DISSERTATIONES CHIMICAE UNIVERSITATIS TARTUENSIS
76

DISSERTATIONES CHIMICAE UNIVERSITATIS TARTUENSIS
76

SIGNE VIGGOR

Impact of biochemical parameters of genetically different pseudomonads at the degradation of phenolic compounds

TARTU UNIVERSITY
PRESS

Institute of Chemistry, University of Tartu, Estonia

Dissertation is accepted for the commencement of the degree of Doctor of Philosophy in Colloidal and Environmental Chemistry on April 24th, 2008 by the council of the Doctoral Committee of the Institute of Chemistry, University of Tartu.

Supervisors: Prof. Ain Heinaru, Institute of Molecular and Cell Biology, University of Tartu
Prof. Toomas Tenno, Institute of Chemistry, University of Tartu

Opponent: Prof. Kalevi Pihlaja, Department of Chemistry, University of Turku, Finland

Commencement: June 18th, 2008 at 18 Ülikooli Str, room 204, 13:00 h.

ISSN 1406–0299
ISBN 978–9949–11–862–5 (trükis)
ISBN 978–9949–11–863–2 (PDF)

Tartu Ülikooli Kirjastus
www.tyk.ee
Tellimus nr. 182

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original papers that will be referred in the text by Roman numerals I-IV.

I. Heinaru E, Viggor S, Vedler E, Truu J, Merimaa M, Heinaru A. 2001. Reversible accumulation of *p*-hydroxybenzoate and catechol determines the sequential decomposition of phenolic compounds in mixed substrate cultivations in pseudomonads. FEMS Microbiol. Ecol. 37 (1), 79–89.

II. Viggor S, Heinaru E, Loponen J, Merimaa M, Tenno T, Heinaru A. 2002. Biodegradation of dimethylphenols by bacteria with different ring-cleavage pathways of phenolic compound. Environ. Sci. Pollut. Res. 9 (Special Issue 1), 19–26.

III. Heinaru E, Merimaa M, Viggor S, Lehiste M, Leito I, Truu J, Heinaru A. 2005. Biodegradation efficiency of functionally important population selected for bioaugmentation in phenol- and oil-polluted area. FEMS Microbiol. Ecol. 51 (3), 363–373.

IV. Viggor S, Heinaru E, Künnapas A, Heinaru A. 2008. Evaluation of different phenol hydroxylase-possessing phenol-degrading pseudomonads by kinetic parameters. Biodegradation. DOI:10.1007/s10532-008-9180-8.

Author's contribution

The author of current PhD work was a principal investigator in papers II and IV. In papers I and III the author contributed to growth experiments and chemical testing and analysis of the substrates.

ABBREVIATIONS

ANT – anthracene
BEN – benzoate
CAT – catechol
CCM – *cis,cis*-muconate
CFU – colony forming units
Crc – carbon repression control
CRE – cresol
C12O – catechol 1,2-dioxygenase, catechol *ortho* cleavage enzyme
C23O – catechol 2,3-dioxygenase, catechol *meta* cleavage enzyme
DGGE – denaturing gradient gel electrophoresis
DMP – dimethylphenol
FEN – phenanthrene
G12O – gentisate 1,2-dioxygenase
GEM – genetically engineered microorganisms
HGT – horizontal gene transfer
4H3MBA – 4-hydroxy-3-methylbenzoic acid
4H2MBA – 4-hydroxy-2-methylbenzoic acid
4H2MB – 4-hydroxy-2-methylbenzaldehyde
LmPH – large subunit of multicomponent phenol hydroxylase
MCI – muconate cycloisomerase, also known as CCM lactonising enzyme
mPH – multicomponent phenol hydroxylase
NAH – naphthalene
ortho^{prot} – protocatechuate *ortho* pathway
PAH – polycyclic aromatic hydrocarbons
PCMH – *p*-cresol methylhydroxylase
PCR – polymerase chain reaction
PC34O – protocatechuate 3,4-dioxygenase, protocatechuate *ortho* cleavage enzyme
PC45O – protocatechuate 4,5-dioxygenase, protocatechuate *meta* cleavage enzyme
PH – phenol hydroxylase
PHE – phenol
POB – *p*-hydroxybenzoate
POBH – *p*-hydroxybenzoate hydroxylase
sPH – single component phenol hydroxylase

1. INTRODUCTION

Pollution of the environment with anthropogenic compounds is a worldwide problem. The main waste in Estonia is produced by oil shale mines, oil shale chemical and energy industries. In 2005 the waste from energy production formed 67.1% from total solid waste produced in Estonia (oil shale ash 5.77 million t, mine waste 5.77 million t, semi-coke and fuses 868 000 t) (Lahtvee, 2007). The mine water (2.23 million m^3 $year^{-1}$) contains high concentrations of sulphates, heavy metals and oil products (Narusk and Nittim, 2003). Thermal processing of oil shale (semi-coking) has resulted in huge dumpsites of semi-coke (about 200 ha and up to 100 million tons) in the areas surrounding oil shale chemical industry plants in northeastern part of Estonia. The pollution is caused by solid wastes and leachate from semi-coke mounds, formed from flushing water and precipitations. These wastes are characterised by high content of several organic and inorganic compounds and high pH. In water contaminated with phenolic leachate the concentrations of phenol and *p*-cresol are higher than that of dimethylphenols, other cresols, resorcinols and polycyclic aromatic hydrocarbons (PAHs) (Ideon, 2007).

Bacteria, yeasts, other fungi and algae are able to degrade aromatic compounds, whereas they can acquire new properties by undergoing genetic evolution resulting in the synthesis of specific enzymes for the catabolism of anthropogenic compounds. Numerous bacterial strains, primarily pseudomonads, have been isolated from a wide range of environments contaminated with aromatic compounds (Mishra *et al*., 2001). The biodegradative bacterial strains can be added to the local microbial community to enhance bioremediation in polluted areas (bioaugmentation). For example combined bioaugmentation (three indigenous strains, also studied in this work) and phytoremediation (plants and trees) field experiments in semi-coke mounds revealed increased plant growth and elimination of pollution compared to the untreated plot (Truu *et al*., 2003; Ostonen *et al*., 2006).

A series of bacterial strains (mainly pseudomonads) from water samples of shale oil industry and surrounding areas continuously polluted with phenolic compounds were isolated and genetically characterised (Heinaru *et al*., 2000; Merimaa *et al*., 2006).

The main aim of the thesis was assessment of the relevance of biochemical parameters of those genetically different indigenous pseudomonads (Heinaru *et al*., 2000) in degradation of phenolic compounds. The role of accumulation of catabolic intermediates on decomposition of phenolic compounds in mixed substrate cultivations, determination of kinetic parameters of genetically different strains and the effectiveness of different phenol/*p*-cresol degrading indigenous bacterial strains on phenolic leachate or shale oil containing microcosm experiments were also studied. Data obtained in this research can be useful for designing effective bioaugmentation technology of phenolic waste.

