



## Environmental Microbiology

## Regional analysis of potential polychlorinated biphenyl degrading bacterial strains from China

Jianjun Shuai<sup>a</sup>, Xurun Yu<sup>a</sup>, Jing Zhang<sup>a</sup>, Ai-sheng Xiong<sup>b</sup>, Fei Xiong<sup>a,\*</sup>

<sup>a</sup> Jiangsu Key Laboratory of Crop Genetics and Physiology/Co-Innovation Center for Modern Production Technology of Grain Crops, Yangzhou University, Yangzhou, China

<sup>b</sup> State Key Laboratory of Crop Genetics and Germplasm Enhancement, College of Horticulture, Nanjing Agricultural University, Weigang, Nanjing, China

## ARTICLE INFO

## Article history:

Received 30 January 2014

Accepted 28 December 2014

Available online 22 April 2016

Associate Editor: Lara Durães Sette

## Keywords:

Polychlorinated biphenyls

Biphenyl

Biodegradation

*Pseudomonas* sp.

HPLC

## ABSTRACT

Polychlorinated biphenyls (PCBs), the chlorinated derivatives of biphenyl, are one of the most prevalent, highly toxic and persistent groups of contaminants in the environment. The objective of this study was to investigate the biodegradation of PCBs in northeastern (Heilongjiang Province), northern (Shanxi Province) and eastern China (Shanghai municipality). From these areas, nine soil samples were screened for PCB-degrading bacteria using a functional complementarity method. The genomic 16S rDNA locus was amplified and the products were sequenced to identify the bacterial genera. Seven *Pseudomonas* strains were selected to compare the capacity of bacteria from different regions to degrade biphenyl by HPLC. Compared to the biphenyl content in controls of 100%, the biphenyl content went down to 3.7% for strain P9-324, 36.3% for P2-11, and 20.0% for the other five strains. These results indicate that a longer processing time led to more degradation of biphenyl. PCB-degrading bacterial strains are distributed differently in different regions of China.

© 2016 Sociedade Brasileira de Microbiologia. Published by Elsevier Editora Ltda. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

## Introduction

Polychlorinated biphenyls (PCBs), the chlorinated derivatives of biphenyl, are highly toxic environmental pollutants that have become ubiquitous throughout the environment and the food web because of their low degradability. Therefore, the removal of PCBs from soil and sediment is a high priority in several industrial countries.<sup>1</sup> PCB molecules consist of a

biphenyl nucleus carrying 1–10 chlorines, which can create >200 possible congeners that differ in chlorine number and position. The less-chlorinated congeners are usually less toxic than the more-chlorinated congeners.<sup>2</sup> The remediation of PCB contamination in the environment has become extremely important.

The microbial degradation of PCBs is one of the most cost-effective and energy-efficient methods for removing them

\* Corresponding author at: Jiangsu Key Laboratory of Crop Genetics and Physiology/Co-Innovation Center for Modern Production Technology of Grain Crops, Yangzhou University, Yangzhou 225009, China.

E-mail: [feixiong@yzu.edu.cn](mailto:feixiong@yzu.edu.cn) (F. Xiong).

<http://dx.doi.org/10.1016/j.bjm.2014.12.001>

1517-8382/© 2016 Sociedade Brasileira de Microbiologia. Published by Elsevier Editora Ltda. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

from the environment. Many PCB-degrading bacteria have been isolated, such as Gram-negative strains belonging to the genera *Pseudomonas*, *Alcaligenes*, *Achromobacter*, *Burkholderia*, *Acinetobacter*, *Comamonas*, *Sphingomonas* and *Ralstonia*, and Gram-positive strains of the genera *Arthrobacter*, *Corynebacterium*, *Rhodococcus* and *Bacillus*.<sup>3</sup> The genes encoding the enzymes for biphenyl degradation are clustered and termed *bph* gene clusters.<sup>4</sup> The enzymes can be divided into four types, including biphenyl dioxygenase (BphA), dihydrodiol dehydrogenase (BphB), 2,3-dihydroxybiphenyl dioxygenase (BphC) and hydrolase (BphD).<sup>5–11</sup>

We previously investigated the distribution of PCB-utilizing bacteria in Shanghai.<sup>12</sup> In this study, we investigated the distribution of PCB-utilizing bacteria from other different areas of China. Moreover, we chose several *Pseudomonas* strains from different soil samples and measured their degradation of biphenyl using HPLC.

