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Isolation and preliminary characterization of a 3-chlorobenzoate degrading bacteria

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Abstract

A study was conducted to compare the diversity of 2-, 3-, and 4-chlorobenzoate degraders in two pristine soils and one contaminated sewage sludge. These samples contained strikingly different populations of mono-chlorobenzoate degraders. Although fewer cultures were isolated in the uncontaminated soils than contaminated one, the ability of microbial populations to mineralize chlorobenzoate was widespread. The 3- and 4-chlorobenzoate degraders were more diverse than the 2-chlorobenzoate degraders. One of the strains isolated from the sewage sludge was obtained. Based on its phenotype, chemotaxonomic properties and 16S rRNA gene, the organism S-7 was classified as *Rhodococcus erythropolis*. The strain can grow at temperature from 4 to 37°C. It can utilize several (halo)aromatic compounds. Moreover, strain S-7 can grow and use 3-chlorobenzoate as sole carbon source in a temperatures range of 10–30°C with stoichiometric release of chloride ions. The psychrotolerant ability was significant for bioremediation in low temperature regions. Catechol and chlorocatechol 1,2-dioxygenase activities were present in cell free extracts of the strain, but no (chloro)catechol 2,3-dioxygenase activities was detected. Spectral conversion assays with extracts from *R. erythropolis* S-7 showed accumulation of a compound with a similar UV spectrum as chloro-*cis*,*cis*-muconate from 3-chlorobenzoate. On the basis of these results, we proposed that S-7 degraded 3-chlorobenzoate through the modified *ortho*-cleave pathway.

Key words: chlorobenzoate; biodegradation; *Rhodococcus erythropolis*; psychrotrophic bacteria; pathway

Introduction

Large amounts of man-made chlorinated organic chemicals are released into the environment as a result of their wide use in agriculture and industry. Due to their chemical structures, these compounds are classified among the most persistent and toxic group. Herbicides and pesticides are the main sources of chlorobenzoates (CBAs) (Yuroff *et al*., 2003), which are also released as a result of the degradation of chlorinated aromatic compounds, such as polychlorinated biphenyls (PCBs), chlorinated phenols, and dichlorodiphenyltrichlorethane (DDT). Adriaens and Focht (1991) reported that chlorobenzoate degradation appeared to be the rate limiting step in the overall PCB-degradation process. Due to their ubiquitous presence, good water solubility and low toxicity, chlorobenzoates have been used as models to study the biodegradation of halogenated aromatic compounds, and to elucidate the microbial strategies implicated in the release of chlorine substituents (Yi *et al*., 2000).

Numbers of reports concerning the bacterial degradation of chlorobenzoates had already been published, such as *Alcaligenes*, *Burkholderia*, *Pseudomonas*, *Bacillus*, *Ralstonia*. Most of these strains are Gram-negative and mesophilic microorganisms. However, in cold climatic regions, temperature often decreases to 20°C at most time of the year. Under such conditions, hydrocarbons are less volatile and become more insoluble, the activity of mesophilic degraders is considerably or completely reduced (Li *et al*., 1991). As a result these chlorinated organic pollutants will remain undegraded in the environment under low-temperature rather than medium or high-temperature conditions (Kato *et al*., 2001). Knowledge is yet limited due to the small number of collections of such bacteria which adapted their metabolism to function optimally at low temperatures.

In this study, we compared the diversity of 2-, 3-, and 4-chlorobenzoate degraders in two pristine soils and one contaminated sewage sludge. A strain of *Rhodococcus erythropolis* was isolated from a waste-water treatment plant. It can use 3-chlorobenzoate as sole carbon and energy source at low temperature; the pathway for degradation was also investigated.

1 Materials and methods

1.1 Samples

Activated sludge and two soil samples were used as starting materials for the isolation of pure cultures. The activated sludge was obtained from the pesticide wastewater treatment plant, Tianjin, China. The soil samples

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were selected from pristine ecosystems of Nanping County Town, Sichuan, China. The samples were pretreated by the method described by de Wever *et al*. (1997).

