Environmental Science

Shree Nath Singh *Editor*

Microbial Degradation of Xenobiotics

Springer

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Shree Nath Singh
Environmental Science Division
National Botanical Research Institute
Rana Pratap Marg
226001 Lucknow
India
e-mail: drsn06@gmail.com

ISSN 1863-5520
ISBN 978-3-642-23788-1 e-ISBN 978-3-642-23789-8
DOI 10.1007/978-3-642-23789-8
Springer Heidelberg Dordrecht London New York

Library of Congress Control Number: 2011938665

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Environmental Science and Engineering

Environmental Engineering

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http://www.springer.com/series/7487

In loving memory of my parents

Preface

Microorganisms are ubiquitous in the environment playing an important role in biogeochemical cycling. However, their ability to metabolize xenobiotic compounds has received much attention in recent years due to their environmental persistence and toxicity. Hence, microbial degradation of xenobiotics is, today, seen as both cost-effective and eco-friendly technology for removing these pollutants by a process known as bioremediation. Earlier researchers have confirmed that microbes are capable of degrading a wide range of organic pollutants. However, process of biodegradation is generally very slow and hence, this process may be accelerated by augmenting pure and mixed cultures of microorganisms in both aerobic and anaerobic conditions. Metabolic intermediates formed in the degradative pathways were also examined for their toxicity assessments using bacteria and higher organisms. Many of degradative genes responsible for xenobiotic metabolism are present on plasmids, transposons or are grouped in clusters on chromosomes. This indicates evolution of degradative pathways and makes the genetic manipulation easier. Development of the transgenic microbial strains highly capable of degrading xenobiotics is now possible through biotechnological approaches. Besides, several catabolic enzymes involved in xenobiotic metabolism have been isolated and characterized. A number of environmental factors, including pH, temperature, bioavailability, nutrient supply and oxygen availability have been shown to affect biodegradation process. These factors have to be optimized to obtain an effective microbial treatment process for the industrial organic wastes at bench and pilot scales. However, in the field scale treatment, all environmental factors cannot be manipulated to enhance the degradation process.

To update the knowledge on bioremediation which is a natural attenuation process, I present before you an edited volume on 'Microbial degradation of xenobiotics' which has focused on different aspects of microbial degradation of xenobiotic compounds, like poly aromatics hydrocarbons, polychlorophenols, polyurethane, dye containing wastewater, water soluble polymers, azo dyes, explosives, chloroorganic pollutants, styrene, trinitrophenol and high molecular weight alkanes. These aspects have been discussed in 17 chapters contributed by the leading scientists drawn from all over the world.

In this endeavor, I am not alone, but assisted by many fellow workers. First of all, I would like to acknowledge all the contributors who responded to my request and very enthusiastically contributed their chapters containing the latest developments on the relevant issues. The services rendered by my own research scholars Mrs. Babita Kumari, Ms. Shweta Mishra, and Mrs. Sadhna Tiwari in this endeavor are remarkable and highly appreciable. Besides, laboratory trainees Ms. Namarata Pandey, Ms. Jyoti, Ms. Rashi Singhal, Ms. Deepika Verma, Ms. Radha Verma, Ms. Shilpi Dupey and Ms. Shilpi Kumari are also duly acknowledged for their multifaceted help and support. Mr. Dilip Chakraborty deserves special appreciation for computer work for preparing the manuscript on the book format.

Lastly, I express my sincere thanks to my family members Mrs. Manorma Singh (wife), Dr. Ragini Singh (daughter), Mr. Pritish Kumar Singh (son) and the little champ Antra for their inspiration, endurance and moral boost up in this endeavor.