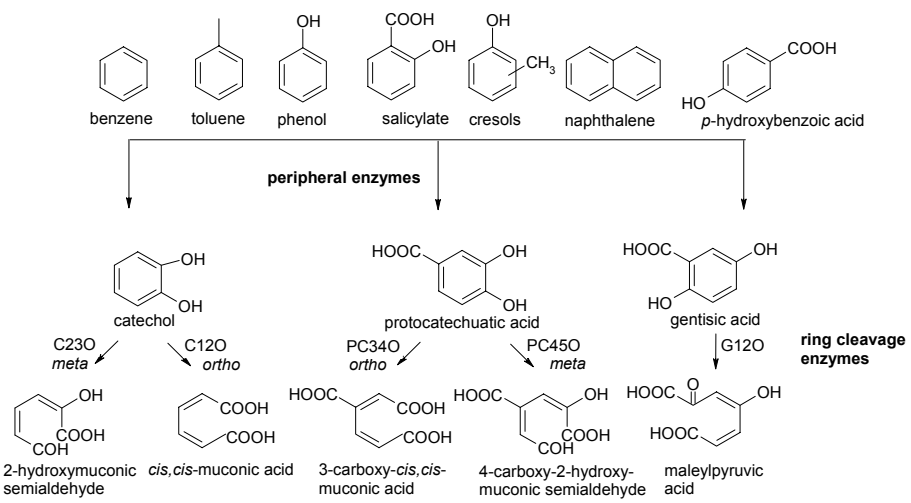
2. LITERATURE OVERVIEW

2.1. CENTRAL PATHWAYS OF AROMATIC CATABOLISM

Aromatic compounds can be either totally or partly degraded by microorganisms depending on the type of substituent(s) and on the number of benzene rings.

Although aromatic compounds can enter cell by passive diffusion, active transport increases the efficiency and rate of substrate acquisition and thus may enhance microbial growth in natural environments where these compounds are present at low concentrations (Nichols and Harwood, 1997). The rates of uptake and mineralization of many organic compounds by microbial populations are related to aqueous solubilities rather than total substrate concentration. Also the temperature, pH, nutrient (nitrogen and phosphorus) availability, salinity and oxygen concentration are essential parameters for successful biodegradation (Leahy and Colwell, 1990).

The next important point in degradation of aromatic compounds is whether substituents (e.g., halogen atoms, nitro groups) remain intact or are either transformed or eliminated before the ring cleavage, – and it depends on the bacterial species. Elimination of side chains can occur before or after ring-fission reactions, whereas substituent(s) often decrease biodegradability of the compounds. However, after mono- or dioxygenation reaction, a limited number of dihydroxylated intermediates such as (substituted) catechol, protocatechuate and gentisate are formed (Scheme 1) (Harwood and Parales, 1996).



Scheme 1. Aerobic routes of aromatic ring cleavage (Harwood and Parales, 1996).

A crucial phase of degradation includes ring fission, catalysed by dioxygenases, which can occur between (intradiol or *ortho* cleavage) or adjacent to one of the hydroxyl groups (extradiol or *meta* cleavage) (Scheme 1). The ring cleavage of gentisate occurs between the carboxyl-substituted carbon and the adjacent hydroxylated carbon. Binuclear compounds, such as naphthalene, are attacked twice. In the first step, one of the rings is cleaved and partially removed, leading to the formation of an intermediate such as benzoate or salicylate. This aromatic ring structure is then cleaved subsequently in the second round of cleavage, catalysed by related but not the same enzymes (van der Meer, 1997). All the described cleavage pathways produce intermediates of central metabolic routs, such as tricarboxylic acid cycle. The generalised scheme of catabolic pathways for aromatic compounds (Harwood and Parales, 1996) suggests that micro-organisms have extended their substrate range by developing peripheral enzymes, which are able to transform initial wide range of substrates into a smaller number of central intermediates (van der Meer *et al*., 1992).

2.1.1. Reactions converting aromatic compounds into ring fission substrates (peripheral enzymes)

Microbial hydroxylation of aromatic compounds involves incorporation of molecular oxygen into the substrate and is accomplished by two different mechanisms (Harayama *et al*., 1992).

2.1.1.1. Aromatic-ring dioxygenases

Aromatic-ring dioxygenases (EC 1.14.12.-, dihydroxylases, also known as Rieske non-heme iron dioxygenases) catalyse incorporation of two adjacent hydroxyl groups into aromatic substrate at the expense of NADH-oxidation, and the dihydrodiol intermediates are formed. In general, aromatic ring dioxygenases are capable of initiating oxidative attack on a very wide range of substrates (monocyclic aromatic and polycyclic aromatic hydrocarbons, chlorinated and heterocyclic aromatic compounds, aromatic acids etc.) using remarkable diversity of reaction types (Parales and Resnick, 2006). For example, in case of halogenated, nitro-, amino- and sulfoaromatic substrates dioxygenation at substituted carbon occurs with elimination of halogen, nitrite, ammonia or sulphite. Two of the best studied dioxygenases naphthalene dioxygenase (*nah* gene cluster) from *Pseudomonas* sp. NCIB 9816-4 (Kurkela *et al*., 1988) and toluene dioxygenase (*tod* gene cluster) from *P. putida* F1 (Zylstra *et al*., 1988) are known to catalyse oxidation of more than 75 and 100 different substrates, respectively (Parales and Resnick, 2006).

Nowadays, over 100 aromatic ring hydroxylating dioxygenases have been identified based on biological activity or nucleotide sequence identity (Parales and Resnick, 2006). Historically dioxygenases were classified based on electron transfer components present in the Rieske non-heme iron oxygenase system (Harayama *et al*., 1992). Due to identification of additional enzyme systems with diverse properties, a new classification system based on amino acid sequence alignments of terminal oxygenase α subunits, that are playing a major role in determining substrate specificity, was introduced (Nam *et al*. 2001). In general, clustering of the enzymes correlated with native substrates oxidised by the members, and following families were described: phthalate, benzoate, naphthalene and toluene/biphenyl. Latest phylogenetic analyses revealed that additional families (for example salicylate) are apparently present, and that several enzymes are quite distantly related to those found in the core families (Parales and Resnick, 2006).

The genes for aromatic-ring dioxygenases usually lay in close proximity to the aromatic-ring cleavage dioxygenases. In *Acinetobacter calcoaceticus* the chromosomally located *benABCD*-genes for benzoate degradation are close to the genes of *ortho* cleavage pathway (*cat*-genes) (Neidle *et al*., 1991). The related *xylXYZL*-genes (encoding toluate dioxygenase) of *P. putida* mt-2 occur on the TOL-plasmid but form here one transcriptional unit with the genes for the *meta* cleavage pathway (*xylTEGFJQKIH*) (Neidle *et al*., 1991).

2.1.1.2. Monooxygenases

Multicomponent and single-component monooxygenases (EC 1.14.13.-, hydroxylases) catalyse hydroxylation of aromatic compounds with a hydroxyl group on the ring (phenol, cresols, *p*- and *m*-hydroxybenzoic acid, salicylic acid etc.) into dihydroxy-derivatives (Harayama *et al*., 1992).

