Journal of Hazardous Materials 191 (2011) 393–396

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Short communication

Novel biodegradation pathways of cyclohexane by *Rhodococcus* sp. EC1

Taewoo Yi[a], Eun-Hee Lee[a], Yun Gyong Ahn[b], Geum-Sook Hwang[b], Kyung-Suk Cho[a,*]

[a] Department of Environmental Science and Engineering, Ewha Womans University, 11-1, Daehyun-dong, Seodaemun-Gu, Seoul 120-750, Republic of Korea
[b] Korea Basic Science Institute, Seoul 136-713, Republic of Korea

ARTICLE INFO

Article history:
Received 6 March 2011
Accepted 18 April 2011
Available online 23 April 2011

Keywords:
Cyclohexane
Rhodococcus sp.
Oxidation pathway
Aromatization

ABSTRACT

The metabolism of cyclohexanes by *Rodococcus* sp. EC1 was investigated using a sequential tracking method of degradation intermediate. Evidence for the formation of cyclohexanol, cyclohexaone, 2-cyclohexen-1-one, and phenol was presented. EC1 metabolized cyclohexane to phenol by aromatization of 2-cyclohexen-1-one, and furthermore gamma-butyrolactone as an intermediate of 2-cyclohexen-1-one was formed. Aromatization by EC1 was confirmed using tetrahydrofuran. Tetrahydrofuran was metabolized through aromatization reaction, involving furan and 2,3-dihydrofuran as key intermediates. EC1 can degrade cyclohexane and tetrahydrofuran in aromatization via desaturation.

1. Introduction

Cyclohexane, a cyclic alkane, originating from petroleum products, is more recalcitrant than even *n*-alkanes or monoaromatic hydrocarbons [1]. Even though two bacterial strain of *Pseudomonas* spp. were reported by Klerk and van der Linden [2] as the first microorganisms which have capability of degradation of cyclohexane, the bacteria could not utilize cyclohexane as a sole carbon and energy source. *Nocardia* sp. was the first microorganism identified as utilizing cyclohexane as a sole carbon and energy source [3].

Two different degradation pathways of cyclic alkanes were known; a lactone formation and an aromatization. In the lactone formation, a well-defined pathway, first cyclohexane was oxidized to cyclohexanol and cyclohexanol is dehydrogenated to cyclohexanone from which epsilon-caprolactone is produced [4]. It is known as Baeyer–Villager oxidation reaction, which is then oxygenated by the action of monooxygenase, called Baeyer–Villiger monooxygenase. Epsilon-caprolactone is further oxidized to adipic acid, and ultimately to CO_2 [5]. Generally, aromatization plays an important role in the biosynthesis of aromatic amino acids and lignin and estrogen. Although the involvement of aromatization in biodegradation is uncommon, in limited number of studies, biodegradation of cyclic alkanes have been reported by aromatization in aerobic bacteria [6]. Kaneda [7] also reported that *Corynebacterium cyclohexanicum* degraded cyclohexanecarboxylic acid through the aromatization.

In this study, the biotransformation of cyclohexane by the pure culture of *Rhodococcus* sp. EC1, which is capable of the co-metabolism of multiple organic contaminants was investigated [8]. The primary metabolites formed during biotransformation of cyclohexane in batch tests were identified using a sequential tracking method of degradation intermediate, and its biodegradation pathways were proposed. In addition, main degradation pathway of cyclohexane, aromatization, was confirmed using tetrahydrofuran. The identities of the metabolites were elucidated using gas chromatography–mass spectrometry (GC/MS).