## Materials and methods

### Chemicals and media

PCB (2,2',3,3'-tetrachlorobiphenyl, CAS No. 38444-93-8) (99.5% purity) and biphenyl (CAS No. 92-52-4) (99.5% purity) were purchased from the J&K Chemical Co Ltd (Shanghai, China) and Shanghai Chemical Agent Co Ltd (Shanghai, China), respectively. The other chemicals used in this study were of the highest purity available. Mineral salt medium (MM)<sup>13</sup> contained (g):  $\text{NH}_4\text{NO}_3$  1.0, KCl 0.7,  $\text{KH}_2\text{PO}_4$  2.0,  $\text{NaHPO}_4$  3.0,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.7,  $\text{CaCl}_2$  0.02 and NaCl 1.0 in 1 L of double-distilled water at pH 7.0. Solid MM was MM containing agar at 16 g/L. After autoclaving, tetrachlorobiphenyl was added as the sole carbon source.

### Isolation of PCB-degrading bacterial strains

Samples 1–3 were collected from Heilongjiang Province in northeast China: Sample 1 from a chemical factory; Sample 2 from a botanical garden; and Sample 3 from a pharmaceutical factory. Samples 4 and 5 were collected from Shanxi Province in northern China: Sample 4 from the Fen River and Sample 5 from a paper mill. Samples 6–9 were collected from Shanghai Municipality in eastern China: Sample 6 from an experimental field; Sample 7 from a riverbed not far from an old chemical factory; Sample 8 from an old chemical factory; and Sample 9 from a riverbed near a residential area (Fig. 1).

For each sample, 10.0 g of soil was put into a sterilized mortar with 50 mL of sterile water, crushed with a sterilized pestle, and then left for 5 min. A sample (1–5 mL) of the clear supernatant liquid was added to an Erlenmeyer flask that contained 100 mL of MM with 0.01% tetrachlorobiphenyl as the sole carbon source. Then, the Erlenmeyer flask was incubated for 3 d at 28 °C in darkness with shaking at 150 rpm. A 1 mL aliquot was transferred to another 100 mL of fresh MM containing 0.01% tetrachlorobiphenyl and incubated under the same conditions. This process was repeated three times. Of the suspension, 20  $\mu\text{L}$  was plated onto solid MM containing 0.01% tetrachlorobiphenyl as the sole carbon source and incubated for 3 d at 28 °C.



Fig. 1 – Geographical locations of the soil samples in China.

### Identification and analysis of 16S rDNA sequences

Genomic DNA was extracted from the PCB-utilizing bacterial strains.<sup>14</sup> The 16S rDNA locus was amplified using two primers (5'-ACG GCTACCTGTACGACTTC-3' and 5'-AGAGTTTGATCCTGGCTCAG-3') and *Ex-taq* polymerase with genomic DNA as the template, separated by 1.5% agarose gel electrophoresis, and cloned into the pMD-18 vector (TaKaRa, Dalian, China) for enzymatic and sequencing identification. The PCR protocol was 94 °C for 10 min, then 35 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 2 min, and a final extension at 72 °C for 10 min in a 50  $\mu\text{L}$  reaction volume. The PCR products were separated by 1.5% agarose gel electrophoresis and quantified using a Model Gel Doc 1000 analyzer (Bio-Rad, Hercules, CA). Phylogenetic analysis was based on the 16S rDNA by MEGA5 software and the neighbor-joining (NJ) method with the following parameters: Poisson correction, pairwise deletion, and bootstrap (1000 replicates).<sup>15</sup>

