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Short communication

Mass spectrometric analysis of isotope effects in bioconversion of benzene to cyclohexanone

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Abstract

Pseudomonas veronii strain PH-03 has been shown to convert benzene to cyclohexanone through phenol. Mass spectrometry results revealed that unusual isotopic effects have been occurred in the transformation product, cyclohexanone. The isotopic composition was strongly depends on the compound specific hydrogen or oxygen source. The exchange of labile deuterium atoms has been investigated through electrospray ionization liquid chromatography mass spectrometry. The mass spectrometric analysis of biotransformation products enabled the proposal of a corresponding bioconversion pathway.

Keywords: Isotope effect; Bioconversion of benzene; GC–MS; ESI/LC–MS

1. Introduction

Mass spectrometry has been applied as a powerful tool for the identification of bioconversion products resulting from microbiological reactions and used extensively in the past for the identification of chemical reactions [1,2]. Currently, bioconversion using whole cells is being increasingly explored to produce industrially important and fine chemicals because it has several advantages over enzymatic reactions [3]. In the biotechnological area, mass spectrometry is an essential technique for detection and identification of various products. Aromatic hydrocarbons, such as BTEX compounds like benzene, toluene, ethylbenzene, and *meta*-, *ortho*-, and *para*-xylenes, are significant constituents of petroleum products. In particular, benzene is a volatile environmental pollutant commonly found in crude petroleum and petroleum products such as gasoline [4,5]. Megatons of benzene are produced every year for use as an industrial solvent or as a starting material in the manufacture of pesticides, plastics, and numerous chemicals [6–8]. Contamination of groundwater systems by petroleum hydrocarbons is widespread due to leaking underground storage tanks, accidental spills, and inappropriate disposal methods. Groundwater contamination by benzene is of particular concern, since benzene is the most water-soluble BTEX compound and a human carcinogen [9,10].

Isotope compounds can be used to directly monitor biodegradation or bioconversion of aromatic hydrocarbons in the environment by measuring the isotopic contents of the remaining contaminant or product as degradation proceeds [11–13]. Isotopes of elements such as carbon [^{12}C and ^{13}C] and hydrogen [^{1}H and ^{2}H] react at slightly dissimilar rates during mass differentiating reactions. During bioconversion, the lighter isotopes are preferentially exchanged, causing the remaining contaminant or producing compound to be enriched in the heavier isotopes [11–13]. A significant isotopic effect can be observed if a bond containing the element of interest is broken or formed in the rate-limiting step [12,14]. Thus, isotope analysis has the ability to identify biodegradation or bioconversion of environmental pollutants in the field and to determine contaminant mass loss or addition due to bioconversion.

In a recent study, we isolated the *Pseudomonas veronii* strain PH-03 from contaminated soils collected near the incinerator of a chemical plant and characterized its ability to degrade chlorinated dibenzo-*p*-dioxins and dibenzofurans [15]. We have also analyzed this strain for the ability to transform benzene and described a novel type of benzene metabolism. In contrast to canonical aerobic biodegradation, this new form of bioconversion produces cyclohexanone from benzene. Here we report on the mass spectrometric analysis using stable isotopes of ben-

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zene and metabolic intermediates of this novel bioconversion. Further, unusual hydrogen isotopic effects were investigated during identification of the hydrogen and oxygen sources in the bioconversion by mass spectrometric techniques, along with gas chromatography and electrospray ionization. The specific effects of individual sources on deuterium ratio were assessed from the isotopic pattern of consecutive dissociation fragments, as observed from mass spectra.

2. Experimental

Benzene-[$^{13}C_6$], 99 at.% [^{13}C] was purchased from ISOTEC (Miamisburg, OH). [^{13}C]-cyclohexanone, deuterated chemicals (benzene-*d*6, cyclohexanone-*d*10, and D_2O), and dibenzofuran were obtained from Sigma–Aldrich (St. Louis, MO). 95 at.% $^{18}O_2$ was purchased from Cambridge Isotope Laboratories (Andover, MA). The solvents and acids employed were obtained from Merck. All chemicals employed were of the highest quality commercially available.