1.2 Media and enumeration of culturable bacteria

The soil extraction solution and activated sludge were serially diluted. Indigenous mono-chlorobenzoate degraders were selectively enumerated on minimal salts medium (MM) plates prepared as described by Kozlovsky *et al*. (1993). The final pH of the medium was 7.0, using 0.2 g/L of 2-, or 3-, or 4-chlorobenzoate (99% purity, Sigma-Aldrich USA) as only carbon and energy source. All plate counts were conducted after incubation of plates at 30°C for 6 d.

1.3 Enrichment procedures for isolation

Enrichments were performed in 250-ml Erlenmeyer flasks containing a liquid volume of 50 ml. Transfers (10% volume) were done every 4–6 weeks into fresh MM containing the 3-chlorobenzoate. After growth the culture suspension was serially diluted and seeded on plates containing mineral medium and 3-chlorobenzoate at the same concentration. After 48 h of incubation at 30°C, colonies that appeared on the plates were isolated on the basis of their morphology. The isolates were grown on mineral medium supplemented with 3-chlorobenzoate for 5 cycles of growth.

1.4 Sequence analysis of 16S rDNA

Pure strain was picked from the plates and sent to TaKaRA Biotechnology (Dalian) Co., Ltd. The sequence of 1427bp of 16S rDNA was compared with the National Center for Biotechnology Information (NCBI) public database using the blast tool (http://www.ncbi.nlm.nih.gov/BLAST/). The phylogenetic tree was done by the soft of MEGA v.3. Other phenotype and chemotaxonomic characters were studied according to the Bergey's Manual of Systematic Bacteriology (Goodfellow, 1986).

1.5 Effect of temperature on growth and chlorobenzoate biodegradation rate

Isolated strain, named S-7, was cultured in nutrient broth at 30°C for 24 h. The cells were collected by centrifugation at 12000×g for 10 min (4°C) and washed with 0.9% NaCl. The cell suspension was added to 250-ml Erlenmeyer flasks containing 50 ml mineral medium amended with 3-chlorobenzoate, and adjusted optical density at 550 nm (OD_{550}) to about 0.16. Then cultivated at 10, 20 and 30°C at 130 r/min.

Growth, chloride and residual 3-chlorobenzoate concentrations were monitored at regular intervals.

1.6 Analytical methods

Growth was measured by the optical density at 550 nm using Cary 100 UV-Visible Spectrophotometer (Varian, USA).

Residual 3-chlorobenzoate was determined by reverse phase high performance liquid chromatography (HPLC) (Agilent 1100, USA), with a Supelcosi™ LC18 column (5 μm, 250 mm×4.6 mm). The mobile phase used was methanol-water-acetic acid (59 : 45 : 5, v/v/v). The flow rate was 1 ml/min and detected by UV-absorption at 235 nm.

Full wavelength scan (200–800 nm) was carried out by Cary 100 UV-Visible Spectrophotometer (Varian, USA).

Chloride ion concentration was measured via ion chromatography (Dionex 600, USA).

1.7 Enzyme assays

Cell free extracts (CFEs): cells were made in 50 mmol/L phosphate buffer (pH 7.5). Then disrupted by ultrasonication at 200 W for 10 min with intermittent bursts of 3 s using ultrasonic crusher. Cell debris was removed by centrifugation at 12000×g for 20 min. All operations were carried out at 4°C.

Catechol and chlorocatechol 1,2-dioxygenase activities were assayed spectrophotometrically according to the method described by Dorn and Knackmuss (1978a).

Catechol and chlorocatechol 2,3-dioxygenase activity were assayed according to Fetzner *et al*. (1989).

Protein in CFE was estimated by the method of Lowry *et al*.(1951) using BSA as the standard. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol product/min under the described conditions.

2 Results and discussion

2.1 Chlorobenzoate biodegradation

Colonies were developed after serially diluted. Number of isolated strain mineralized different mono-chlorobenzoate is shown in Table 1.