### HPLC analytical assay of biphenyl degradation

To verify and compare the extent of degradation, we chose seven *Pseudomonas* strains: P1-5, P2-11, P3-24, P4-38, P5-31, P7-245 and P9-324 from seven soil samples. The details of detecting biphenyl degradation by HPLC were as follows: each of the seven strains (100  $\mu\text{L}$ ) and a control (no strain) were cultured in an Erlenmeyer flask (500 mL) containing 100 mL of LB medium at 28 °C in a rotary shaker at 120 rpm overnight. The next day, 1 mL of biphenyl solution (50 mg/mL, dissolved in methyl alcohol) was added into each Erlenmeyer flask and the culture was continued. After 2 and 4 d, 500  $\mu\text{L}$  of each solution was placed into an Eppendorf tube and then 500  $\mu\text{L}$  of ether was added. The tubes were deposited on an oscillator for 5–10 min so that the biphenyl was fully extracted by the ether. The supernatant of the two tubes was withdrawn and mixed together in a new tube, 500  $\mu\text{L}$  of methyl alcohol was added and the mixture was ready for rotary evaporation. The ether was fully evaporated and the biphenyl was kept in the methyl

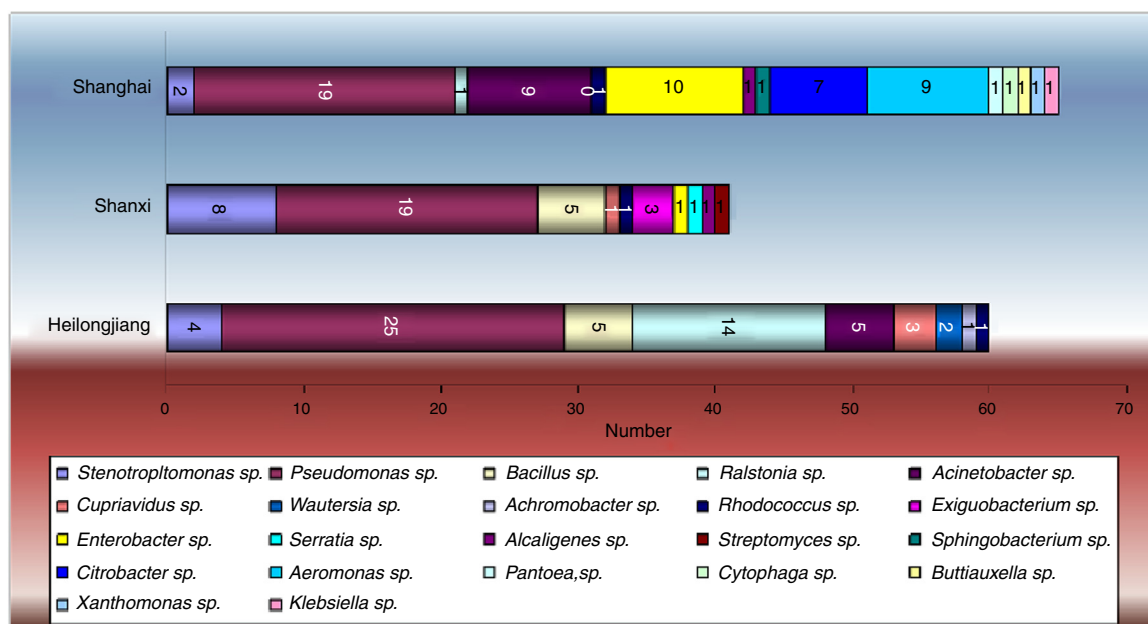


Fig. 2 – The overall distribution of PCB-degrading bacterial strains in different areas of China.

alcohol to keep it from adhering to the wall of the round-bottomed bottle.<sup>12</sup>

Before HPLC analysis, each sample was membrane-filtered. A 10- $\mu$ L sample of the resulting solution was subjected to reverse-phase HPLC (1100 series, Agilent Co, USA). The separation column for the HPLC (4.6 mm  $\times$  150 mm  $\times$  5  $\mu$ m) was filled with Kromail 100-5C18. The mobile phase contained acetonitrile/water (70:30, v/v) and the flow rate was 1.0 mL/min. Biphenyl was detected at a wavelength of 254 nm.<sup>4</sup> A solution of pure biphenyl (100  $\mu$ L/mL) was used to determine the peak retention time.