P. veronii strain PH-03 employed in this study was routinely grown in the dark (160 rpm, 28 °C) in minimum salts medium (MSM) amended with 1 mM dibenzofuran (DF) as the sole carbon source, from resting cells which were prepared as previously described [15]. Pre-grown strain PH-03 culture was inoculated into 250 ml of MSM containing 0.5 g of DF in 2-l Erlenmeyer flasks. After overnight incubation, the entire culture was harvested (11,000 × *g* for 10 min at 4 °C) and washed three times with 20 mM phosphate buffer. The OD_{600} of the resulting culture was adjusted to 8.0 with 20 mM phosphate buffer (pH 7.0). For bioconversion of benzene, 10 ml portions of a resting cell suspension were added to 100-ml Erlenmeyer flasks containing 87.9 mM of the benzene and incubated at 160 rpm and 28 °C for 24 h. The bioconversion was monitored and quantified by taking samples of the culture suspensions over time. Every 2 h, a set of triplicate flasks was removed, and the samples were immediately analyzed by HPLC after centrifugation and filtration. In addition, we collected all HPLC analytes using prep-HPLC. The analytical system consisting of a reverse-phase HPLC (Agilent 1100; Agilent, Waldbronn, Germany) equipped with a Lichrocart RP-18 column (125 mm × 30 mm; 5 μm; Merck, Darmstadt, Germany). The aqueous solvent system (flow rate, 1.0 ml/min) contained 0.01% (w/v) of 85% *ortho*-phosphoric acid and 20% CH_3CN. After collection of the purified samples from HPLC, the contents were extracted and analyzed as described below. The samples and the corresponding controls were extracted four times with 0.2 volumes of dichloromethane and hexane. Extracts were dried over anhydrous sodium sulfate, and the solvents were evaporated under reduced pressure.

Substrate and products present in the extracts obtained from the bioconversion experiments were detected and characterized initially using a liquid chromatography–mass spectrometry (Qstar Pulsar; Applied Biosystems, Foster City, CA) system consisting of a reverse-phase high-performance liquid chromatograph (Agilent 1100; Agilent, Waldbronn, Germany) equipped with a Lichrocart RP-18 column (125 mm × 30 mm; 5 μm; Merck, Darmstadt, Germany) and then by using mass and UV–vis light detection after filtration of the samples with 2 ml syringe through a 0.45 μm syringe filter (Millipore). The aqueous solvent system (flow rate, 1.0 ml/min) contained 0.1% (w/v) acetic acid and 40% methanol. In addition, the extracts of bioconversion experiments with labeled and unlabeled benzene and cyclohexanone were analyzed by nanospray ESI (Electro Spray Ionization)-MS/MS in positive or negative mode by using an ESI-mass spectrometry quadruple-time of flight (Qstar Pulsar; Applied Biosystems, Foster City, CA) system. Then, the extracted samples obtained were analyzed by high-resolution gas chromatography–ion trap mass spectrometry. For gas chromatography–mass spectrometry analyses, we used a Trace GC 2000 system (Thermoquest, Austin, TX) linked to a Finnigan Polaris Q mass spectrometer (Thermoquest) with a 60-m DB-5 mass spectrometry column. The initial temperature, 100 °C, was maintained for 2 min, and then programmed to reach 280 °C at a rate increase of 10 °C/min, and held for 5 min. The temperatures of the injector port and the interface were set at 250 and 280 °C, respectively. The carrier gas (helium) flow rate was 1.0 ml/min. In all experiments, additional controls were employed; these controls consisted of cells that were heat inactivated (75 °C for 20 min) and cultures that were poisoned (10 mM NaN_3).