Table 1 Number of total degraders in different samples after 6 d incubation at 30°C

Sample	2-Chl	3-Chl	4-Chl
Soil 1	2.4×10^3	4.5×10^4	3.7×10^4
Soil 2	3.2×10^2	6.1×10^4	7.9×10^3
Sewage sludge	6.1×10^5	4.7×10^6	1.8×10^6

Chl: chlorobenzoate.

Usually, researchers isolated chlorobenzoate degraders from agricultural or industrially contaminated sites. According to those research, researchers formed the hypothesis that environments have not been exposed to chloro-compounds do not harbor chloroaromatic degraders (Brunsbach and Reineke, 1993; Theim *et al*., 1994; Hernandez *et al*., 1995). However, Roberta *et al*. (1996) found the ability of microbial populations in undisturbed and uncontaminated soils to mineralize chlorobenzoate was widespread. Gentry *et al*. (2001) also studied the diversity of 2-, 3-, and 4-chlorobenzoate degraders in two pristine soils and indicated that 2-, 3-, and 4-chlorobenzoate are degraded by different bacterial populations.

Our results showed that although fewer cultures were isolated in the uncontaminated soils than contaminated

one, the ability of microbial populations in undisturbed soils to mineralize chlorobenzoate was widespread, which was in accordance with literature (Roberta *et al*., 1996; Gentry *et al*., 2001). The degradation of unnatural chloroaromatics might be caused by the pre-existing enzymes that used in the degradation of non-substituted analogs, or enzymes specifically involved in the mineralization of natural halogenated compounds (e.g. 2,4-dichlorophenol), or caused by the evolution of new catabolic pathways.

The result also indicated that the 3- and 4-chlorobenzoate degraders in sewage sludge and two soils were more diversity than the 2-chlorobenzoate degraders. Such substrate selection may cause by the steric hindrance plus the effect of halogen atoms on the electron density at the *ortho* position of the benzene ring, which hampered the attack of the dioxygenase responsible of the first step in chlorobenzoate catabolism (Bernard *et al*., 1996). Our result was consistent with the data obtained from chlorobenzoate degraders, which were isolated in separated studies and from different contaminated environments (Ajithkumar and Kunhi, 2000; Suzuki *et al*., 2001; Bott and Kaplan, 2002). Such species diversity in the soil has potential implications for successful microbial adaptation following environmental contamination with chlorobenzoates or PCBs (Gentry *et al*., 2001). However, to our knowledge, this is the first report that analyzed the difference between undisturbed soils and contaminated sewage sludge.

One of the isolated cultures from the sewage sludge, named S-7, showed higher 3-chlorobenzoate degradation ability than others and this strain was used for further analysis.

2.2 Characterization and identification of strain S-7

Bacterium S-7 was Gram-positive, aerobe, catalase-positive, urease-positive, oxidase-negative, non-motile, and growth on glucose without acidification. It was not acid-fast and its growth was not inhibited by 6% (w/v) NaCl. It was typically round, convex with a smooth margin, muccoid and light red. Microscopy revealed the presence of rod-shaped cells that formed V-shaped arrangements in the process of division. The strain had amylolytic and proteolytic activities, but lack the cellulolytic activity. It cannot reduce nitrate to nitrite, either. The pH range for growth was wide (5.0–12.0), and maximum growth occurred at pH 7.0. Strain S-7 grew at temperatures from 4°C to 37°C; optimum growth was observed at 30°C. Based on chemotaxonomic analysis, isolate S-7 was identified as a species of the *Rhodococcus*.

16S rDNA sequence (accession No. in NCBI gene bank was DQ306923) showed that it was identical with *R. erythropolis* CV71b and showed 99.9% and 99.8% similarity to *R. erythropolis* EPWF, *R. erythropolis* DSM43188, respectively. Phylogenetic tree (data not shown) indicated that strain S-7 had the closest relationship with *R. erythropolis*. Thus, strain S-7 was classified as *Rhodococcus erythropolis*.