The operons encoding multicomponent monooxygenases that have similar electron transport system as those of multicomponent dioxygenases (Harayama *et al*., 1992), contain the genes of *meta* (*Pseudomonas* sp. CF600, Shingler *et al*., 1989) or *ortho* (*A. calcoaceticus* NCIB8250, Ehrt *et al*., 1995) cleavage pathway. Based on structural, biochemical and genetic data multicomponent hydroxylases belong to the class of soluble diiron monooxygenses together with other soluble multicomponent oxygenases such as methane, toluene and alkane monooxygenases (Leahy *et al*., 2003). Multicomponent hydroxylases have usually broad substrate specificity. Both phenol hydroxylase encoded by *Pseudomonas* sp. strain CF600 gene cluster *dmpKLMNOP* (Shingler *et al*., 1989) and toluene *o*-monooxygenase encoded by *Burkholderia cepacia* G4 gene cluster *tomA012345* (Newman and Wackett, 1995) catalyze the oxidation of phenol and certain methyl-substituted phenols. The latter enzyme is able to oxidize a variety of other substrates, including diethyl ether, trichloroethylene

and three isomers of dichloroethylene, vinyl chloride, benzene and naphthalene (Newman and Wackett, 1995).

Single component monooxygenases are flavoproteins, that use NAD(P)H and O_2 as co-substrates for hydroxylation of phenolic compounds. They are also able to catalyse transformation of benzoic acids (salicylate, *p*-hydroxybenzoate, etc.), some of them being particularly useful in degradation of halogenated compounds. Analysis of the organization of genes of single component monooxygenases revealed that they are distantly related to one another (van der Meer, 1997). The two highest related ones are plasmid-located *pheA* for phenol monooxygenase in *P. putida* EST1001 (Nurk *et al*., 1991) and *tfdB* for 2,4-dichlorophenol hydroxylase in *Alcaligenes eutrophus* JMP134 (Perkins *et al*., 1990). Another group is formed by *pcpB* for pentachlorophenol-4-monooxygenase of *Flavobacterium* (Orser *et al*., 1993), *tbuD* for phenol/cresol hydroxylase of *Ralstonia pickettii* PKO1 (Kukor and Olsen, 1990), *pobA* for *p*-hydroxybenzoate hydroxylase of *A. calcoaceticus* (DiMarco *et al*., 1993) and *nahG* for salicylate hydroxylase in *P. putida* PpG7 (You *et al*., 1991), the latter two are more distantly related to the previous ones (van der Meer, 1997). Most flavoprotein aromatic hydroxylases have narrow substrate specificity and can only be used for the synthesis of a limited range of products (Moonen *et al*., 2003).

2.1.2. Aromatic ring cleavage pathways

The aromatic ring dioxygenases are classified into two groups based on the cleavage site of *o*-dihydroxylated substrates (catechol, protocatechuate) - *ortho* or intradiol cleavage and *meta* or extradiol cleavage enzymes. The use of either *ortho* or *meta* cleavage pathway is dependent upon the microbial species and/or the nature of the growth substrate (Shingler *et al*., 1992). There is also a third group of dioxygenases that cleave *p*-dihydroxylated substrates (gentisate or 2,5-dihydroxybenzoate) between the carboxyl and proximal hydroxyl groups (Scheme 1). Phylogenetic analyses indicate that these three groups of ring fission dioxygenases form structurally different clusters and originate from different ancestors (Harayama *et al*., 1992).

2.1.2.1. o-Dihydroxylated aromatic ring cleavage pathways

2.1.2.1.1. *ortho* Cleavage pathway

o-Dihydroxylated aromatic compounds are cleaved in *ortho* pathway by intradiol dioxygenases, such as catechol 1,2-dioxygenase (C12O, EC 1.13.11.1) and protocatechuate 3,4-dioxygenase (PC34O, EC 1.13.11.3), which contain Fe^{3+} in catalytic centre. The cleavage of the aromatic ring between the two hydroxyl groups produces *cis,cis*-muconate (or a derivative), that is transformed

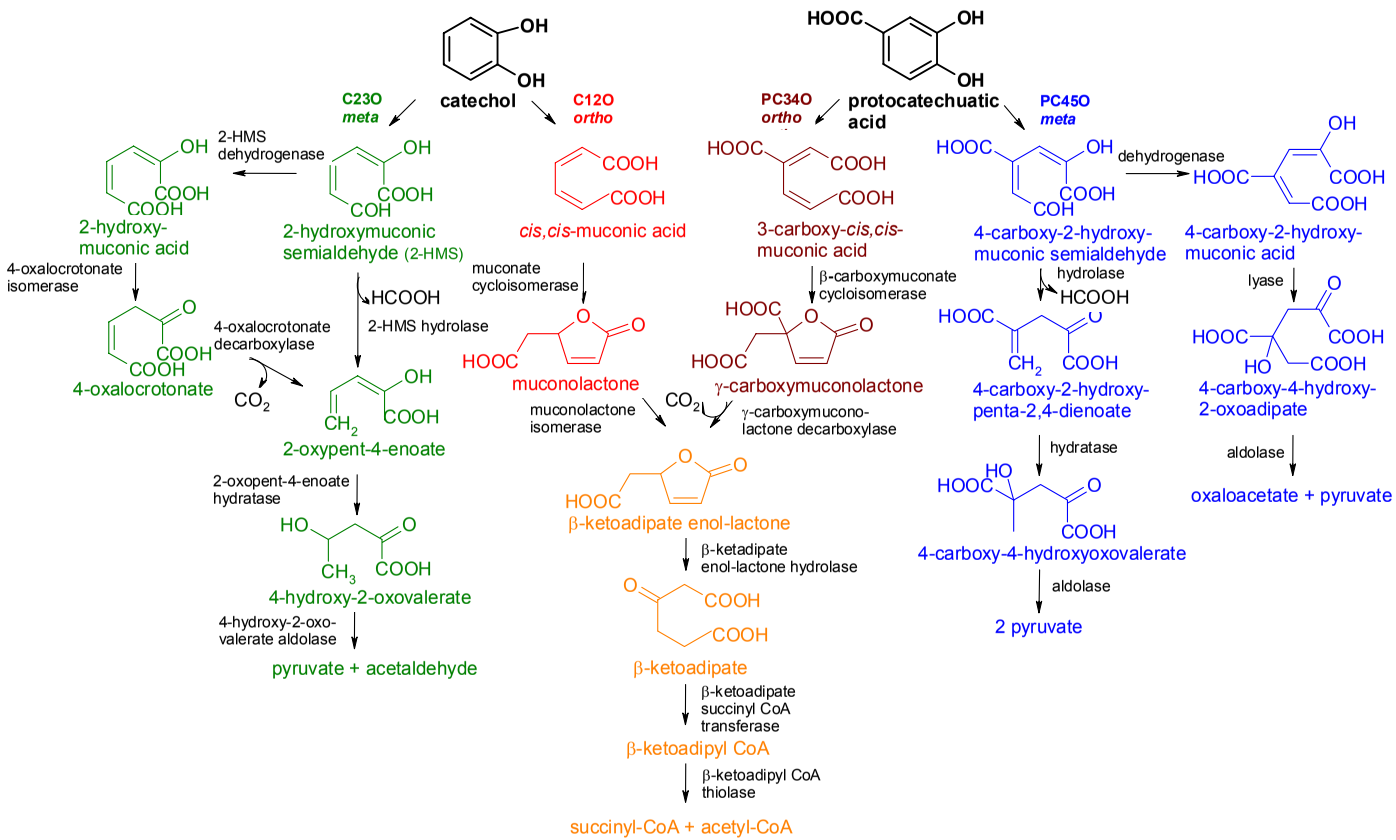
to a common intermediate, β-ketoadipate, which is further converted to succinate and acetyl coenzyme A (Scheme 2) (Ornston and Stanier, 1966). The *ortho* or β–ketoadipate pathway is usually chromosomally encoded in bacteria, which degrade *p*-cresol, *p*-hydroxybenzoate, phenol, benzoate etc. (Harwood and Parales, 1996). The chlorinated aromatic compounds are converted to chloro-substituted catechols, which are only *ortho* cleaved by a chlorocatechol 1,2-dioxygenase. The enzymes of this pathway have wider substrate specificities than ordinary *ortho* cleavage pathway enzymes; therefore it is called the modified *ortho* cleavage pathway (van der Meer *et al*., 1992).

