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Citation	大阪大学, 2009, 博士論文
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PhD Thesis

ACCELERATED DEGRADATION OF PHENOLS IN THE RHIZOSPHERE OF GIANT DUCKWEED

(ウキクサ根圏における各種フェノール類の分解促進)

HOANG HAI

Osaka University

Graduate School of Engineering

Division of Sustainable Energy and Environmental Engineering

September 2009

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(ウキクサ根圏における各種フェノール類の分解促進)

by

Hoang Hai

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Engineering

Nationality	Vietnamese
Previous Degree	Master of Science Vietnam National University, Hanoi
Scholarship donor	Ministry of Education, Science, Sports and Culture, Government of Japan (Monbukagakusho)

Osaka University

Graduate School of Engineering

Division of Sustainable Energy and Environmental Engineering

Osaka, Japan

September 2009

Contents

Chapter	Title	Page
	Contents	i
	Acknowledgements	v
	List of Publications and Conferences	vi
	List of Abbreviations	vii
	List of Figures	ix
	List of Tables	xi
1	**General Introduction**	1
	1.1 Characteristics and application of aquatic plant treatment system	1
	1.2 Concept of rhizodegradation applicable to hazardous chemical compounds	1
	1.3 Selective accumulation bacterial population responsible for aromatic compounds degradation in the rhizosphere of floating aquatic plants	2
	1.4 Objectives of the study	3
	References	5
2	**Enrichment of bacteria possessing catechol dioxygenase genes in the rhizosphere of *Spirodela polyrrhiza*: A mechanism of accelerated biodegradation of phenol**	7
	2.1 Introduction	7
	2.2 Materials and Methods	7
	2.2.1 *Spirodela polyrrhiza*	7
	2.2.2 Environmental water samples	7
	2.2.3 DNA extraction from bulk water and rhizosphere fractions	8

Chapter	Title	Page
	2.2.4 Quantification of 16S rDNA, C12O and C23O DNA by MPN-PCR	9
	2.2.5 T-RFLP analysis of 16S rDNA	9
	2.2.6 PCR-DGGE analyses of 16S rDNA, C12O and C23O DNA	9
	2.2.7 Microbial community analyses in original environmental water and in rhizosphere of *S. polyrrhiza* after acclimation to environmental water samples	10
	2.2.8 Microbial community analyses in bulk water fraction and in rhizosphere fraction of *S. polyrrhiza* during phenol degradation	11
	2.2.9 Analysis of root exudates of *S. polyrrhiza*	12
	2.2.10 Data analysis	13
	2. 3 Results	13
	2.3.1 Comparison of the bacterial communities in original environmental water and in rhizosphere fraction of *S. polyrrhiza* after acclimation	13
	2.3.2 Phenol degradation in various environmental water samples with/without *S. polyrrhiza*	18
	2.3.3 Changes in the numbers of 16S rDNA, C12O and C23O DNA during phenol degradation	19
	2.3.4 Change in the bacterial community structure in the test systems during phenol degradation	22
	2.3.5 Estimation of root exudates released by *S. polyrrhiza*	23
	2.4 Discussion	24
	2.5 Conclusion	26
	References	27

Chapter	Title	Page
3	**Accelerated degradation of a variety of aromatic compounds by *Spirodela polyrrhiza*-bacterial associations and contribution of root exudates released from *S. polyrrhiza***	29
	3.1 Introduction	29
	3.2 Materials and methods	29
	3.2.1 Chemicals	29
	3.2.2 *Spirodela polyrrhiza*	29
	3.2.3 Aromatic compound removal experiments	30
	3.2.4 Characterization of root exudates	31
	3.3 Results and discussion	32
	3.3.1 Aromatic compound degradation	32
	3.3.2 Root exudates analysis	35
	3.4 Conclusions	38
	References	39
4	**Characterization of novel 4-*n*-butylphenol degrading *Pseudomonas veronii* strains isolated from rhizosphere of *Spirodela polyrrhiza***	40
	4.1 Introduction	40
	4.2 Materials and methods	40
	4.2.1 Chemicals	40
	4.2.2 Aquatic plant	41
	4.2.3 Culture media	41
	4.2.4 4-*t*-BP degradation tests in river water microcosms with and without *S. polyrrhiza*	41
	4.2.5 Enrichment, isolation and identification of 4-*n*-butylphenol degrading bacteria	42

Chapter	Title	Page
	4.2.6 Biodegradation assays using isolated 4-*n*-BP-degrading bacteria	42
	4.2.7 Analytical procedures	43
	4.2.8 Nucleotide sequence accession numbers	44
	4.3 Results	44
	4.3.1 Accelerated degradation of 4-*t*-BP in the presence of *S. polyrrhiza*	44
	4.3.2 Enrichment, isolation and identification of BP-degrading bacteria	45
	4.3.3 4-*n*-BP degradation by strain nBP5	47
	4.3.4 Identification of metabolites and 4-*n*-BP degradation pathway	49
	4.3.5 Degradability of other APs	52
	4.4 Discussion	53
	4.5 Conclusions	54
	References	55
5	**General conclusion**	56

Acknowledgments

The author would like to express his sincere thanks and deep gratefulness to Professor Michihiko Ike for his valuable supervision, constant encouragement and support during the course of this work.

Sincerely thanks are also given to Professor Akikazu Kaga and Associate Professor Satoshi Soda for their scientific evaluation, constructive comments, and serve as members of the thesis committee.

The author would like to acknowledge Assistant Professor Kazunari Sei and Doctor Daisuke Inoue for their helpful guidance and encouragement throughout the experiments and writing phase of articles and this thesis.

The support of Associate Professor Lai Thuy Hien (Vietnamese Academy of Science and Technology, Institute of Biotechnology) and Professor Pham Hung Viet (Vietnam National University, Hanoi) is highly appreciated; it was they who constantly encouraged the author in his scientific career.

The author wishes to thank Mrs. Yuriko Yamaoka, Ms. Sali Masumoto and all the members of the Bio-Environmental Engineering Laboratory for their patience and assistance with administrative procedures and the working in the laboratory.

Many thanks are also due to Doctor Tadashi Toyama (Muroran Institute of Technology) and graduated students (Ms. Ning Yu, Mr. Naonori Momotani) for their splendid assistance and cooperation.

The support of Professor Masanori Fujita (Kochi National College of Technology) and Associate Professor Masafumi Tateda (Toyama Prefecture University) is highly appreciated during the author's stay in Japan.

Sincere thanks are given to the Ministry of Education, Science, Sports and Culture, Government of Japan (Monbukagakusho) for the awards of scholarship, which allowed the author to study in Japan.

Last but not least, the author wishes to thank his parent, parent-in-law, sister, his wife Le Thi Thanh Thuy, his daughter Hoang Ha and his Vietnamese friends for their love, sacrifice, and continual emotional support. This thesis is dedicated to his family.

List of Publications and Conferences

Publications (peer-reviewed)

1. Hoang H, Inoue D, Momotani N, Yu N, Toyama T, Sei K, Ike M, 2009. Characterization of novel 4-*n*-butylphenol degrading *Pseudomonas veronii* strains isolated from rhizosphere of giant duckweed, *Spirodela polyrrhiza*. *Japanese Journal of Water Treatment Biology*, 45, 83-92.

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3. Hoang H, Yu N, Toyama T, Inoue D, Sei K, Ike M, 2009. Accelerated degradation of a variety of aromatic compounds by *Spirodela polyrrhiza* bacterial associations and contribution of root exudates released from *S. polyrrhiza*. *Journal of Environmental Sciences*, in press.

4. Ike M, Inoue D, Toyama T, Matsunaga Y, Momotani N, Hoang H, Sei K, Soda S, 2009. Biodegradation of nonylphenol by rhizosphere of *Spirodela polyrrhiza*: isolation and characterization of nonylphenol-degrading bacteria isolated from the rhizosphere. *Journal of Environmental Conservation Engineering*, in press (in Japanese).

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2. Hoang H, Yu N, Toyama T, Inoue D, Sei K, Soda S, Ike M, 2008. Accelerated aromatic compounds degradation in the rhizosphere of *Spirodela polyrrhiza* and analysis of root exudates. *The 45th Annual Conference of Japanese Society of Water Treatment Biology*, November 12-14, Akita, Japan.

3. Inoue D, Yu N, Toyama T, Hoang H, Sei K, Soda S, Ike M, 2009. Accelerated degradation/removal of aromatic compounds by *Spirodela polyrrhiza*-rhizobacterial association, *International Symposium on Frontiers of Environmental and Industrial Biotechnology*, January 21-22, Osaka, Japan

List of Abbreviations

2,4-DCP	2,4-dichlorophenol
2-*i*-ProP	2-*i*-propylphenol
2-*n*-ProP	2-*n*-propylphenol
2-*s*-BP	2-*s*-butylphenol
2-*t*-BP	2-*t*-butylphenol
3-*i*-ProP	3-*i*-propylphenol
3-*t*-BP	3-*t*-butylphenol
4-AAP	4-aminoantipyrine
4-EP	4-ethylphenol
4-*i*-ProP	4-*i*-propylphenol
4-*n*-AmyP	4-*n*-amylphenol
4-*n*-APs	4-*n*-alkylphenols
4-*n*-BP	4-*n*-butylphenol
4-*n*-HepP	4-*n*-heptylphenol
4-*n*-HexP	4-*n*-hexylphenol
4-*n*-OP	4-*n*-octylphenol
4-*n*-NP	4-*n*-nonylphenol
4-*n*-ProP	4-*n*-propylphenol
4-*s*-BP	4-*s*-butylphenol
4-*t*-AmyP	4-*t*-amylphenol
4-*t*-BP	4-*t*-butylphenol
4-*t*-OP	4-*t*-octylphenol
APs	Alkylphenols
APTS	aquatic plant treatment system
BPs	Butylphenols
BPA	bisphenol A
BSTFA	*N*,*O*-bis(trimethylsilyl)trifluoroacetamide
C120	catechol 1,2-dioxygenase
C230	catechol 2,3-dioxygenase
CFU	colony forming unit

DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetracetic acid
GC-MS	gas chromatography-mass spectrometry
IP	Inukai Pond
KR	Katura River
LA	Lake Amagase
LB	Lake Biwa
MPN-PCR	most probable number-polymerase chain reaction
MSM	mineral salts medium
N	Nitrogen
$NH4^{+}$-N	Ammonium nitrogen
NO_3-N	nitrate nitrogen
NP	Nonylphenol
O_2	Oxygen
OD_{600}	optical density at 600 nm
OPs	Octylphenols
P	Phosphorus
PCR	Polymerase chain reaction
PO_4^{3-}-P	phosphate phosphorus
PCBs	Polychlorinated biphenyls
RE	rhizosphere effect
SPMEs	secondary plants metabolites
TAE	Tris-acetate-EDTA
TOC	total organic carbon
TMS	Trimethylsilylated
t-NP	technical nonylphenol
T-RFLP	terminal restriction fragment length polymorphism
YR	Yodo River
ZP	Zuion Pond

List of Figures

Figure	Title	Page
1.1	The schematic diagram of "rhizodegradation"	2
1.2	Schematic flow of this dissertation	3
2.1	The numbers of 16S rDNA, C12O and C23O DNA in the 6 original environmental water samples, and in rhizosphere fractions of *S. polyrrhiza* after acclimation to each environmental water sample	14
2.2	The 16S rDNA-based T-RFLP patterns of bacterial communities in the 6 original environmental water samples, in the rhizosphere fractions of *S. polyrrhiza* after acclimation, in the bulk water fractions of test system A after phenol degradation, in the rhizosphere fractions of test system A after phenol degradation, and in the bulk water fractions of test system B after phenol degradation	16
2.3	Cluster analysis and principal component analysis of the microbial communities in the 6 original environmental water samples, in the rhizosphere fractions of *S. polyrrhiza* after acclimation, in the bulk water fractions of test system A after phenol degradation, in the rhizosphere fractions of test system A after phenol degradation, and in the bulk water fractions of test system B after phenol degradation	17
2.4	PCR-DGGE banding patterns of 16S rDNA, C12O and C23O DNA of bacterial communities in Inukai Pond water and in the rhizosphere fraction of *S. polyrrhiza* after acclimation to Inukai Pond water.	18
2.5	Degradation profiles of phenol in microcosms with and without *S. polyrrhiza*	19
2.6	Changes in the numbers of 16S rDNA, C12O and C23O DNA in the bulk water and the rhizosphere fractions during phenol degradation test.	20
2.7	Chromatogram of the phenolic compounds extracted from the root exudate of sterile *S. polyrrhiza*	23
3.1	Degradation profiles of phenol, aniline, 2,4-DCP, NP and BPA in test systems A, B, C and D	33

Figure	Title	Page
3.2	HPLC chromatograms of phenolic compounds in root exudates.	37
4.1	4-*t*-BP degradation profile in river water microcosms with and without *S. polyrrhiza*	44
4.2	4-*n*-BP degradation profile in enrichment culture	45
4.3	A phylogenetic tree constructed by the neighbor-joining method based on 16S rRNA gene sequences of strains nBP2, nBP3 and nBP5, type strains of *Pseudomonas* and *Escherichia coli* and 4-alkylphenols degrading bacterial strains	46
4.4	Typical time course of 4-*n*-BP degradation and cell growth of strain nBP5 preincubated on 4-*n*-BP	47
4.5	Effect of temperature, salinity and pH on specific growth rate of strain nBP5 grown on 4-*n*-BP as the sole carbon source	47
4.6	Effect of initial 4-*n*-BP concentration on specific degradation rate of 4-*n*-BP by strain nBP5	48
4.7	Mass spectra of metabolites formed during 4-*n*-BP degradation by strain nBP5	50
4.8	Proposed 4-*n*-BP degradation pathway by strain nBP5	51
4.9	Degradability of APs of strain nBP5	52

List of Tables

Table	Title	Page
2.1	Properties of the environmental water samples used in this study	8
2.2	Changes in the relative abundance of C12O and C23O DNA before and after acclimation, and RE values for acclimation	15
2.3	Changes in the relative abundance of C12O and C23O DNA before and after phenol degradation, and RE values for phenol degradation	21
2.4	Characteristics of root exudates released by sterile *S. polyrrhiza* into the bulk water fraction and rhizosphere fraction	23
3.1	Distribution of nonylphenol in bulk water and root surface fractions and efficiency of NP removal during experimental period	34
3.2	Specific release rates of total organic carbon and phenolic compounds in the root exudates of *S. polyrrhiza*	36

Chapter 1

General introduction

1.1 Characteristics and application of aquatic plant treatment system

Finishing treatment of secondary effluent from wastewater treatment plants (WWTPs) or on-site water purification in aquatic bodies using aquatic plants (aquatic plant treatment system; APTS) is a cost-effective and environmentally-friendly technology. Especially in tropical Asia like Vietnam, as the rapid and hearty biomass growth can be expected, APTS is one of the practical and promising options for the conservation of aquatic environments. APTS has been applied all over the world, especially developing countries, to the removal of nitrogen and phosphorus to prevent eutrophication (Tripathi *et al.*, 1991; Greenway, 2003; Li *et al.*, 2009) and heavy metals to purify the contaminated sediment (Keskinkan *et al.*, 2004; Miretzky *et al.*, 2004; Mishra and Tripathi, 2008). It has also been reported that aquatic plants can contribute to the degradation/removal of easily biodegradable organic compounds (Körner *et al.*, 1998; Al-Nozaily *et al.*, 2000).

1.2 Concept of rhizodegradation applicable to hazardous chemical compounds

Some studies, mainly by our research group, have revealed that the effective degradation/removal of hazardous chemical compounds can occur at the rhizosphere of a certain kinds of aquatic plants (Federle and Schwab, 1989; Mori *et al.*, 2005; Toyama *et al.*, 2005; Toyama *et al.*, 2006). This suggests that applicability of APTS can be expanded to treat hazardous chemical compounds as well as nitrogen and phosphorus. Although the exogenous enzymatic degradation of such chemical compounds by plants themselves can not be excluded, the main mechanism is considered to be the activation of microbial degradation in the rhizosphere. As general characteristics, plants have ability to transport oxygen and to secrete physiologically active substances, such as carbohydrates, amino acids and organic acids into the rhizosphere as root exudates (Anderson *et al.*, 1993; Shaw and Burns, 2003). Phenolic compounds are also a major group of secondary plant metabolites (SPMEs) in plant root exudates (Singer *et al.*, 2003). This ability of plants, so called "rhizosphere effect", is well documented elsewhere (Kuiper *et al.*, 2004), and stimulate the bacterial chemical compounds degradation activity in the rhizosphere

(rhizodegradation). The schematic diagram of "rhizodegradation" is shown in Fig. 1.1. Recent results in our research group exhibit the superior ability of aquatic plants in aromatic compound degradation (Toyama *et al.*, 2005; Toyama *et al.*, 2006).

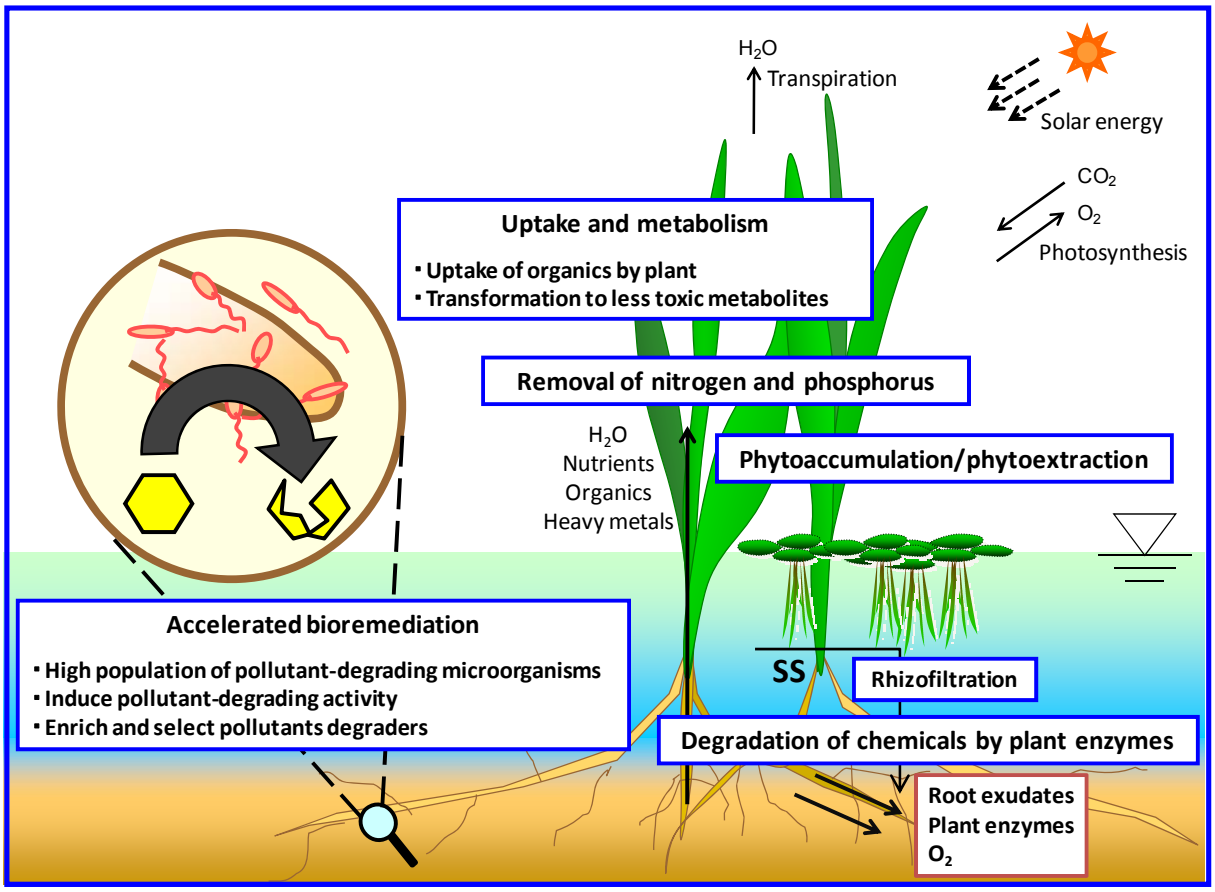

Fig. 1.1. The schematic diagram of "rhizodegradation"

1.3 Selective accumulation bacterial population responsible for aromatic compounds degradation in the rhizosphere of floating aquatic plants

One of the quite interesting observation on rhizodegradation, especially effective degradation of aromatic compounds, is the fact that bacterial population responsible for the aromatic compounds degradation are selectively and highly accumulated in the rhizosphere of giant duckweed (*Spirodela polyrrhiza*), a floating aquatic plant, even without historical exposure to anthropogenic aromatic compounds (Toyama *et al.*, 2006). As a similar phenomenon was also observed for water lettuce (*Pistia stratiotes* L.), this accelerated aromatic compounds degradation potential can be considered as a general ability of floating aquatic plants (Toyama *et al.*, 2005).