## Results

### Isolation and classification of PCB-degrading bacteria

From all the soil samples, a total of 22 PCB-utilizing bacterial strains were detected, including the following genera: *Stenotrophomonas*, *Pseudomonas*, *Bacillus*, *Ralstonia*, *Acinetobacter*, *Cupriavidus*, *Wautersia*, *Achromobacter*, *Rhodococcus*, *Exiguobacterium*, *Enterobacter*, *Serratia*, *Alcaligenes*, *Streptomyces*, *Sphingobacterium*, *Citrobacter*, *Aeromonas*, *Pantoea*, *Cytophaga*, *Buttiauxella*, *Xanthomonas* and *Klebsiella* (Fig. 2). There were 66 strains identified from Shanghai, more than 60 from Heilongjiang, and 45 from Shanxi. Furthermore, the strains from Shanghai encompassed 15 categories, more than the other two regions. Some bacterial strains were found in all three regions, e.g., *Stenotrophomonas*, *Pseudomonas* and *Rhodococcus*. However, some bacteria only existed in a particular soil, e.g., *Achromobacter* was only in soil samples from Heilongjiang; *Exiguobacterium*, *Serratia* and *Streptomyces* were only in soil samples from Shanxi; *Sphingobacterium*, *Citrobacter*, *Aeromonas*, *Pantoea*, *Cytophaga*, *Buttiauxella*, *Xanthomonas* and *Klebsiella* were only in soil samples from Shanghai.

### Phylogenetic reconstruction of the selected strains

A multiple alignment analysis using full 16S rDNA sequences was used to study the phylogenetic relationships among the seven *Pseudomonas* strains selected from different soil samples and other *Pseudomonas* strains. Strains P7-245 and P9-324 were found to belong to one branch, and P3-24 and P4-38 belong to a separate branch, with each of these showing the closest relationship (Fig. 3). Moreover, strains P1-5 and P5-31 occupy large sub-branches of the two above-mentioned branches. Strain P2-11 is in a different branch from the other six *Pseudomonas* strains.