The catechol 1,2-dioxygenase and protocatechuate 3,4-dioxygenase share moderate amino acid sequence identity (about 25% between *P. putida catA* and *pcaG* gene products) and both have conserved tyrosyl and histidyl residues at positions proposed to ligate Fe^{3+} at the active site (Nakai *et al*., 1995).

The *pcaB* and *catB* genes that encode the next enzymes in *ortho* pathway, β-carboxy-*cis,cis*-muconate cycloisomerase (EC 5.5.1.2) and *cis,cis*-muconate cycloisomerase (MCI, EC 5.5.1.1) catalyse analogous reactions but are not phylogenetically similar (Scheme 2). Mechanistic distinctions between the two branches of the *ortho* pathway extend to γ–carboxymuconolactone decarboxylase (EC 4.1.1.44) and muconolactone isomerase (EC 5.3.3.4). The genes (*pcaC* and *catC*) encoding these proteins share little sequence similarity (Harwood and Parales, 1996).

The *ortho* pathways branch convergence points and the existence of isoenzymes may differ between the bacteria. For example, in *P. putida*, the pathway converges at β–ketoadipate enol-lactone, and one set of enzymes is present to complete the conversation to TCA intermediates (Ornston, 1966). *A. eutrophus* has two isofunctional hydrolases that convert β-ketoadipate enol-lactone to β-ketoadipate, the branch convergence point (Johnson and Stanier, 1971). In *A. calcoaceticus*, the two branches never converge: two independently regulated sets of genes encode isofunctional enzymes for the last three steps of the pathway (Kowalchuk *et al*., 1994).

The genes of the *ortho* pathway are inducible by pathway intermediates. Three enzymes of the catechol *ortho* pathway (C12O, MCI and muconolactone isomerase) are induced by *cis,cis*-muconate in *P. putida* (Ornston, 1966). In the protocatechuate pathway, PC34O is induced by both its substrate and product (*p*-hydroxybenzoate and protocatechuate) in *P. aeruginosa* and *P. putida*. The remainder of the enzymes of the protocatechuate branch (β-carboxymuconate cycloisomerase and γ-carboxymuconolactone decarboxylase) and the common enzymes of the two branches (β-ketoadipate enol-lactone hydrolase and β-ketoadipate-succinyl coenzyme A (CoA) transferase) are coordinately induced by β-ketoadipate (Ornston, 1966; Kemp and Hegeman, 1968). Lately Matsumura *et al*. (2006) showed that the genes for catechol catabolism (*catABC*) in the *Rhodococcus* sp. strain AN-22 are constitutively expressed.



Scheme 2. *ortho* and *meta* degradation pathways of catechol and protocatechuate in bacteria (Dagley et al., 1960; Ornston and Stanier, 1966; Shingler *et al*., 1992; Harwood and Parales, 1996).

2.1.2.1.2. *meta* Cleavage pathway

Cleavage of the aromatic ring adjacent to two hydroxyl groups (*meta* cleavage) produces 2-hydroxymuconic semialdehyde (or derivative) and is catalysed by extradiol dioxygenases, such as catechol 2,3-dioxygenase (C23O, EC 1.13.11.2) and protocatechuate 4,5-dioxygenase (PC45O, EC 1.13.1.18), which contain Fe^{2+} in catalytic centre (Dagley *et al*., 1960). Chemical structures of the substrates of intradiol and extradiol enzymes are identical or similar, but the reaction mechanisms are completely different (Scheme 2). There are differences in structure (extradiol enzymes contain ferrous ion as a prosthetic group), in substrate specificities (*meta* pathways in general are the routes by which aromatic rings with alkyl substituents can be metabolised without chemical modification of the substituents), and location of genes (most of *meta* pathway genes have been found in plasmids) (Harayama and Rekik, 1989). It is generally accepted that extradiol oxygenases are evolutionarily unrelated to intradiol cleavage enzymes (Harayama *et al*., 1992).

The most studied catechol *meta* cleavage pathways are coded by the *xyl* operon of the TOL plasmid pWW0 of *P. putida* mt-2 (Harayama and Rekik, 1990) and the *dmp* operon of the pVI150 of *Pseudomonas* sp. CF600 (Shingler *et al*., 1989). The nucleotide sequence determination, biochemical and polypeptide analyses revealed that the catechol 2,3-dioxygenases (C23O) encoded by *xylE* and *dmpB* are closely related (Shingler *et al*., 1992). The *xylE* and *dmpB* genes show also sequence similarity to other extradiol ring cleavage enzymes, such as 1,2-dihydroxy-naphthalene dioxygenase encoded by the *nahC* gene in the *nah* operon of the plasmid NAH7 of *P. putida* (van der Meer, 1997), and they share a common origin (Harayama and Rekik, 1989).

Noda *et al*. (1990) compared the amino acid sequences of LigA and LigB, the subunits of protocatechuate 4,5-dioxygenase (PC45O) in *Sphingomonas paucimobil*is (formerly *Pseudomonas paucimobilis*) SYK-6 with other extradiol enzymes and concluded that they have different origin. The determination of the crystal structure of LigAB from *S. paucimobilis* SYK-6 (Sugimoto *et al*., 1999) gave a preliminary evidence that this group of extradiol cleavage enzymes could be attributed to new class.

Both catechol and protocatechuate *meta* pathway diverge after forming of 2-hydroxymuconic semialdehyde or corresponding substituted derivative which may then either undergo hydrolysis or NAD^+-dependent dehydrogenation (Scheme 2). In case of catechol pathway the reactions from 2-oxopent-4-enoate are common to both pathways, and it was proposed that the two branches complemented rather than duplicated each other (Assinder and Williams, 1990). The 3-methylcatechol (from *m*-toluate) is catabolised only through hydrolytic route, while 4-methylcatechol and catechol (from *p*-toluate or benzoate, respectively) are dissimilated preferentially in the 4-oxalocrotonate branch (Harayama *et al*., 1987).

The regulation of *meta* pathway is different from *ortho* cleavage pathway: the whole pathway is induced by its substrate. So, the *P. putida* strain mt-2 having both catechol *ortho* and *meta* pathways degrades benzoate preferentially by the plasmid-coded *meta* pathway rather than through the product-induced chromosomal *ortho* pathway (Assinder and Williams, 1990).