In $^{18}O_2$ incorporation studies, reaction mixtures contained resting cells (OD_{600} = 8.0) and benzene in 20 mM phosphate buffer (pH 7.0). Reactions were carried out with $^{18}O_2$ (Cambridge Isotope Laboratories, Andover, MA) purging into 100-ml baffled Erlenmeyer flask fitted with Teflon-lined septa and screw-on caps in 10-ml portions of a cell suspension after N_2 flushing. Reaction products were analyzed by LC–MS or GC–MS after filtration or extraction as described above.

3. Results and discussion

In this study, unusual isotopic effects were observed and characterized during the novel benzene ring reduction by *P. veronii* strain PH-03. When determining the hydrogen source of the bioconversion of strain PH-03, we observed unpredicted effects that occurred consistently. In our recent study, the bioconversion was monitored by a HPLC to determine the conversion rate and the HPLC profiles revealed a decrease in the benzene concentration with time, and a concomitant increase in the concentration of a new product (Fig. 1). The newly formed product was identified by GC and ESI-MS analysis as cyclohexanone through phenol; these were confirmed by comparing the data with those of authentic standards. Importantly, these results were a novel bioconversion of benzene by a bacterium under aerobic conditions that utilizes oxidation and reduction concomitantly.

In order to confirm that the detected cyclohexanone carbon source originated from benzene, experiments were conducted with stable isotope [^{13}C]-labelled benzene. As shown in Fig. 2, the result clearly shows [^{13}C]-cyclohexanone originated from [^{13}C]-benzene. In other sets of bioconversion experiments, however, unpredicted isotopic effects were detected by GC and ESI-MS analysis in the case of deuterium-labelled compounds (Fig. 3). This suggests that the deuterium-labelled compound is more unstable than the [^{13}C]-labelled compound in the biological reaction. [D5]-labelled cyclohexanone (m/z = 103) was

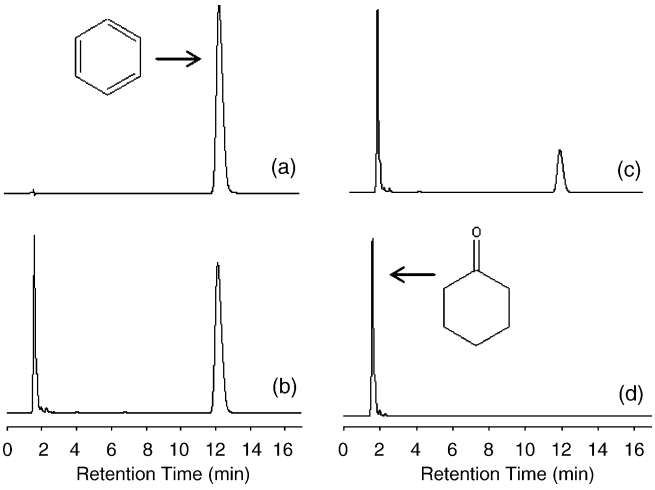


Fig. 1. HPLC chromatograms illustrating cyclohexanone formation from benzene with time. (a) 30 min; (b) 8 h; (c) 12 h; (d) 24 h.

expected as the bioconversion product, but the major ion had an *m/z* ratio of 105, as shown in Fig. 3(b). In addition, similar isotopic effects were detected in ESI-MS spectra in both positive (Fig. 4) and negative (Fig. 5) ionization modes. We examined whether the isotopic effects observed in the previous