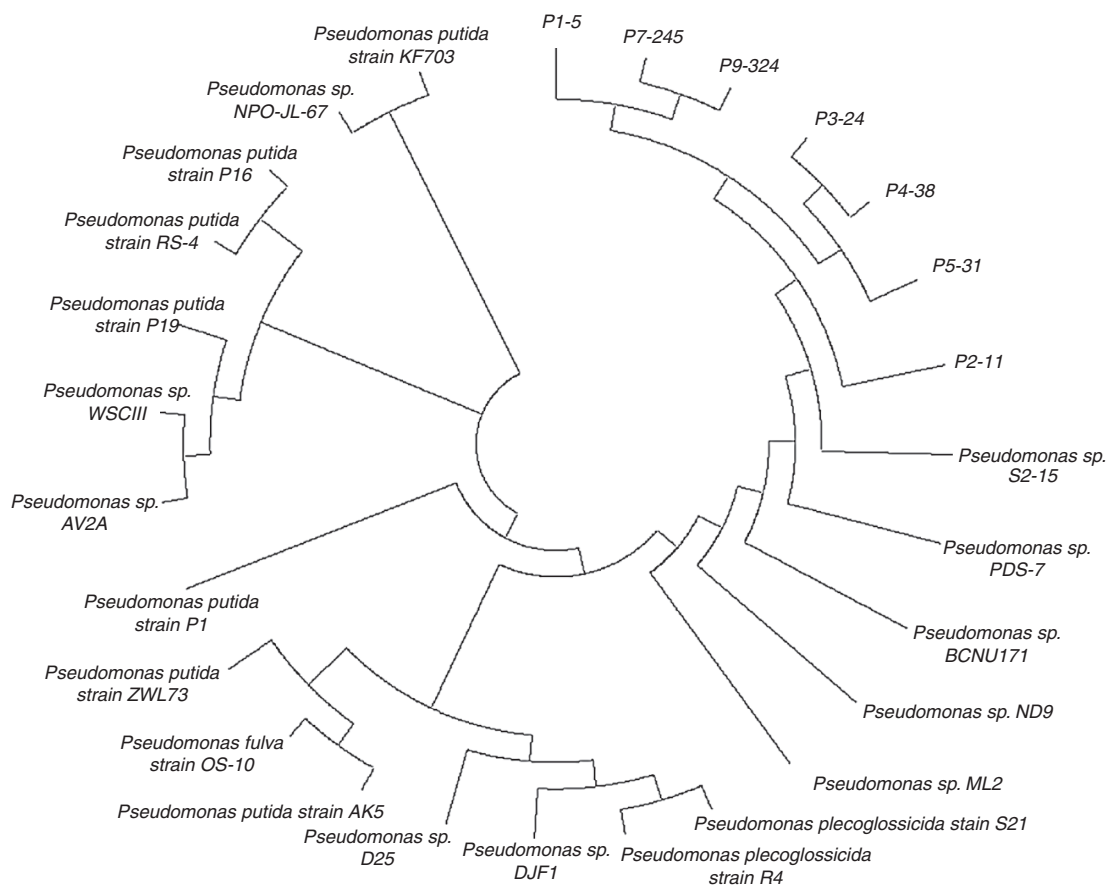
### Degradation of biphenyl

HPLC was used to detect the efficiency of the seven *Pseudomonas* strains at biphenyl degradation. When pure biphenyl was subjected to HPLC, the highest peak appeared at a retention time of  $\approx$ 5.8 min, indicating the point of biphenyl detection.<sup>12</sup> The peak's area is directly proportional to the sample concentration. In our assay, there was only one large peak.

After 2 d, assay results showed that each strain could degrade biphenyl with the degradation rate ranging from 5.9% (strain P6-245) to 65.3% (strain P4-38) (Table 1). After 4 d, strain P9-324 had degraded the maximum amount of biphenyl (96.3%), whereas the other strains all degraded amounts varying from 63.7% (strain P2-11) to 83.6% (strain P4-38) (Fig. 4). However, the amount of biphenyl in the controls had changed little by either 2 or 4 d.

## Discussion

PCBs refer to a large group of chlorinated biphenyls with 209 possible congeners.<sup>16</sup> Due to their hydrophobic properties,



**Fig. 3 – Phylogenetic analysis of *Pseudomonas* sp. strains as revealed by 16S rDNA sequence comparisons.**

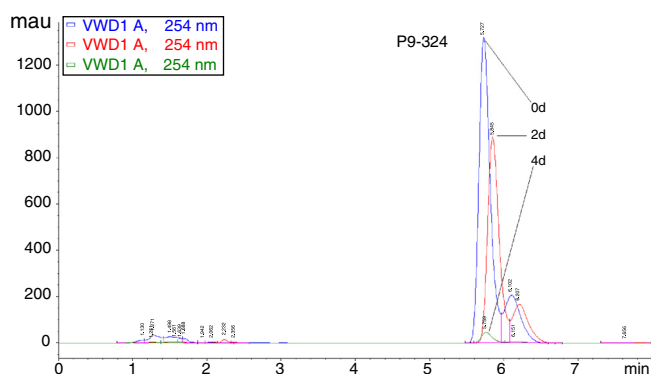
PCBs tend to become absorbed by natural organic matter in soil, sediments and sludges. PCBs are a serious environmental problem due to their toxicity and persistence in the environment. Natural biodegradation of PCBs in contaminated soil and sediment occurs at low rates,<sup>17</sup> and various attempts to accelerate biodegradation have been made.<sup>18–21</sup>

Despite active research in developed nations spanning three decades, extensive regulatory action, and an effective ban on their production since the late 1970s, PCBs remain a focus of research and environmental attention.<sup>22</sup> China

is a developing country in which pollution prevention and control is most likely less effective compared to developed countries. Moreover, PCBs are widespread in the environment and are found globally as a result of ocean currents and atmospheric deposition.<sup>23,24</sup> Thus, it is urgent to study PCB contamination and biodegradation in China and across the globe.

The PCB-biodegrading bacterial strains in Shanghai, the biggest industrial metropolis in China, have been characterized in detail.<sup>12</sup> The objective of this study was to investigate

<b>Table 1 – Comparison of the degradation of biphenyl for controls and <i>Pseudomonas</i> sp. strains.</b>			
Sample	Biphenyl content (%)		
	0 d	2 d	4 d
Control	100	97.6	92.3
P1-5	100	40.8	23.7
P2-11	100	77.4	36.3
P3-24	100	36.6	21.1
P4-38	100	68.4	16.5
P5-31	100	59.1	19.9
P7-245	100	94.1	23.4
P9-324	100	69.1	3.7



**Fig. 4 – HPLC results of strain P9-324. The processing time from top to bottom (d): 0, 2 and 4.**

and compare the PCB-degrading bacteria in northeastern (Heilongjiang Province), northern (Shanxi Province) and eastern China (Shanghai Municipality). Thus, research on PCB contamination and biodegradation in large-scale regions is urgently needed. Three regions were selected to investigate PCB-degrading bacteria. Heilongjiang Province, located in northeast China, is a main agricultural (rice and corn production) region. Shanxi Province, located in northern China, is a main energy (coal production) region. Shanghai has developed rapidly in recent decades and along with growth of the economy, environmental pollution is a subject worthy of concern. Soil samples were collected from northeastern, northern and eastern China to investigate the biodegradation of PCBs. Seven categories of strains were identified in the soil samples from Heilongjiang and Shanxi Provinces but not in the soil samples of Shanghai.