1.4 Objective of this study

Although high potential of the floating aquatic plants for the effective degradation of aromatic compounds has been suggested, there have been only a few related studies reported so far. Little is known for the practical application and for rational strategy development to make better use of APTS. Accumulation of the knowledge on the mechanisms underlying the accelerated degradation of aromatic compounds including selective accumulation of aromatic compound–degrading bacteria in the rhizosphere of aquatic plants is needed. To know what kinds of aromatic compounds can be treated by this mechanism is also a quite important information. These areas remain the focus of research for the development of novel and effective APTS for the removal of hazardous chemical compounds in addition to nitrogen, phosphorous from the aquatic environment.

This study, by selecting *S. polyrrhiza* as a model floating aquatic plant, was performed to accumulate the basic knowledge on the degradation of a variety of aromatic compounds in the rhizosphere of aquatic plants from various aspects. Aromatic compounds were selected in this study because they are frequently a major category of pollutants in aquatic environment.

The structure of this study is shown in Fig. 1.2.

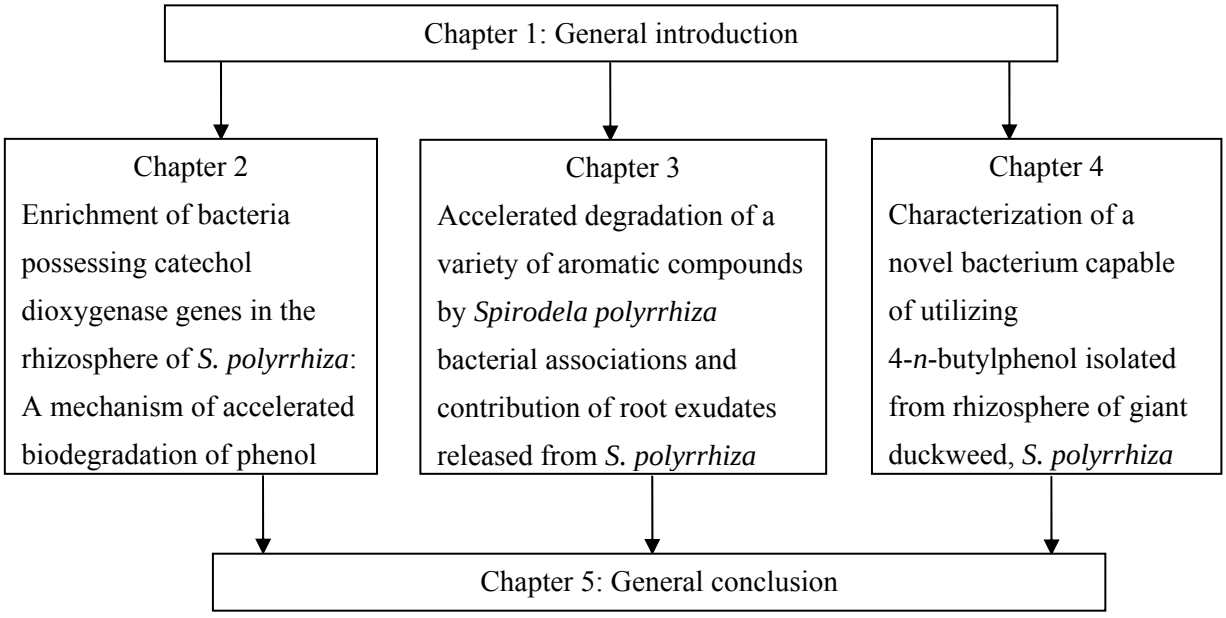

Fig. 1.2 Schematic flow of this dissertation

In Chapter 2, phenol degradation tests were performed using 6 environmental water samples with/without *S. polyrrhiza*. Accelerated phenol degradation in the rhizosphere of *S. polyrrhiza* was confirmed as general phenomena due to the selective accumulation of a variety of aromatic compounds degrading bacteria in the rhizosphere of *S. polyrrhiza*.

In Chapter 3, based on the results obtained in Chapter 2 that a variety of aromatic compounds degrading bacteria were accumulated in the rhizosphere of *S. polyrrhiza*, the possibility of degrading various aromatic compounds by use of those bacteria in the rhizosphere of *S. polyrrhiza* was investigated. Five aromatic compounds were subjected to the degradation tests with/without *S. polyrrhiza* in a natural pond water. Accelerated degradation/removal was confirmed in 4 substrates, and root exudates of the *S. polyrrhiza* were considered to stimulate the growth and activity of the bacteria responsible for their degradation.

In Chapter 4, an attempt was made to degrade 4-*tert*-butylphenol (4-*t*-BP), which is hardly degradable under the natural aquatic environment, by rhizodegradation using *S. polyrrhiza*. Seven-cycle batch degradation experiments of 4-*t*-BP in river water microcosms were performed with/without *S. polyrrhiza* and revealed that the significant 4-*t*-BP degradation occurred in the presence of *S. polyrrhiza*, which was not observed in microcosms without *S. polyrrhiza*. Further attempt to isolate 4-*t*-BP degrading bacteria, which had not been reported yet, was performed. Instead of 4-*t*-BP degrading bacteria, 4-*n*-BP degrading bacteria which had been reported only 3 strains so far (Ajithkumar *et al.*, 2003; Jeong *et al.*, 2003; Takeo *et al.*, 2006) were successfully isolated and one of the isolates, named as *Pseudomonas veronii* strain nBP5, was taxonomically identified and characterized on its 4-*n*-BP degrading ability.

In Chapter 5, the results obtained in this study were summarized with providing some new findings on rhizodegradation, and concluded that rhizodegradation by use of *S. polyrrhiza* is effective for various aromatic compounds due to the selective accumulation of a variety of aromatic compounds degrading bacteria in the rhizosphere.

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Chapter 2

Enrichment of bacteria possessing catechol dioxygenase genes in the rhizosphere of *Spirodela polyrrhiza*: A mechanism of accelerated biodegradation of phenol

2.1 Introduction

In this chapter, to obtain the useful information for elucidating the mechanisms underlying the selective accumulation of aromatic compound-degrading bacteria in the rhizosphere of *S. polyrrhiza*, degradation tests of phenol as a model aromatic compound were performed using 6 environmental water samples with/without *S. polyrrhiza* and the bacterial community structure and dynamics both in bulk water and in rhizosphere fractions was investigated in detail using PCR-based molecular methods. The C12O and C23O DNA were selected as the indexes for phenol-degrading bacteria because they encode the key enzymes for the degradation of aromatic compounds (Harayama and Rekik, 1989; Smith, 1990; Harwood and Parales, 1996; Sei et al., 1999).

2.2 Materials and methods

2.2.1 Spirodela polyrrhiza

Sterile *S. polyrrhiza* was maintained in 300-mL Erlenmeyer flasks containing 200 mL of sterile modified Hoagland nutrient solution until used for experiments (Toyama et al., 2006). *S. polyrrhiza* were statically grown in an incubation chamber at 28 ± 1 °C under fluorescent lamp at 8,000 lux (16 h of light and 8 h of dark condition).

2.2.2 Environmental water samples

Six different environmental water samples were collected from 2 lakes (Lake Biwa [LB] and Lake Amagase-dam [LA]), 2 rivers (Katura River [KR] and Yodo River [YR]) and 2 ponds (Inukai Pond [IP] and Zuion Pond [ZP]) located in Osaka, Kyoto or Shiga Prefecture, Japan. The pH and dissolved oxygen were measured at the sampling point using potable water quality checker (WQC-22A, TOA-DKK, Japan). Five hundred milliliter of the water samples were filtered (Isopore Membrane Filter, pore size 10.0 μm, Millipore, Tokyo, Japan) for analysis of the water quality (Table 2.1).

Table 2.1 Properties of the environmental water samples used in this study

Environmental water	pH [a]	Dissolved oxygen [a]	Nitrogen [b] (mg-N/L)			Phosphorus [b] (mg-P/L)		Colony forming units [b]
		(mg-O_2/L)	NH_4^+-N	NO_3^--N	Total-N	PO_4^{3-}-P	Total-P	(CFU/mL)
LB	8.7	9.7	0.05	0.02	0.61	0.01	0.02	2.2×10^4
LA	7.8	8.2	0.07	0.21	0.49	0.01	0.06	1.0×10^4
KR	7.3	7.3	0.11	2.09	2.5	0.15	0.18	1.3×10^5
YR	7.4	7.6	0.13	0.97	1.3	0.05	0.08	3.4×10^4
IP	8.6	7.6	0.05	0.79	1.16	0.01	0.02	8.0×10^3
ZP	8.3	8.6	0.06	0.57	0.76	0.02	0.04	7.2×10^3

[a] pH and dissolved oxygen concentration were measured at sampling points.

[b] Nitrogen and phosphorus concentrations and colony forming units were measured at our laboratory after filtration. Ten times diluted LB plate (1.0 g/L Bacto Peptone, 0.5 g/L Yeast Extract, 1.0 g/L NaCl, pH 7.0, 1.5 % Agar) was used for CFU measurement.

The water quality (i.e., nitrogen and phosphorus concentration) was analyzed following Standard Methods (APHA, 1998). For instance, phenate method (NH_4^+-N), ultraviolet spectrophotometric method after persulfate oxidation (total-N), and ascorbic acid method with (total-P) / without potassium peroxydisulphate oxidation (PO_4-P) were performed. Colorimetric brucine method was applied for NO_3^--N analysis following USEPA Method #352.1. The colony forming units (CFU) in the samples was measured using ten times diluted LB plate (1.0 g/L Bacto Peptone, 0.5 g/L Yeast Extract, 1.0 g/L NaCl, pH 7.0, 1.5 % Agar). These water samples were stored at 4 °C until used for experiments.

2.2.3 DNA extraction from bulk water and rhizosphere fractions

Microbes were collected from bulk water and rhizosphere fractions of *S. polyrrhiza* as previously described (Toyama et al., 2006). Briefly, 3 fronds of *S. polyrrhiza* were collected and then gently washed to remove microbes into the water sample (bulk water fraction). Then the roots were cut, transferred to a test tube containing 10 mL of 50 mg/L tripolyphosphate solution, and homogenized by vortex mixing and ultrasonication (rhizosphere fraction). DNA was extracted from each sample by the proteinase K method (Sei et al., 2000) and purified using a QIAquick PCR purification kit (Qiagen, CA, USA).

2.2.4 Quantification of 16S rDNA, C12O and C23O DNA by MPN-PCR

The copy numbers of 16S rDNA, C12O and C23O DNA in each bacterial community were measured by MPN-PCR as described by Sei et al. (2004) except that the eubacterial universal primer EUB-933f (Iwamoto et al., 2002) was used as a forward primer for 16S rDNA enumeration. The copy number of 16S rDNA was used as the index of total bacteria while the numbers of C12O and C23O DNA were used as the indices of phenol-degrading bacteria. The gene copy numbers were determined as MPN-DNA copies per milliliter for bulk water fraction and MPN-DNA copies per gram of root (wet weight) for rhizosphere fraction with the 95% confidence interval based on the cut-off probability theory of Kohno and Fukunaga (1998).

2.2.5 T-RFLP analysis of 16S rDNA

PCR was conducted using the forward primer 27F, a eubacterial universal primer labeled at the 5'-end with 6-carboxyfluorescein (6-FAM), together with the reverse primer 1392R (Amann et al., 1995). The following thermocycling conditions were used: initial denaturation at 95 °C for 5 min, 20–26 cycles of denaturation at 95 °C for 1 min, annealing at 57 °C for 1 min, extension at 72 °C for 3 min, and final extension at 72 °C for 10 min. The PCR products were purified using Microcon PCR (Qiagen) and digested with *Hha*I at 37 °C for 5 h. The resulting product (1 μL) was mixed with 12 μL of deionized formamide and 0.5 μL of GeneScan 2500 TAMRA Size Standard (Applied Biosystems, CA, USA), denatured at 95 °C for 3 min, immediately placed on ice, and analyzed by electrophoresis using an ABI PRISM 310 Genetic analyzer (Applied Biosystems) with GeneScan POP-4^{TM} capillary column (47 cm × 50 μm, Applied Biosystems). The size and the fluorescence intensity of each T-RF were automatically calculated by the GeneScan Analysis Software (version 3.7, Applied Biosystems). Bacterial community similarities were analyzed by cluster analysis with unweighted pair groups with mathematical averages (UPGMA) of Dice's coefficient of similarity (S_D) and principal component analysis (PCA) using NTSYS-pc 2.1 software (Exeter software, NY, USA).

2.2.6 PCR-DGGE analyses of 16S rDNA, C12O and C23O DNA

For PCR-DGGE analysis of 16S rDNA, PCR was conducted using the universal eubacterial primers GC-clamped-EUB-933f (EUB-933f-GC) and EUB-1387r (Iwamoto et al., 2000) as described by Sei et al. (2004). For PCR-DGGE analyses of C12O and C23O

DNA, two-step PCR was performed. The first step PCR was conducted with C12Of/C12Or or C23Of/C23Or primer sets using the following conditions: initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 1 min, annealing at 60 °C (20 cycles)/ 57 °C (15 cycles) for 30 s, extention at 72 °C for 2 min, and final extension at 72 °C for 10 min. The resultant PCR products were used as templates for the second-step PCR. The second-step PCR was conducted with GC-clamped-C12Of (C12Of-GC)/C12Or or GC-clamped-C23Of (C23Of-GC)/C23Or primer sets using the following cycling conditions: initial denaturation at 95ºC for 5 min, 35 cycles of denaturation at 95ºC for 1 min, annealing at 60ºC for 30 s (decreasing by 0.25 ºC for each cycle until 55ºC), extension at 72ºC for 2 min, and final extension at 72ºC for 10 min. The PCR products (15 μL) were loaded onto a 6% [w/v] polyacrylamide gel with denaturing gradient ranging from 25% to 50% (for 16S rDNA) or from 20% to 70% (for C12O and C23O DNA) in 0.5 × TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0). The 100% denaturant consisted of 7 M urea and 40% [v/v] formamide. The electrophoresis was conducted using the D-Code system (Bio-Rad Laboratories, CA, USA) at 60 ºC and 200 V for 5 h in 0.5 × TAE buffer. After the electrophoresis, the gel was stained with 0.5 μg/mL of ethidium bromide solution and analyzed for the PCR-DGGE banding pattern.

2.2.7 Microbial community analyses in original environmental water and rhizosphere fractions of S. polyrrhiza *after acclimation to environmental water samples*

Twenty fronds of sterile *S. polyrrhiza* were cultured in 200 mL of 6 environmental water samples in 300-mL Erlenmeyer flasks. The fronds were then statically incubated in the incubation chamber at 28 ± 1 ºC under fluorescent lamp at 8,000 lux (16 h of light and 8 h of dark condition) for 7 days to allow acclimation to each environmental water sample. The copy numbers of 16S rDNA, C12O, and C23O DNA in the original environmental water samples and in the rhizosphere of *S. polyrrhiza* after acclimation to each environmental water sample were enumerated by MPN-PCR. The relative abundances of C12O and C23O DNA was calculated as the ratios of their copy numbers of them to the copy number of 16S rDNA. The rhizosphere effect of *S. polyrrhiza* during acclimation was calculated by Equation 1, and was defined as RE value for acclimation. This value indicates the ability of *S. polyrrhiza* to accumulate bacteria possessing C12O and C23O genes in the rhizosphere during acclimation.

RE values for acclimation

$$= \frac{\text{Relative abundance of C12O and C23O DNA in the rhizosphere fraction after acclimation}}{\text{Relative abundance of C12O and C23O DNA in the original environmental water sample}} \quad (1)$$

The microbial community structure was analyzed by T-RFLP. Furthermore, PCR-DGGE analyses of 16S rDNA, C12O and C23O DNA were performed to the original Inukai Pond water sample and the rhizosphere fraction of *S. polyrrhiza* after acclimation to Inukai Pond water.

2.2.8 Microbial community analyses in the bulk water fraction and rhizosphere fraction of S. polyrrhiza *during phenol degradation*

Test system A was constructed in the 300-mL Erlenmeyer flask with 100 mL of each environmental water sample and with 20 fronds of *S. polyrrhiza*, which had been acclimated to the respective environmental water sample for 7 days as described above. Test system B was constructed with 200 mL of environmental water samples and served as the control. Phenol was added to each flask in the test systems at a final concentration of 10 mg/L. All the test systems were prepared in triplicate and statically incubated in the incubation chamber at 28 ± 1 °C under fluorescent lamp at 8,000 lux (16 h of light and 8 h of dark condition) for 3 days. The phenol concentration was periodically measured by HPLC as previously described (Toyama et al., 2006). The copy numbers of 16S rDNA, C12O and C23O DNA were enumerated by MPN-PCR as described above. The copy numbers of total DNA in the test systems were also calculated as follows: (MPN-DNA copies/mL) × (total water volume in test system; 100 mL) or (MPN-DNA copies/g-root) × (total weight of root of *S. polyrrhiza* in test system). The rhizosphere effect of *S. polyrrhiza* during phenol degradation was calculated by Equation 2 as previously described (Anderson et al., 1993, Shaw and Burns, 2003) with slight modification, and was defined as RE value for phenol degradation. This value indicates the ability of *S. polyrrhiza* to accumulate bacteria possessing C12O and C23O genes in the rhizosphere during phenol degradation.

RE values for phenol degradation

$$= \frac{\text{Relative abundance of C12O and C23O DNA in the rhizosphere fraction of test system A after phenol degradation}}{\text{Relative abundance of C12O and C23O DNA in the bulk water fraction of test system B after phenol degradation}} \quad (2)$$

2.2.9 Analysis of root exudates of S. polyrrhiza

Root exudates were collected from triplicate sterile cultures of *S. polyrrhiza*. Twenty fronds of sterile *S. polyrrhiza* were gently shaken for 10 min using rotary shaker (120 rpm) in 200 mL of sterile deionized water to flush initial root exudates from the root surface. This washing process was repeated twice, each time with 200 mL of fresh sterile deionized water. The fronds were then transferred to a 300-mL Erlenmeyer flask containing 200 mL of sterile deionized water and statically incubated in the incubation chamber for 1 day. The root exudates of *S. polyrrhiza* in bulk water and rhizosphere fractions was collected and analyzed separately. For the analysis of bulk water fraction, 200-mL sample of bulk water was collected, and 10 mL of this sample was subjected to total organic carbon (TOC) analysis. The remaining 190 mL was freeze-dried and then dissolved in 9.5 mL of deionized water for total phenolic compounds analysis. For the analysis of rhizosphere fraction, 20 fronds of *S. polyrrhiza* were transferred to a 100-mL Erlenmeyer flask containing 20 mL of sterile deionized water and gently shaken (120 rpm) for 3 min. Ten milliliter of the sample was subjected to TOC analysis, and the remaining 10 mL was subjected to total phenolic compounds analysis. The TOC concentrations were measured using a TOC analyzer (TOC-5000A, Shimadzu, Kyoto, Japan). The total concentration of phenolic compounds was measured by the 4-aminoantipyrine (4-AAP) method (APHA, 1998) with minor modifications. Briefly, each 10-mL sample was mixed with 0.06 mL of 4-AAP solution (2.0 g/L), 0.2 mL of potassium ferricyanide solution (2.0 g/L), and 0.1 mL of ammonium chloride buffer (67.5 g/L NH_4Cl, 14 % (v/v) NH_4OH, pH 10.0). The mixtures were shaken for 3 min, and the absorbance was then measured at 460 nm (A_{460}) after 5 min. Results are the mean values of triplicate samples from one experiment and are expressed as phenol equivalent using a calibration curve constructed from a standard phenol solution. The ability of *S. polyrrhiza* to secrete phenolic compounds was showed as milligrams of TOC or phenol per gram of wet root per day (mg-TOC or mg-phenolic compounds/d/g root (wet weight)). For HPLC analysis of phenolic compounds in root exudates, the Oasis HLB polymeric cartridge (500 mg/6 mL, Waters, MA, USA) was used to concentrate phenolic compounds from root exudates. The Oasis HLB cartridge was conditioned by 6 mL of *n*-hexane, ethyl acetate and methanol in turn at a flow rate of 1

mL/min. Then 50 mL of the root exudates mixture (1 : 1 of rhizosphere and bulk water fractions) acidified to pH 3.0 with 1 N HCl was passed through the cartridge at a flow rate of 5-10 mL/min. After the sorbent bed was air dried for 1.5 h, the phenolic compounds were eluted from the extraction cartridge by 6 mL of *n*-hexane, ethyl acetate, and methanol, in turn, under positive pressure at a flow rate of 1 mL/min. The sample was dried under a nitrogen atmosphere and dissolved in 200 μL of acetonitrile. The solution was subjected to HPLC analysis with ten Chromolith RP-18 columns (100 × 4.6 mm [i.d.] each; particle size, 2 μm; Merck, NJ, USA) connected in series. The mobile phase was 20 % acetonitrile, and the flow rate was 1.0 mL/min. Phenolic compounds were detected at a wavelength of 254 nm.