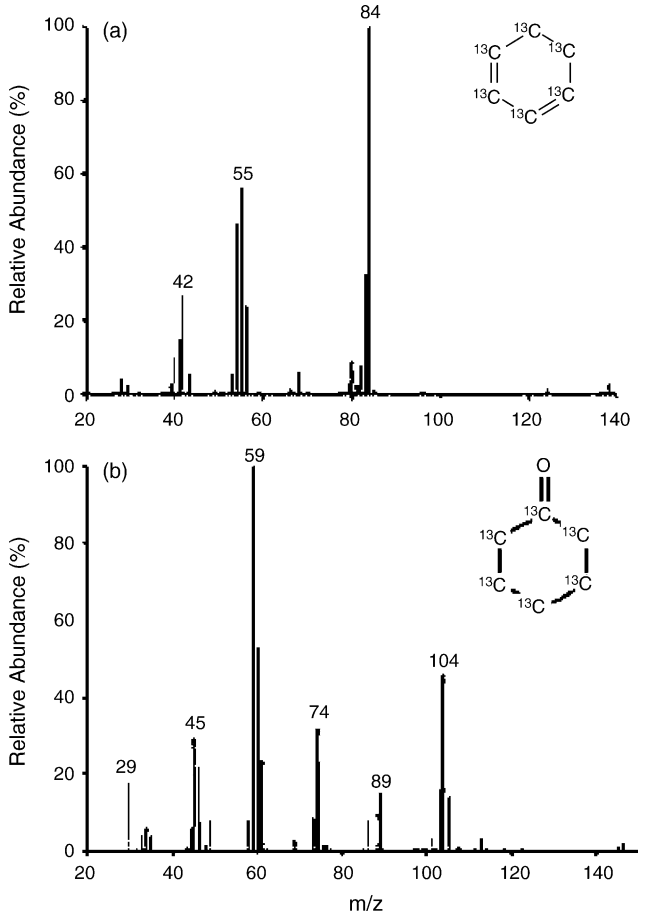


Fig. 2. GC–MS spectra of authentic [^{13}C]-labelled benzene and cyclohexanone produced through bioconversion by *Pseudomonas veronii* PH-03.

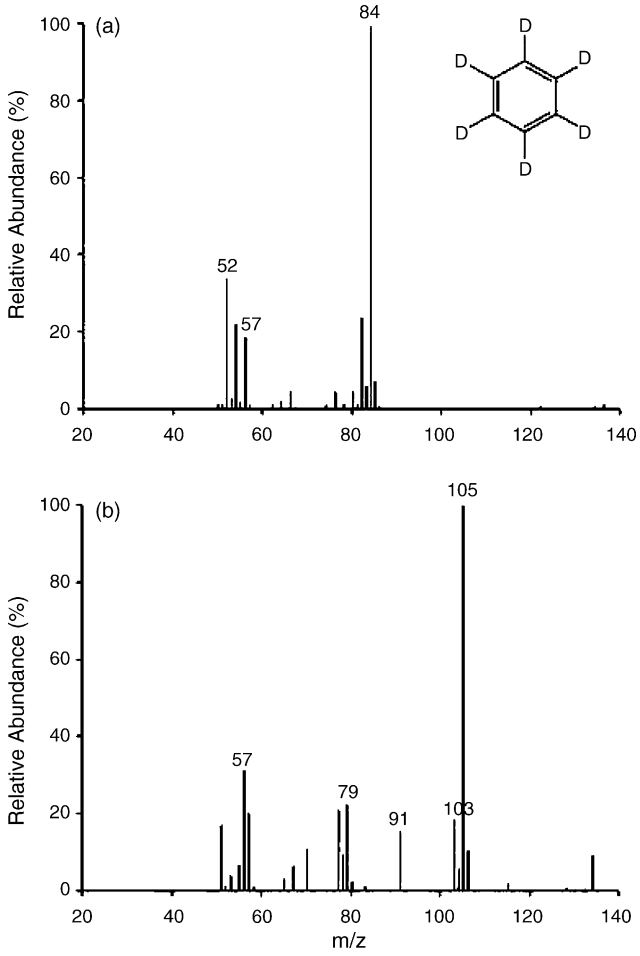


Fig. 3. GC–MS spectra of authentic [D6]-labelled benzene and cyclohexanone product with isotopic effects.