R. erythropolis S-7 was able to utilize many aromatic compounds as sole carbon and energy source. It grew well on D-sorbitol, oleic acid, sodium benzoate, 4-chlorocatechol, and slowly on creatine, tetrahydrofuran, anthraquinone, benzidine and 3-hydroxybenzoic acid. No growth was observed with 2-chlorobenzoate, 4-chlorobenzoate, heptane, dodecylamine, toluene, starch, petroleum ether, nitrobenzene, dichlorvo, phenol, or aminophenol as sole carbon source.

2.3 Growth of *R. erythropolis* S-7 on 3-chlorobenzoate at different temperature

Degradation of 3-chlorobenzoate by the strain was tested at 10, 20, and 30°C, respectively (Fig.1). The organism grew exponentially with 3-chlorobenzoate as sole source of carbon and energy. The 3-chlorobenzoate degradation rate and the chloride release rate were found to be somehow dependent on the biomass production rate.

Strain S-7 completely degraded 3-chlorobenzoate in a temperature range of 10–30°C. At 20°C and 30°C, degradation of 3-chlorobenzoate started immediately after cultured. And the strain degraded all 3-chlorobenzoate within 60 h. However the degradation rate was faster

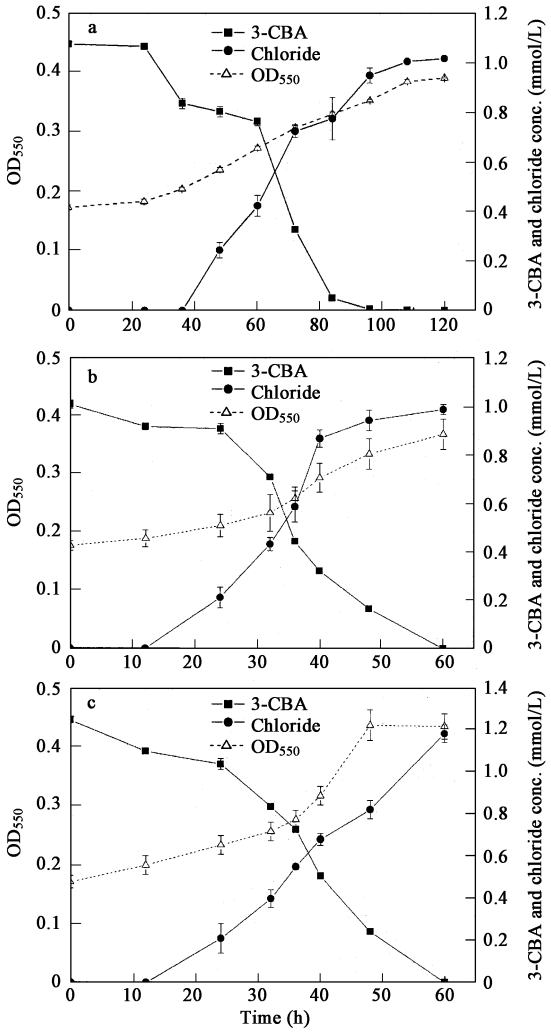

Fig. 1 Growth of *R. erythropolis* S-7 in minimal salts medium contained 3-chlorobenzoate at 10°C (a), 20°C (b) and 30°C (c) respectively. 3-CBA: 3-chlorobenzoate; OD_{550}: optical density at 550 nm.

at 20°C than at 30°C, which may indicate the enzymes involved in the degradation showed higher activities at 20°C. Although, strain can completely consume the 3-chlorobenzoate within 5 d at 10°C, it need more time to adapt the cold environment, and spend much longer time to totally degrade substrate. These data suggested that the strain had adapted their 3-chlorobenzoate degradation system to low-temperature conditions. Although there are a few reports on psychrotrophic benzenes analog degrading bacteria (Whyte *et al*., 1998; Bott and Kaplan, 2002; Peter and Lotte, 2003; Lambo and Patel, 2006), this is the first reported *R. erythropolis* can degrade 3-chlorobenzoate at 10°C. As most of the microorganisms cannot grow at 10°C, *R. erythropolis* S-7 showed great potential application for bioremediation at cold regions.