2.2.10 Data analysis

All results were indicated as the mean values with standard deviations (± 95% confidence interval) of triplicate experiments. Significant difference was determined by Student's *t* test with $p < 0.05$.

2.3 Results

2.3.1 Comparison of the bacterial communities in original environmental water and in rhizosphere fraction of S. polyrrhiza after acclimation

Sterile *S. polyrrhiza* was cultured in the 6 environmental samples. Bacterial communities accumulated in the rhizosphere fractions of *S. polyrrhiza* after 7-day acclimation to the 6 environmental water samples were compared with the communities in the original environmental water samples. The relative abundances of C12O and C23O DNA to 16S rDNA in the rhizosphere fractions were $1.0 \times 10^1 – 9.3 \times 10^3$ and $1.7 \times 10^2 – 1.5 \times 10^4$ times as high, respectively, as those in the original water environmental samples (RE values for acclimation) (Fig. 2.1 and Table 2.2). After acclimation, the bacterial communities in the original environmental water and in the rhizosphere fractions of *S. polyrrhiza* were assessed by T-RFLP analysis based on 16S rDNA. Although some of the T-RFs, such as 79 bp and 197 – 201 bp T-RFs, were commonly detected both in the original environmental water and in the rhizosphere fraction, the total patterns for the

rhizosphere fractions were different from those of the corresponding original environmental water. The dominant T-RFs in the rhizosphere fractions were different among the environmental water samples (Fig. 2.2). The dendrogram, representing the similarities of the T-RFs patterns among the samples, showed a clear distinction between the bacterial communities of the original environmental water and those from the rhizosphere fractions (Fig. 2.3A). The first component axis (PC1) of PCA score plots confirmed the clear distinction between the bacterial communities of the original environmental water samples and those from the rhizosphere fractions (Fig. 2.3B).

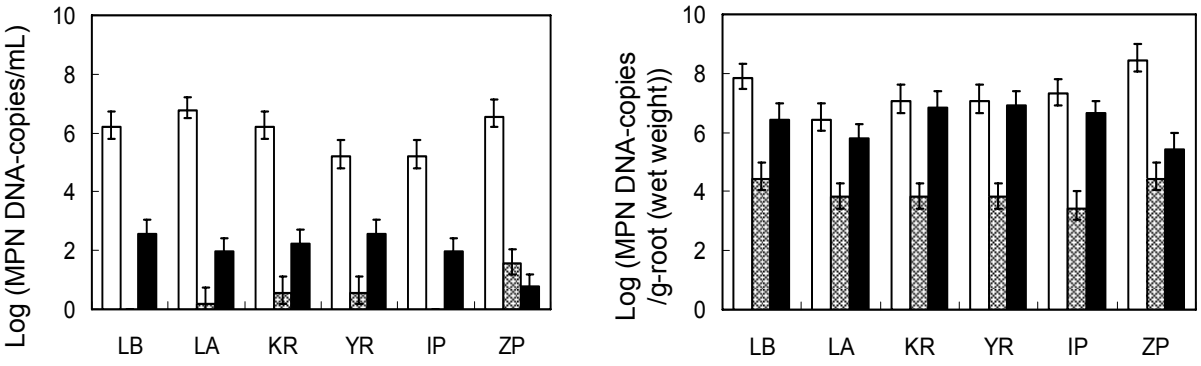

Fig. 2.1 The numbers of 16S rDNA (open bar), C12O (shaded bar) and C23O DNA (closed bar) in the 6 original environmental water samples (left), and in rhizosphere fractions of *S. polyrrhiza* after acclimation to each environmental water sample (right). LB: Lake Biwa, LA: Lake Amagese, KR: Katsura River, YR: Yodo River, IP: Inukai Pond and ZP: Zuion Pond. Error bars represent 95 % confidence intervals (n = 3).

Table 2.2 Changes in the relative abundance of C12O and C23O DNA before and after acclimation, and RE values for acclimation

	Relative abundance of C12O DNA			Relative abundance of C23O DNA			Relative abundance of C12O + C23O DNA		
	Original Water[a]	Rhizosphere fraction after acclimation[a]	RE for acclimation[b]	Original Water[a]	Rhizosphere fraction after acclimation[a]	RE for acclimation[b]	Original water[a]	Rhizosphere fraction after acclimation[a]	RE for acclimation[b]
LB	N.D.	4.0×10^{-4}	N.A.	2.3×10^{-4}	4.0×10^{-2}	1.7×10^{2}	2.3×10^{-4}	4.0×10^{-2}	1.7×10^{2}
LA	2.7×10^{-7}	2.5×10^{-3}	9.3×10^{3}	1.5×10^{-5}	2.3×10^{-1}	1.5×10^{4}	1.6×10^{-5}	2.3×10^{-1}	1.4×10^{4}
KR	2.3×10^{-6}	5.7×10^{-4}	2.5×10^{2}	1.1×10^{-4}	5.6×10^{-1}	5.1×10^{3}	1.1×10^{-4}	5.6×10^{-1}	5.1×10^{3}
YR	2.3×10^{-5}	5.7×10^{-4}	2.5×10^{1}	2.3×10^{-3}	7.1×10^{-1}	3.1×10^{2}	2.3×10^{-3}	7.1×10^{-1}	3.1×10^{2}
IP	N.D.	1.3×10^{-4}	N.A.	5.8×10^{-4}	2.1×10^{-1}	3.6×10^{2}	5.8×10^{-4}	2.1×10^{-1}	3.6×10^{2}
ZP	1.0×10^{-5}	1.0×10^{-4}	1.0×10^{1}	1.7×10^{-6}	1.0×10^{-3}	5.9×10^{2}	1.2×10^{-5}	1.1×10^{-3}	9.2×10^{1}

[a] Results are shown as MPN-C12O, -C23O or -(C12O + C23O) DNA copies / MPN-16S rDNA copies. N.D.: Not detected.

[b] Results are shown as Relative abundance of C12O, C23O or (C12O + C23O) DNA in the rhizosphere fraction / Relative abundance of C12O, C23O or (C12O + C23O) DNA in the original water. N.A.: Not available.

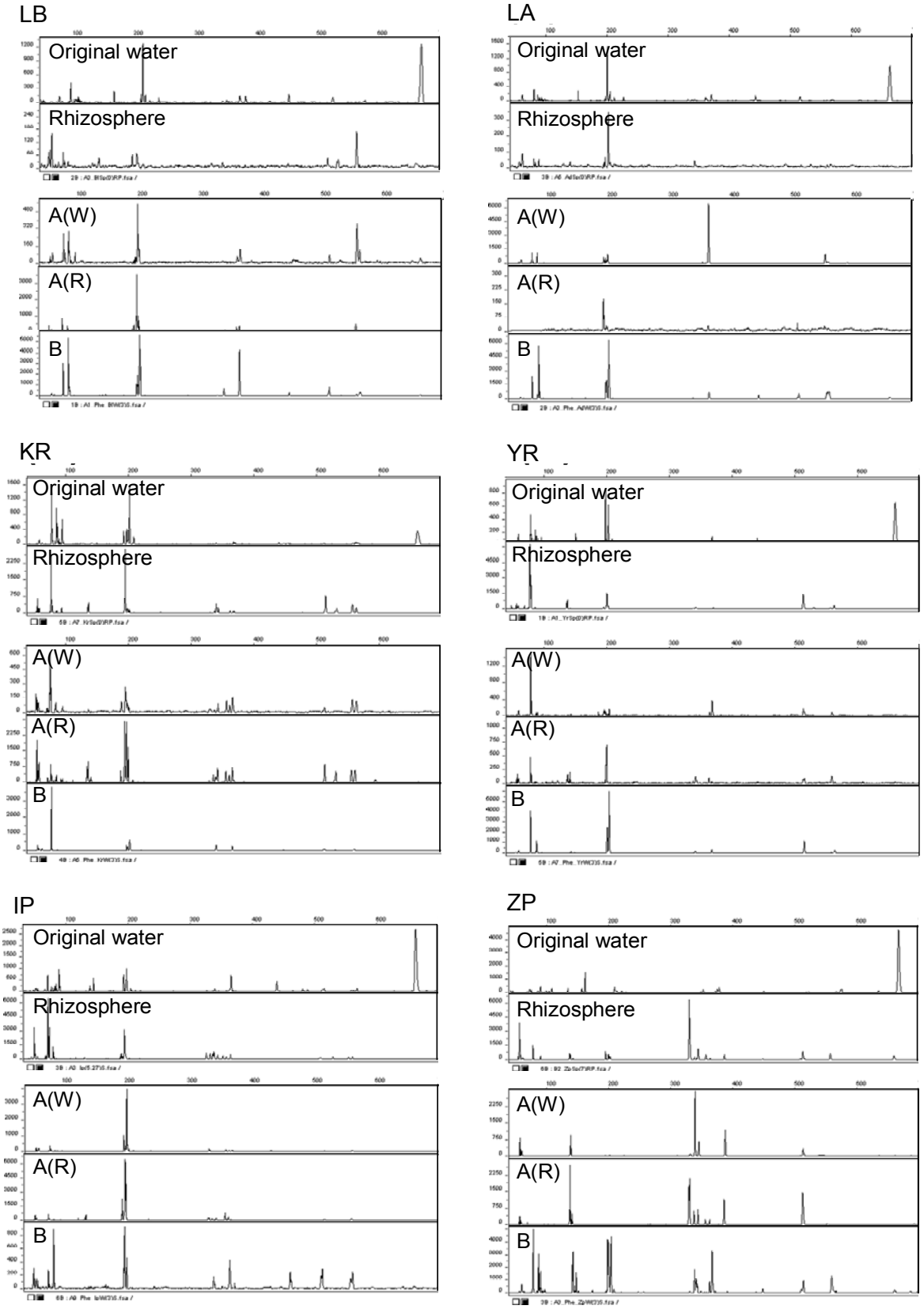

Fig. 2.2 The 16S rDNA-based T-RFLP patterns of bacterial communities in the 6 original environmental water samples (Original water), in the rhizosphere fractions of *S. polyrrhiza* after acclimation (Rhizosphere), in the bulk water fractions of test system A after phenol degradation (A(W)), in the rhizosphere fractions of test system A after phenol degradation (A(R)), and in the bulk water fractions of test system B after phenol degradation (B). LB: Lake Biwa, LA: Lake Amagese, KR: Katsura River, YR: Yodo River, IP: Inukai Pond and ZP: Zuion Pond.

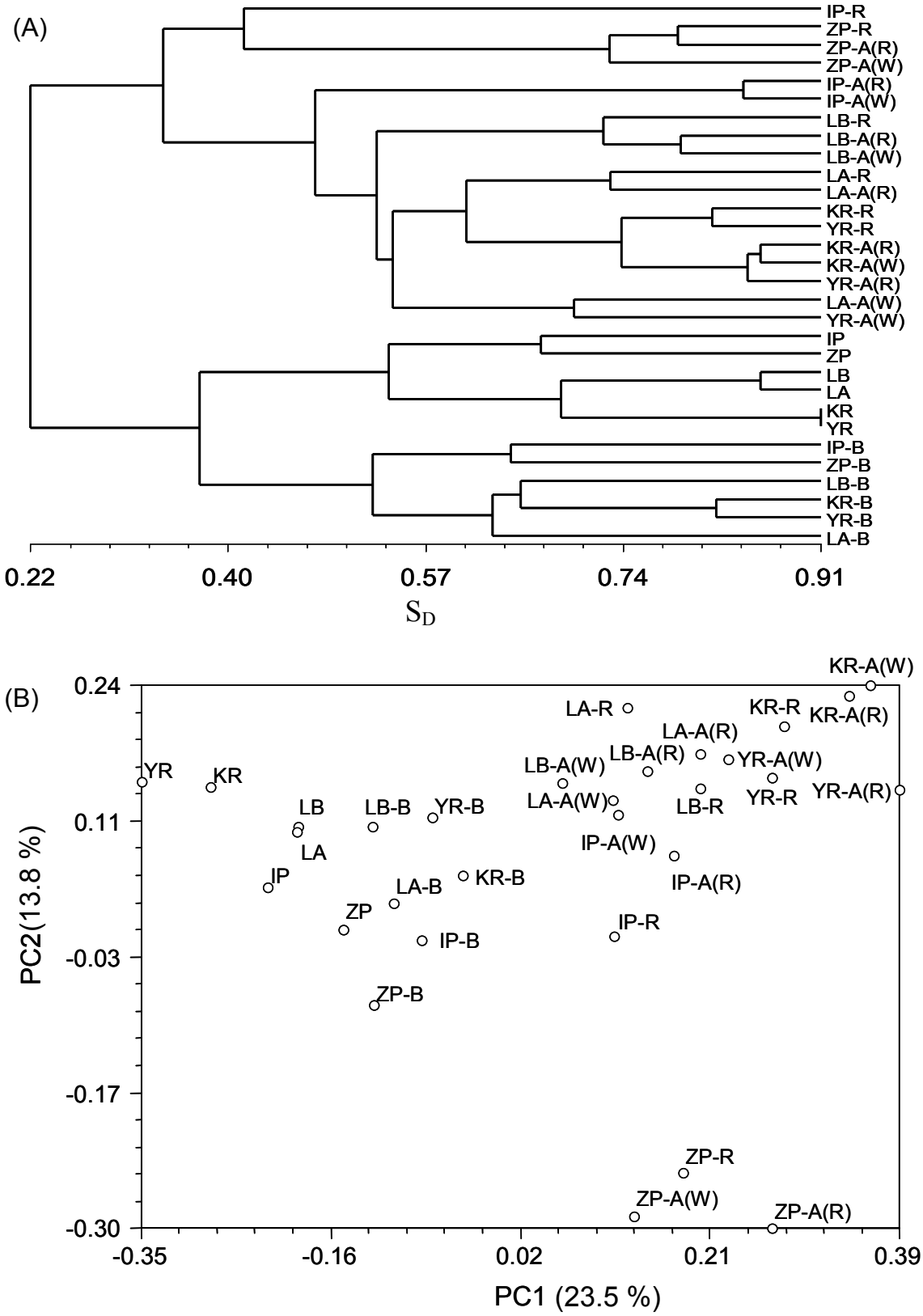

Fig. 2.3 Cluster analysis (A) and principal component analysis (B) of the microbial communities in the 6 original environmental water samples, in the rhizosphere fractions of *S. polyrrhiza* after acclimation (-R), in the bulk water fractions of test system A after phenol degradation (-A(W)), in the rhizosphere fractions of test system A after phenol degradation (-A(R)), and in the bulk water fractions of test system B after phenol degradation (-B). LB: Lake Biwa, LA: Lake Amagese, KR: Katsura River, YR: Yodo River, IP: Inukai Pond and ZP: Zuion Pond

The bacterial communities in the original water sample of the Inukai Pond and the rhizosphere fractions of *S. polyrrhiza*, acclimated to Inukai Pond water for 7 days in duplicate, were also assessed by PCR-DGGE analyses of 16S rDNA, C12O, and C23O DNA (Fig. 2.4). The PCR-DGGE banding patterns of 16S rDNA, C12O and C23O DNA in the rhizosphere fraction were notably different from those in the original pond water. Furthermore, the PCR-DGGE profiles of C12O and C23O DNA showed a relatively higher number of bands in the rhizosphere fraction than in the pond water. These phenomena showed a similar trend in the independent duplicate trials.

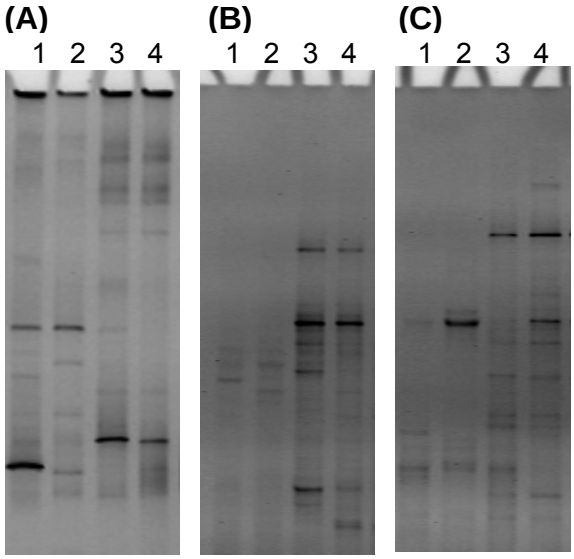

Fig. 2.4 PCR-DGGE banding patterns of 16S rDNA (A), C12O (B) and C23O DNA (C) of bacterial communities in Inukai Pond water and in the rhizosphere fraction of *S. polyrrhiza* after acclimation to Inukai Pond water. Duplicate PCR-DGGE banding patterns from the same DNA template were indicated. Lanes 1 and 2: Inukai Pond water, lanes 3 and 4: Rhizosphere fractions of *S. polyrrhiza*.

2.3.2 Phenol degradation in various environmental water samples with/without S. polyrrhiza

Phenol degradation tests were performed in 6 environmental water samples with/without *S. polyrrhiza* (Fig. 2.5). Accelerated phenol degradation was confirmed in all the 6 environmental water samples with *S. polyrrhiza* (test system A) in comparison with those without *S. polyrrhiza* (test system B). The phenol degradation profiles were similar, despite differences in the environmental origin of the water samples. Furthermore, the phenol degradation rates for the two test systems were almost the same, and added phenol (10 mg/L) was completely removed from all samples in both test systems. However, the lag time of starting phenol degradation differed between the two test systems: 6–15 h in test system A and 40–48 h in test systems B.

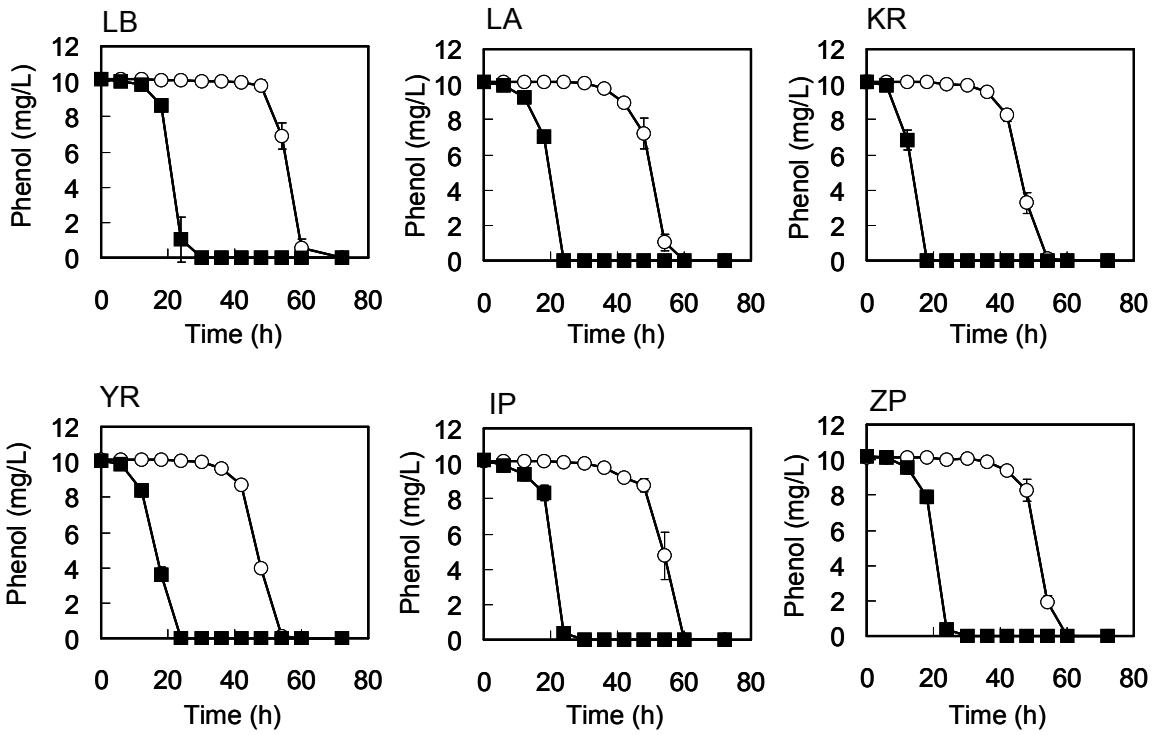

Fig. 2.5 Degradation profiles of phenol in microcosms with (test system A, closed squares) and without (test system B, open circles) *S. polyrrhiza*. Error bars represent 95 % confidence intervals (n = 3). LB: Lake Biwa, LA: Lake Amagese, KR: Katsura River, YR: Yodo River, IP: Inukai Pond and ZP: Zuion Pond.