[D6]-labelled benzene experiment come from the buffer by using deuterated water (D_2O) instead of H_2O. The results revealed that cyclohexanone formation was similar to that observed in H_2O-based buffer systems (Figs. 4(a) and 5(a)). Also, the D_2O-based buffer systems spectra showed 1–2 mass unit larger fragments than the corresponding peak in H_2O-based buffer systems spectra, which suggests that the source of isotopic effects is from deuterium-labelled chemicals such as D_2O and there are multiple hydrogen sources. Finally we confirmed that a partial source of hydrogen was H_2O. From the magnified inset Figs. 4(b) and 5(b), the deuterium ratio of individual specific effect was assessed from the isotopic pattern of consecutive dissociation fragments. The ratio of presented peaks in the positive mode of ESI-MS was [*m/z*, 99.0942:100.0998 = 2:1]. This result suggested that the isotopic effect potential value was about 50% for 1 unit large mass. Meanwhile, the ratio of negative mode of ESI-MS was [*m/z*, 96.9700:97.9742:98.9802 = 1:3:4.5].

The source of oxygen for this oxygenation should be molecular oxygen, which clearly proves that the present bioconversion is occurring under aerobic conditions. It has been reported that water is the source of oxygen to form phenol from benzene in anaerobiosis [16] whereas molecular oxygen is incorporated in aerobic transformation [17,18]. We determined that the strain

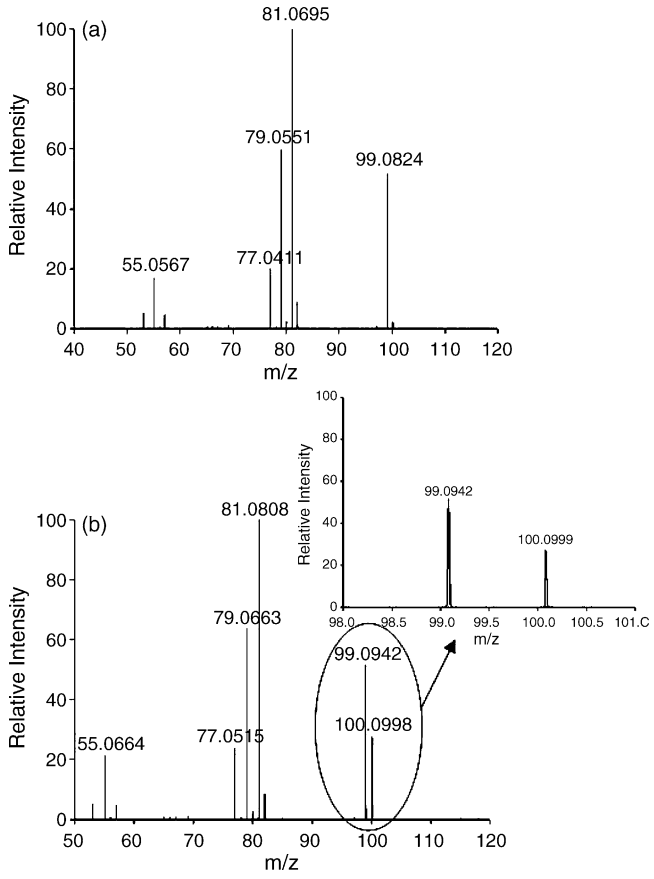


Fig. 4. Positive LC/ESI-MS spectra of liquid suspensions in H_2O (a) and in D_2O (b) from the bioconversion reaction by *Pseudomonas veronii* PH-03 supplemented with benzene. In figure (a) diagnostic peaks are $[M+H]=99.0824$ (cyclohexanone), $[M+H-H_2O]=81.0695$, and $[M+H]=79.0551$ (benzene). In figure (b) diagnostic peaks are $[M+H]=99.0942$ and 100.0998 (cyclohexanone), $[M+H-H_2O]=81.0808$, and $[M+H]=79.0663$ (benzene).The isotopic effects for the spectrum are shown in the figure.