The degradation of chlorobenzoate involved the stoichiometric release of chloride ions as inorganic ions. Chloride ions release was due to oxygenolytic mechanisms with the involvement of dioxygenases, more or less specific for the halogenated compounds. From the chloride concentrations, we can conclude that all the organically bound chlorine was released as chloride ions. Meantime there was a slight lag period between 3-chlorobenzoate disappearance and chloride ions release.

2.4 Enzyme activities

(Chloro) catechol 2,3-dioxygenase activity, which was responsed for extradiol cleavage of the meta-pathway in microorganisms, was not detected in CFEs obtained from cell grown on 3-chlorobenzoate.

Specific activity of dioxygenase detected is shown in Table 2. Catechol and chlorocatechol 1,2-dioxygenase activities were present in CFEs obtained. The strain S-7, like some chlorocatechol-degrading bacteria described in the literature (Dorn and Knackmuss, 1978a) seems to have two different pyrocatechases, i.e., a catechol 1,2-dioxygenase, capable of catabolizing catechol, and a chlorocatechol 1,2-dioxygenase, which has relaxed substrate specificity and is thus active against both catechol and substituted catechols (Hartmann *et al*., 1979). This type of dioxygenase would spontaneously remove a chloride from the *ortho*-carbon atom that bears the OH group.

Table 2 Specific activity of dioxygenases detected in CFEs (U/mg protein)

Enzyme substrate	Grown on 3-chlrobenzoate (200 mg/L)
Catechol	68
4-Chlorocatechol	27

2.5 Conversion of 3-chlorobenzoate by cell extracts of S-7

Full wavelength scan of cell extracts led to the appearance of a peak at 260 nm, which later disappeared (Fig.2). Incubation of S-7 with 3-chlorobenzoate produced a peak at 260 nm at 24 h, with a maximum at 40 h, and disappeared after 96 h. For the characteristic absorption maxima (λ_{max}) of 3-chloro-*cis*,*cis*-muconate was 260 nm (Dorn and Knackmuss, 1978b), we propose that this compound was chloro-*cis*,*cis*-muconate, the only compound in the 3-chlorocatechol degradation pathway with a maximum at 260 nm.

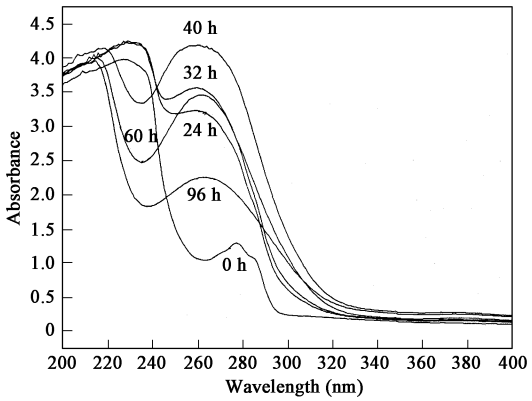

Fig. 2 Full wavelength scan of S-7 at different culture times.

2.6 Elucidation of the *R. erythropolis* S-7 chlorobenzoate biodegradative pathway

During growth on 3-chlorobenzoate, no yellow meta-cleavage products were produced as observed by Parsons *et al*. (1988). This suggested that S-7 might degrade 3-chlorobenzoate either using a (modified) *ortho*-cleavage pathway or first dehaloged to form a 4-hydroxybenzoate as described by Keil *et al*. (1981). However, the slight lag period between 3-chlorobenzoate disappearance and complete chloride ions release suggested that the initial degradation step does not result in chloride release. Thus *R. erythropolis* S-7 may degrade the 3-chlorobenzoate through the formation of chlorocatechol as intermediate, and the chlorocatechol degraded via the modified *ortho*-pathway. This suggestion was confirmed by the measurements of chlorocatechol 1,2-dioxygenase activity.