2.1.2.2. *p*-Dihydroxylated aromatic ring cleavage pathway

Gentisic acid (2,5-dihydroxybenzoic acid) is one of a key intermediates in the aerobic degradation of naphthalene, salicylate, anthranilate and 3-hydroxybenzoate. Degradation of gentisate is initiated by gentisate 1,2-dioxygenase (G12O, EC 1.13.11.4), which cleaves the aromatic ring between the carboxyl and proximal hydroxyl group to form maleylpyruvate (Lack, 1959) employing Fe^{2+} as cofactor (Harpel and Lipscomb, 1990) (Scheme 1). The maleylpyruvate can be converted to central metabolites either by cleavage to pyruvate and maleate (Bayly *et al*., 1980) or by isomerization to fumarylpyruvate and subsequent cleavage to fumarate and pyruvate (Lack, 1961).

All isolated G12Os have very low similarity to other known ring-cleaving dioxygenases and the oxidative cleavage of *p*-dihydroxylated aromatic ring is probably less common route for bacterial aromatic catabolism than either of the more extensively studied pathways through *o*-dihydroxybenzenes (Zhou *et al*., 2001).

2.2. DEGRADATION OF PHENOLIC COMPOUNDS

2.2.1. Phenol

The first step in phenol degradation pathway is conversion of phenol into catechol by phenol hydroxylase (PH, phenol 2-monooxygenase, EC 1.14.13.7), which may determine the kinetic constants for cellular phenol-oxygenating activity (Hino *et al*., 1998). This flavoprotein monooxygenase uses NAD(P)H and O_2 as co-substrates and is composed of either one (single-component phenol hydroxylase, sPH) or several (multicomponent phenol hydroxylase, mPH) components (Pessione *et al*., 1999). Both catechol *ortho*- and *meta*-cleavage types can be combined with either sPH or mPH. The mPHs are classified into two groups according to genetic organisation of the operon – the *dmp* type (from *Pseudomonas* sp. CF600) and the *mop* type (from *Acinetobacter calcoaceticus* NCIB 8250), in which the mPH genes are linked to the genes for either *meta* (Shingler *et al*., 1989) or *ortho* pathway (Ehrt *et al*., 1995), respectively. In addition to the plasmid-borne *dmp* gene cluster (Shingler *et al*., 1989) almost identical chromosome-encoded mPHs (encoded by *phhN*

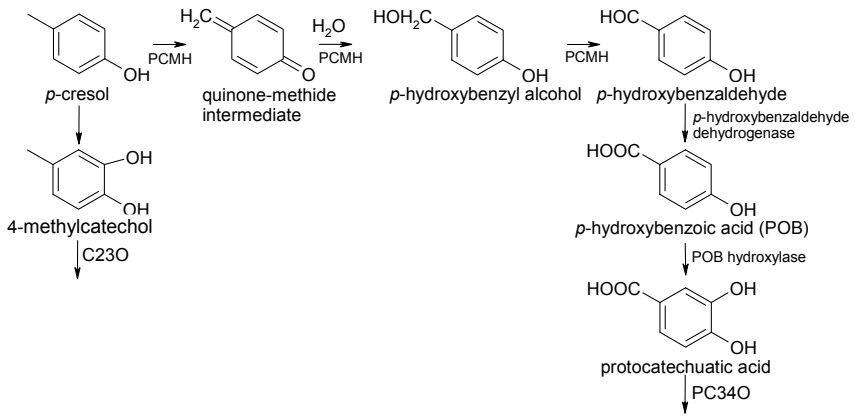
and *pheA* genes) have been found in *P. putida* strains P35X and BH, respectively (Ng *et al*., 1994; Takeo *et a*l., 1995). Merimaa *et al*. (2006) have characterised different types of mPHs in *P. putida*, *P. mendocina* and *P. fluorescens* strains and have shown that the strains phylogenetically close to those with the *mop* type of mPH contain genes for *ortho* cleavage (Merimaa *et al*., 2006). Among the same set of strains, the catechol *ortho*-cleavage type was also characteristic to the sPH-possessing strains, in which the structure of the corresponding operon was similar to the *pheBA* operon of *Pseudomonas* sp. EST1001 (Kivisaar *et al*., 1991; Nurk *et al*., 1991). However, in *Ralstonia* (formerly *Pseudomonas) pickettii* PKO1 the sPH gene *tbuD* was shown to be co-transcribed with the C23O (*tbuE*) gene (Kukor and Olsen, 1990; 1991).

Merimaa *et al*. (2006) demonstrated co-presence of genes for mPH and sPH combined with the cleavage of aromatic ring via *ortho* pathway in some *Pseudomonas* strains. The presence of two different monooxygenases, which are involved in catalysis of identical or similar reactions in the same micro-organism, was shown by Cafaro *et al*. (2004). These authors assumed that presence of two different enzymes confers a selective advantage for the strains, – the ability to optimise the use of aromatic compounds (Cafaro *et al*., 2005).

2.2.2. *p*-Cresol

Two distinct catabolic routes have been described for 4-methylphenol (*p*-cresol) (Scheme 3). In one of these pathways the hydroxyl-group is added to *p*-cresol and the resulting 4-methylcatechol is cleaved by catechol *meta* pathway enzymes (Bayly *et al*., 1966). In the alternative pathway the methyl group of *p*-cresol is oxidised stepwise to carboxyl group (Dagley and Patel, 1957). The first steps in this rout are catalysed by *p*-cresol methylhydroxylase (PCMH, EC 1.17.99.1), which converts *p*-cresol to *p*-hydroxybenzyl alcohol (intermediate quinone methide) and later to *p*-hydroxybenzaldehyde (Hopper, 1976; Hopper and Taylor, 1977). Last compound is converted into *p*-hydroxybenzoate (POB) by dehydrogenation reaction. The formation of protocatechuate from POB is catalysed by *p*-hydroxybenzoate hydroxylase.

PCMHs have been isolated from several *Pseudomonas* species and from some other organisms. Hopper *et al*. (1985) showed the periplasmic location of PCMH in *P. putida* strains NCIMB9869 and NCIMB9866. In *P. putida* NCIMB9869, A (plasmid-encoded) and B (chromosome-encoded) form of the enzyme are expressed when the organism is grown on *p*-cresol, 3,5-dimethyphenol, glutamate or succinate and on *p*-cresol, respectively (Kim *et al*., 1994). At the same time, *P. putida* NCIMB9866 produces only one plasmid-encoded single form of PCMH (Hopper and Kemp, 1980; Kim *et al*., 1994).



