar radiation in alkaline 2-propanol (0.05%, v/v) in the presence of phenothiazine (PT) as a sensitizer (1.5 m*M*). Fig. 2 shows the predominant formation of BP together with trace amounts of 3-chlorobiphenyl (3-Cl-BP). The traces of 3-CL-BP were later removed by extending the time of photolysis. In fact we have already reported that photoreductive dechlorination of PCBs is regioselective favoring *ortho*- and *para*- C–Cl cleavage over that of the *meta*-isomer [8]. Eventually the *meta*-isomer (3-Cl-BP) was dechlorinated to **1**. Based on the initial concentration of Aroclor 1254

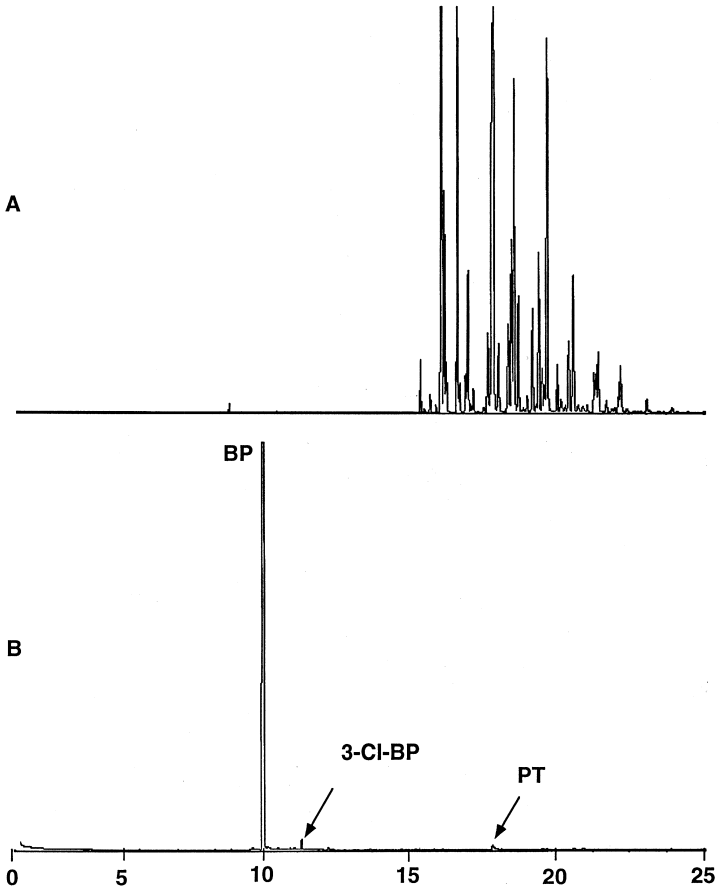


Fig. 2. SPME–GC–MS total ion chromatogram of: (A) Aroclor 1254 before photolysis and (B) after solar irradiation (12 h) in alkaline 2-propanol (0.05 *M*) in the presence of phenothiazine (PT) as a sensitizer (1 m*M*).

and taking into account that 54% (w/w) of commercial Aroclor 1254 is chlorine, the chloride ion recovery was found to be quantitative. Further details on the photodegradation of PCBs can be found in Hawari et al. [8].

The BP product from the above photolysis was subjected to aerobic degradation using either *Pseudomonas pseudoalcaligenes* KF707 and *Burkholderia* sp LB400. A typical SPME–GC–MS total ion chromatogram of metabolites formed from BP biodegradation using *Pseudomonas* culture is shown in Fig. 3, $t=4$ h. The intermediates were identified by comparison with their corresponding standards using retention times (t_R), molecular (m/z), and base peak (bp) mass ions. The parameters (t_R=min, m/z=amu, and bp=amu) for BP (**1**) and the three detected intermediates were (10.05, 154, 154), 2-hydroxybiphenyl (2-OH-BP) (**2**), (11.7, 170, 170), 2,3-dihydroxybiphenyl (2,3-di-OH-BP) (**3**), (12.53, 186, 186) and 1-methylethylbenzoate (MeEt-Bz) (**4**), (8.05, 164, 105), respectively. Metabolite **4** was identified by its mass spectrum (no commercial source is available for this product), as shown in Fig. 4. By excluding 2-propanol from the biodegradation experiment we were unable to detect **4** but instead benzoic acid (**iv**) whose presence was confirmed by the formation of its acetyl derivative (m/z=136) and by comparison with a reference compound. Benzoic acid (**iv**) is usually considered the last stable metabolite of the aerobic degradation of BP [16]. Apparently, the presence of 2-propanol and phenothiazine with PCBs dechlorinated product BP (brought for-