Full wavelength scan showed that during the degradation, the chloromuconic acid was generated as intermediates. Chloromuconic acid could only be formed in the growth medium if 3-chlorobenzoate was first hydroxylated to generate chlorocatechol, followed by ring cleavage by (chloro)catechol 1,2-dioxygenase. All the results allowed us to suggest that *R. erythropolis* S-7 utilized the xenobiotic via the chlorocatechol branch of the modified *ortho*-cleavage pathway (Fig.3).

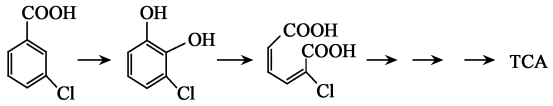

Fig. 3 Proposed pathway for the degradation of 3-chlorobenzoate by *R. erythropolis* S-7.

Therefore, *R. erythropolis* S-7 degrades 3-chlorobenzoate via a pathway where the substrate was first converted to chlorocatechol, which was then opened by a chlorocatechol 1,2-dioxygenase. This pathway resembles the ones carried out by some other researchers (Ajithkumar and Kunhi, 2000; Brenner *et al*., 2004; Rodrigues *et al*., 2006).

The chlorocatechol was further transformed by modified *ortho*-pathway enzymes.

Bacterial degradation of chlorobenzoate via chlorocatechol is supposed to be one of the typical degradation pathways for chlorobenzoates. Most of the studied strains were Gram-negative bacteria, such as *Alcaligenes*, *Peducomonas*, *Burkholderia*, *Ralstonia eutropha*. Assimilation of chlorosubstituted aromatic compound was first identified in Gram-positive strains of *Arthrobacter* utilizing chlorosubstituted benzoates or phenoxyacetates (Bollag *et al*., 1968). *Rhodococcus*, the Gram-positive strain, present a broad catabolic diversity and unique enzymatic capabilities that result in environmental and biotechnological importance (Bell, 1998; van der Geize and Dijkhuizen, 2004; de Carvalho and da Fonseca, 2005; Larkin *et al*., 2005). They show remarkable ability to degrade hydrophobic natural compounds and xenobiotics. However, to our knowledge, this is the first report *Rhodococcus erythropolis* can degrade 3-chlorobenzoate at 10°C, which showed well potential use for low temperature regions, such as surface water, groundwater and soil environments in temperate climates exhibit constantly temperatures around 10–20°C.

3 Conclusions

We investigated the difference in the ability of microbial populations in uncontaminated soils and contaminated sewage sludge to mineralized 2-, 3-, and 4-chlorobenzoate. Results showed that although fewer cultures were isolated in the uncontaminated soil than contaminated one, the ability of microbial populations to mineralize chlorobenzoate is widespread and 3-chlorobenzoate degraders are more easily obtained from these sources than 2- or 4-chlorobenzoate.

We isolated one Gram-positive strain S-7, which can use 3-chlorobenzoate as sole carbon and energy sources. It was identified by morphological, biochemical and 16S rDNA sequence analysis as *Rhodococcus erythropolis*.

R. erythropolis S-7 can fully degrade 3-chlorobenzoate with all the organically bound chlorine released as chloride ions in a temperature range of 10–30°C. Although the cell growth was maximal at 30°C, the strain showed comparable or even higher degradability of 3-chlorobenzoate at 20°C. What is most important is that it can completely degrade 3-chlorobenzoate at 10°C. Thus the strain should be useful for biodegradation of 3-chlorobenzoate under temperature conditions lower than 30°C.

Based on the chloride released rate, enzymes activities and full scan of cell extract results, a pathway for degradation of 3-chlorobenzoate was proposed: 3-chlorobenzoate was converted initially to chlorocatechol, and then degraded by modified *ortho*-pathway. Product entered the TCA cycle.

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References

Adriaens P, Focht D D, 1991. Continuous co-culture degradation of selected polychlorinated biphenyl congeners by *Acinetobacter* spp. in an aerobic reactor system[J]. Environ Sci Technol, 24: 1042–1049.