2. Materials and methods

2.1. Bacterial growth conditions

Rhodococcus sp. EC1 (AY878707) was isolated from oil-contaminated soil in our previous study [8]. This bacterium was precultured in a 1.2-L serum bottle containing 50 mL of Bushnell–Hass (BH) medium (0.409 g/L $MgSO_4 \cdot 7H_2O$, 0.0265 g/L $CaCl_2 \cdot 2H_2O$, 1 g/L KH_2PO_4, 1 g/L NH_4NO_3, 6 g/L $Na_2HPO_4 \cdot 12H_2O$, and 0.0833 g/L $FeCl_3 \cdot 6H_2O$) and 50 μL trace elements (17 g/L $FeCl_3$, 0.6 g/L $CaCl_2$, 0.2 g/L $ZnSO_4$, 0.2 g/L $CuSO_4 \cdot 7H_2O$, 0.2 g/L $MnSO_4$, 0.8 g/L $CoCl_2$, 0.1 g/L H_3BO_3, and 0.3 g/L $Na_2MoO_4 \cdot 2H_2O$). After the serum bottle was sealed using a butyl rubber and aluminum cap, 0.1 mL cyclohexane (99%; Duksan Pure Chemical Co., Ansan, Korea) was injected using a 1-mL syringe, and the culture was incubated at 30 °C with shaking at 180 rpm.

For measurement of growth on cyclohexane by EC1, 0.5 mL of precultured EC1 was inoculated in a 1.2-L serum bottle containing 50 mL of BH medium with 30 μL cyclohexane (5.52 mM) under the

* Corresponding author. Tel.: +82 2 3277 2393; fax: +82 2 3277 3275.
E-mail address: kscho@ewha.ac.kr (K.-S. Cho).

0304-3894/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jhazmat.2011.04.080

conditions described above. Cell concentrations were determined by measuring the absorption at 600 nm ($OD_{600\,nm}$, Agilent 8453, Agilent, USA).

2.2. Examination of cyclohexane degradation pathway using a sequential tracking method of degradative intermediates

The following experiments were conducted to characterize the cyclohexane biodegradation pathway using EC1. Initially, 50 mL of precultured EC1, grown in BH medium supplemented with cyclohexane, was centrifuged (8900 g, 10 min) with the supernatant discarded and the collected cells resuspended with 50 mL of distilled water to wash the cells. After washing twice, the cells were resuspended in 20 mL of BH medium. Two milliliters of the newly suspended cell culture was inoculated into each of ten sets of 10-mL serum bottles; the bottle was sealed with butyl rubber and an aluminum cap; 1 μL of cyclohexane was injected into the bottles and then cultivated at 30 °C and 180 rpm. During the tests, all the bottles were horizontally positioned to avoid the contact between the top of bottle and culture solution. The intermediates by EC1 were extracted by solid-phase microextraction (SPME, Supelco, Bellefonte, PA, USA) installed with carboxenTM/polydimethyl siloxane (CAR/PDMS, Supelco, Bellefonte, PA, USA) fiber in each bottle. The fiber was exposed in the headspace of test bottles and then the bottle was heated in the heat block at 75 °C for 10–15 min for adsorption of evaporated intermediates onto the fiber. The adsorbed intermediates were analyzed using GC/MS (HP6890 series plus, Agilent, Palo Alto, CA, USA) with a DB-5 column (30 m × 0.32 mm × 0.25 μm, J&W Scientific, CA, USA) and model 5973 Network Mass Selective Detector (Agilent, Palo Alto, CA, USA). For desorption of the intermediates from the fiber, SPME was put in the injector for 5 min. The initial oven temperature was 40 °C for 1 min, which was increased to 280 °C at 10 °C/min and held for 2 min. The injector and detector temperatures were 270 °C and 280 °C, respectively. Helium was used as the carrier gas. The Wiley 275 and Nist 98 libraries proposed for the GC/MS system were used for homology searches of the mass spectra of the intermediates.

Cyclohexanone (1 μL) was added to 2 mL of precultured EC1, as a sole carbon source, as the intermediate had been identified in the 1st step of the degradation process and then cultivated at 30 °C and 180 rpm. All the other steps (2nd to 4th) followed were the same as the 1st step for extraction and analysis. The intermediates at each step were cyclohexanone, 2-cyclohexen-1-one, and tetrahydrofuran (THF), in that order, which were used as sole carbon sources in the next step. Therefore, the degradative pathway of cyclohexane due to EC1 was verified through a sequential qualitative analysis method. For all experiments, control tests without the inoculation of EC1 were simultaneously carried out.