2.3.3 Changes in the numbers of 16S rDNA, C12O and C23O DNA during phenol degradation

Figure 2.6 shows the change in the bacterial communities in the bulk water and rhizosphere fractions over the course of phenol degradation tests based on the enumeration of 16S rDNA, C12O and C23O DNA. The copy number of 16S rDNA in bulk water fraction in test system A remained $1.6 \times 10^7 – 6.0 \times 10^8$ MPN-DNA copies/test system for all the 6 environmental water samples over the 3 days of the experiment. By comparison, the copy number of 16S rDNA in the rhizosphere fraction had increase by 10^1 to 10^2 times, to almost the same level found in the bulk water fraction after 3 days. The copy numbers of C12O and C23O DNA increased markedly in both the bulk water and rhizosphere fractions within 3 days. The copy numbers of C23O DNA were always higher than those of C12O DNA in all the 6 environmental water samples.

In test system B, the 16S rDNA copy number remained relatively stable. C12O DNA was not detected in LB and ZP on day 0 but was detected in all 6 samples on day 3. The numbers of C23O DNA significantly increased within 3 days.

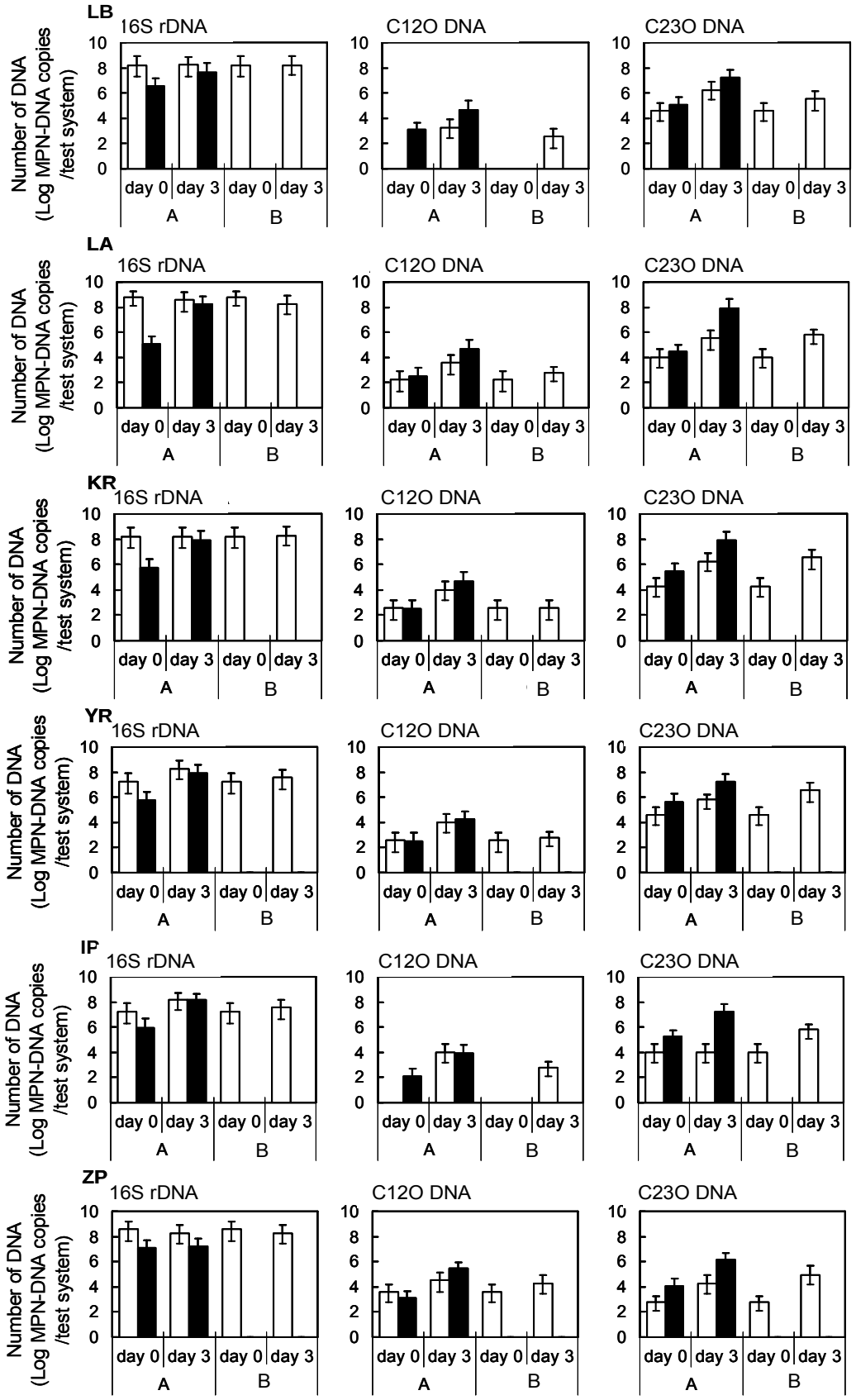

Fig. 2.6 Changes in the numbers of 16S rDNA, C12O and C23O DNA in the bulk water (open bars) and the rhizosphere (closed bars) fractions during phenol degradation test. Error bars represent 95 % confidence intervals (n = 3). LB: Lake Biwa, LA: Lake Amagese, KR: Katsura River, YR: Yodo River, IP: Inukai Pond and ZP: Zuion Pond..

Table 2.3 Changes in the relative abundance of C12O and C23O DNA before and after phenol degradation, and RE values for phenol degradation

		Relative abundance of C12O DNA					Relative abundance of C23O DNA					Relative abundance of C12O + C23O DNA				
		Day 0		Day 3		RE for degradation[b]	Day 0		Day 3		RE for degradation[b]	Day 0		Day 3		RE for degradation[b]
		W[a]	R[a]	W[a]	R[a]		W[a]	R[a]	W[a]	R[a]		W[a]	R[a]	W[a]	R[a]	
LB	A	N.D.	4.0×10^{-4}	9.4×10^{-6}	1.0×10^{-3}	4.3×10^{2}	2.3×10^{-4}	4.0×10^{-2}	9.4×10^{-3}	4.0×10^{-1}	1.7×10^{2}	2.3×10^{-4}	4.0×10^{-2}	9.4×10^{-3}	4.0×10^{-1}	1.7×10^{2}
	B	N.D.	N.A.	2.3×10^{-6}	N.A.		2.3×10^{-4}	N.A.	2.3×10^{-3}	N.A.		2.3×10^{-4}	N.A.	2.3×10^{-3}	N.A.	
LA	A	2.7×10^{-7}	2.5×10^{-3}	1.0×10^{-5}	2.5×10^{-4}	7.1×10^{1}	1.5×10^{-5}	2.3×10^{-1}	1.0×10^{-3}	4.8×10^{-1}	1.4×10^{2}	1.6×10^{-5}	2.3×10^{-1}	1.0×10^{-3}	4.8×10^{-1}	1.4×10^{2}
	B	2.7×10^{-7}	N.A.	3.5×10^{-6}	N.A.		1.5×10^{-5}	N.A.	3.5×10^{-3}	N.A.		1.6×10^{-5}	N.A.	3.5×10^{-3}	N.A.	
KR	A	2.3×10^{-6}	5.7×10^{-4}	5.8×10^{-5}	5.3×10^{-4}	2.5×10^{2}	1.1×10^{-4}	5.6×10^{-1}	1.1×10^{-2}	9.5×10^{-1}	4.5×10^{1}	1.1×10^{-4}	5.6×10^{-1}	1.1×10^{-2}	9.5×10^{-1}	4.5×10^{1}
	B	2.3×10^{-6}	N.A.	2.1×10^{-6}	N.A.		1.1×10^{-4}	N.A.	2.1×10^{-2}	N.A.		1.1×10^{-4}	N.A.	2.1×10^{-2}	N.A.	
YR	A	2.3×10^{-5}	5.7×10^{-4}	5.4×10^{-5}	2.2×10^{-4}	1.3×10^{1}	2.3×10^{-3}	7.1×10^{-1}	3.5×10^{-3}	2.2×10^{-1}	2.2×10^{0}	2.3×10^{-3}	7.1×10^{-1}	3.6×10^{-3}	2.2×10^{-1}	2.2×10^{0}
	B	2.3×10^{-5}	N.A.	1.7×10^{-5}	N.A.		2.3×10^{-3}	N.A.	1.0×10^{-1}	N.A.		2.3×10^{-3}	N.A.	1.0×10^{-1}	N.A.	
IP	A	N.D.	1.3×10^{-4}	5.8×10^{-5}	6.1×10^{-5}	3.6×10^{0}	5.8×10^{-4}	2.1×10^{-1}	5.8×10^{-5}	1.3×10^{-1}	7.6×10^{0}	5.8×10^{-4}	2.1×10^{-1}	1.1×10^{-4}	1.3×10^{-1}	7.6×10^{0}
	B	N.D.	N.A.	1.7×10^{-5}	N.A.		5.8×10^{-4}	N.A.	1.7×10^{-2}	N.A.		5.8×10^{-4}	N.A.	1.7×10^{-2}	N.A.	
ZP	A	1.0×10^{-5}	1.0×10^{-4}	2.1×10^{-4}	1.7×10^{-2}	1.7×10^{2}	1.7×10^{-6}	1.0×10^{-3}	1.0×10^{-4}	7.8×10^{-2}	1.4×10^{2}	1.2×10^{-5}	1.1×10^{-3}	3.1×10^{-4}	9.5×10^{-2}	1.5×10^{2}
	B	1.0×10^{-5}	N.A.	1.0×10^{-4}	N.A.		1.7×10^{-6}	N.A.	5.4×10^{-4}	N.A.		1.2×10^{-5}	N.A.	6.5×10^{-4}	N.A.	

[a] Results are shown as MPN-C12O, -C23O or -(C12O + C23O) DNA copies / MPN-16S rDNA copies. W: Bulk water fraction, R: Rhizosphere fraction, N.D.: Not detected, N.A.: Not available.

[b] Results are shown as Relative abundance of C12O, C23O or (C12O + C23O) DNA in the rhizosphere fraction on Day 3 in test system A / Relative abundance of C12O, C23O or (C12O + C23O) DNA in the bulk water fraction on Day 3 in test system B.

The relative abundances of C12O and C23O DNA to 16S rDNA on days 0 and 3 were summarized in Table 2.3. During the 3-day phenol degradation experiments, in most of the test systems, the relative abundance of C12O and C23O DNA, both in bulk water and rhizosphere fractions, increased by 10^1 to 10^2 times. The relative abundance of C23O DNA was always higher than that of C12O DNA.

The rhizosphere effect of *S. polyrrhiza* during phenol degradation processes is summarized in Table 2.3. The RE values for phenol degradation by the different water samples were $3.6 \times 10^0 - 4.3 \times 10^2$ for C12O DNA and $2.2 \times 10^0 - 1.7 \times 10^2$ for C23O DNA. These results indicate that *S. polyrrhiza* has the ability to selectively accumulate bacteria possessing C12O and C23O DNA during phenol degradation, although this effect is greater during acclimation.

2.3.4 Change in the bacterial community structure in the test systems during phenol degradation

T-RFLP analyses of the bacterial community structure during phenol degradation (Fig. 2.2) showed that the T-RFs common to most samples of original environmental water and the rhizosphere fraction of *S. polyrrhiza* after acclimation (i.e., 79 bp and 197-201 bps) were maintained in most samples in test systems A and B after phenol degradation. Interestingly, the total T-RFs patterns were similar in the bulk water fraction and in the rhizosphere fraction in test system A, but differed markedly from those of test system B. The dendrogram showed clear differences in the T-RFLP pattern between the samples in test system A and those in test system B (Fig. 2.3(A)). The bacterial community structures in test system A were closely related to those in the rhizosphere fraction of *S. polyrrhiza* after acclimation (i.e., before phenol degradation). By comparison, the bacterial community structures in test system B were closely related to those in the original environmental water samples. PCA score plots again showed a clear distinction in the bacterial community structure between test systems A and B by PC1 (Fig. 2.3(B)). PC1 clearly showed that the bacterial community structures of samples in test system B clearly differed from those in the original environmental water after acclimation, while there was no clear difference in the bacterial community structures of samples in the rhizosphere fraction of *S. polyrrhiza* after acclimation, and in the bulk water and rhizosphere fractions of test system A after phenol degradation.

2.3.5 Estimation of root exudates released by S. polyrrhiza

The root exudate released by sterile *S. polyrrhiza* not exposed to any anthropogenic compound was analyzed (Table 2.4). Specific release rates of TOC in bulk water and rhizosphere fractions were 1,270 ± 110 and 250 ± 75 mg/d/g-root (wet weight), respectively, while those of phenolic compounds in bulk water and rhizosphere fractions were 16.8 ± 2.8 and 197 ± 29 mg/d/g-root (wet weight), respectively. HPLC analysis of phenolic compounds in root exudate showed more than 100 peaks, indicating that a highly diverse phenolic compounds were released by *S. polyrrhiza* (Fig. 2.7).

Table 2.4 Characteristics of root exudates released by sterile *S. polyrrhiza* into the bulk water fraction and rhizosphere fraction

	Distribution of released root exudates (mg/d/g-root (wet weight))[a]		Root exudates releasing rate (mg/d/g-root (wet weight))[a]
	Bulk water fraction	Rhizosphere fraction	
TOC	1,270 ± 110	250 ± 75	1,520 ± 185
Phenolic compounds[b]	16.8 ± 2.8	197 ± 29	214 ± 32

[a]Results are shown as mg carbon or phenolic compounds per g of wet root per day (mg/d/g-root (wet weight); specific release rate) and as mean ± 95% confidence interval (n = 3).

[b] Total phenolic compounds are shown as mg of phenol equivalent

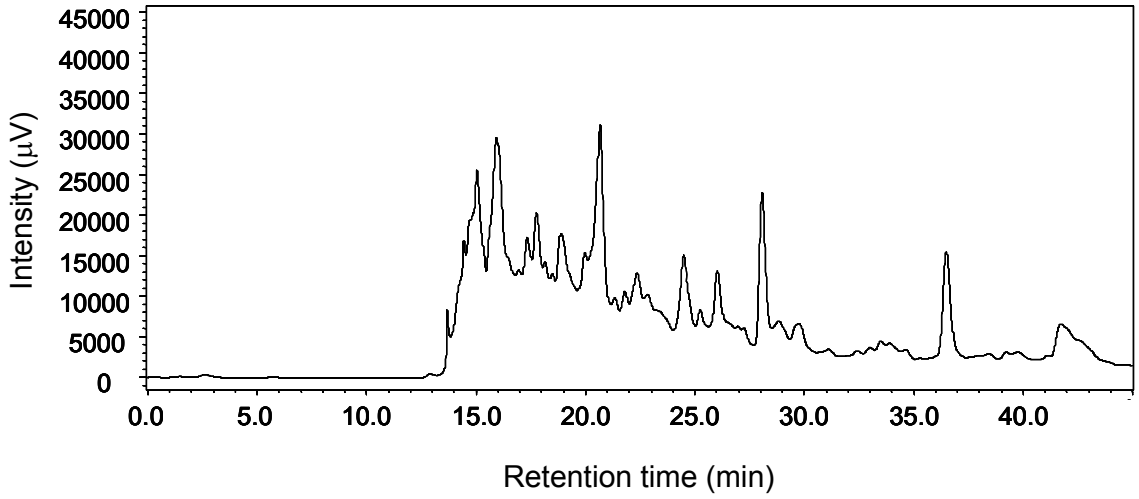

Fig. 2.7 Chromatogram of the phenolic compounds extracted from the root exudate of sterile *S. polyrrhiza*.

2.4 Discussion

Aromatic compound-degrading bacteria were highly and selectively accumulated in the rhizosphere of *S. polyrrhiza* following acclimation to the original environmental water samples (Fig. 2.1). The bacterial communities in the rhizosphere of *S. polyrrhiza* were different from those in the original environmental water samples. The dendrogram and principal component analyses clearly showed that the bacterial communities differed between the rhizosphere and original environmental water samples, and cluster analysis revealed the formation of distinct bacterial groups in the rhizosphere of *S. polyrrhiza* (Figs. 2.2 and 2.3). Interestingly, although the taxonomic profile of the bacterial communities in the rhizosphere varied according to the origin of the water samples, selective accumulation of bacteria harboring C12O and C23O DNA with considerable degree of sequence diversity was commonly observed, regardless of the water sample origin (Table 2.3 and Fig. 2.4). In other words, the rhizosphere of *S. polyrrhiza* has the ability to selectively accumulate a diverse rang of aromatic compound-degrading bacteria under natural condition, i.e., in the absence of phenol.

Accelerated phenol degradation was evident in all environmental water samples with *S. polyrrhiza* (Fig. 2.5). The main contribution of *S. polyrrhiza* was to shorten the lag time of starting phenol degradation. *S. polyrrhiza* itself has the ability to degrade/adsorb phenol but to a lesser extent that contributed by bacteria (Toyama et al., 2006). The 3-day phenol degradation test revealed that the copy numbers of C12O and C23O DNA increased both in the bulk water and rhizosphere fractions, while the copy numbers of 16S rDNA increased in the rhizosphere fraction but not in the bulk water fraction. Thus, *S. polyrrhiza* has the ability to selectively accumulate bacteria harboring C12O and C23O DNA during the phenol degradation as well as under natural conditions. Especially the copy number of C23O DNA was found to be markedly higher than that of C12O DNA in the rhizosphere fraction in all samples in both test systems (Fig. 2.6 and Table 2.3). This finding suggests that *S. polyrrhiza* tends to selectively accumulate bacteria harboring C23O DNA. Interestingly, after phenol degradation, the bacterial communities in the bulk water fraction in test system A were much affected by those in the rhizosphere fraction (Figs. 2.2 and 2.3). This results suggest that the rhizosphere of *S. polyrrhiza* functions as the accumulation

zone and provides a source of aromatic compound-degrading bacteria. Thus, it is possible that once *S. polyrrhiza* is introduced into an aquatic environment, bacteria possessing C12O and C23O DNA would accumulate in the rhizosphere and then repopulate the surrounding water.

Further, the bacterial communities after phenol degradation in the bulk water fraction without *S. polyrrhiza* (test system B) were unique and differed markedly from those in the original environmental water samples, from those in rhizosphere fraction of *S. polyrrhiza* after acclimation to each environmental water samples, or from those in the bulk water and rhizosphere fractions in test system A after phenol degradation. Thus, phenol degradation in the absence of *S. polyrrhiza* in test system B is due to indigenous phenol-degrading bacteria, and the differences in the level of phenol-degrading bacteria between test systems A and B was most likely the result of the effect of the root exudates. These differences in the bacterial communities between test systems A and B would explain the observed differences in the performance of phenol degradation.