S. N. Singh

Contributors

R. Balasubramanian Department of Civil and Environmental Engineering, National University of Singapore, 1 Engineering Drive 2, Singapore, 117576, Singapore

Anat Bernstein Department of Environmental Hydrology and Microbiology, Zuckerberg Institute for Water Research, Blaustein Institutes for Desert Research, Ben-Gurion University of the Negev, Sede Boqer Campus, 84990 Beer-Sheva, Israel

Tomáš Cajthaml Laboratory of Environmental Biotechnology, Institute of Microbiology, v.v.i., Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague 4, Czech Republic, e-mail: cajthaml@biomed.cas.cz

Pritam Chattopadhyay Plant Biotechnology Laboratory, Department of Botany, Visva-Bharati, Santiniketan, India

Indrani Ghosh Centre for Environmental Science and Engineering, Indian Institute of Technology, Powai, Mumbai 400 076, India

Gary T. Howard Department of Biological Sciences, Southeastern Louisiana University, Hammond, Louisiana 70402, USA, e-mail: ghoward@selu.edu

Leela Iyengar Centre for Post graduate Studies, Jain University, Bangalore, India

Stefan R. Kaschabek Environmental Microbiology, Department of Biological Sciences, Faculty of Chemistry and Physics, TU Bergakademie Freiberg, Leipziger Str. 29, 09599 Freiberg, Germany

Fusako Kawai Center for Nanomaterials and Devices, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606-8585, Japan, e-mail: fkawai@kit.ac.jp

Christian Kennes Faculty of Sciences, Campus da Zapateira, University of La Coruña, Rua da Fraga 10, 15008 La Coruña, Spain, e-mail: kennes@udc.es

B. Kumari Environmental Science Division, National Botanical Research Institute, Rana Pratap Marg, Lucknow 226001, India

Toshinari Maeda Department of Biological Functions and Engineering, Graduate School of Life Science and Systems Engineering, Kyushu Institute of Technology, 2-4 Hibikino, Wakamatsu-ku, Kitakyushu 808-0196, Japan, e-mail: toshi.maeda@life.kyutech.ac.jp

Ernest Marco-Urrea Department of Chemical Engineering and Institute of Environmental Science and Technology, Autonomous University of Barcelona, 08193 Bellaterra, Spain

S. Maruthamuthu Corrosion Protection Division, Biocorrosion Group, Central Electrochemical Research Institute, Karaikudi 630 006, India

Shweta Mishra Environmental Science Division, National Botanical Research Institute, Rana Pratap Marg, Lucknow 226001, India

Pijush Kanti Mondal Environmental Research laboratory, Department of Applied Chemistry, Faculty of Engineering and Technology, Aligarh Muslim University, Aligarh, Uttar Pradesh 202 002, India, e-mail: pijushamu@gmail.com

Suparna Mukherji Centre for Environmental Science and Engineering, Indian Institute of Technology, Powai, Mumbai 400 076, India

Hiroaki I. Ogawa Department of Biological Functions and Engineering, Graduate School of Life Science and Systems Engineering, Kyushu Institute of Technology, 2-4 Hibikino, Wakamatsu-ku, Kitakyushu 808-0196, Japan

Anjali Pandey Department of Chemistry, Indian Institute of Technology, Kanpur, India, e-mail: anjali@iitk.ac.in

José A. Perales-Vargas-Machuca Department of Environmental Technologies, Andalusian Centre of Marine Science and Technology (CACYTMAR), University of Cadiz, Puerto Real, 11510, Cadiz, Spain

Pattanathu K. S. M. Rahman Chemical and Bioprocess Engineering Group, School of Science and Engineering, Teesside University, Middlesbrough, Tees Valley, TS13BA, UK, e-mail: p.rahman@tees.ac.uk

A. Rajasekar Department of Civil and Environmental Engineering, National University of Singapore, 1 Engineering Drive 2, Singapore, 117576, Singapore, e-mail: e-mail: esera@nus.edu.sg

Department of Chemical and Biomolecular Engineering, National University of Singapore, 4 Engineering Drive 4, Singapore, 117576, Singapore, e-mail: rajasekar.aruliah@gmail.com