3. Results and discussion

3.1. Degradation of cyclohexane by EC1

Our previous study [8] revealed that EC1 has the ability to degrade various kinds of hydrocarbons including cyclohexane. As shown in Fig. 1, EC1 reached the exponential growth phase in the presence of cyclohexane as a sole carbon source after lag periods of 30 h. The accumulation of metabolites after cell growth reached stationary phase was analyzed by GC/MS and any intermediates was not detected (data not shown). It was shown that cyclohexane was converted to cell components and degraded to CO_2.

The intermediates were analyzed to propose the degradation pathway by EC1. After 6-h cultivation, the intermediates derived from the biotransformation of cyclohexane were analyzed by

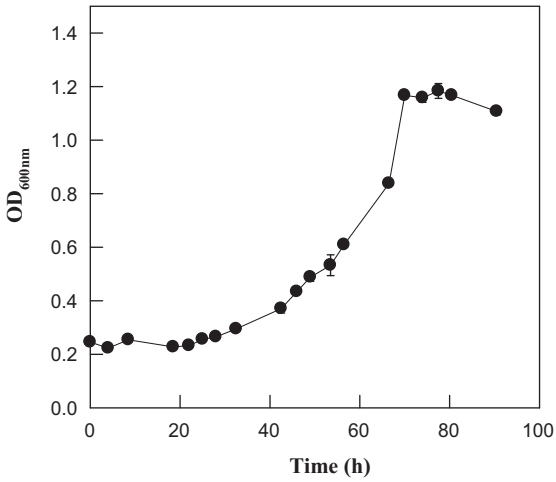

Fig. 1. Cell growth of EC1 in the presence of cyclohexane as a sole carbon and energy source.

mass spectra by GC/MS (Fig. 2) and confirmed by their retention time of reference compounds. During cyclohexane degradation experiments, transiently cyclohexanol appeared followed by cyclohexanone (Fig. 2a). When cyclohexanone as a sole carbon source was incubated with EC1, 2-cyclohexen-1-one was accumulated (Fig. 2b). When cyclohexanone was supplied into the EC1 culture both as a sole carbon substrate, 2-cyclohexen-1-one and phenol were detected (Fig. 2c). THF were used as a sole carbon for EC1, furan and tetrahydrofuran-2-ol were formed as the intermediate of THF (Fig. 2d). Based on our best knowledge, this study is the first to report the formation of THF intermediates during cyclohexane degradation.

3.2. Degradation pathway of cyclohexane and metabolites

On the basis of the results shown in Fig. 2, the biodegradation pathways of cyclohexane by EC1 were proposed (Fig. 3). Cyclohexane was oxidized primary to cyclohexanol and then further dehydrogenated to cyclohexanone. In previous studies [4], [9], ring cleavage via lactone formation has been considered as a main removal mechanism of cyclohexane; cyclohexanone is further cleaved to adipic acid via 2-oxepanone formation. In this study, the formation of 2-cyclohexen-1-one showed that desaturation reaction occurred. Desaturation has been observed in the reactions catalyzed by cytochrome P450 [10] or dioxygenase extracted from *P.* sp. strain 9816-4 incubated with naphthalene and from *P. putida* F1 with toluene [11]. However, most of common pathway of P450-mediated metabolisms, including hydroxylation, epoxidation, and dealkylation, are known. In addition, the detection of phenol confirmed that the aromatization of cyclohexane by EC1 was one of main biodegradation mechanisms, although biodegradation of cyclic hydrocarbon by aromatase is rare [7]. The well-known key intermediate resulting from the metabolism of phenol is catechol [12]. Thus, phenol would be further degraded to catechol.