The "rhizosphere effect" of plants to accumulate and activate bacteria in their rhizosphere is defined as the ability of plants to transport oxygen and to secrete physiologically active substances such as sugars and amino acids (Anderson et al., 1993; Shaw and Burns, 2003). However, this ability can not explain the selective accumulation of aromatic compound-degrading bacteria, especially those possessing C23O DNA in the rhizosphere of *S. polyrrhiza*. For this reason, the root exudates of *S. polyrrhiza*, which should be one of the key factors in the ability of the rhizosphere of *S. polyrrhiza* to selectively accumulate aromatic compound-degrading bacteria, were analyzed quantitatively and qualitatively. Phenolic compounds were analyzed because they represent a major group of secondary plants metabolites (SPMEs) in plant root exudates (Singer et al., 2003). The amount of organic carbon and phenolic compounds released by terrestrial plants is estimated to be 0.4–27.7 mg-TOC and 0.24–8.5 mg-phenolic compounds/g-root (wet weight) (salicylate equivalent), respectively (Kamath et al., 2004). In this study, *S. polyrrhiza* released root exudates into the rhizosphere at the specific release rate of 1,520 mg-TOC and 214 mg-phenolic compounds/d/g-root (wet weight). These results indicate that the root exudates of *S. polyrrhiza* are rich in phenolic compounds that appear to

stimulate bacteria in the rhizosphere to degrade aromatic compounds. In addition, the specific release rate of phenolic compounds on the root surface (250 mg-TOC and 197 mg-phenolic compounds/d/g-root (wet weight)) was significantly higher than those in the bulk water fraction (1,270 mg-TOC and 16.8 mg-phenolic compounds/d/g-root (wet weight)). This finding indicates that phenolic compounds released by *S. polyrrhiza* do not readily diffuse into the surrounding water and tend to remain on the root surface. This might cause the selective enrichment of bacteria possessing C12O and C23O DNA on the root surface and the accelerated degradation of phenol in the rhizosphere. Moreover, root exudates of *S. polyrrhiza* comprised a diverse range of phenolic compounds (Fig. 2.7). The accumulation of bacteria harboring a diverse range of C12O and C23O DNA in the rhizosphere of *S. polyrrhiza* in this study might have been caused by the high diversity of phenolic compounds released in root exudates. Since difference in the gene sequence results in different kinetic parameters for phenol degradation and substrate specificity, depending on their upstream or downstream genes which is correlated with phenol catabolic pathway (Nishihara et al., 1994; Watanabe et al., 1996), it is possible that *S. polyrrhiza* might accumulate a range of bacterial species with different substrate specificity for aromatic compounds and different abilities to degrade these compounds. This ability of *S. polyrrhiza* provides the possibility to isolate different kinds of aromatic compound-degrading bacteria from its rhizosphere. Aromatic ring cleavage by C12O and C23O is an essential step in the degradation of a wide range of aromatic compounds (Harayama and Rekik, 1989; Harwood and Parales, 1996; Smith, 1990). Thus, the different types of bacteria possessing C12O and C23O DNA in the rhizosphere provide a potential source of bacteria to purify aquatic environments contaminated by a range of aromatic compounds.

2.5 Conclusion

In this chapter, phenol degradation tests were performed using 6 environmental samples with/without *S. polyrrhiza*. Bacterial community structure in bulk water and in rhizosphere fractions of *S. polyrrhiza* was investigated at the same time to elucidate the mechanisms of the accelerated degradation of phenol and the selective accumulation of

phenol-degrading bacteria in its rhizosphere. Accelerated phenol degradation in the rhizosphere of *S. polyrrhiza* was confirmed as general phenomena. *S. polyrrhiza* secreted root exudates which were rich in highly diverse phenolic compounds into its rhizosphere. This ability of *S. polyrrhiza* could selectively recruit highly-diverse aromatic compounds degrading bacteria and resulted in the accelerated biodegradation of phenol. Thus *S. polyrrhiza* seemed to be very useful device for the enrichment of bacteria which can degrade various aromatic compounds.

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Chapter 3

Accelerated degradation of a variety of aromatic compounds by *Spirodela polyrrhiza*-bacterial associations and contribution of root exudates released from *S. polyrrhiza*

3.1 Introduction

Results in chapter 2 demonstrated that *S. polyrrhiza* has a great ability to selectively accumulate bacteria responsible for aromatic compounds degradation in its rhizosphere fraction. This suggests that *S. polyrrhiza*-bacterial associations may be applied for accelerated degradation of various aromatic compounds. Therefore, this chapter aimed to determine what kinds of aromatic compounds can be removed effectively by the *S. polyrrhiza*-bacterial association. To this end, the effect of planting *S. polyrrhiza* on the accelerated degradation of phenol, aniline, 2,4-dichlorophenol (2,4-DCP), nonylphenol (NP) and bisphenol A (BPA), which have often been detected in the aquatic environment and are of concern due to their toxicity, was examined. In addition, the root exudates of *S. polyrrhiza* was characterized as a possible factor that causes the accelerated degradation of these aromatic compounds.

3.2 Materials and methods

3.2.1 Chemicals

Phenol and aniline were purchased from Kishida Chemical (Osaka, Japan). 2,4-DCP, NP and BPA were purchased from Tokyo Chemical Industry (Tokyo, Japan). *n*-Hexane and ethyl acetate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile was purchased from Kanto Kagaku (Tokyo, Japan).

3.2.2 Spirodela polyrrhiza

Intact plants of *S. polyrrhiza* were obtained from an existing laboratory stock culture. They were maintained in pond water collected from Inukai Pond without significant contamination by chemicals in Osaka University Suita Campus (Osaka, Japan). Sterile *S. polyrrhiza* were obtained and maintained as described by Toyama et al. (2006). *S.*

polyrrhiza were statically grown in an incubation chamber at 28 ± 1 °C under a fluorescent lamp at 8,000 lux (16 h-light and 8 h-dark condition).

3.2.3 Aromatic compound removal experiments

Pond water used in the aromatic compound removal experiments was collected from Inukai Pond. Four test systems were constructed for each aromatic compound. Test system A was made up of natural pond water with 30 fronds of intact *S. polyrrhiza*, to evaluate the accelerated degradation effect by whole plant-bacterial associations. Test system B consisted of natural pond water with 30 fronds of sterile *S. polyrrhiza*, to exclude the effect of bacteria in the rhizosphere (rhizobacteria) of *S. polyrrhiza*, so that the contribution of *S. polyrrhiza* to the accelerated degradation of the aromatic compounds by the stimulation of pond water microbes could be evaluated. Test system C was made up of only natural pond water, so that the contribution of pond water bacteria to the aromatic compound degradation could be evaluated. This test system was used as a control for the other test systems. Test system D was made up of sterile pond water with 30 fronds of sterile *S. polyrrhiza*, so that the contribution of *S. polyrrhiza* alone to the aromatic compound removal could be evaluated. All test systems were constructed in duplicate using 300 mL of pond water in 500-mL Erlenmeyer flasks. Phenol, aniline and BPA were amended to a final concentration of 10 mg/L, while 2,4-DCP and NP were amended to 5 mg/L. Control microcosms without the addition of the aromatic compounds were also prepared. All microcosms were statically incubated in an incubation chamber at 28 ± 1 °C under a fluorescent lamp at 8,000 lux (16 h-light and 8 h dark condition) for 3 (phenol and aniline) or 5 (2,4-DCP, NP and BPA) days.

During the experiments, the concentrations of the aromatic compounds were monitored periodically by HPLC. For phenol, aniline and BPA amended systems, an aliquot (1 mL) of sample from each microcosm was centrifuged (20,000×*g*, 4 ºC, 10 min), and the supernatant was subjected to HPLC analysis. For NP amended systems, 0.5 mL of the bulk water fraction was mixed with an equal volume of acetonitrile and centrifuged, and the supernatant was subjected to HPLC analysis. In addition, three fronds of *S. polyrrhiza* were shaken (300 rpm, 10 min) in 5 mL of acetonitrile and NP adsorbed on the surface of *S. polyrrhiza* was dissolved in acetonitrile. The solution was then centrifuged

(20,000×*g*, 4 ºC, 10 min), and the supernatant was subjected to HPLC analysis. HPLC analysis was conducted using a Shimadzu LC-10A*vp* HPLC system (Shimadzu, Kyoto, Japan) equipped with a Shim-pack VP-ODS column (150 (phenol, aniline, 2,4-DCP and BPA) or 250 (NP) × 4.6 mm [i.d.]; particle size, 5 μm; Shimadzu). Mobile phases of 50%, 70%, 90% and 50% acetonitrile were used for phenol, aniline, NP and BPA analyses, respectively, while 2% acetic acid in 60% acetonitrile was used for 2,4-DCP analysis. The flow rate of the mobile phase was 0.7 mL/min for NP analysis and 1.0 mL/min for the other compounds. Detection was carried out at 270, 254, 225, 277 and 254 nm for phenol, aniline, 2,4-DCP, NP and BPA, respectively.

3.2.4 Characterization of root exudates

To characterize the root exudates of *S. polyrrhiza* exposed to aromatic compounds, root exudates were collected from triplicate sterile cultures of *S. polyrrhiza* with and without exposure to phenol, aniline, 2,4-DCP or BPA. Twenty fronds of sterile *S. polyrrhiza* gently washed in sterile MilliQ water to flush initial root exudates were statically incubated in 200 mL of sterile modified Hoagland nutrient solution (Toyama *et al.*, 2006) amended with 5 mg/L of phenol, aniline, 2,4-DCP or BPA for 3 days. Then *S. polyrrhiza* was washed twice with sterile MilliQ water and statically incubated in 50 mL of sterile MilliQ water for 1 day. Root exudates were also obtained from sterile *S. polyrrhiza* without any chemical exposure. The root exudates of *S. polyrrhiza* in bulk water and rhizosphere fractions were collected and analyzed separately. For the analysis of the bulk water fraction, a 200 mL sample of bulk water was collected, and 10 mL of the sample was subjected to total organic carbon (TOC) analysis. The remaining 190 mL was freeze-dried, dissolved in 9.5 mL of MilliQ water and subjected to total phenolic compounds analysis. For the analysis of the rhizosphere fraction, 20 fronds of *S. polyrrhiza* were shaken in 20 mL of sterile MilliQ water on a rotary shaker (120 rpm) for 3 min. Ten milliliters of the sample was subjected to TOC analysis, and the remaining 10 mL was subjected to total phenolic compounds analysis. The TOC concentration was measured using a TOC analyzer (TOC-5000A, Shimadzu). The total phenolic compounds concentration was measured by the 4-aminoantipyrine method (APHA, 1998) with minor modifications, using a calibration curve drawn for the standard phenol solution. The ability of *S.*

polyrrhiza to secrete phenolic compounds was shown as milligrams of TOC or phenol per gram of wet root per day (mg-C or mg-phenolic compounds/d/g root (wet weight)). For HPLC analysis of the phenolic compounds in root exudates, 50 mL of root exudates mixture (1 : 1 of rhizosphere and bulk water fractions) acidified to pH 3.0 with 1 N HCl was passed through an Oasis HLB cartridge (500 mg/6 cc, Waters, MA, USA), which was conditioned by 6 mL of *n*-hexane, ethyl acetate and methanol in sequence, at a flow rate of 5-10 mL/min. After the sorbent bed was air dried, the phenolic compounds were eluted with 6 mL of *n*-hexane, ethyl acetate and methanol in sequence. The eluent was dried under a gentle stream of nitrogen, dissolved in 500 μL of acetonitrile and subjected to HPLC analysis with ten Chromolith RP-18 columns (100 × 4.6 mm [i.d.] each; particle size, 2 μm; Merck, NJ, USA) connected in series. The mobile phase was 20% acetonitrile with a flow rate of 1.0 mL/min. The detection was carried out at 254 nm.

3.3 Results and discussion

3.3.1 Aromatic compound degradation

Degradation profiles of the five aromatic compounds in the test systems are shown in Fig. 3.1. Accelerated removal of all the aromatic compounds except BPA was confirmed in test systems A and B, where intact and sterile *S. polyrrhiza*, respectively, were planted, in comparison to the bacterial degradation in the natural pond water (test system C) which served as a control. That is, the *S. polyrrhiza*-bacterial association can effectively remove a wide variety of aromatic compounds.

For phenol and aniline removal, test system B showed more effective removal than test system C (Figs. 3.1A and 3.1B). Because no significant removal was observed in test system D, where sterile *S. polyrrhiza* was planted in sterile pond water, over the experimental period (72 hours), this accelerated removal should be due not to adsorption or enzymatic degradation by *S. polyrrhiza* but to activation of phenol and aniline degrading bacteria in pond water and rhizospheres by *S. polyrrhiza*. Interestingly, more effective degradation of phenol and aniline was confirmed in test system A, where the intact rhizobacteria were maintained in the rhizosphere, than in test system B, where only the indigenous bacteria in the pond water were stimulated by *S. polyrrhiza*. Thus, a high

degradation ability of rhizobacteria with respect to these compounds was suggested.

In the case of 2,4-DCP, the removal tendency differed from that for phenol and aniline (Fig. 3.1C). No degradation was confirmed in the natural pond water (test system C), whereas a linear decline in the 2,4-DCP concentration was observed in test system D. This suggests that significant removal of 2,4-DCP was caused by *S. polyrrhiza* itself. Here it was confirmed that the decrease in the 2,4-DCP concentration did not depend on adsorption by *S. polyrrhiza*, suggesting degradation occurred by secretion of enzymes from the roots and not by adsorption. The removal rate of 2,4-DCP in test systems B and D was the same, while it was a little faster in test system A, suggesting the ability of rhizobacteria to degrade 2,4-DCP to a certain extent.

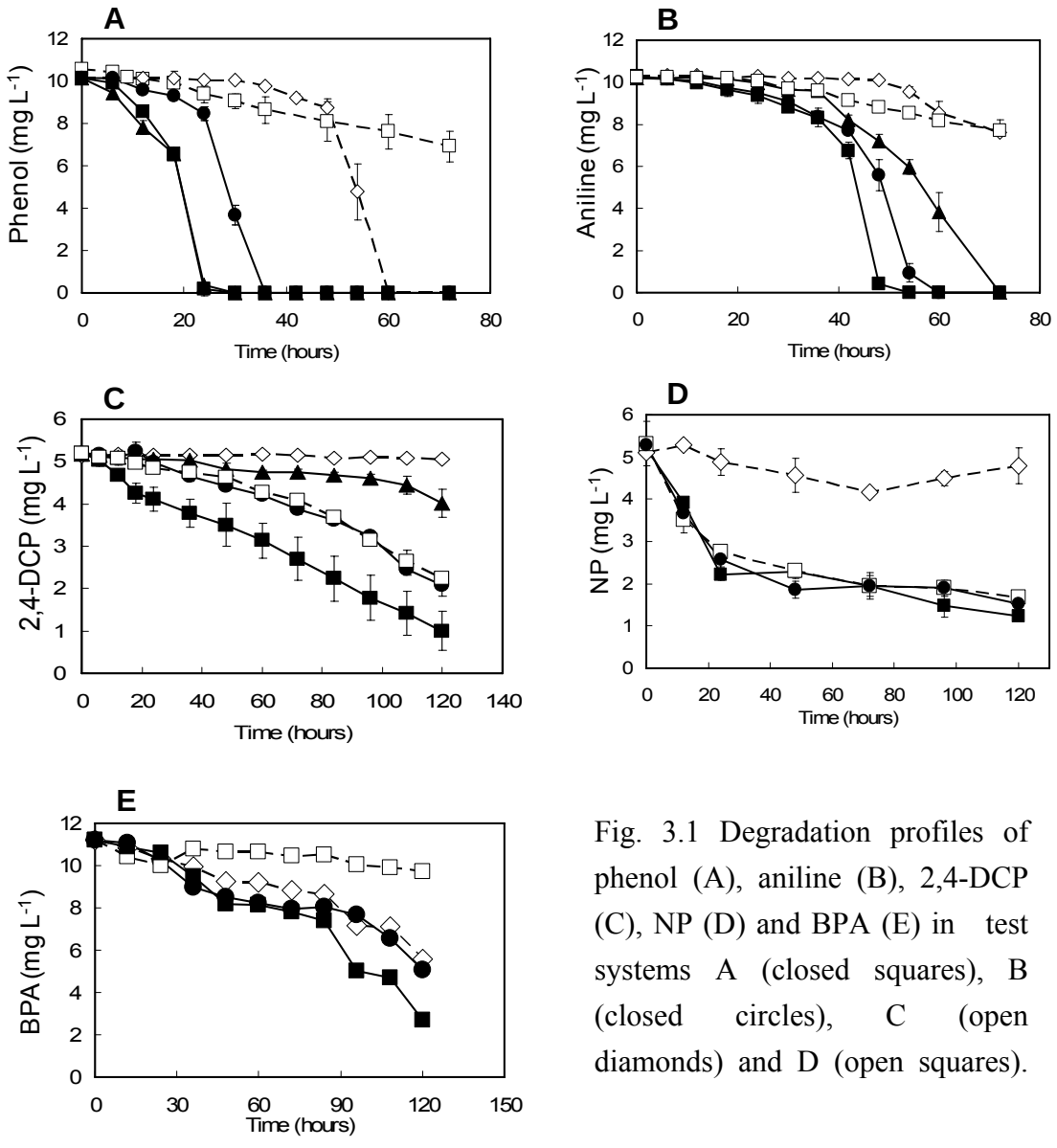

Fig. 3.1 Degradation profiles of phenol (A), aniline (B), 2,4-DCP (C), NP (D) and BPA (E) in test systems A (closed squares), B (closed circles), C (open diamonds) and D (open squares).

In the NP removal test, 48-57% of the initial NP was removed after 24 hours in test systems A, B and D, which contained sterile or non-sterile *S. polyrrhiza*. On the other hand, no significant decrease in the NP concentration in test system C was observed over the experimental period (120 hours) (Fig. 3.1D). Because the adsorptive removal of NP was suggested, the distribution of NP in bulk water and rhizosphere fractions was also analyzed, to evaluate the removal of NP by adsorption. No obvious NP removal by natural pond water (test system C) was observed during the experimental period (120 hours), whereas slight (13%) NP removal by *S. polyrrhiza* itself (test system D) was confirmed (Table 3.1). This suggests that the enzymatic degradation of NP by *S. polyrrhiza* also occurred after adsorption. When bacteria were also present (test systems A and B), the NP removal ratios from the test system were considerably higher (42% and 21% for test systems A and B, respectively) than for test system D. Thus it was confirmed that *S. polyrrhiza* activated bacterial NP degradation activity in pond water and rhizospheres, and indigenous rhizobacteria seem to have a higher ability to degrade NP.

Table 3.1 Distribution of nonylphenol (NP) in bulk water and root surface fractions and efficiency of NP removal during experimental period

Test system	Time (day)	Distribution of NP in the test system (mg-NP/L)		NP removal efficiency (%)		
		Bulk water fraction	Root surface fraction	Removal from water	Removal by adsorption	Removal by Degradation
A	0	5.2	0	0	0	0
	1	2.2	2.9	57.7	55.8	1.9
	3	2.0	1.9	61.5	36.5	25.0
	5	1.2	1.8	76.9	34.6	42.3
B	0	5.3	0	0	0	0
	1	2.6	2.7	50.9	50.9	0
	3	2.0	2.9	62.3	54.7	7.6
	5	1.5	2.7	71.7	50.9	20.8
C	0	5.1	-	0	-	0
	1	4.9	-	3.9	-	3.9
	3	4.2	-	17.6	-	17.6
	5	4.8	-	5.9	-	5.9
D	0	5.3	0	0	0	0
	1	2.8	2.6	47.2	47.2	0
	3	2.0	2.9	62.3	54.7	7.6
	5	1.7	2.9	67.9	54.7	13.2

BPA was degraded in natural pond water (test system C) while *S. polyrrhiza* (test system D) did not remove significant amounts of BPA over the experimental period (120 hours) (Fig. 3.1E). In addition, there was no obvious difference between test systems B and C, suggesting that *S. polyrrhiza* could not activate bacterial BPA degradation, although indigenous rhizobacteria might slightly degrade BPA (test system A). The first step of bacterial BPA degradation has recently been reported to require cytochrome P450 (Sasaki et al., 2005), while it is well known that hydroxylase or dioxygenase is the key enzyme for the degradation of phenol, aniline, 2,4-DCP and NP. Thus the difference between the degradation pathways should be one possible reason why accelerative degradation of BPA did not take place.

It is interesting that the accelerative degradation of all the tested aromatic compounds apart from BPA depended, at least partly, depended on the bacteria activated by *S. polyrrhiza*. These results indicate that *S. polyrrhiza* can contribute to activation of the bacterial degradation of a variety of aromatic compounds.

3.3.2 Root exudates analysis

Table 3.2 shows the amounts of TOC and phenolic compounds in root exudates of *S. polyrrhiza* exposed to the different aromatic compounds. Corresponding HPLC chromatograms of phenolic compounds are shown in Fig. 3.2. The cases in which *S. polyrrhiza* was exposed to phenol and aniline are shown as examples of the cases showing obvious activation of bacterial degradation by *S. polyrrhiza*, whereas those of 2,4-DCP and BPA are shown as examples of the case showing less and insignificant activation, respectively. Because NP was adsorbed on the rhizoplane, it was difficult to clearly characterize the root exudates from *S. polyrrhiza* exposed to NP and the results are not shown here. The root exudates of *S. polyrrhiza* not exposed to aromatic compounds were also analyzed.