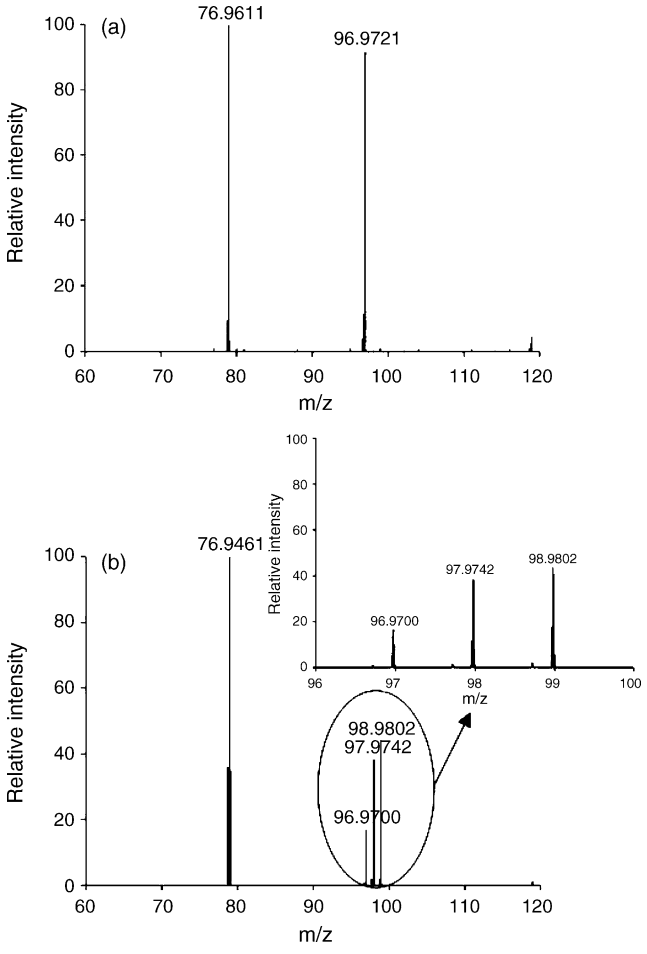


Fig. 5. Negative LC/ESI-MS spectra of liquid suspensions in H_2O (a) and in D_2O (b) from the bioconversion reaction by *Pseudomonas veronii* PH-03 supplemented with benzene. In figure (a) diagnostic peaks are $[M-H]=96.9721$ (cyclohexanone) and $[M-H]=76.9611$ (benzene). In figure (b) diagnostic peaks are $[M-H]=96.9700$, 96.9742, and 98.9802 (cyclohexanone) and $[M-H]=76.9461$ (benzene). The isotopic effects for the spectrum are shown in the figure.

PH-03 utilized molecular oxygen at a higher rate in the presence of benzene than in the presence of phenol. This result suggests that molecular oxygen is consumed for the production of phenol. During phenol formation, the oxygen level decreased, so specific ring reducing enzymes might be involved in the subsequent cyclohexanone formation. To verify the oxygen source of the bioconversion, we performed additional experiments with $^{18}O_2$. As shown in Fig. 6, the results showed that $^{18}O_2$ is the oxygen source in the bioconversion. Even-though we removed the $^{16}O_2$ by N_2 purging from resting cells suspension, [^{16}O]-cyclohexanone fragmentation occurred. This might be due to involvement of trace of $^{16}O_2$ from the buffer. Finally, we determined the bioconversion is completely aerobic and the oxygen originated from molecular oxygen.