C. A. Reddy Department of Microbiology and Molecular Genetics and The NSF Center for Microbial Ecology, Michigan State University, East Lansing, Michigan 48824-4320, USA, e-mail: reddy@msu.edu

Elisa Rojo-Nieto Department of Environmental Technologies, Andalusian Centre of Marine Science and Technology (CACYTMAR), University of Cadiz, Puerto Real, 11510, Cadiz, Spain, e-mail: elisa.rojo@uca.es

Zeev Ronen Department of Environmental Hydrology and Microbiology, Zuckerberg Institute for Water Research, Blaustein Institutes for Desert Research, Ben-Gurion University of the Negev, Sede Boqer Campus, 84990 Beer-Sheva, Israel, e-mail: zeevrone@bgu.ac.il

Sukanta K. Sen Microbiology Division, Department of Botany, Visva-Bharati, Santiniketan, India, e-mail: sksenvb@rediffmail.com

Antaryami Singh Defence Laboratory, Defence Research and Development Organization, Jodhpur 342 011, India

Poonam Singh Division of Toxicology, Central Drug Research Institute, Lucknow, India

Rita Singh Defence Laboratory, Defence Research and Development Organization, Jodhpur 342 011, India, e-mail: singhritadr@yahoo.com

S. N. Singh Environmental Science Division, National Botanical Research Institute, Rana Pratap Marg, Lucknow 226 001, India, e-mail: drsn06@gmail.com

Shelly Sinha Rabindra Mahavidyalaya, Champadanga, Hooghly, West Bengal, India

Kateřina Svobodová Laboratory of Environmental Biotechnology, Institute of Microbiology, v.v.i., Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague 4, Czech Republic

Y. P. Ting Department of Chemical and Biomolecular Engineering, National University of Singapore, 4 Engineering Drive 4, Singapore, 117576, Singapore

Dirk Tischler Environmental Microbiology, Department of Biological Sciences, Faculty of Chemistry and Physics, TU Bergakademie Freiberg, Leipziger Str. 29, 09599 Freiberg, Germany, e-mail: Dirk-Tischler@email.de

María C. Veiga Faculty of Sciences, Campus da Zapateira, University of La Coruña, Rua da Fraga 10, 15008 La Coruña, Spain

Luying Xun School of Molecular Biosciences, Washington State University, Life Sciences Building, Room 202, Pullman, WA 99164-7520, USA, e-mail: xun@mail.wsu.edu

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Chapter 1
Microbial Degradation of Polychlorophenols

Luying Xun

1.1 Introduction

Polychlorophenols are major environmental pollutants, and their degradation by microorganisms has been extensively studied for the purpose of bioremediation. Three different metabolic pathways for aerobic degradation of polychlorophenols have been completely worked out, revealing the metabolic diversity for these structurally similar compounds. Substituted quinols, rather than catechols, are key metabolic intermediates of polychlorophenol biodegradation. Substituted quinols and quinones are also called as *p*-hydroquinones and *p*-benzoquinones, reflecting the reduced and oxidized forms. For example, tetrachloroquinol is the same as tetrachloro-*p*-hydroquinone, and tetrachloroquinone is often referred as tetrachloro-*p*-benzoquinone. Characterization of individual enzymes has led to the discoveries of novel dechlorination mechanisms. The genes coding for the enzymes have been cloned and sequenced, and the gene organization and regulation suggest that recent gene recruitments have occurred for the degradation of some polychlorophenols.