Here, we chose nine soil samples from northeastern, northern and eastern China, based on areas where industry has quickly developed and were thus more likely to have polluted the environment. There were >2 sampling locations in each region to make the results more representative. The PCB-degrading bacterial strains were varied, with strains of genera *Stenotrophomonas*, *Pseudomonas* and *Rhodococcus* present in each region; these three PCB-utilizing genera have been reported in previous studies.<sup>25</sup> In contrast, some bacteria were found to exist only in a single region, possibly due to the different geographical locations. Thus, the distribution of PCB-degrading bacterial strains may be closely linked to geographic location, and the state of development in the region may also play a role in the evolutionary selection.

According to the phylogenetic tree (Fig. 3), the relationships among the seven *Pseudomonas* strains were closer than with the others. HPLC confirmed that the seven *Pseudomonas* strains could degrade biphenyl. The sampling results at 2 d showed that all bacteria had started to degrade biphenyl. By 4 d, the degradation of biphenyl by all strains was not the same. Strain P9-324 had degraded nearly 96.0% of the biphenyl compared with the control, and strain P2-11 had degraded 63.7%, which was less than the other six *Pseudomonas* strains. In addition to strains P9-324 and P2-11, the other five *Pseudomonas* strains degraded approximately 80.0% of the biphenyl. These results are relatively consistent with the phylogenetic tree. Strain P2-11 came from Sample 2, which was from a botanical garden in Heilongjiang. Botanical gardens are well protected and less susceptible to outside contamination; this balanced ecosystem may cause the resistance of bacteria there to be relatively weaker than in other places, e.g., strain P2-11 2 degraded only 63.7% of the biphenyl. Strain P9-324 isolated from Sample 9 was from a riverbed near residential quarters and had the strongest ability to degrade biphenyl of approximately 96.3%. It is most likely that the river was seriously polluted many years ago, and these strains remained in the mud and retained their strong ability to degrade biphenyl.

In the present study, different areas in China were screened for the presence of PCB-degrading bacteria due to interest in geographical influences. We combined molecular biology and chemistry together in the experiments. Our future work will include searching for the sources of environmental pollution, solving the problems of pollution by biodegradation and combining multi-disciplinary methods to prevent pollution.

## Conflict of interest

The authors declare no conflicts of interest

## Acknowledgments

The research was supported by A Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