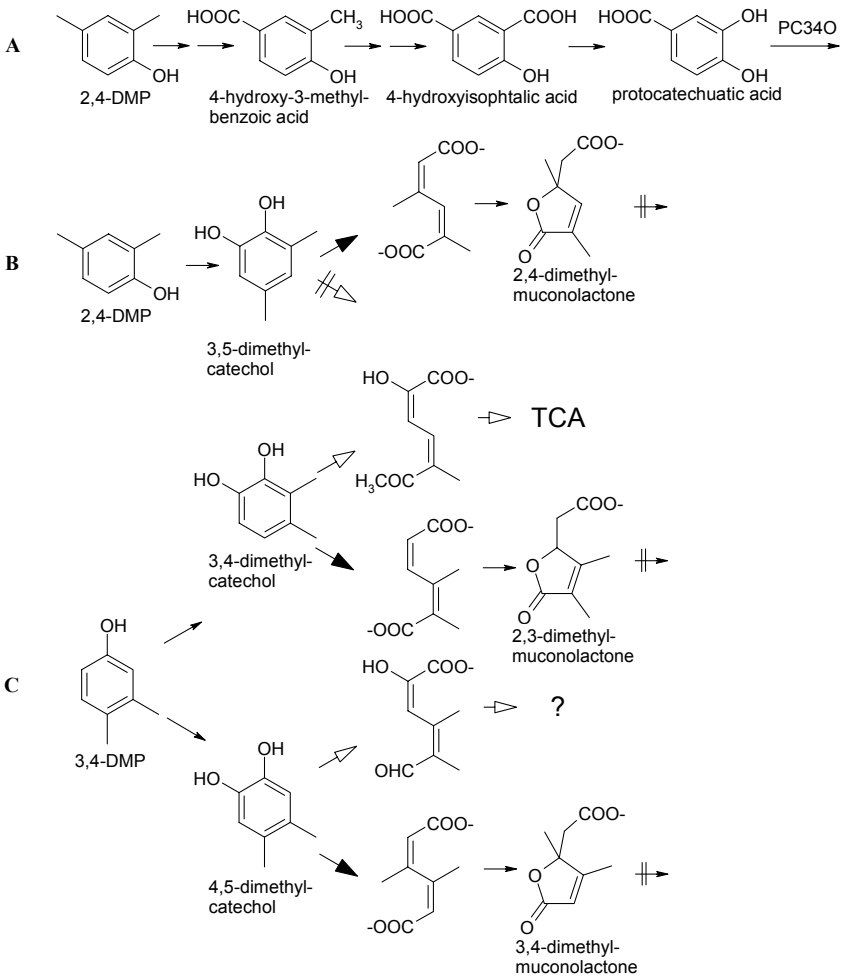
Scheme 3. Two distinct catabolic routes for degradation of *p*-cresol (Dagley and Patel, 1957; Bayly *et al*., 1966).

Also, a third route exists. In some *Bacillus* strains *p*-cresol is converted into POB, which is further metabolised through gentisate pathway (Crawford, 1976; Tallur *et al*., 2006).

2.2.3. Dimethylphenols

The first step in degradation of dimethylphenols (DMPs or xylenols) involves oxidation of methylgroup(s) or hydroxylation of aromatic ring by adding a second hydroxyl group in *ortho* position to the one already present.

Chapman and Hopper (1968) proved that metabolism of 2,4-DMP by *Pseudomonas* sp. NCIMB9866 was initiated by stepwise oxidation of the methyl group *para* to the hydroxyl group, followed by *ortho*–substituent oxidation to carboxyl groups. The formed 4-hydroxyisophtalic acid in the presence of reduced nicotine nucleotides was converted into protocatechuatic acid by an oxidative decarboxylation reaction. The latter compound was cleaved by PC34O and metabolised further to *β*–ketoadipatic acid (Scheme 4A). On the other hand, in *Comomonas testosteroni* JH5 2,4- and 3,4-DMP were transformed into dead-end metabolites 4-hydroxy-3-methylbenzoic acid and 4-hydroxy-2-methylbenzoic acid, respectively. However, through catechol *meta* cleavage, this strain also mineralised 2,3-DMP completely and 2,5- and 3,5-DMP partially (Hollender *et al*., 1994).



Scheme 4. Metabolism of 2,4-dimethylphenol or 3,4-dimethylphenol by A – *Pseudomonas* sp. NCIMB9866 (Chapman and Hopper, 1968) and by B and C – *Alcaligenes eutrophus* JMP134 (Pieper *et al*., 1995). ➡ activities of catechol *ortho* cleavage pathways. ⇨, activities of catechol *meta* cleavage pathway.

The oxidation of methyl group *meta* to hydroxyl group, hydroxylation of aromatic ring *para* to the first hydroxyl group and cleavage of formed methylgentisic acid by G12O are the steps of degradation pathway of 2,5- and 3,5-DMP described in *Pseudomonas putida* strains NCIMB9867 and NCIMB9869 (Hopper and Chapman 1971; Hopper et al., 1971).

The action of phenol hydroxylase to DMP will lead to the formation of substituted catechols, which can be subjected to either *ortho* or *meta* cleavage

(Scheme 4BC). Mineralization of 2,3- and 3,4-DMP has been reported to occur via catechol *meta* pathway, with 3,4-dimethylcatechol as an intermediate (Shingler *et al*., 1989; Pieper *et al*., 1995). According to Pieper *et al*. (1995), in case of *ortho* cleavage of dimethylcatechols, dimethylmuconolactones are formed as dead-end products in *Alcaligenes eutrophus* JMP134 degrading 2,3-, 2,4-, 2,5-, 3,4-and 3,5-DMPs (Scheme 4BC). Accumulation of methylmuconolactones was explained with different affinities of *meta* and *ortho* cleavage enzymes towards the methylcatechols and high specificity of methylmuconolactone methylisomerases to attack the dimethyl structure (Pieper *et al*., 1995).

2.3. DEGRADATION OF MIXED SUBSTRATES. CATABOLITE REPRESSION

Study of microbial activities under mixed-substrate conditions is the starting point for several biotechnological applications (Pieper and Reineke, 2000). Expression of many bacterial catabolic pathways is controlled not only by the presence or absence of the compound to be assimilated (a specific control response), but also by several global regulatory proteins that link the induction of the pathway genes to the physiological status of the cell (a global control response). The global control is usually dominant over the specific control (Ruiz-Manzano *et al*., 2005).

Although certain mixtures are degraded more rapidly than compounds present individually (Jahnke *et al*., 1993), the biodegradation rate of aromatic mixtures may be much lower under natural conditions, mostly due to catabolite repression, but also due to insufficient nutrient concentrations or a limited bioavailability of the contaminant as a result of binding to soil particles or low water solubility (Holtel *et al*., 1994; Duetz *et al*., 1996; Müller *et al*., 1996).

In pseudomonads, genes encoding the enzymes involved in catabolism of aromatic compounds are not expressed if certain other growth-supporting substrates such as succinate, lactate, citrate, pyruvate, acetate, glutamate, gluconate, glucose (Zylstra *et al*., 1989; Duetz *et al*., 1994; 1996; Holtel *et al*., 1994) or benzoate (Nichols and Harwood, 1995) are available. Plasmid-encoded degradative pathways for aromatics may also be subject to catabolite repression by organic acids. It was found that organic acids (e.g., succinate, lactate, acetate) and carbohydrates (e.g., glucose) could repress phenol degradation in *P. putida* H (Müller *et al*., 1996). In this case, the carbon catabolite repression was achieved at the transcriptional level of plasmid-encoded *phl* genes. It was also shown that the catechol *meta* cleavage pathway was less susceptible to catabolite repression than the TOL upper pathway (Duetz *et al*., 1996). On the other hand, Basu *et al*. (2006) reported that in *P. putida* CSV86, a naphthalene-degrading organism, aromatic compounds repressed the degradation of glucose,

whereas organic acids, which suppressed glucose utilization, were cometabolised with aromatic compounds.