S. polyrrhiza has a great ability to release phenolic compound-rich root exudates and this seemed crucial for the accelerated degradation of aromatic compounds by stimulating the catabolic bacteria in the rhizosphere (Table 3.2). In the control system, while *S. polyrrhiza* secreted 90% of the root exudates into the bulk water fraction (126.5 mg-TOC/d/g-wet root), 95% (9.9 mg-phenolic compounds/d/g-wet root) of the phenolic compounds in the root exudates were found in the rhizosphere fraction.

Table 3.2 Specific release rates of total organic carbon (TOC) and phenolic compounds in the root exudates of *S. polyrrhiza*

	TOC[a,b] (mg-C/d/g-root [wet weight])			Phenolic compounds[a,b,c] (mg-Phenol/d/g-root [wet weight])		
	Bulk water fraction	Root surface fraction	Total	Bulk water fraction	Root surface fraction	Total
Control	126.5 ± 12.4	12.5 ± 3.7	139.0 ± 16.1	0.5 ± 0.1	9.9 ± 1.5	10.3 ± 1.5
Phenol	137.0 ± 9.3	17.4 ± 2.2	154.4 ± 11.5	0.5 ± 0.1	14.2 ± 2.2	14.7 ± 2.3
Aniline	134.7 ± 15.0	19.6 ± 4.0	154.3 ± 19.0	0.7 ± 0.1	13.5 ± 1.6	14.2 ± 1.7
2,4-DCP	121.7 ± 5.9	15.4 ± 1.8	137.1 ± 7.7	0.5 ± 0.1	10.9 ± 1.2	11.4 ± 1.3
BPA	130.4 ± 5.4	9.4 ± 2.6	139.8 ± 8.0	1.7 ± 0.1	7.6 ± 1.6	9.3 ± 1.7

[a] Results are shown as mean ± 95% confidence interval (n=3).

[b] Results are shown as mg carbon or phenolics compounds per g of wet root per day (mg/d/g of wet root, specific release rate).

[c] Total phenolics compounds are shown as mg of phenol equivalent.

When *S. polyrrhiza* was exposed to phenol and aniline, the specific release rates of phenolic compounds significantly increased in the rhizosphere fraction, and as a result, the specific release rates of root exudates (TOC) in the rhizosphere fraction also increased. The HPLC chromatograms of the phenolic compounds showed almost the same pattern as the control (Figs. 3.2A, 3.2B and 3.2C).

When *S. polyrrhiza* was exposed to 2,4-DCP, no significant change was observed in the specific release rates of TOC and phenolic compounds in either the bulk water or the rhizosphere fractions. The HPLC chromatogram of phenolic compounds in the root exudates revealed a pattern that had some similarities to the control, but some major peaks that were completely different from the control were also observed (Figs. 3.2A and 3.2D).

In contrast, BPA significantly lowered the specific release rate of phenolic compounds in the rhizosphere fraction, increasing that in the bulk water fraction instead. The specific release rate of root exudates (TOC) was almost the same as that of the control (Table 3.2).

These results suggest that *S. polyrrhiza* changed the components of the phenolic compounds in the root exudates into more soluble substrates. The HPLC chromatogram of phenolic compounds in the root exudates was quite different from that of the control, which confirmed that the components of the phenolic compounds secreted by *S. polyrrhiza*

exposed to BPA were quite different from those under natural conditions. These results indicate an interesting tendency, that the amount of phenolic compounds in the root exudates increased in the rhizosphere fraction when *S. polyrrhiza* was exposed to the aromatic compounds that were effectively degraded by bacterial activation. Meanwhile, they decreased when exposed to the aromatic compounds that resulted in less effective or insignificant bacterial activation. In addition, the HPLC chromatograms imply another interesting phenomenon: when *S. polyrrhiza* is exposed to aromatic compounds that can be effectively degraded by bacterial activation, it secretes phenolic compounds with almost the same components that unexposed *S. polyrrhiza*, which is free from chemical stress, does. When *S. polyrrhiza* is exposed to aromatic compounds that result in less effective or insignificant bacterial activation, it secretes new, different phenolic compounds from those secreted by unexposed *S. polyrrhiza*.

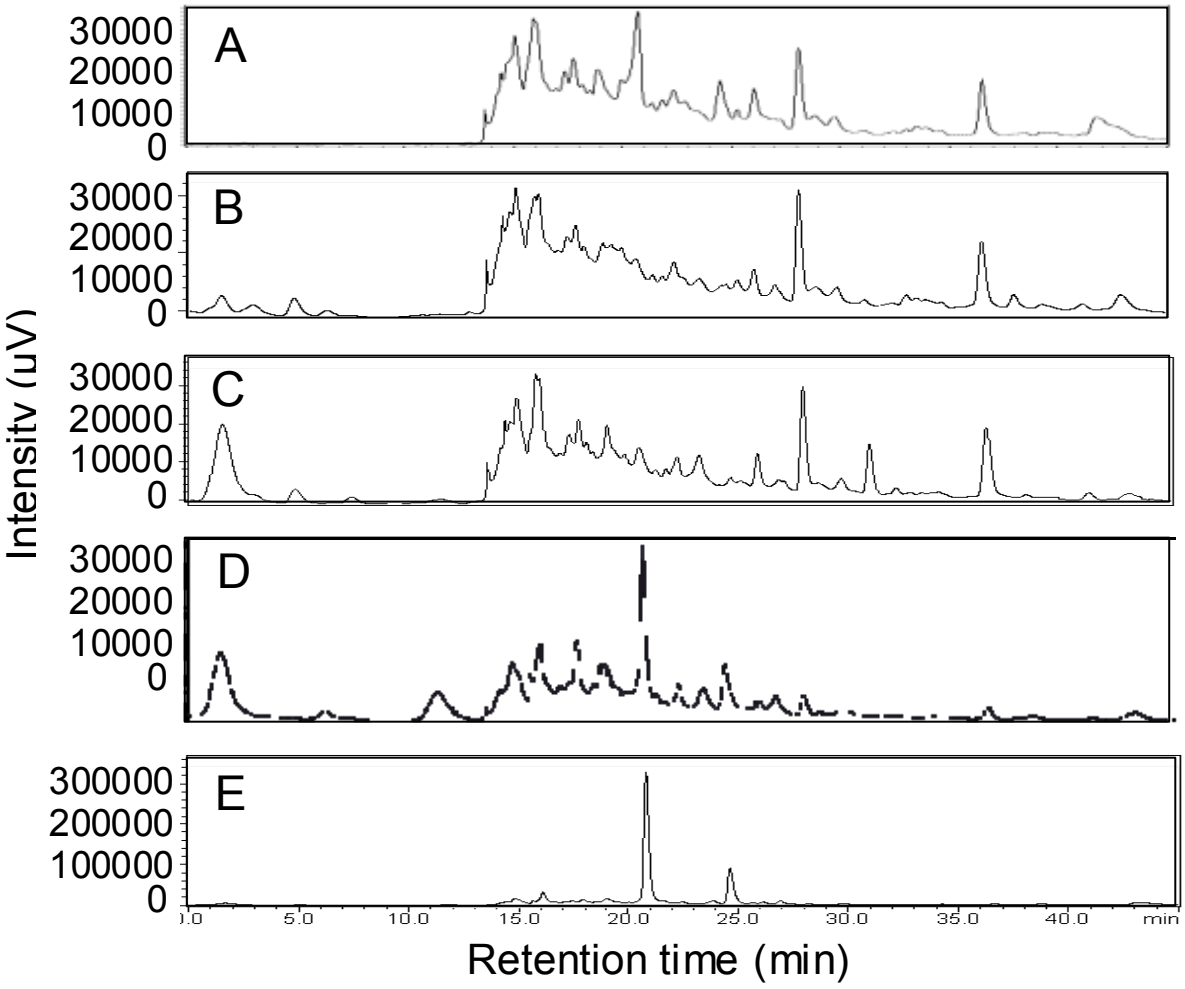

Fig. 3.2 HPLC chromatograms of phenolic compounds in root exudates. The root exudates released by sterile *S. polyrrhiza* free from chemical exposure (control) (A) and by *S. polyrrhiza* exposed to phenol (B), aniline (C), 2,4-DCP (D) and BPA (E), respectively.

These observations imply a significant contribution by the root exudates to the accelerated bacterial degradation of aromatic compounds. Identification of the key components in the root exudates is desirable to open the way for the development of accelerated bacterial degradation technologies for a variety of aromatic compounds.

3.4 Conclusions

In this chapter, the effect of planting *S. polyrrhiza* on the accelerated degradation/removal of 5 aromatic compounds, phenol, aniline, 2,4-DCP, NP and BPA, was examined to determine what kinds of aromatic compounds can be effectively removed by using an *S. polyrrhiza*-bacterial association. The root exudates of *S. polyrrhiza* were also characterized as a possible factor that causes the accelerated degradation of these aromatic compounds. Accelerated removal of all the aromatic compounds except BPA by aquatic plant-bacterial associations was confirmed. This shows that an *S. polyrrhiza*-bacterial association can effectively remove a wide variety of aromatic compounds. The acceleration of bacterial degradation by *S. polyrrhiza* was confirmed for phenol and aniline. However, enzymatic degradation and adsorption were the main factors for 2,4-DCP and NP, respectively. In these two cases, accelerated bacterial degradation was less effective. Analysis of the root exudates revealed that *S. polyrrhiza* has a great ability to release phenolic compound-rich root exudates and this seemed crucial for the accelerated degradation/removal of various aromatic compounds by stimulating/recruiting the key-role bacteria in the rhizosphere. The amounts of phenolic compounds in the root exudates increased in the rhizosphere fraction when *S. polyrrhiza* was exposed to the aromatic compounds that were effectively degraded by bacterial activation. At the same time, the HPLC chromatograms implied that *S. polyrrhiza*, when exposed to the aromatic compounds that are effectively degraded by bacterial activation, secretes phenolic compounds with almost the same components as unexposed *S. polyrrhiza*, which is free from chemical stress, secretes.

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Chapter 4

Characterization of novel 4-*n*-butylphenol degrading *Pseudomonas veronii* strains isolated from rhizosphere of *Spirodela polyrrhiza*

4.1 Introduction

4-*t*-BP is a member of alkylphenols with tertiary branched side chain of four carbon atoms (C4) at *para* position of phenol and an industrially important chemical used widely as the primary raw material in the production of synthetic phenol resins, polycarbonate, molecular weight regulator of polycarbonate resins and surfactants. The chemical has been frequently detected in the aquatic environment (Heemken et al., 2001) and has acute and chronic toxicities and cytotoxicity as well as estrogenic activity on specific aquatic organisms (Routledge and Sumpter, 1997; Hasselberg et al., 2004). However, biodegradation of 4-*t*-BP has been rarely documented, and the chemical has been believed to be very tough against biodegradation in the aquatic environment.

On the other hand, results in chapter 3 indicated that various kinds of aromatic compounds can be acceleratively degraded/removed by *S. polyrrhiza*-bacterial associations. Therefore, in this chapter, seven cycle-batch degradation experiments of 4-*t*-BP in various environment water samples were performed with/without *S. polyrrhiza* with the primary aim of evaluating the applicability of *S. polyrrhiza*-bacterial associations to 4-*t*-BP degradation. Because accelerated biodegradation occurred in the presence of *S. polyrrhiza*, further experiments were carried out for isolation and characterization of bacteria responsible for the 4-*t*-BP degradation in *S. polyrrhiza* rhizosphere. Although the enrichment culture lost the 4-*t*-BP-degrading ability by eliminating *S. polyrrhiza*, it maintained the 4-*n*-BP-degrading ability. Consequently, 3 different 4-*n*-BP-degraging bacteria were isolated from rhizosphere of *S. polyrrhiza* cultured in Kita river water sample. Therefore, one of the strains. *P. veronii* strain nBP5, was characterized on its 4-*n*-BP-degrading ability.

4.2 Materials and methods

4.2.1 Chemicals

4-ethylphenol (4-EP), 4-*n*-propylphenol (4-*n*-ProP), 4-*i*-ProP, 3-*i*-ProP, 2-*i*-ProP, 4-*s*-BP, 4-*t*-BP, 3-*t*-BP, 2-*s*-BP, 2-*t*-BP, 4-*n*-amylphenol (4-*n*-AmyP), 4-*t*-AmyP, 4-*n*-hexylphenol (4-*n*-HexP), 4-*n*-heptylphenol (4-*n*-HepP), 4-*t*-octylphenol (4-*t*-OP), technical nonylphenol (t-NP) and BSTFA (*N*,*O*-bis(trimethylsilyl)trifluoroacetamide) were purchased from Tokyo Chemical Industry (Tokyo, Japan). 4-*n*-NP was purchased from Wako Pure Chemical Industries (Osaka, Japan). 2-*n*-ProP and 4-*n*-OP were purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals were of highest grade commercially available.

4.2.2 Aquatic plant

The sterile *S. polyrrhiza* was maintained in 300 m*l* Erlenmeyer flasks containing 200 m*l* of sterile modified Hoagland nutrient solution until used for experiments as described in 2.2.1. They were statically grown in an incubation chamber at 28 ± 1 °C under fluorescent lamp at 8000 lux (16 h of light and 8 h of dark condition).

4.2.3 Culture media

Mineral salts medium (MSM), which was used for enrichment and isolation of 4-*t*-BP and 4-*n*-BP degrading bacteria from aquatic plant rhizosphere and biodegradation tests of APs, contained (per liter of water) 1.0 g of $(NH_4)_2SO_4$, 1.0 g of K_2HPO_4, 0.2 g of NaH_2PO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of NaCl, 0.05 g of $CaCl_2$, 8.3 mg of $FeCl_3 \cdot 6H_2O$, 1.4mg of $MnCl_2 \cdot 4H_2O$, 1.17 mg of $NaMoO_4 \cdot 2H_2O$, and 1mg of $ZnCl_2$. Unless otherwise noted, the pH of MSM was adjusted to 7.2. The isolates were routinely maintained on MSM containing 1.0 mM 4-*n*-BP as the sole carbon source. Solid agar medium was prepared with 20 g/L of agar.

*4.2.4 4-***t***-BP degradation tests in river water microcosms with and without* **S. polyrrhiza**

A river water sample was collected from the subsurface zone (30-to 50-cm depth) of Kita River (Fukui, Japan). The quality of the river water sample was as follows: pH, 7.3; dissolved oxygen, 7.3 mg/L; total nitrogen, 2.5 mg/L; total phosphorus, 0.18 mg/L; heterotrophic bacteria, 1.3×10^5 CFU/mL. The water sample after membrane filteration (pore size 10.0 μm; Millipore, Tokyo, Japan) was used for 4-*t*-BP degradation experiments.

Degradation tests of 4-*t*-BP were conducted in the river water microcosms with and without 20 fronds of *S. polyrrhiza*. Control microcosm made up of sterilized river water sample was also prepared. All microcosms were constructed using 100 mL of river water

sample in 300 mL-Erlenmeyer flasks. 4-*t*-BP was added to give a final concentration of 0.03 mM. Microcosms were statically incubated at 28 ± 1 °C under fluorescent lamp at 8000 lux (16 h light and 8 h dark condition) for 3 days × 7 cycles (test microcosms A and B) or 7 days × 1 cycle (control microcosm). Aliquot (1 m*l*) of each microcosm was periodically sampled and subjected to HPLC analysis to measure the concentration of 4-*t*-BP.

*4.2.5 Enrichment, isolation and identification of 4-*n*-butylphenol degrading bacteria*

First degradation tests revealed that 4-*t*-BP was degraded only in the presence of *S. polyrrhiza* (test microcosm A). Thus, enrichment of 4-*t*-BP and 4-*n*-BP degrading bacteria was carried out using the test microcosm A. The *S. polyrrhiza* roots and 10% of the river water from the 7th cycle of test microcosm A were inoculated in 300 mL-Erlenmeyer flasks containing 100 mL of MSM supplemented with 0.2 mM of 4-*t*-BP or 4-*n*-BP. The enrichment culture was at 28 °C on a rotary shaker at 120 rpm. After degradation of BP was confirmed, 10 mL of the culture was transferred to fresh MSM. In the second, third and forth subculturings, BP was added at 0.2, 0.3 and 0.5 mM, respectively. Because no obvious degradation was observed for 4-*t*-BP in the first enrichment culture, these subculturings were carried out only for 4-*n*-BP.

The final subculture was serially diluted and spread onto MSM plates amended with 1.0 mM of 4-*n*-BP. The plates were incubated at 28 ºC, and morphologically different colonies were screened for their ability to degrade 4-*n*-BP in an axenic culture. Consequently, three bacterial strains, designated strains nBP2, nBP3 and nBP5, were isolated as 4-*n*-BP-degrading bacteria. The isolated bacterial strains were characterized and identified by physiologic and phylogenetic analyses (Inoue et al., 2008).

*4.2.6 Biodegradation assays using isolated 4-*n*-BP-degrading bacteria*

Biodegradation assays were conducted using the growing cells and whole cells. Unless otherwise indicated, cultivation was carried out on a rotary shaker (120 rpm) at 28 °C. Cells of isolated 4-*n*-BP-degrading bacteria were grown to the late logarithmic phase in MSM containing 1.0 mM of 4-*n*-BP as the sole carbon source. The cells were harvested by centrifugation (9,600×*g*, 4 ºC, 10 min) and washed twice with 50 mM potassium phosphate buffer (pH 7.5). The cells were added to the turbidity, that is, the optical density at 600 nm (OD_{600}), of 0.01 (for the growing cell assays) or 0.5 (for whole cell assays) to MSM containing 1.0 mM of 4-*n*-BP or one of the following compounds as

the sole carbon source: 4-EP, 4-*n*-ProP, 4-*i*-ProP, 3-*i*-ProP, 2-*n*-ProP, 2-*i*-ProP, 4-*s*-BP, 4-*t*-BP, 3-*t*-BP, 2-*s*-BP, 2-*t*-BP, 4-*n*-AmyP, 4-*t*-AmyP, 4-*n*-HexP, 4-*n*-HepP, 4-*n*-OP, 4-*t*-OP, 4-*n*-NP or t-NP. To examine the effects of temperature, pH and salinity on the growth of isolated 4-*n*-BP-degrading bacteria, conditions in the degradation assays were changed as follows: temperature was set at 10, 15, 20, 25, 30, 35 and 40 °C; pH of MSM was adjusted to 4-8; NaCl concentration was adjusted to 0.1, 0.5, 1, 1.5, 2 and 3 % (w/v). Effect of 4-*n*-BP concentratin on the 4-*n*-BP degradation activity was examined using the whole cells at 4-*n*-BP concentrations of 0.001 to 1.0 mM. At appropriate intervals during the degradation assays, the cell density and the concentrations of substrate were measured. All tests were performed in triplicate.

4.2.7 Analytical procedures

Bacterial growth was monitored as the increase in OD_{600} of culture with a plate reader (Viento XS, Dainippon Sumitomo Pharma, Osaka, Japan). Concentration of substrates was determined by HPLC, and metabolites of 4-*n*-BP were analyzed by gas chromatography-mass spectrometry (GC-MS). For HPLC, the collected culture was acidified with phosphoric acid, centrifuged (9,600×*g*, 4 ºC, 10 min), and then the supernatant was used. HPLC analysis was conducted using a Shimadzu LC-10A*vp* HPLC system consisting of an SIL-10AF automatic sampler, LC10AD*vp* solvent delivery units, a CTO-10A*vp* column oven, and an SPD-10A*vp* UV/VIS detector (Shimadzu, Kyoto, Japan). The instrument was equipped with a Shim-pack VP-ODS column (250 × 4.6 mm [i.d.]; particle size, 5 μm; Shimadzu). Acetonitrile and water at a ratio of 8:2 was used as the mobile phase. The detection was carried out at 277 nm. For GC-MS analysis of metabolites produced by 4-*n*-BP biodegradation, the collected culture was acidified with 1 N HCl, shaken for 3 min with an equal volume of 1:1 (vol/vol) ethyl acetate/*n*-hexane and centrifuged (9,600×*g*, 4 ºC, 10 min); the organic layer was then extracted. The extract was dried under nitrogen flow and trimethylsilylated (TMS) by a BSTFA-acetonitrile solution at 60 °C for 1 h. The GC-MS analysis was conducted using a Shimadzu GC-MS system (GCMS-QP2010; Shimadzu) and a Rxi-5ms capillary column (30 m × 0.25 mm [i.d.]; 1.00 μm [df]; Nonpolar 5 % diphenyl 95 % dimetylpolysiloxane phase; Restek, Bellefonte, PA, USA). Samples were injected in splitless mode for 1 min. Helium gas (99.9999 %) was used as the carrier gas at a flow rate of 1.2 m*l*/min. The oven temperature programs were as follows: 60 ºC isothermal for 2 min, 60 to 300 ºC at 20 ºC/min and 300 ºC hold for 6

min. The MS analysis was performed at 70 eV.