As shown in Fig. 2b, when EC1 was incubated with 2-cyclohexen-1-one, gamma-butyrolactone as well as phenol was detected. Though tetrahydrofuran was not directly detected as a metabolite of cyclohexane, degradation pathway of THF was investigated to confirm the aromazation as main removal mechanism of aromatic hydrocarbons by EC1since gamma-butyrolactone was generally considered as an intermediate of THF [13]. Therefore, further degradation test was performed with THF, and 2,3-dihydrofuran and furan were detected as intermediates (Fig. 2c). Based on the results in Fig. 2b and c, it is considered that EC1 has the ability to degrade cyclohexane and THF using

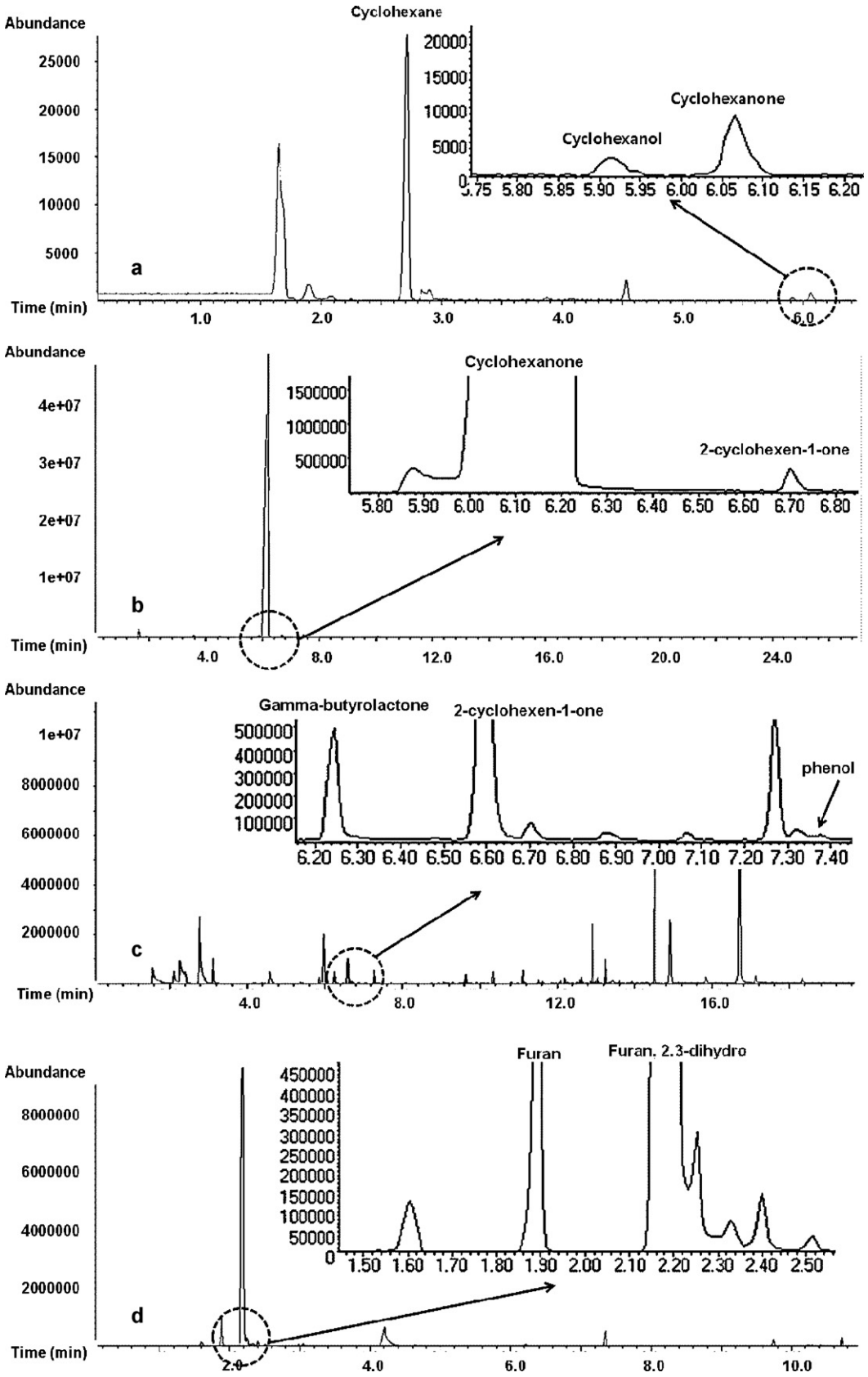

Fig. 2. The schematic diagrams of the GC/MS chromatograms using the sequential tracking method. Detection of the metabolites from cyclohexane, (a) Cyclohexanol (retention time (RT), 5.9 min; *m*/*z* fragments (F_s), 44.1, 57.1, 67, 82) and cyclohexanone (RT 6.06 min; *m*/*z* (F_s), 39.2, 42.2, 55, 69, 83, 98), (b) 2-cyclohexen-1-one (RT 6.707 min; *m*/*z* F_s, 39.1, 47.2, 55.1, 68.1, 81, 96), (c) Gamma-butyrolactone (RT 6.232 min; *m*/*z* F_s, 36.1, 42.2, 56.1, 69.1, 77.2, 86.1) and phenol (RT 7.322 min; *m*/*z*: 39.2, 47.1, 55.2, 66.1, 77.1, 85.1, 94.1), (d) Furthermore, 2,3-dihydrofuran (RT 2.19 min; *m*/*z* F_s, 38.9, 41.2, 43.2, 50.2, 53.1, 55.1, 66.1, 70.2) and furan (RT 1.887 min; *m*/*z* F_s, 37.2, 39.2, 42.1, 44.1, 48.1, 50.2, 53.1, 58.2, 66.1, 68.1).

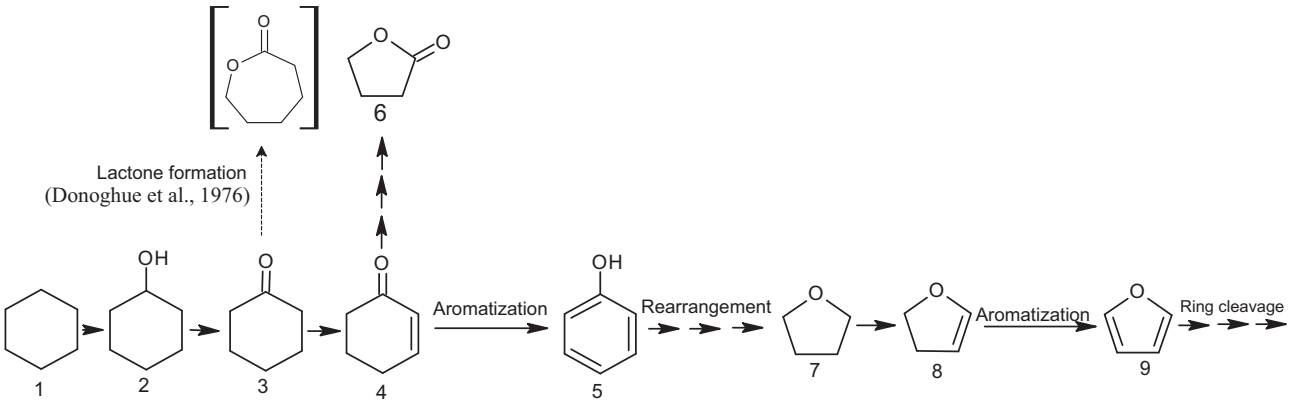

Fig. 3. Proposed cyclohexane degradation pathways by *Rhodococcus* sp. EC1; (1) cyclohexane (2) cyclohexanol (3) cyclohexanone (4) 2-cyclohexen-1-one (5) phenol (6) gamma-butyrolactone (7) tetrahydrofuran (8) 2, 3-hydrofuran (9) furan.

the aromatization; THF to furan via 2,3-hydrofuran and lactone formation, ring-cleavage of THF via tetrahydrofuran-2-ol and gamma-butyrolactione [13]. Previously aromatization of cyclic alkanes has been reported in limited number of studies; by aerobic bacteria [7], by denitrifying bacteria [6], and by animal liver cells [14].