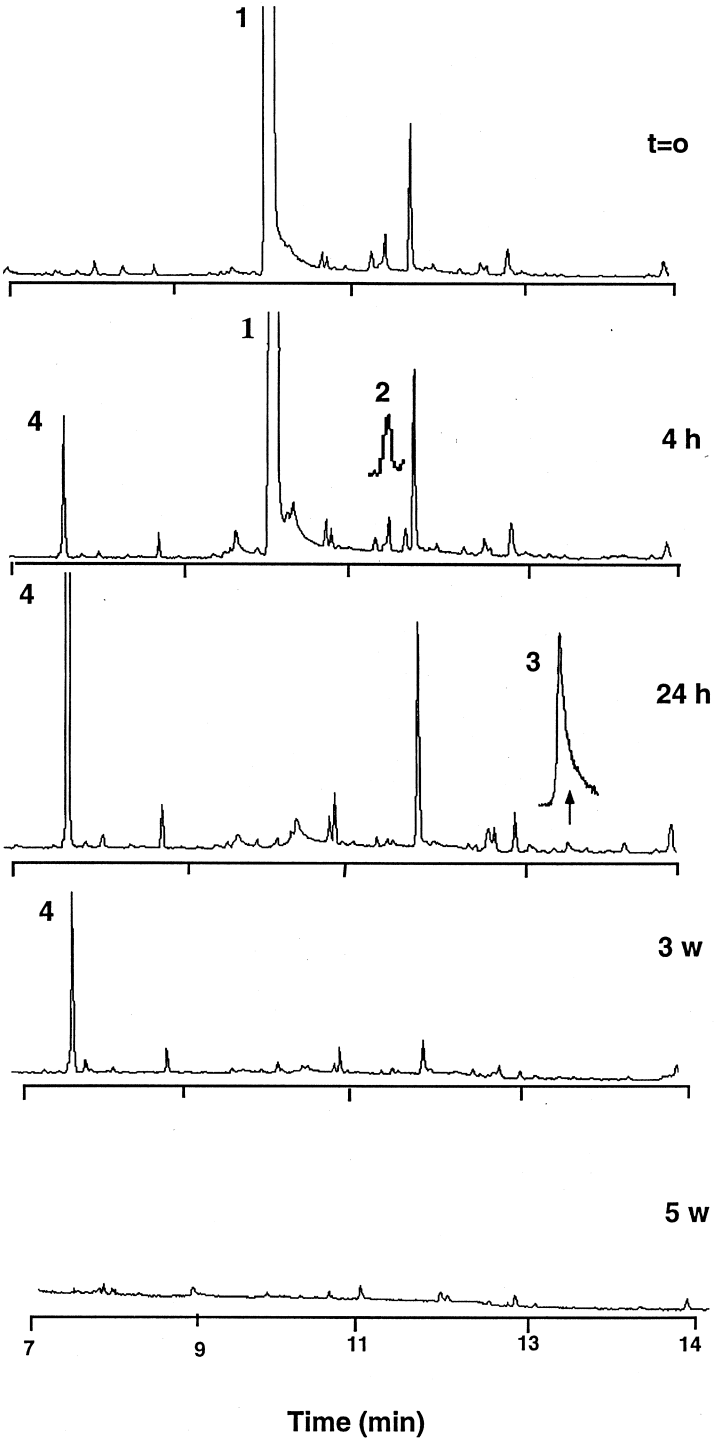


Fig. 3. Profile of biphenyl (BP) (1) and metabolites detected during degradation by *Pseudomonas pseudoalcaligenes* KF707 showing the metabolites: (2) 2-hydroxybiphenyl (2-OH-BP), (3) 2,3-dihydroxybiphenyl (2,3-di-OH-BP) and (4) 1-methylethylbenzoate.