4.2.8 Nucleotide sequence accession numbers

The partial 16S rRNA gene sequences of strains nBP2, nBP3 and nBP5 were registered in GenBank/EMBL/DDBJ as accession numbers AB494443, AB494444 and AB494445, respectively.

4.3 Results

4.3.1 Accelerated degradation of 4-t-BP in the presence of S. polyrrhiza

Seven cycle-batch degradation experiments of 4-*t*-BP in river water microcosms with and without *S. polyrrhiza* revealed that approximately 20% of the amended 4-*t*-BP was degraded in the presence of *S. polyrrhiza*, while no significant decrease of 4-*t*-BP was observed in microcosms without *S. polyrrhiza* (Fig. 4.1). This suggests that 4-*t*-BP can be acceleratedly degraded in the presence of *S. polyrrhiza* possibly due to the selective accumulation of organic pollutants degrading bacteria in the rhizosphere of *S. polyrrhiza* (Mori et al., 2005, Toyama et al., 2006). Such selective accumulation of specific bacteria in the *S. polyrrhiza* rhizosphere would be attributable to the rhizosphere effects such as transportation of oxygen and secretion of physiologically active root exudates.

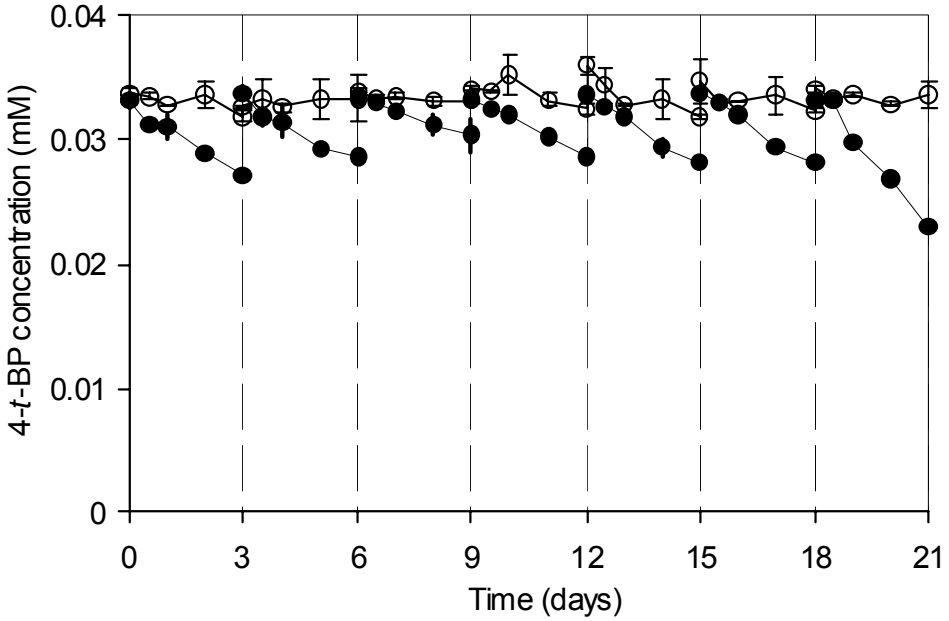

Fig. 4.1 4-*t*-BP degradation profile in river water microcosms with (closed circle) and without (open circle) *S. polyrrhiza*. Experiments were carried out for 3 days × 7 cycles. Error bars represent 95 % confidence intervals.

4.3.2 Enrichment, isolation and identification of BP-degrading bacteria

To enrich BP-degrading bacteria from the microcosm with *S. polyrrhiza* where significant 4-*t*-BP degradation was observed, roots of *S. polyrrhiza* and 10 mL of liquid were transferred to MSM supplemented with 0.2 mM of 4-*t*-BP or 4-*n*-BP at the end of the 7th cycle of degradation experiment. In the enrichment culture, no obvious reduction of 4-*t*-BP was detected, although 4-*n*-BP decreased rapidly and disappeared within 48 h (Fig. 4.2). The results suggested that the 4-*t*-BP degrading ability of our test system was largely dependent on the presence of *S. polyrrhiza* and thus the enrichment lost the 4-*t*-BP degrading ability by eliminating *S. polyrrhiza*. Therefore, we tried to obtain pure cultures of 4-*n*-BP degrading bacteria. Enrichment by successive transfers to fresh MSM containing 4-*n*-BP at 0.2, 0.3 and 0.5 mM showed that 4-*n*-BP was completely degraded without lag period (Fig. 4.2). Finally, three morphologically different colonies that were capable of utilizing 4-*n*-BP as the sole carbon source were isolated and designated strains nBP2, nBP3 and nBP5.

Three isolated strains nBP2, nBP3 and nBP5 were gram-negative, rod-shaped, motile, oxidase- and catalase-positive bacteria. Phylogenetic analysis based on partial 16S rRNA gene sequence showed that the three bacteria had the greatest 16S rRNA gene similarity with *Pseudomonas veronii* CIP104663^T (99.8%; Fig. 4.3). Based on the result, we identified the three strains as *P. veronii*.

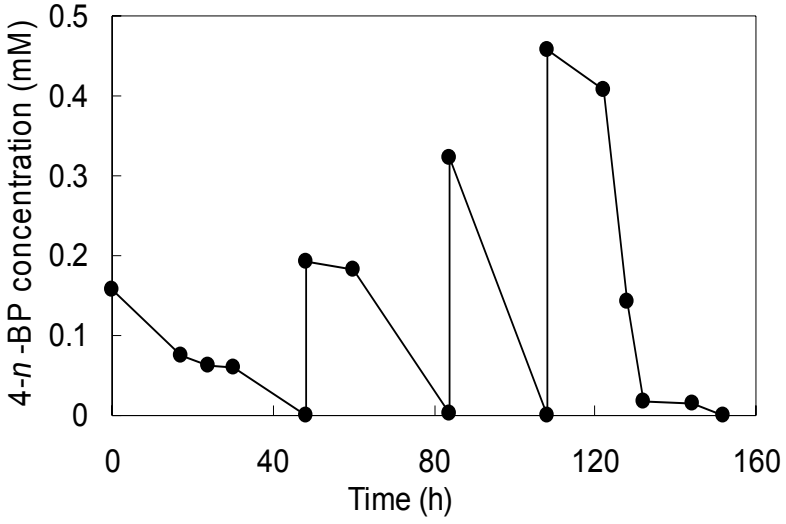

Fig. 4.2 4-*n*-BP degradation profile in enrichment culture. 4-*n*-BP concentration in the 1st, 2nd, 3rd and 4th subculturings were 0.2, 0.2, 0.3 and 0.5 mM, respectively.

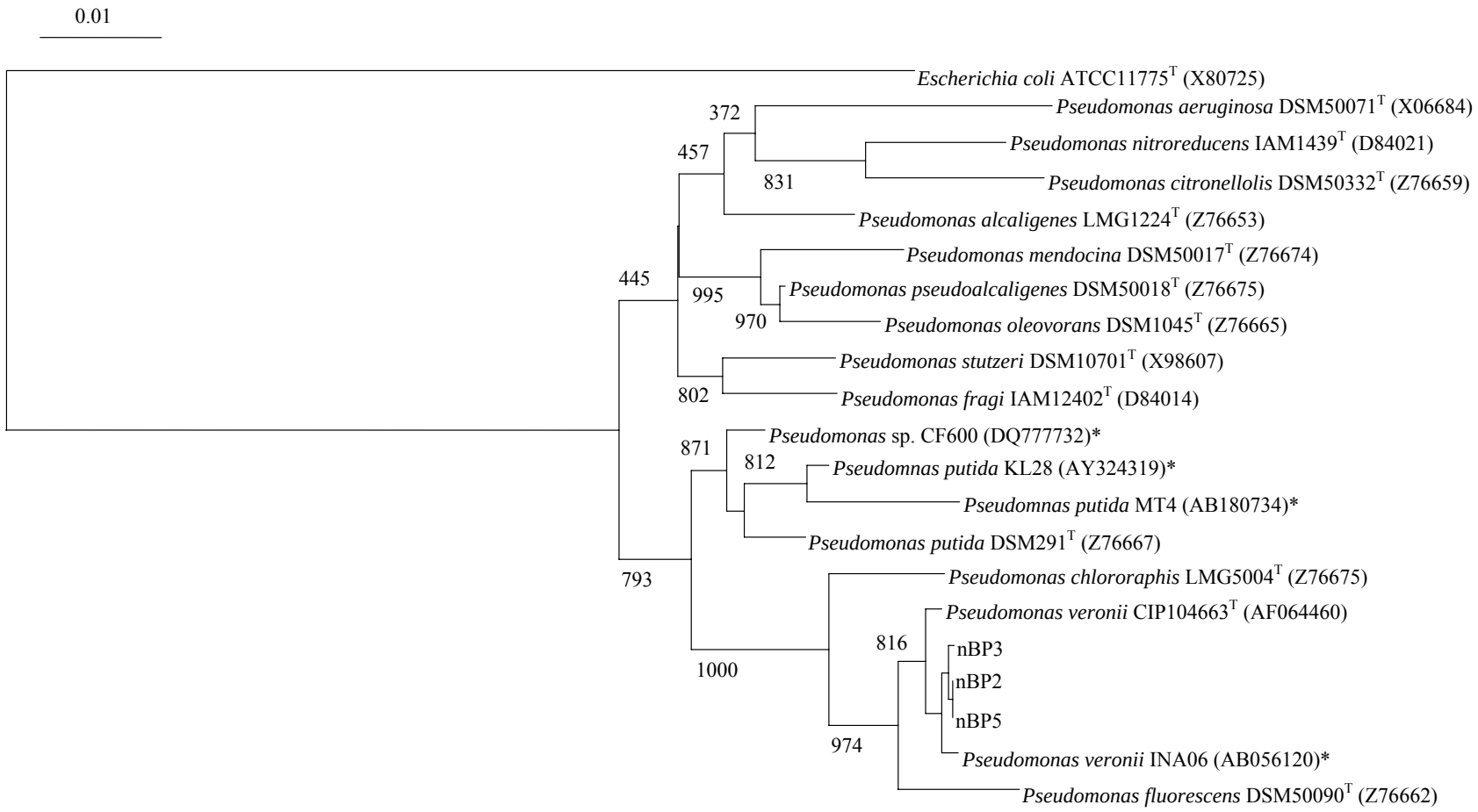

Fig. 4.3 A phylogenetic tree constructed by the neighbor-joining method based on 16S rRNA gene sequences of strains nBP2, nBP3 and nBP5, type strains of *Pseudomonas* and *Escherichia coli* and 4-alkylphenols degrading bacterial strains. 4-APs degrading strains are marked with asterisks. Numbers on the branches indicate bootstrap confidence estimates obtained with 1000 replicates. The scale bar represents evolutionary distance of 0.01.

*4.3.3 4-*n*-BP degradation by strain nBP5*

Strain nBP5 was selected for further studies because of its highest 4-*n*-BP degrading ability among the three isolates. A typical time course of cell growth and 4-*n*-BP degradation of strain nBP5 at an initial 4-*n*-BP concentration of 0.5 mM is shown in Fig. 4.4. After preincubation on 4-*n*-BP, strain nBP5 completely degraded 0.5 mM of 4-*n*-BP within 5 h without any lag period. The turbidity of the culture (OD_{600}) increased from 0.03 to 0.15 concomitantly with the degradation of 4-*n*-BP.

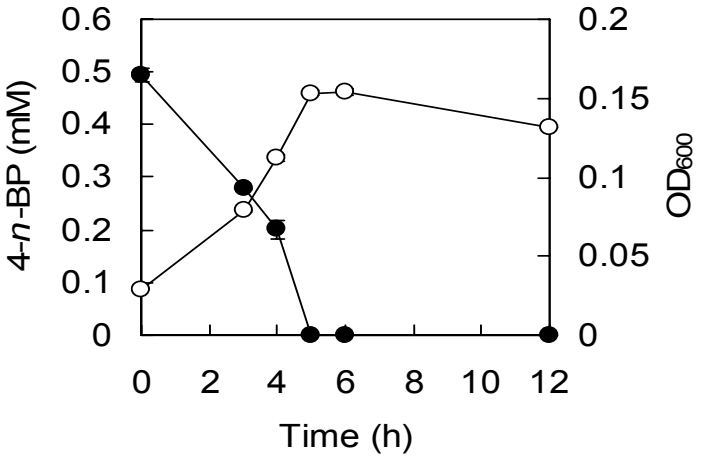

Fig. 4.4 Typical time course of 4-*n*-BP degradation (closed circle) and cell growth (open circle) of strain nBP5 preincubated on 4-*n*-BP. Error bars indicate the standard deviation of triplicate experiments.

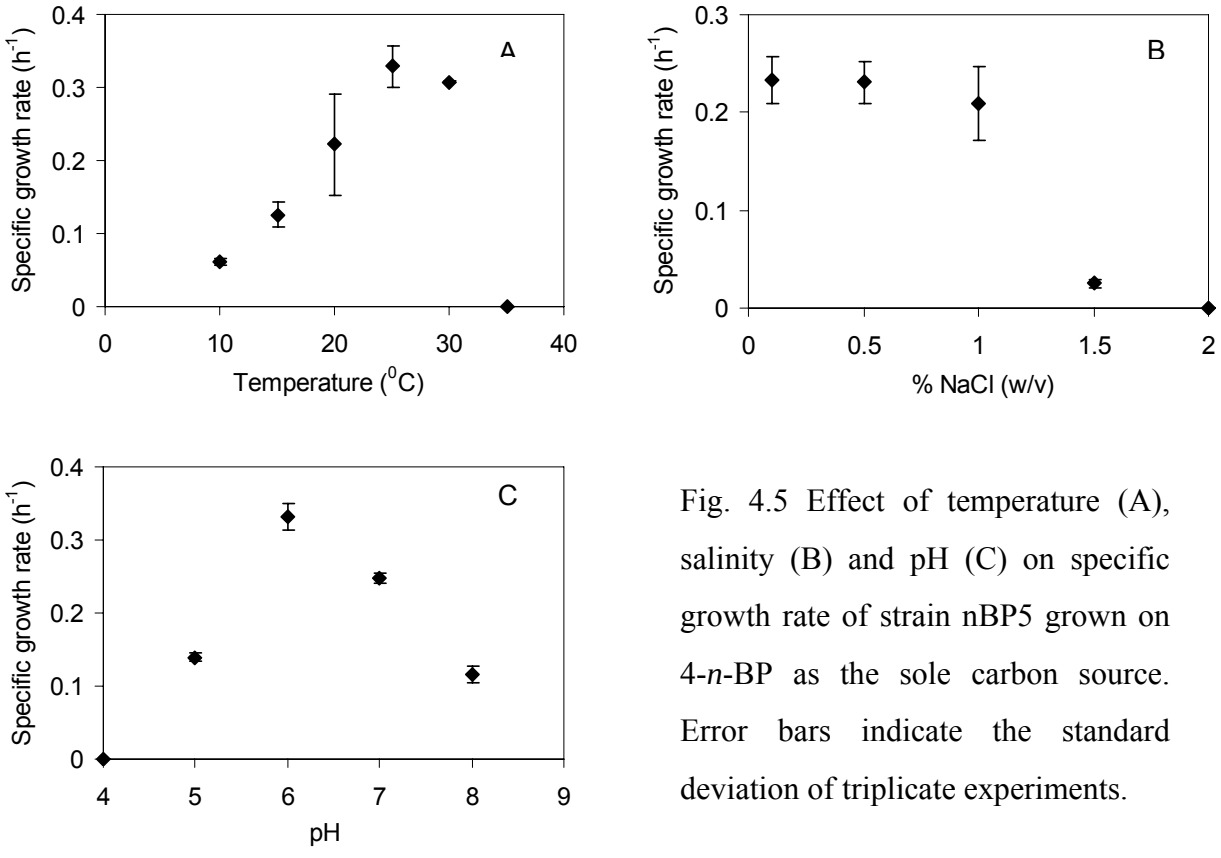

Fig. 4.5 Effect of temperature (A), salinity (B) and pH (C) on specific growth rate of strain nBP5 grown on 4-*n*-BP as the sole carbon source. Error bars indicate the standard deviation of triplicate experiments.

Effects of temperature, pH and salinity (NaCl concentration) on the growth of strain nBP5 in MSM containing 4-*n*-BP (1.0 mM) as the sole carbon source were investigated. The specific growth rate was calculated by deviding the increment of OD_{600} by time on logarithmic growth phase, and the specific growth rate under a condition was calculated as the mean in triplicate experiments. Specific growth rate of strain nBP5 under different conditions are presented in Fig. 4.5. Strain nBP5 was capable of growing on 4-*n*-BP at temperature of 10–30 °C, pH of 5–8 and NaCl concentration of 1.5 % or less. The optimal temperature, pH and NaCl concentration for growth of strain nBP5 on 4-*n*-BP were 25 °C, 6.0 and 0.1 %, respectively.

Effect of substrate (4-*n*-BP) concentration on the 4-*n*-BP degradation by strain nBP5 was examined by whole-cell assays (Fig. 4.6). Experiments were carried out at initial 4-*n*-BP concentrations of 0.001–1.0 mM. Strain nBP5 completely degraded 1.0 mM or less of 4-*n*-BP. The 4-*n*-BP degradation rate increased with increasing substrate concentration between 0.001 and 0.05 mM, and reached the highest value of 0.246 mmol/mg-cell/h at 0.05 mM. However, the degradation rate declined at 4-*n*-BP concentration of 0.075 mM or higher. The degradation rate at 1.0 mM was only 0.007 mmol/mg-cell/h (2.8 % of the highest rate). These results suggested that 4-*n*-BP concentrations more than 0.075 mM were inhibitory to the 4-*n*-BP degradation by strain nBP5.

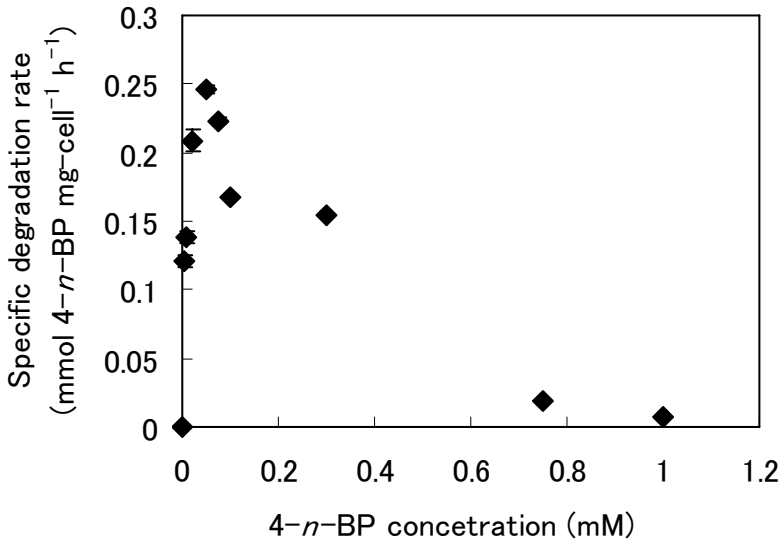

Fig. 4.6 Effect of initial 4-*n*-BP concentration on specific degradation rate of 4-*n*-BP by strain nBP5. Error bars indicate the standard deviation of triplicate experiments.

Experimental data were further analyzed to elucidate the degradation and substrate inhibition kinetics of 4-*n*-BP degradation by strain nBP5. For less than 0.075 mM of 4-*n*-BP, substrate inhibition was negligible, and the 4-*n*-BP degradation patterns could be represented by the Michaelis-Menten equation:

$$V = \frac{V_{\max} S}{K_m + S}$$

where V and V_{max} are the actual and maximum degradation rate of 4-*n*-BP degradation (mmol/mg-cell/h), S is the initial 4-*n*-BP concentration (mM) and K_m is the saturation constant (mM). From the experimental results, V_{max} and K_m were estimated to be 0.258 mmol/mg-cell/h and 0.0062 mM, respectively.