4. Conclusions

Aromatization as a cyclohexane degradation pathway by EC1 proposed in this research is different from lactone formation, known as a main degradation pathway of cyclohexane. It was shown that microorganisms have various degradation mechanisms for cyclic alkanes such as cyclohexane. This research increases the deep understanding and provides the answer for fundamental questions related to various degradation mechanisms of cyclohexane. For further study, it is required to study the enzymes and their activity related to each degradation pathway of cyclohexane.

Acknowledgements

The paper (author) was supported by RP-Grant 2010 of Ewha Womans University, and the Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (R0A-2008-000-20044-0).

References

[1] R.C. Prince, T.F. Parkerton, C. Lee, The primary aerobic biodegradation of gasoline hydrocarbons, Environ. Sci. Technol. 41 (2007) 3316–3321.
[2] H. Klerk, A.C. van der Linden, Bacterial degradation of cyclohexane participation of a co-oxidation reaction, Ant. van Leeuw. 40 (1974) 7–15.
[3] L.A. Stirling, R.J. Watkinson, Microbial metabolism of alicyclic hydrocarbons: isolation and properties of a cyclohexane-degrading bacterium, J. Gen. Microbiol. 99 (1977) 119–125.
[4] W.H. Lee, J.B. Park, K. Park, M.D. Kim, J.H. Seo, Enhanced production of epsilon-caprolactone by overexpression of NADPH-regenerating glucose 6-phosphate dehydrogenase in recombinant *Escherichia coli* harboring cyclohexanone monooxygenase gene, Appl. Microbiol. Biotechnol. 76 (2007) 329–338.
[5] A. Baeyer, B. Villiger, Einwirkung des Caro'schen Reagens auf ketone, Ner. Dtsch. Chem. 32 (1899) 3625–3651.
[6] D. Koma, Y. Sakashita, K. Kubota, Y. Fujii, F. Hasumi, S.Y. Chung, M. Kubo, Degradation pathways of cyclic alkanes in *Rhodococcus* sp. NDKK48, Appl. Microbiol. Biotechnol. 66 (2005) 92–99.
[7] T. Kaneda, Enzymatic aromatization of 4-ketocyclohexanecarboxylic acid to p-hydroxybenzoic acid, Biochem. Biophys. Res. Commun. 58 (1974) 140–144.
[8] E.H. Lee, K.S. Cho, Characterization of cyclohexane and hexane degradation by *Rhodococcus* sp. EC1, Chemosphere 71 (2008) 1738–1744.
[9] J.J. Perry, Microbial metabolism of cyclic alkanes, in: R.M. Atlas (Ed.), Petroleum Microbiology, Macmillan, NY, 1984, pp. 61–67.
[10] C.J.C. Whitehouse, S.G. Bell, L.L. Wong, Desaturation of alkylbenzenes by cytochrome P450 (BM3) (CYP102A1), Chem. Eur. J. 14 (2008) 10905–10908.
[11] D.T. Gibson, S.M. Resnick, K. Lee, J.M. Brand, D.S. Torok, L.P. Wackett, M.J. Schocken, B.E. Haigler, Desaturation, dioxygenation, and monooxygenation reactions catalyzed by naphthalene dioxygenase from *Pseudomonas* sp Strain 9816-4, J. Bacteriol. 177 (1995) 2615–2621.
[12] M. Stiborová, V. Suchá, M. Mikanová, J. Páca Jr., J. Páca, Hydroxylation of phenol to catechol by *Candida tropicalis*: involvement of cytochrome P450, Gen. Physiol. Biophys. 22 (2003) 167–179.
[13] B. Thiemer, J.R. Andreesen, T. Schrader, Cloning and characterization of a gene cluster involved in tetrahydrofuran degradation in *Pseudonocardia* sp. strain K1, Arch. Microbiol. 179 (2003) 266–277.
[14] B.M. Babior, K. Bloch, Aromatization of cyclohexane-carboxylic acid, J. Biol. Chem. 241 (1966) 3643–3651.