Scheme 1 summarizes the isotope effect scheme involved in this bioconversion. In organic synthesis, aromatic ring reduction is a energetically and mechanistically difficult process that requires solvated electrons generated by dissolving alkali metals in liquid ammonia. Through a so-called Birch mechanism, aromatic ring reduction proceeds in single electron and proton transfer steps by means of radical intermediates [19]. A similar

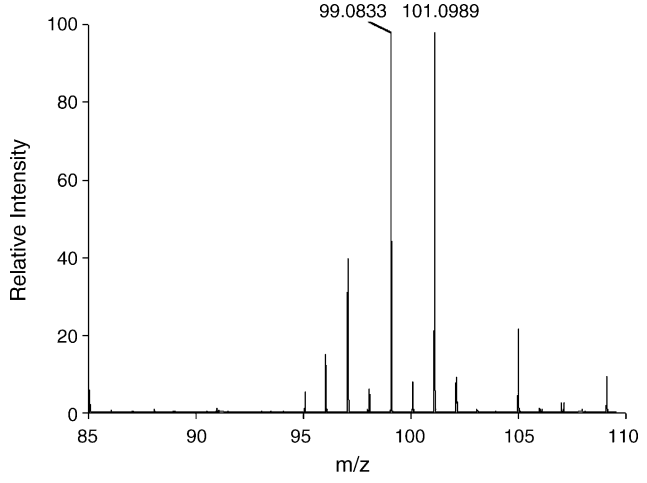
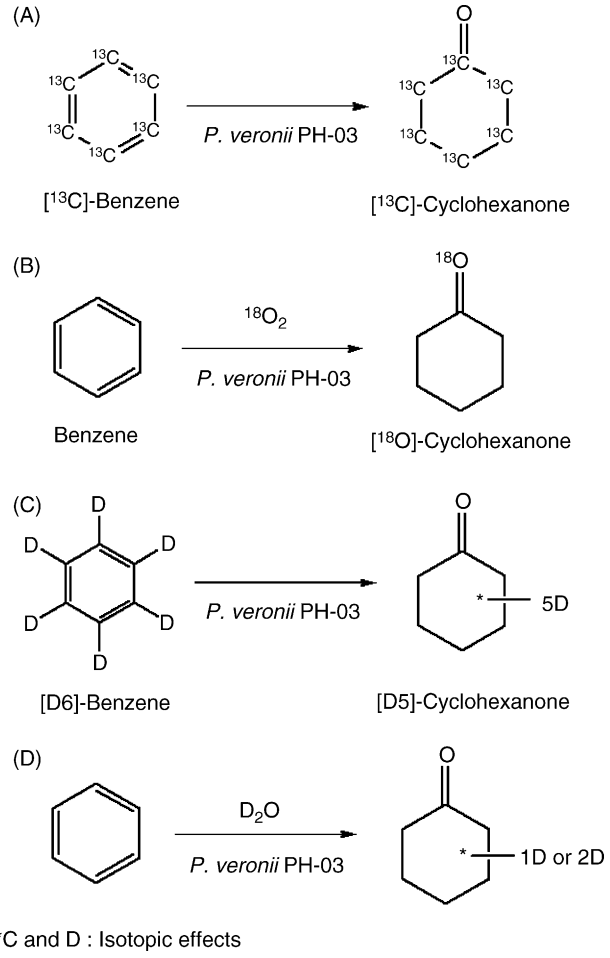


Fig. 6. Positive LC/ESI-MS spectra of culture suspensions purged with $^{18}O_2$ from the bioconversion reaction by *Pseudomonas veronii* PH-03 supplemented with benzene.



Scheme 1.