*4.3.4 Identification of metabolites and 4-*n*-BP degradation pathway*

During the 4-*n*-BP degradation by strain nBP5, no peaks responsible for metabolites were detected in HPLC-UV/VIS analysis. However, in GC-MS analysis of the ethyl acetate/*n*-hexane extracts from the cultures during the 4-*n*-BP degradation by strain nBP5, several peaks responsible for the metabolites were detected in addition to the parental compound. By interpreting the mass spectral pattern, five compounds including 4-*n*-butylcatechol (Fig. 4.7A) and its *meta*-cleavage products (Figs. 4.7B to 4.7E) were identified. Based on the metabolites identified, the pathway of 4-*n*-BP degradation by strain nBP5 was proposed as presented in Fig. 4.8. 4-*n*-BP is initially hydroxylated to 4-*n*-butylcatechol. Subsequently, 4-*n*-butylcatechol is metabolized via the *meta*-cleavage pathway. The *meta*-cleavage products are further degraded and finally mineralized. During the 4-*n*-BP degradation by strain nBP5, the culture turned yellow, and concomitantly a peak at 381 nm was detected in scanning the UV/VIS spectra (data not shown). This would be due to the accumulation of hydroxy muconic acid semialdehide derivatives (Ajithkumar et al., 2003), and the fact supported the involvement of the *meta*-cleavage of aromatic ring in the 4-*n*-BP biodegradation by strain nBP5.

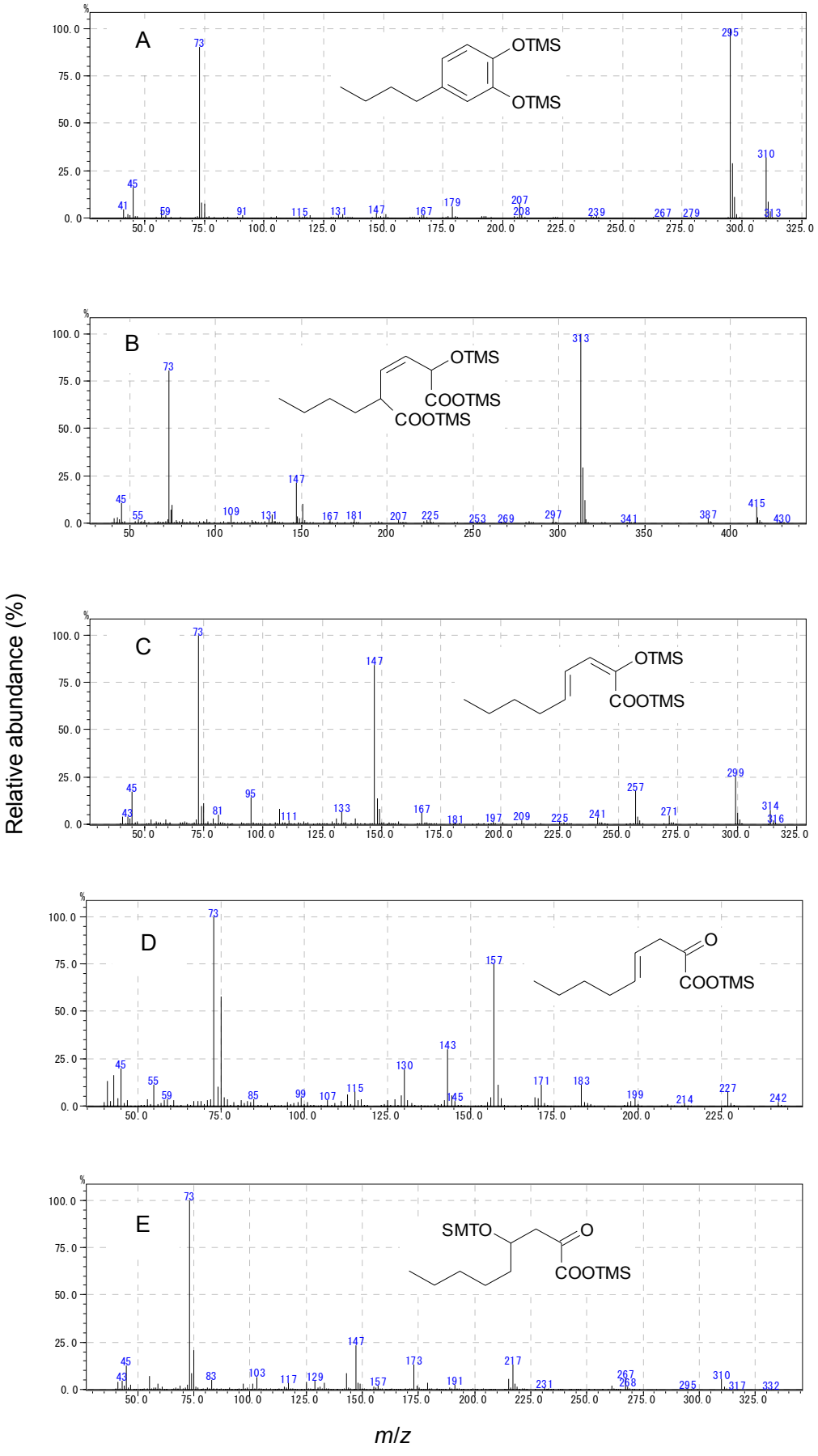

Fig. 4.7 Mass spectra of metabolites formed during 4-*n*-BP degradation by strain nBP5

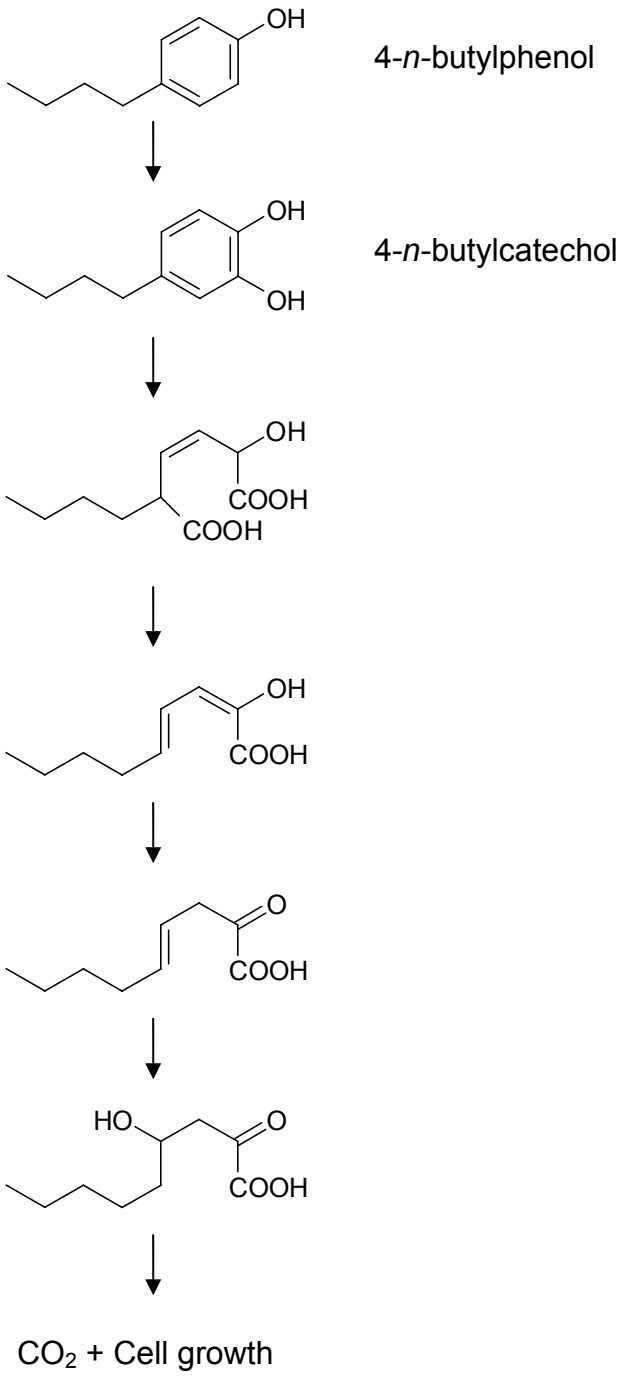

Fig. 4.8 Proposed 4-*n*-BP degradation pathway by strain nBP5

4.3.5 Degradability of other APs

Twenty APs (4-*n*-BP and 19 other APs) were tested for their degradability by strain nBP5 in the whole-cell assays (Fig. 4.9). Within 72 h of the test period, 4-EP, 4-*n*-ProP and 4-*n*-AmyP in addition to 4-*n*-BP were completely degraded. Degradation ratio of the other 4-*n*-APs declined with increase of the length of the alkyl chain. Branched 4-ProPs and 4-BPs were also degraded; however, degradation ratios of branched 4-ProPs and 4-BPs were lower than those of 4-*n*-ProPs and 4-*n*-BPs, respectively. In addition, degradation ratios of *ortho*- or *meta*-substituted ProPs and BPs were generally low (<20%), except for 3-*i*-ProP whose degradation ratio was 63%. Degradation ratios of OPs and NPs, which have a long alkyl chain, were very low, and 4-*t*-OP and t-NP were never degraded.

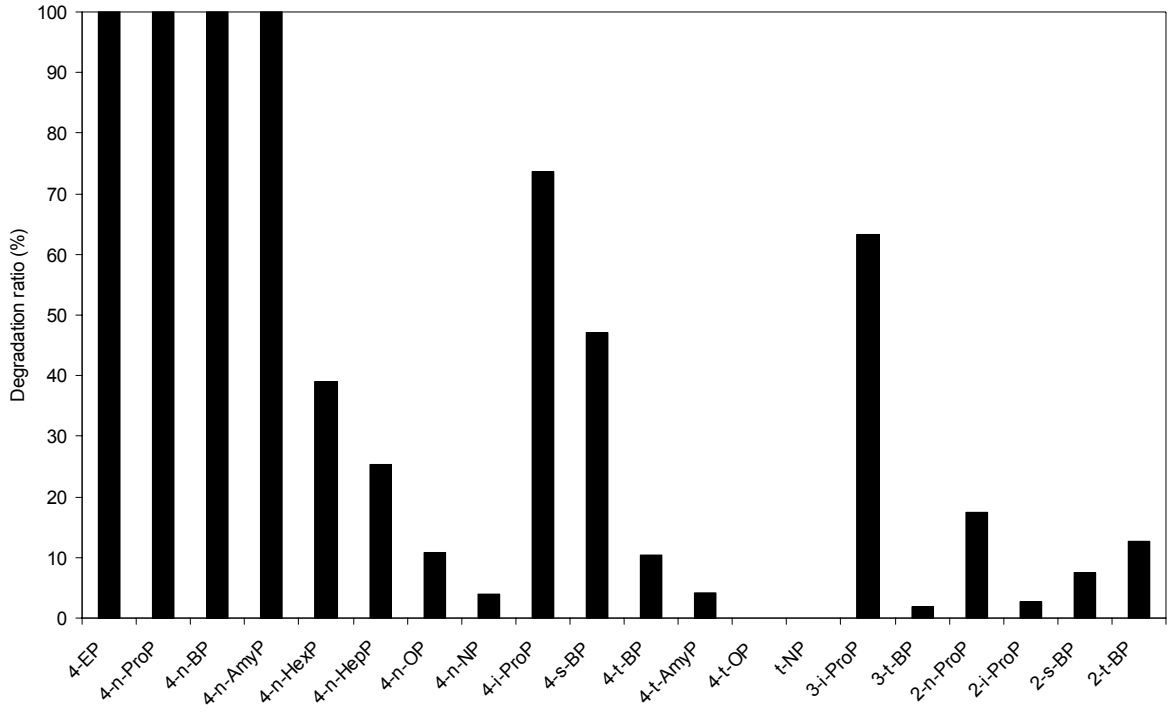

Fig. 4.9 Degradability of APs of strain nBP5. The degradation ratios were calculated using substrate concentration of test vial and sterile control vial in 72 h as follows: Degradation ratio (%) = 100 × (1 – substrate concentration in test vial/substrate concentration in sterile control vial).

4.4 Discussion

It is of great importance to degrade BP isomers in the aquatic environment due to their toxicity. This study demonstrated that 4-*t*-BP, the most recalcitrant BP isomer, can be degraded by the associations of *S. polyrrhiza* and its rhizobacteria (Fig. 4.1). During the enrichment of BP-degrading bacteria from *S. polyrrhiza* rhyzosphere, the culture lost the 4-*t*-BP degrading activity, while 4-*n*-BP-degrading bacteria, *P. veronii* strains nBP2, nBP3 and nBP5, were successfully isolated. It was thus suggested that complex mechanism might be involved in the 4-*t*-BP degradation in the *S. polyrrhiza* rhizosphere. However, 4-*n*-BP-degrading bacteria isolated here showed a slight degrading activity against 4-*t*-BP (Fig. 4.9). Therefore, these bacteria would partially contribute to the 4-*t*-BP degradation in the *S. polyrrhiza* rhizosphere.

To date, 3 strains, *Pseudomonas* sp. strain KL28 (Jeong et al., 2003), *P. veronii* strain INA06 (Ajithkumar et al., 2003) and *P. putida* strain MT4 (Takeo et al., 2006) have been reported to degrade 4-*n*-BP. However, no information is available on the optimal conditions and kinetics for 4-*n*-BP biodegradation. This study first determined the optimal conditions (temperature, pH and NaCl concentration) and kinetics for 4-*n*-BP biodegradation using *P. veronii* strain nBP5.

Among 4-*n*-BP-degrading bacteria isolated previously, strain KL28 was isolated from soil, and the others were from activated sludge. Therefore, strains nBP2, nBP3 and nBP5 isolated here were the first 4-*n*-BP degrading bacteria from the natural aquatic environment. All of the 4-*n*-BP degrading bacteria reported previously and isolated in this study were members of the genus *Pseudomonas*. Thus, this genus seems to play a major role in the depletion of 4-*n*-BP regardless of the type of environment.

Earlier studies have reported that 4-*n*-BP biodegradation proceeded through an initial hydroxylation of 4-*n*-BP to 4-*n*-butylcatechol and the *meta*-cleavage of 4-*n*-butylcatechol (Takeo et al., 2006; Jeong et al., 2003). In this study, 4-*n*-butylcatechol and its *meta*-cleavage products were also detected during the 4-*n*-BP degradation by our strain nBP5. Thus, it was indicated that the 4-*n*-BP degradation pathway of strain nBP5 was similar to that of these strains.

Strain nBP5 isolated in this study completely degraded 4-*n*-APs with short and medium alkyl chains (C2–C5) within 72 h under the inductive condition. This strain also

degraded 4-*n*-APs with long alkyl chains (C6–C9) although the degradation ratio declined with increase of length of alkyl chain. In addition, 4-*n*-BP-degrading enzyme of strain nBP5 was effective (>60 % of degradation ratio within 72 h) to 3- and 4-*i*-ProPs, but not to other branched APs and *ortho*- and *meta*-substituted APs. Previous studies showed that *Pseudomonas* sp. strain KL28, *P. veronii* strain INA06 and *P. putida* strain MT4 could degrade 4-*n*-APs with alkyl chain of C1–C5 (Jeong et al., 2003), C3–C6 (Ajithkumar et al., 2003) and C1–C4 (Takeo et al., 2006), respectively. Thus, the range of degradable 4-*n*-APs by our strain nBP5 appeared to be wider than that for other 4-*n*-BP-degrading bacterial strains.

Rhizosphere of *S. polyrrhiza* would be attractive as a promising source of useful bacteria for the treatment of wastewater containing recalcitrant compounds and the remediation of natural aquatic environment contaminated with recalcitrant compounds. Considering the versatile APs degrading ability, 4-*n*-BP-degrading bacteria in this study can be applied for the treatment of industrial wastewaters containing complex mixture of APs.

4.5 Conclusions

In this chapter, 4-*t*-BP degradation tests in river water microcosms were performed with/without *S. polyrrhiza*. Although 4-*t*-BP could not be biodegraded in the natural river water only, the presence of *S. polyrrhiza* could cause a significant 4-*t*-BP degradation. This suggests that *S. polyrrhiza*-bacterial association is also feasible for 4-*t*-BP removal from the aquatic environment. Further attempt for isolation of bacteria responsible for the 4-*t*-BP degradation in the rhizosphere of *S. polyrrhiza* was unfortunately failed. However, 3 different strains of 4-*n*-BP-degrading bacteria, designated strains nBP2, nBP3 and nBP5, were successfully isolated using the same river water microcosm planting *S. polyrrhiza*. Characterization of strain nBP5 revealed that this strain metabolizes 4-*n*-BP via the *meta*-cleavage pathway and is capable of degrading 4-*n*-BP up to 1.0 mM although some inhibitory effect occurred at 0.075mM or higher of 4-*n*-BP. In addition, strain nBP5 seems capable of degrading a wider range of 4-*n*-APs than other 4-*n*-BP degrading bacteria previously reported.

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Chapter 5

General conclusion

Application of APTS for finishing treatment of secondary effluent from wastewater from WWTPs or on-site water purification in aquatic bodies is a cost-effective and environmentally-friendly technology especially in tropical Asia like Vietnam. APTS has been mainly applied for the removal of nutrient, such as nitrogen and phosphorus, to prevent eutrophication, and for the removal of heavy metal to purify the contaminated sediment. Recent researches revealed the possibility for the application of APTS, especially rhizodegradation, to purify chemically contaminated aquatic environment. Accumulation of bacterial population responsible for the degradation of aromatic compounds in the rhizosphere of a certain aquatic plants such as *S. polyrrhiza* (giant duckweed), *P. stratiotes* L. (water lettuce) and *Phragmites australis* (reed) has been reported, and results in accelerated degradation of aromatic compounds.

This study was performed to accumulate the basic knowledge on the degradation of a variety of aromatic compounds in the rhizosphere of a fast-growing giant duckweed *S. polyrrhiza*, which is worldwide distributed and often used in ATPS, from various aspects.

In Chapter 2, phenol degradation tests were performed using 6 environmental samples with/without *S. polyrrhiza*. Bacterial community structure in bulk water and in rhizosphere fractions of *S. polyrrhiza* was investigated at the same time to elucidate the mechanisms of the accelerated degradation of phenol and the selective accumulation of phenol-degrading bacteria in its rhizosphere. Accelerated phenol degradation in the rhizosphere of *S. polyrrhiza* was confirmed as general phenomena. *S. polyrrhiza* secreted root exudates which were rich in highly diverse phenolic compounds into its rhizosphere. This ability of *S. polyrrhiza* could selectively recruit highly-diverse aromatic compounds degrading bacteria and resulted in the accelerated biodegradation of phenol. Thus *S. polyrrhiza* seemed to be very useful device for the enrichment of bacteria which can degrade various aromatic compounds.

In Chapter 3, based on the results from Chapter 2, the possibility of degrading various aromatic compounds by use of a variety of aromatic compounds degrading bacteria, which are accumulated in the rhizosphere of *S. polyrrhiza*, was evaluated. The effect of planting *S.*

polyrrhiza on the accelerated degradation/removal of phenol, aniline, 2,4-DCP, NP and BPA was evaluated using natural pond water. The root exudates of *S. polyrrhiza* were also characterized as a possible factor that causes the accelerated degradation of these aromatic compounds. Accelerated removal of all the aromatic compounds except BPA was confirmed by planting *S. polyrrhiza*, suggesting that an *S. polyrrhiza*-bacterial association can effectively remove a wide variety of aromatic compounds. Analysis of the root exudates revealed that *S. polyrrhiza* has a great ability to release phenolic compound-rich root exudates and this seemed crucial for the accelerated degradation/removal of various aromatic compounds by stimulating/recruiting the key-role bacteria in the rhizosphere.

In Chapter 4, an attempt was made to degrade 4-*t*-BP by rhizodegradation using *S. polyrrhiza*. Cycle batch 4-*t*-BP degradation tests in river water microcosms showed the significant 4-*t*-BP degradation in the presence of *S. polyrrhiza*. Although further attempt to isolate 4-*t*-BP degrading bacteria was unfortunately failed, 4-*n*-BP degrading bacteria were successfully isolated for the first time from aquatic environment. The bacterium, identified and named as *P. veronii* nBP5, was revealed capable of degrading a wider range of 4-*n*-alkylphenols than previously reported three 4-*n*-BP degrading bacteria. Thus, 4-*n*-BP-degrading bacteria in this study can be applied for the treatment of industrial wastewaters containing complex mixture of APs.

From the results obtained through this study and other related studies, it can be concluded that rhizodegradation by use of aquatic plant-bacterial associations seem effective for the accelerated degradation of various recalcitrant compounds, particularly aromatic compounds. Thus, although APTS has been mainly used for the removal of nutrients to prevent eutrophication in the aquatic environment so far, it may be also applied for the finishing process to remove recalcitrant compounds that cannot be removed in the secondary treatment in wastewater treatment plants. Further study on the treatment system utilizing aquatic plants is desirable for the development of a novel contamination control system especially in developing countries in the tropical region as an alternative to high-cost physical/chemical treatment technologies.