During the last decade many works (Hester *et al*., 2000; O'Toole *et al*., 2000; Ruiz-Manzano *et al*., 2005; Aranda-Olmedo *et al*., 2006) have been published exploring molecular mechanisms of catabolite repression in pseudomonads, which in many cases is different from that in *Escherichia coli*. Catabolite repression control (Crc) protein is a master regulator of carbon metabolism in *Pseudomonas*. Available data suggest that Crc is a component of a signal transduction pathway modulating carbon metabolism and other phenomena such as biofilm development (Hester *et al*., 2000; O'Toole *et al*., 2000). Crc ultimately affects the expression of target genes, although the precise molecular mechanism underlying this effect remains to be elucidated. The scarce information obtained so far suggests that Crc does not bind to DNA, so it would not be a classical DNA-binding repressor (MacGregor *et al*., 1996; Hester *et al*., 2000). Workgroup of Ruiz-Manzano (2005) showed that induction of the *alk* pathway by alkanes is severely inhibited when cells grow in rich LB medium, transcription of the *crc* gene varies according to the growth phase, being three- to fourfold more efficient during exponential growth than in stationary phase. Lately, Aranda-Olmedo *et al*. (2006) concluded that no single response mechanism is suitable for all environmental conditions; instead, cells may resort to more than one strategy to appropriately respond to the changing environment. Catabolite repression trigged by carbon and/or energy excess and repression produced by some components of rich medium are sensed differently. In the first case, a mechanism involving σ^{54}-dependent transcriptional machinery plays a central role, while *crc* seems to be involved only in negative modulation of catabolic pathways when cells are growing exponentially on a complex rich medium.

The *ortho* and *meta* pathways are alternatives, whereby simultaneous operation of these pathways in degradation of mixed substrates yields toxic intermediates and causes accumulation of dead-end metabolites which may increase the degradation time and cause a sequential degradation of substrates (Hollender *et al*., 1994; Pieper *et al*., 1995; Erb *et al*., 1997).

During batch growth of *Ralstonia eutropha* on a benzoate-phenol mixture, benzoate (induces the catechol *ortho* pathway) completely inhibited phenol degradation (induces the catechol *meta* pathway, respectively) causing diauxic growth, and repression of phenol utilisation was attributed to the presence of benzoate (Ampe *et al*., 1998). Benzoate was also a preferred substrate during growth of same bacteria on a benzoate-acetate mixture (Ampe and Lindley, 1995). In this case, the actual triggerer of the repression signal was not benzoate, but catechol, transiently accumulating in the medium when high specific rates of benzoate consumption were reached (Ampe and Lindley, 1995; 1996).

It was also shown that *P. putida* cells degrade benzoate in preference to *p*-hydroxybenzoate (POB) by repressing POB transport by transcriptional downregulation of *pcaK*, the gene encoding POB permease (Nichols and Harwood, 1995; Cowles *et al*., 2000). In *Acinetobacter calcoaceticus* ADP1 benzoate and POB are degraded via the catechol and protocatechuate *ortho* pathways, respectively. When a mixture of both compounds was provided, benzoate was preferentially metabolised. In this case, the product of catechol ring cleavage, *cis,cis*-muconate, inhibited the utilization of POB (Gaines *et al*., 1996).

2.4. BIODEGRADATION EFFICIENCY

Under selective pressure of environmental pollution, microbial capacity for degradation of recalcitrant xenobiotics is developing that might be used for pollutant removal by biotechnological processes. Another way is design of improved biocatalysts by creating new metabolic routes; by expanding the substrate ranges of existing pathways, by improving the substrate and intermediate flux through pathway to avoid the accumulation of inhibitory intermediates and misrouting into unproductive routes or by increasing the genetic stability of catabolic activities (Timmis and Pieper, 1999).

The types and mechanisms of genetic changes in bacteria may be very slight as a result of random mistakes which occur during DNA replication and repair, or large as a result of DNA recombination. In the latter case, the genes can become deleted, replaced at different positions, reversed, duplicated, or transported on mobile DNA elements (insertion elements, transposons, integrons or retro-elements) (van der Meer, 2006). The third way is acquisition of DNA – the exchange of genetic information between the cells – well known as horizontal gene transfer (HGT). Besides the involvement in the evolution of bacterial genomes (Gogarten and Townsend, 2005), HGT is important for genetic adaptation of bacteria to persistent pollutants, for example chlorinated aromatic compounds (van der Meer, 1997). Based on obtained data, Peters *et al*. (1997) suggested that HGT of the released *pheBA* operon (*P. putida* PaW85) had occurred in natural conditions.

In laboratory experiments using mixed microbial communities under continuous low exposure of pollutants such as chlorobenzenes the adaptation times were between 6 and 12 months (van der Meer *et al*., 1987). In the field, *de novo* chlorobenzene adaptation has been found to occur over the order of a few years (van der Meer *et al*., 1998). Often adaptation times are even longer and then other bioremediation techniques such as biostimulation (addition of specific compounds), bioaugmentation (addition of specific microorganisms), and phytoremediation (addition of specific plants) to enhance microbial metabolism are advisable. A number of opportunities for improving degradation

performance using genetically engineered (GEM) or natural microorganisms, surfactants, nutrients or cosubstrates have been described (Timmis and Pieper, 1999).

Genetic engineering is a tool for production of strains with increased pollutant degradation rates and substrate range. Sayler and Ripp (2000) gave an overview of field application where GEM *P. fluorescens* HK44 (contains a transposon-based bioluminescence-producing *lux* gene fused with a promoter for the naphthalene catabolic genes) was used for bioremediation of contaminated soil. With this strain the efficiency of natural consortium was increased and the process of bioremediation was easily followed by measurement of luminescence. Recombinant *E. coli* harbouring and expressing the phenol hydroxylase gene from *P. putida* BH could efficiently degrade trichloroethylene (Fujita *et al*., 1995). Cloning of the *todABC* genes encoding toluene dioxygenase into *Deinococcus radiodurans* enabled the bacterium to oxidize toluene, chlorobenzene, 3,4-dichloro-1-butene and indole in the presence of radionucleides (Lange *et al*., 1998). Erb *et al*. (1997) showed that GEM *Pseudomonas* sp. B13 SN45RE was able to simultaneously degrade mixtures of chloro- and methylphenols and also protect the indigenous bacteria from shock loads of phenolic mixtures.

Due to the fact that only a minority of microorganisms are cultivable the exploitation of diversity and degradative potential of uncultivable microorganisms for bioremediation is desirable. It can be achieved by genetic engineering via construction of metagenomic libraries and subsequent transfer to culturable bacteria (Debarati *et al*., 2005).

The use of GEMs usually results in higher degradative capability and utility but application *in situ* has been limited by the risks associated with horizontal gene transfer and uncontrolled proliferation of the introduced GEMs (Boopathy, 2000). Paul *et al*. (2005) gave a survey of possibilities to construct GEMs in such a way that after destroying of the pollutant foreign cells will be killed by the induction of a controlled 'suicide' system. In most cases GEMs are made with conjugal transfer of a catabolic plasmid from a host to an appropriate recipient. Stability of such GEMs (and also natural isolates) is important, because the plasmids on which the catabolic genes reside often segregate in the absence of selective pressure. Therefore new methods are developed to insert genes stably into chromosomes of host bacteria (Erb *et al*., 1997) and the use of antibiotic resistance markers is not advisable.