1.1.1 Sources of Polychlorophenols

Trichlorophenols can be naturally produced, but pentachlorophenol (PCP) is anthropogenic in origin. Hoekstra et al. (1999) have reported the production of 2,4,6-trichlorophenol (2,4,6-TCP) and 2,4,5-trichlorophenol (2,4,5-TCP) as well as

L. Xun (✉)
School of Molecular Biosciences, Washington State University,
Life Sciences Building, Room 202, Pullman, WA 99164-7520, USA
e-mail: xun@mail.wsu.edu

S. N. Singh (ed.), *Microbial Degradation of Xenobiotics*,
Environmental Science and Engineering, DOI: 10.1007/978-3-642-23789-8_1,

less chlorinated phenols from spiked $Na^{37}Cl$ in soils of a Douglas fir forest. However, tetrachlorophenols and pentachlorophenol (PCP) are not produced from the spiked ^{37}Cl after one year in situ incubation. There is no evidence of natural production of PCP. PCP is manufactured either by phenol chlorination with chlorine gas or alkaline hydrolysis of hexachlorobenzene, producing a technical grade of PCP that contains other polychlorophenols as impurities (WHO 1987). Merz and Weith first synthesized PCP in 1872 (Merz and Weith 1872). The massive release of PCP into the environment is mainly associated with its use as a wood preservative, a practice starting in the 1930s (Crosby 1981). PCP-treated lumbers are commonly used for outdoor structures, but some have been used to build wine cellars and log houses. The vapors of polychlorophenols released from the building materials can contaminate wine, giving it a corky taste (Suckling et al. 1999), probably due to the formation of chloroanisoles (Coque et al. 2003). People living in PCP-treated log houses have elevated blood levels of PCP over control groups (Cline et al. 1989). Further, polychlorophenol derivatives are often used as herbicides and fungicides. 2,4,5-Trichlorophenoxyacetate (2,4,5-T), a derivative of 2,4,5-TCP, is a potent herbicide and is a major ingredient of "Agent Orange" used for defoliation during the Vietnam War in the 1960s (Firestone 1978). Prochloraz, a derivative of 2,4,6-TCP, is an effective fungicide for plant pathogens (Birchmore and Meneley 1979). Consequently, a wide usage of polychlorophenols and their derivatives have resulted in environmental contamination.

The main sources of polychlorophenol contamination are from their production, application and discharge. The previously uncontrolled disposal has resulted in a widespread contamination of polychlorophenols, e.g. at least 415 locations of former wood preserving facilities are contaminated with polychlorophenols (Middaugh et al. 1994). Their hazardous nature has promoted many countries to regulate their use. In the United States, the release of polychlorophenols requires registration with the Environmental Protection Agency, and the data are published in Toxic Release Inventory: Public Data Release (EPA 2006).

1.1.2 Toxicity of Polychlorophenols

Polychlorophenols are notorious for several reasons. First, they are harmful to all life forms because they disrupt the integrity and function of biological membranes (Cunarro and Weiner 1975; Escher et al. 1996). Second, their metabolites are also toxic. Human uptake of polychlorophenols is rapid via three mechanisms: skin absorption, inhalation, and ingestion (WHO 1986; Proudfoot 2003). High dose leads to hyperthermia, convulsions, and rapid death. The effects of low dose are unclear, resulting in elevated blood chlorophenol levels, which can be metabolized to chloroquinols or conjugated to polychlorophenol glucuronides for renal excretion (Uhl et al. 1986). The oxidation of chloroquinols and reduction of chloroquinones lead to the formation of reactive oxygen species, causing DNA damage (Dahlhaus et al. 1995) and other oxidative stresses (Wang et al. 2001).

Third, technical-grade polyclorophenols contain impurities, e.g. chlorinated dibenzo-*p*-dioxins and dibenzofurans, which are highly carcinogenic (Firestone 1978; Kaiser 2000). They are produced from polychlorophenols during manufacturing processes (Crosby 1981), and they can also be formed via biotransformation in soils (Hoekstra et al. 1999).