Ajithkumar P V, Kunhi A A, 2000. Pathways for 3-chloro- and 4-chlorobenzoate degradation in *Pseudomonas aeruginosa* 3mT[J]. Biodegradation, 11: 247–261.

Bell K, Philip J, Aw D W *et al*., 1998. The genus *Rhodococcus*[J]. J Appl Microbiol, 85: 195–210.

Bernard J, van der Woude, Jan G *et al*., 1996. Extent of reductive dechlorination of chlorobenzoates in anoxic sediment slurries depends on the sequence of chlorine removal[J]. Environ Sci Technol, 30: 1352–1357.

Bollag J M, Briggs G G, Dawson J E *et al*., 1968. 2,4-D metabolism: enzymatic degradation of chlorocatechols[J]. J Agric Food Chem, 16: 829–833.

Bott T L, Kaplan L A, 2002. Autecological properties of 3-chlorobenzoate-degrading bacteria and their population dynamics when introduced into sediments[J]. Microb Ecol, 43: 199–216.

Brenner V, Rucka L, Totevova S *et al*., 2004. Efficiency of chlorocatechol metabolism in natural and constructed chlorobenzoate and chlorobiphenyl degraders[J]. J Appl Microbiol, 96: 430–436.

Brunsbach F R, Reineke W, 1993. Degradation of chlorobenzoates in soil slurry by special organisms[J]. Appl Microbiol Biotechnol, 39: 117–122.

de Carvalho C C, da Fonseca M M, 2005. The remarkable *Rhodococcus erythropolis*[J]. Appl Microbiol Biotechnol, 67: 715–726.

de Wever H, de Cort S, Noots I *et al.*, 1997. Isolation and characterization of *Rhodococcus rhodochrous* for the degradation of the wastewater component 2-hydroxybenzothiazole[J]. Appl Microbiol Biotechnol, 47: 458–461.

Dorn E, Knackmuss H J, 1978a. Chemical structure and biodegradability of halogenated aromatic compounds: Two catechol 1, 2-dioxygenases from 3–chlorobenzoate-grown Pseudomonad[J]. Biochem J, 174: 73–84.

Dorn E, Knackmuss H J, 1978b. Chemical structure and biodegradability of halogenated aromatic compounds. Substituent effects on 1,2-dioxygenation of catechol[J]. Biochem J, 174: 85–94.

Fetzner S, Muller R, Lingens F, 1989. Degradation of 2-chlorobenzoate by *Pseudomonas cepacia* 2CBS[J]. Biol Chem Hoppe Seyler, 370: 1173–1182.

Häggblom M M, 1992. Microbial breakdown of halogenated aromatic pesticides and related compounds[J]. FEMS Microbiol Rev, 9: 29–71.

Hartmann J, Reineke W, Knackmuss H J, 1979. Metabolism of 3-chlor, 4-chloro, and 3,5-dichlorobenzoate by a *Pseudomonad*[J]. Appl Environ Microbiol, 37: 421–428.

Hernandez B S, Arensdorf J J, Focht D D, 1995. Catabolic characteristics of biphenyl-utilizing isolates which cometabolize PCBs[J]. Biodegradation, 6: 75–82.

Gentry T J, Newby D J, Josephson K L, 2001. Soil microbial population dynamics following bioaugmentation with a 3-chlorobenzoate-degrading bacterial culture[J]. Biodegradation, 12: 349–357.

Goodfellow M, 1986. Genus *Rhodococcus* Zopf 1891, 28AL[M]. In: Bergey's manual of systematic bacteriology (Sneath P. H. A., Mair N. S., Sharpe M. E *et al.,* ed.). Williams and Wilkins. 1472–1481.

Kato T, Haruki M, Imanaka T *et al*., 2001. Isolation and characterization of long-chain-alkane degrading *Bacillus thermoleovorans* from deep subterranean petroleum reservoirs[J]. J Biosci Bioeng, 91: 64–70.