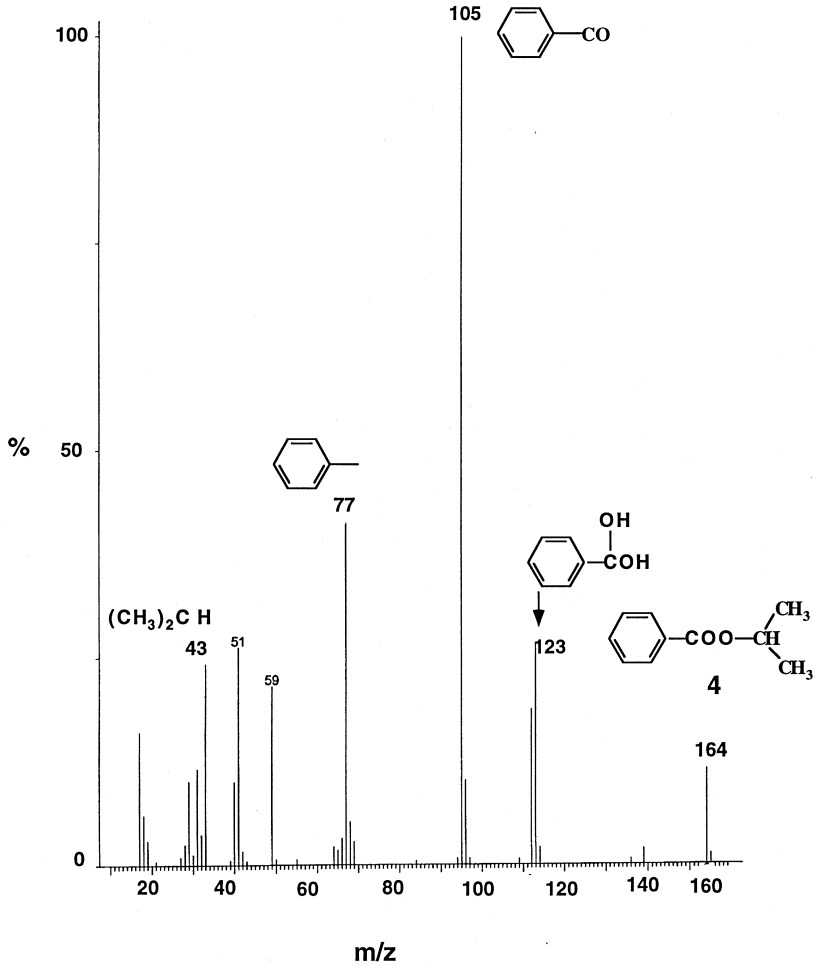


Fig. 4. A typical mass spectrum of 1-methylethylbenzoate (**4**) formed by treatment of BP (**1**) with *Pseudomonas pseudoalcaligenes* KF707.

ward from the photolysis part of the experiment) lead to the conversion of benzoic acid to benzoic acid 1-methylethyl ester (**4**). However, as Fig. 3 shows, BP (**1**), 2-OH-BP (**2**), 2,3-di-OH-BP (**3**) and benzoic acid derivative with 2-propanol (**4**) had their peaks highly resolved and posed no identification problem.

3.2. Time profiles of metabolites detected during the biodegradation of bp: metabolic pathway

After establishing the suitability of SPME–GC–MS for the direct detection of metabolites formed during biodegradation, fresh microcosms were set up with either of the two strains *Pseudomonas pseudoalcaligenes* KF707 and *Burkholderia* sp LB400 to look for the formation and disappearance of the corresponding metabolites using SPME–GC–MS with time. However, the principle behind the performance of the SPME analytical technique is that the target analyte must first achieve a thermodynamic equilibrium in its distribution between the polymeric coating of the SPME fiber and the bulk aqueous phase (Fig. 1). We found stirring to be extremely important to achieve the thermodynamic equilibration considered necessary for quantitative analysis. Furthermore, in this particular study we found that a stirring time of 40 min was needed to achieve such equilibrium.