mechanism has been proposed for anaerobic, enzymatic benzene ring reduction in benzoyl CoA, a central metabolite of anaerobic biodegradation of aromatic compounds [20]. However, the complete ring reduction yields a substituted cyclohexanone such as cyclohexanoneacyl-CoA. We examined whether the hydrogen ion comes from water by replacing water with deuterated water (D_2O). Cyclohexanone formation was slightly different from that observed in H_2O-based buffer systems (see Figs. 4 and 5) suggesting that H_2O is a partial source of hydrogen although the main hydrogen source is another reductant. Recently, NADPH-dependent xenobiotic ring reductase has been characterized from *Pseudomonas fluorescens*, which is able to mediate the partial ring reduction of xenobiotic compounds by hydride addition [21]. Finally, in this bioconversion, the proposed oxygen source is molecular oxygen from aerated conditions and the hydrogen source is partially the H_2O-buffered system. Although the effects investigated from GC–MS and ESI-MS were not identical for mass units, obvious results showed the compound-specific isotopic effects from bioconversion according to different experimental conditions. Scheme 1 illustrates the individual bioconversions examined in this study using stable isotopes and clearly shows the effects of deuterium isotope specifically.

4. Conclusions

The specific isotopic effects in this study should help elucidate the oxygen and hydrogen sources in the biological conversion of some chemicals of interest. Deuterium [^{2}H], [^{13}C]-labelled, and [^{18}O]-labelled compounds can be efficiently used for this purpose. To our knowledge, this is the first report on isotope effects in the biological conversion of environmentally hazardous compounds such as benzene.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijms.2006.01.033.

References

[1] R.E. Clement, H.M. Tosine, Mass Spectrom. Rev. 7 (1988) 593.
[2] R.E. Clement, P.W. Yang, Anal. Chem. 73 (2001) 2761.
[3] R.E. Parales, N.C. Bruce, A. Schmid, L.P. Wackett, Appl. Environ. Microbiol. 68 (2002) 4699.
[4] J. Kazumi, M.E. Caldwell, J.M. Suflita, D.R. Lovley, L.Y. Young, Environ. Sci. Technol. 31 (1997) 813.
[5] C.A. Nicholson, B.Z. Fathepure, Appl. Environ. Microbiol. 70 (2004) 1222.
[6] A. Monero, L. Lanza, M. Zilli, L. Sene, A. Converti, Biotechnol. Prog. 19 (2003) 676.
[7] J.D. Coates, R. Chakraborty, J.G. Lack, S.M. O'Connor, K.A. Cole, K.S. Bender, L.A. Achenbach, Nature 411 (2001) 1039.
[8] C.S. Harwood, J. Gibson, J. Bacteriol. 179 (1997) 301.
[9] R.J. Snyder, Toxicol. Environ. Health 61 (2000) 339.
[10] B.J. Dean, Mutat. Res. 154 (1985) 153.
[11] D. Schaumölffel, R. Lobinski, Int. J. Mass Spectrom. 242 (2005) 217.
[12] S.A. Mancini, A.C. Ulrich, G. Lacrampe-Couloume, B. Sleep, E.A. Edwards, B.S. Lollar, Appl. Environ. Microbiol. 69 (2003) 191.
[13] S.P. Mirza, P. Krishna, S. Prabhakar, M. Vairamani, D. Giblin, M.L. Gross, Int. J. Mass Spectrom. 230 (2003) 175.
[14] P.C. Wilkins, H. Dalton, C.J. Samuel, J. Green, Eur. J. Biochem. 226 (1994) 555.
[15] H.B. Hong, I.H. Nam, K. Murugesan, Y.S. Chang, Biodegradation 15 (2004) 303.
[16] T.M. Vogel, D. Grbic-Galic, Appl. Environ. Microbiol. 52 (1986) 200.
[17] D.T. Gibson, G.E. Cardini, F.C. Maseles, R.E. Kallio, Biochemistry 9 (1970) 1631.
[18] G.R. Johnson, R.H. Olsen, Appl. Environ. Microbiol. 61 (1995) 3336.
[19] A.J. Birch, G.S. Rao, Adv. Org. Chem. 8 (1972) 1.
[20] W. Buckel, R. Keese, Angew. Chem. Int. Edit. 34 (1995) 1502.
[21] J.W. Pak, K.L. Knoke, D.R. Noguera, B.G. Fox, G.H. Chambliss, Appl. Environ. Microbiol. 66 (2000) 4742.