1.2 Microbial Degradation of Polychlorophenols

The most efficient and economical approach to the removal of low concentrations of polychlorophenols from contaminated soils and aquifers is bioremediation (Crawford and Mohn 1985; Lamar and Evans 1993; Miethling and Karlson 1996). The position of the chlorine substitution and the number of chlorines influence how the chlorophenols are degraded by microorganisms. Because of the presence of six isomers of trichlorophenols, three isomers of tetrachlorophenols and one pentachlorophenol, various microorganisms have evolved different strategies for the degradation of selected isomers. Bacteria can degrade polychlorophenols under both aerobic and anaerobic conditions, and fungi are able to aerobically metabolize them.

1.2.1 Pentachlorophenol Degradation by Aerobic Bacteria

Chu and Kirsch (1972) reported the first aerobic PCP-degrading bacterium in 1972. Since then, numerous aerobic bacteria that degrade PCP have been isolated from different regions around the globe. The early isolates were originally assigned to various genera, such as *Arthrobacter*, *Pseudomonas*, *Flavobacterium*, *Sphingomonas*, *Rhodococcus*, and *Mycobacterium*. The gram-positive *Rhodococcus* spp. and *Mycobacterium* spp. have been reclassified as *Mycobacterium chlorophenolicum* (Briglia et al. 1994; Haggblom et al. 1994). All the gram-negative, PCP-degrading bacteria, previously known as *Arthrobacter*, *Pseudomonas*, and *Flavobacterium*, were subsequently reclassified as *Sphingomonas chlorophenolica* strains (Crawford and Ederer 1999; Takeuchi et al. 2001), but have been subsequently renamed as *Sphingobium chlorophenolicum* strains (Takeuchi et al. 2001). A PCP-degrading *Sphingomonas* sp. strain UG30A is related to *S. chlorophenolicum* strains, but remains as a *Sphingomonas* sp. (Habash et al. 2009). A related psychrophilic PCP-degrader is *Novosphingobium lentum* MT1 (Tiirola et al. 2005). *S. chlorophenolicum* strains are the most frequently isolated bacteria that degrade PCP; however, other PCP-degrading bacteria have also been reported (Golovleva et al. 1992; Sharma et al. 2009).

1.2.2 2,4,6-Trichlorophenol Degradation by Aerobic Bacteria

Although *S. chlorophenolicum* degrades both PCP and 2,4,6-TCP (Steiert et al. 1987), *Azotobacter* sp. GP1 (Li et al. 1991) and *Ralstonia* (ex. *Pseudomonas*) *pickettii* (Kiyohara et al. 1992) use only 2,4,6-TCP as a sole carbon source. More 2,4,6-TCP degraders have since been identified and isolated: *Cupriavidus necator* (ex. *Ralstonia eutrapha*) JMP134 (Clement et al. 1995), *Sphingopyxis chilensis* (ex. *Pseudomonas paucimobilis*) S37 (Aranda et al. 1999), *Aureobacterium* sp. C964 (Bock et al. 1996), *Rhodococcus percolatus* MBS1T (Briglia et al. 1996), *Sphingobium subarctica* (Puhakka et al. 1995; Nohynek et al. 1996), *Pseudomonas* sp., *Agrobacterium* sp. (Wang et al. 2000), *Nocardioides* sp. (Mannisto et al. 1999), *Flavobacterium* sp. and *Caulobacter* sp. (Mannisto et al. 1999). It appears that the 2,4,6-TCP degrading ability is widespread among the soil bacteria.

1.2.3 2,4,5-Trichlorophenol Degradation by Aerobic Bacteria

Several bacteria are known to degrade 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). *Burkholderia* (ex *Pseudomonas*) *cepacia* AC1100, isolated from an enrichment culture, is a gram-negative bacterium that uses 2,4,5-T as a sole carbon source for the growth (Kilbane et al. 1982). The bacterium degrades 2,4,5-T with 2,4,5-TCP as the first metabolic intermediate (Karns et al. 1983). Two other *Burkholderia* spp. that degrade 2,4,5-T have recently been reported (Lü et al. 2003; Rice et al. 2005). A different 2,4,5-T degrader is *Nocardioides simplex* 3E that is a gram-positive actinomycete, able to grow on 2,4,5-T as a sole carbon source (Golovleva et al. 1990). This microorganism may have two pathways for 2,4,5-T degradation: one with 2,4,5-TCP as the first metabolic intermediate, and the other with dichlorohydroxyphenoxyacetate as the first metabolic intermediate. Since 2,4,5-TCP degradation is an integral part of 2,4,5-T degradation, the characterized pathway for 2,4,5-T degradation is reviewed here.