The SPME–GC–MS data shown in Fig. 3 repre-

sent the biotransformation of BP with time. After 4 h of incubation the SPME–GC–MS area count of BP (**1**) decreased and in return other new peaks appeared which included 2-OH-BP (**2**), and 1-methylethylbenzoate (**4**). After 24 h there was an obvious build up in the concentration of the benzoate (**4**), and a small amount of 2,3-di-OH-BP (**3**) was also detected with a continued reduction in the concentration of BP (**1**). After three weeks of incubation, BP (**1**) disappeared completely and the GC area count of the benzoate metabolite (**4**) started to disappear. Finally after five weeks of incubation neither the starting material (**1**) nor any of the metabolites (**2**, **3** and **4**) could be detected. Mineralization (liberated $^{14}CO_2$) at this time reached its maximum value of 86 and 83% for the two microorganisms *Pseudomonas pseudoalcaligenes* KF707 and *Burkholderia* sp LB400.

The present time study that showed the relationship among the various detected intermediates is best described in Scheme 1 which showed the following sequence: BP (**1**)>2-OH-BP (**2**)>2,3-di-OH-BP (**3**)>BA (**4**) >CO_2. Although there is a general consensus that degradation of BP proceeds in a stepwise fashion until CO_2 is formed, there seems to be a variation among the literature reports on the nature of the metabolites and the order in which they appear. The present SPME–GC–MS method provided convincing evidence on the presence of three key metabolites in the degradation of BP, namely, 2-OH-BP (**2**), 2,3-di-OH-BP (**3**) and benzoic acid detected as its 1-methylethylbenzoate derivative (**4**). However, the presence of the monohydroxylated BP (**2**) has not been considered as an important biotransformation product of BP [12,17–19].

We are not sure at this stage whether 2-OH-BP (**2**) was first formed to produce 2,3-di-OHBP (**3**) or if the two metabolites were formed by two different routes. Presumably, an attack on BP (**1**) by a monooxygenase enzyme produced metabolite **2** via the unstable oxirane derivative (**ii**) and by a dioxygenase produced product, **3**, via the dioxetane derivative (**i**). On the other hand, monohydroxylated chlorobiphenyl and dihydroxybiphenyl were both identified by Massé et al. [12] in a study that involved the bacterial strain B-206. However, the degradation of hydroxylated biphenyls to 2,3-dihydroxybiphenyl by *Pseudomonas* sp. Strain HBP1 has been addressed by Kohler et al. [20] and Higgson and Focht [21]. Furthermore, it was noticed that under certain conditions the enzyme dioxygynase can function as a monooxygenase [22].

The formation of benzoic acid derivative metabolite **4** is a clear indication of the ring opening of the hydroxylated ring in **2** and **3**. This metabolite, which was the last stable intermediate product to be detected, was finally transformed to carbon dioxide after three weeks. However, we were unable to observe the intermediate that would be formed after the ring cleavage of either **2** or **3** to produce benzoic acid or its derivatized ester, **4**. However, a yellow color (λ_{max}=434 nm) typical of that of muconic acid 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienate (Scheme 1, **iii**), was observed and measured during the degradation of **1** by the strain KF707. One possible explanation of the yellow color (λ_{max}=434 nm) detection may be attributed to the formation of this *meta*-cleavage product [23,24]. The formation of muconic acid has been considered as a precursor to benzoic acid by the *meta*-ring cleavage of either **2** or **3,** although no measurement for this unstable metabolite was attempted in the present SPME study.

A similar time profile of metabolites was also obtained for the degradation of BP using *Burkholderia* sp. LB400 (data not shown). It is of particular interest to note that the catabolic pathway of BP in these two strains are nearly identical in terms of gene organization and nucleotide sequence [25–28], implying that both organisms exercise the same degradation pathway for BP. Interestingly, both strains produced approximately the same mineralization amounts as measured by liberated $^{14}CO_2$, i.e., 86 and 83%, respectively. The remaining ^{14}C-BP was probably incorporated into the biomass.

This is the first time that the metabolites from biodegradation of BP, a photoproduct of PCBs, by *Pseudomonas pseudoalcaligenes* KF707 and *Burkholderia* sp. LB400 was attempted using SPME–GC–MS. The speed and the sensitivity of the analytical technique allows the detection of trace amounts of products with relatively short life times. A time study for the appearance and disappearance of these metabolites was successfully used to construct a metabolic pathway for BP biodegradation as shown in Scheme 1.

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