Keil H, Klages U, Lingens F, 1981. Degradation of 4-chlorobenzoate by *Pseudomonas* sp. CBS3: induction of catabolic enzymes[J]. FEMS Microbiol Lett, 10: 213–215.

Kozlovsky S A, Zaitsev G M, Kunc F *et al*., 1993. Degradation of 2-chlorobenzoic and 2,5-dichlorobenzoic acids in pure culture by *Pseudomonas stutzeri*[J]. Folia Microbiol, 38: 371–375.

Lambo A J, Patel T R, 2006. Isolation and characterization of a biphenyl-utilizing psychrotrophic bacterium, Hydrogenophaga taeniospiralis IA3-A, that cometabolize dichlorobiphenyls and polychlorinated biphenyl congeners in Aroclor 1221[J]. J Basic Microbiol, 46: 94–107.

Larkin M J, Kulakov L A, Allen C C, 2005. Biodegradation and *Rhodococcus*-masters of catabolic versatility[J]. Curr Opin Biotechnol, 16: 282–290.

Li D Y, Eberspächer Y, Wagner B *et al*., 1991. Degradation of 2,4,6-trichlorophenol by *Azotobacter* sp. strain GP1[J]. Appl Environ Microbiol, 57: 1920–1928.

Lowry O H, Rosebrough N J, Farr A L *et al*., 1951. Protein measurement with the Folin- phenol reagent[J]. J Biol Chem, 193: 265–275.

Parsons J R, Sijm D T H M, van Laar A *et al*., 1988. Biodegradation of chlorinated biphenyls and benzoic acid by a *Pseudomonas* strain[J]. Appl Microbiol Biotechnol, 29: 81–84.

Peter P, Lotte H E G, 2003. Degradation of alkanes and highly chlorinate benzenes, and production of biosurfactants, by a psychrophilic *Rhodococcus* sp. and genetic characterization of its chlorobenzene dioxygenase[J]. Microbiol, 149: 2879–2890.

Roberta R F, Albert N R, James M T, 1996. Pristine soils mineralize 3-chlorobenzoate and 2,4–dichlorophenoxyacetate via different microbial populations[J]. Appl Environ Microbiol, 62: 1159–1166.

Rodrigues J L, Kachel C A, Aiello M R *et al*., 2006. Degradation of aroclor 1242 dechlorination products in sediments by *Burkholderia xenovorans* LB400 (ohb) and *Rhodococcus* sp. strain RHA1 (fcb)[J]. Appl Environ Microbiol, 72: 2476–2482.

Suzuki K, Ogawa N, Miyashita K, 2001. Expression of 2-halobenzoate dioxygenase genes (cbdSABC) involved in the degradation of benzoate and 2-halobenzoate in *Burkholderia* sp. TH2[J]. Gene, 262: 137–145.

Theim S M, Krumme M L, Smith R L *et al*., 1994. Use of molecular techniques to evaluate the survival of a microorganism injected into an aquiferb[J]. Appl Environ Microbiol, 60: 1059–1067.

Whyte L G, Hawari J, Zhou E *et al*., 1998. Biodegradation of variable-chain-length alkanes at low temperatures by a psychrotrophic *Rhodococcus* sp.[J]. Appl Environ Microbiol, 64: 2578–2584.

van der Geize R, Dijkhuizen L, 2004. Harnessing the catabolic diversity of rhodococci for environmental and biotechnological applications[J]. Curr Opin Microbiol, 7: 255–261.

Yi H, Min K, Kim C *et al*., 2000. Phylogenetic and phenotypic diversity of 4-chlorobenzoate-degrading bacteria isolated from soils[J]. FEMS Microbiol Ecol, 31: 53–60.

Yuroff S A, Sabat G, Hickey J W, 2003. Transporter-mediated uptake of 2-chloro- and 2-hydroxybenzoate by *Pseudomonas huttiensis* strain D1[J]. Appl Environ Microbiol, 69: 7401–7408.