## REFERENCES

1. Vasilyeva GK, Strijakova ER. Bioremediation of soil and sediment contaminated by polychlorobiphenyls. *Microbiology*. 2007;76:639–653.
2. Jia LY, Zheng AP. Isolation and characterization of comprehensive polychlorinated biphenyl-degrading bacterium, *Enterobacter* sp. LY402. *J Microbiol Biotechnol*. 2008;18:952–957.
3. Sakai M, Ezaki S. Isolation and characterization of a novel polychlorinated biphenyl-degrading bacterium: *Paenibacillus* sp. KBC101. *Appl Microbiol Biotechnol*. 2005;68:111–116.
4. Hong Q, Dong XJ, He LJ, Li SP. Isolation of a biphenyl-degrading bacterium: *Achromobacter* sp. BP3, and cloning of the bph gene cluster. *Int Biodeter Biodegr*. 2009;63:365–370.
5. Furukawa K. Microbial degradation of polychlorinated biphenyls. In: Chakrabarty AM, ed. *Biodegradation and Detoxification of Environmental Pollutants*. Boca Raton: CRC Press; 1982 [chapter 2].
6. Furukawa K. Molecular genetics and evolutionary relationship of PCB-degrading bacteria. *Biodegradation*. 1994;5:289–300.
7. Furukawa K, Fujihara H. Microbial degradation of polychlorinated biphenyls: biochemical and molecular features. *J Biosci Bioeng*. 2008;105:433–449.
8. Abramowicz DA. Aerobic and anaerobic biodegradation of PCBs: a review. *Crit Rev Biotechnol*. 1990;10:241–251.
9. Unterman R. A history of PCBs biodegradation. In: Crawford RL, Crawford RL, Crawford DL, eds. *Bioremediation: Principles and Applications*. New York, Cambridge: University Press; 1996.
10. Xiong F, Shuai JJ, Peng RH, et al. Expression, purification and functional characterization of a recombinant 2,3-dihydroxybiphenyl-1,2-dioxygenase from *Rhodococcus rhodochrous*. *Mol Biol Rep*. 2011;38:4303–4308.
11. Bian L, Shuai JJ, Xiong F, Peng RH, Yao QH, Xiong AS. Expression: purification, and characterization of a 2,3-dihydroxybiphenyl-1,2-dioxygenase from *Bacillus* sp. JF8 in *Escherichia coli*. *Biochem Biophys Res Commun*. 2012;419:339–343.
12. Shuai JJ, Tian YS, Yao QH, Peng RH, Xiong F, Xiong AS. Identification and analysis of polychlorinated biphenyls (PCBs)-biodegrading bacterial strains in Shanghai. *Curr Microbiol*. 2010;61:477–483.
13. Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning: A Laboratory Manual*. 2nd ed. New York: Cold Spring Harbor; 1989 [chapter 2].
14. Yoon JH, Lee ST, Kim SB, Kim WY, Goodfellow M, Park YH. Restriction fragment length polymorphisms analysis of PCR-amplified 16S ribosomal DNA for rapid identification of *Saccharomonospora* strains. *Int J Syst Bacteriol*. 1997;47:111–114.
15. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using

- maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol.* 2011;28:2731–2739.
16. Jim AF, Reyes SA. Microbial transformation and degradation of polychlorinated biphenyls. *Environ Pollut.* 2008;155:1–12.
  17. Young LY, Cerniglia C. *Microbial Transformation and Degradation of Toxic Organic Chemicals*. New York: Wiley-Liss; 1995 [chapter 1].
  18. Mildaziene V, Nauciene Z, Baniene R, Demin O, Krab K. Analysis of effects of 2,2',5,5'-tetrachlorobiphenyl on the flux control in oxidative phosphorylation system in rat liver mitochondria. *Mol Biol Rep.* 2002;29:35–40.
  19. Fava F, Bertin L, Fedi S, Zannoni D. Methyl-beta-cyclodextrin-enhanced solubilization and aerobic biodegradation of polychlorinated biphenyls in two aged-contaminated soils. *Biotechnol Bioeng.* 2003;81: 381–390.
  20. Manzano MA, Perales JA, Sales D, Quiroga JM. Enhancement of aerobic microbial degradation of polychlorinated biphenyl in soil microcosms. *Environ Toxicol Chem.* 2003;22:699–705.
  21. Singer AC, Smith DW, Jury A, Hathuc K, Crowley DE. Impact of the plant rhizosphere and augmentation on remediation of polychlorinated biphenyl contaminated soil. *Environ Toxicol Chem.* 2003;22:1998–2004.
  22. Sunday AA, Flynn WP, Matthew OI, Olukayode OA. Evidence of aerobic utilization of di-ortho-substituted trichlorobiphenyls as growth substrates by *Pseudomonas* sp. SA-6 and *Ralstonia* sp. SA-4. *Environ Microbiol.* 2008;10:1165–1174.
  23. Aono S, Tanabe S, Fujise Y, Kato H, Tatsukawa R. Persistent organochlorines in minke whale (*Balaenoptera acutorostrata*) and their prey species from the Antarctic and the North Pacific. *Environ Pollut.* 1997;98:81–89.
  24. Breivik K, Alcock R, Li YF, Bailey RE, Fiedler H, Pacyna JM. Primary sources of selected POPs: regional and global scale emission inventories. *Environ Pollut.* 2004;128:3–16.
  25. Kensuke F, Hidehiko F. Microbial degradation of polychlorinated biphenyls: biochemical and molecular features. *J Biosci Bioeng.* 2008;105:433–449.