1.2.4 Anaerobic Degradation of Polychlorophenols

Microorganisms also degrade polychlorophenols under anaerobic conditions. Reductive dechlorination of PCP to tetrachlorophenols, trichlorophenols, dichlorophenols, and monochlorophenols was first observed in anaerobic paddy soils in the 1970s (Ide et al. 1972). The degradation has been confirmed by studies with enrichment cultures and bacterial isolates. An anaerobic bacterial consortium completely dechlorinates PCP to phenol and then mineralizes the produced phenol (Mikesell and Boyd 1986). *Desulfitobacterium frappieri* converts PCP by sequential reductive dehalogenation to 3-chlorophenol (Bouchard et al. 1996). These anaerobic bacteria use polychlorophenols as terminal electron acceptors for anaerobic

respiration to produce less substituted chlorophenols and phenol (Crawford and Mohn 1985), and these phenols are further degraded by other organisms in enrichment cultures or in the environment (Mikesell and Boyd 1986). Progress has been made towards understanding the biochemistry and genetics of reductive dechlorination of polychlorophenols (Boyer et al. 2003; Bisaillon et al. 2010).

1.2.5 Fungal Degradation of Polychlorophenols

Fungal degradation of PCP was reported as early as 1960s (Duncan and Deverall 1964), and the non-specific breakdown of PCP by fungal laccase, tyrosinase, and peroxidase was implied (Lyr 1963). Research on fungal degradation of polychlorophenols has progressed rapidly since then, especially with white-rot fungi (Reddy et al. 1998; Reddy and Gold 2000). The metabolic pathways of 2,4,6-TCP and PCP degradation have been studied with cell extracts of white-rot fungus *Phanerochaete chrysosporium* (Reddy et al. 1998; Reddy and Gold 2000), and a glutathione conjugate reductase involved in PCP degradation has been purified and characterized (Reddy and Gold 2001). *Phanerochaete* spp. have been used for the removal of PCP from contaminated soils (Lamar and Dietrich 1990; Lamar and Evans 1993) and for the disposal of PCP-treated woods (Lamar and Dietrich 1992).

1.3 Biochemistry of Polychlorophenol Degradation

The aerobic breakdown of aromatic compounds starts with monooxygenases or dioxygenases that introduce hydroxyl groups into the aromatic rings. Many aromatic compounds, including phenol, benzene and anthranilate, are converted to catechol or substituted catechols. Then intradiol or extradiol catechol dioxygenases break the aromatic rings to produce aliphatic compounds, which are further channelized into the tricarboxylic acid cycle for the complete mineralization (Harwood and Parales 1996). However, polychlorinated phenols are converted to substituted quinols before ring-cleavage: *S. chlorophenolicum* L-1 (ex. *S. chlorophenolicum* ATCC 39723) metabolizes PCP to 2,6-dichloroquinol (Cai and Xun 2002), *C. necator* JMP134 converts 2,4,6-TCP to 6-chlorohydroxyquinol (Louie et al. 2002), and *B. cepacia* AC1100 channels 2,4,5-TCP to hydroxyquinol (Zaborina et al. 1998).

1.3.1 Pentachlorophenol Metabolic Pathway of S. Chlorophenolicum L-1

PCP degradation pathways have been thoroughly investigated in *S. chlorophenolicum* L-1 and partially studied in *Mycobacterium chlorophenolicum*. Studies with cell