Important components in biodegradation are bioavailability and entry of xenobiotics into cells. To enhance substrate range of otherwise specialised energy-dependent transport systems of many aromatic compounds can be changed using genetic manipulations. For example, based on known three-dimensional structures, Vollmer *et al*. (1998) constructed variants of muconate cycloisomerase changing the amino acid positions in the binding cavity, increasing thereby specificity constants for some chloromuconates. As bio-

surfactants increase degradation of hydrophobic pollutants (through enhancing bioavailability) (Deziel *et al*., 1996), efforts are made to design recombinant biocatalysts that exhibit a desired catabolic trait and produce at the same time a suitable biosurfactant. Also, chemotaxis is a selective advantage for microbes, enabling them to sense and locate environmental pollutant, and this property can be successfully used for bioremediation (Pandey and Jain, 2002).

Although a wide range of strategies has been proposed for bioremediation, selecting the most appropriated to treat a specific site is sometimes problematic. The amenability of a pollutant to biological transformation to less toxic products (biochemistry), the accessibility of the contaminant to microorganisms (bioavailability) and the opportunity for bioprocess optimisation (bioactivity) are the three basic principles, which must be considered before making decision (Dua *et al*., 2002).

2.5. GROWTH KINETICS OF MICROORGANISMS

Microbial growth kinetics, i.e. relationship between specific growth rate of a microbial population and substrate concentration, is an indispensable tool in all fields of microbiology, be it physiology, genetics, ecology or biotechnology (Kovárová-Kovar and Egli, 1998). The growth of microbes on noninhibitory compounds can be described using the Monod (Michaelis-Menten) equation. Among substrate inhibition models Andrews (Haldane) model is most widely used. Several models have been developed to describe substrate inhibition that causes a complete loss of activity at the finite critical concentration.

Evaluation of substrate inhibition on growth becomes an important consideration in the treatment of toxic compounds but the mechanism of growth inhibition is more complicated than that of single enzyme-catalysed reaction (Kovárová-Kovar and Egli, 1998). Toxicity of aromatic compounds is frequently attributed to disruption of membrane structure by hydrophobic interactions with the lipid bilayer structure by lipophilic compounds (Sikkema *et al*., 1994a). In addition to significant changes in the structure and the integrity of membranes, the function of enzymes directly associated with these membranes is also influenced (Sikkema *et al*., 1994b). In case of phenol, the experiments by Leonard and Lindley (1999) on *Ralstonia eutropha* and by Fialová *et al*. (2004) on *Candida maltosa* have shown that phenol-related inhibition was primary caused by the effect of phenol on substrate consumption rate, being attributed to inhibition of phenol hydroxylase activity rather than the modified expression of the genes encoding this enzyme. The authors also speculated that the phenol hydroxylase that is highly sensitive to hydrophobic stress is located in cell membrane, thereby avoiding penetration of phenol into the cytosol.

There are two extensively used laboratory cultivation methods for investigation of microbial growth kinetics – batch and continuous cultures. During

batch experiments, the composition of cells and their physiological state change. However, in continuous culture bacteria grow at fixed dilution rate by maintaining stable environmental growth conditions and the same physiological state. Under natural conditions, an organism most probably resides somewhere between the closed batch-culture and open continuous-culture systems (Jannasch and Egli, 1993). Measuring of the absorbance of the suspension is rapid, inexpensive and relatively easy to automate technique to follow growth, but in case of batch culture it makes the estimations at low substrate concentrations inaccurate. Thus, maximum specific growth rate (μ_{max}) is the only parameter that rigidly fixes the growth behaviour in batch culture (Kovárová-Kovar and Egli, 1998). Determination of the flow rate in continuous cultures can be made accurately and, consequently, estimation of the half saturation constant (K_{SG}) is precise. However, estimation of the μ_{max} may be less accurate in case of continuous cultivations, thereby the results may be affected by wall growth and mutation over the long cultivation periods required in case of this technique. The variation of kinetic parameters is probably caused by the culture history, parameter identifiability, and quality of experimental data (Grady *et al*., 1996).

The length of the lag phase (λ), an adjustment period during which bacterial cells modify themselves in order to take advantage of a new environment and initiate exponential growth, is an important parameter in describing the growth (Swinnen *et al*., 2004). Several models (modified Gompertz equation (Zwietering *et al*., 1990), the model of Baranyi and Roberts (1994) etc.) have been developed to calculate λ from growth curve data. The variability of growth parameter estimates (especially estimates of lag time) is, however, not only due to the technique used to monitor bacterial growth (measuring absorbance or total viable count) but also due to the model applied to fit the growth data (Dalgaard and Koutsoumanis, 2001). The factors that also influence the duration of the lag time are (changes in) environmental conditions, the identity and the phenotype of the bacterium (Buchanan and Cygnarowicz, 1990) and the growth stage and physiological history of the cells (Dens *et al*., 2005).

Occurrence of environmental contaminants in mixtures is an important problem because degradation of one component can be inhibited by other compounds of the mixture, and because different conditions may be required to treat different compounds within the mixture (Reardon *et al*., 2000). The effects of the mixture of substrates on biodegradation of a chemical can be either positive (increased growth rate, induction of degradative enzymes) or negative (toxicity, formation of toxic intermediates, competitive inhibition). Moreover, biodegradation of individual pollutants in mixtures has been shown to be different from their degradation as single carbon sources (Reardon *et al*., 2000). Several models have been proposed for modelling of microbial growth on mixed homologous substrates (six sugars (Lendenmann *et al*., 1996), five BTEX (benzene, toluene, ethylbenzene, and xylenes) compounds (Bielefeldt

and Stensel, 1999), three PAHs (Guha *et al*., 1999)). The effect of excreted metabolites on growth has also been considered in design of kinetic equations (Sanchez *et al*., 1998).

If a microbial population is growing on a mixture of substrates sequential or simultaneous utilization patterns can be observed. In case of sequential utilization pattern characterized by the diauxie, one compound inhibits degradation of the other by exerting toxicity, catabolite repression, competitive inhibition of enzymes, or depletion of electron acceptors (Smith *et al*., 1991). The simultaneous mechanism is either due to unspecificity of enzyme induction for degradation of similar substrates or convergence of catabolic pathways for the utilization of many substrates. In the latter case the removal efficiencies become enhanced compared to growth of bacteria on individual compounds, but usually the degradation rates of compounds are not same. At low substrate concentrations otherwise sequentially degraded substrates are degraded simultaneously (Kovárová-Kovar and Egli 1998).

In nature, usually only a fraction of indigenous population eliminates the pollution. Recombinant organisms may not be necessary for bioremediation because different members of an appropriate indigenous microbial consortium might best handle different metabolic tasks associated with the breakdown of pollutants. This view may be correct or not depending on the situation. Apart from decreased efficiency and reduced degradation rates anticipated when intermediates must be shuttled between different organisms in complex environmental matrices, released intermediates may be misrouted by microorganisms into unproductive dead-end pathways, or even transformed into toxic products that may destabilise the community and inhibit biodegradative processes (Erb *et al*., 1997).

3. AIMS OF THE STUDY

The general objective of this thesis was to assess the relevance of biochemical and genetic parameters of indigenous pseudomonads isolated from river water continuously polluted by phenolic compounds in degradation of aromatic compounds.

The specific aims were:

1. To study the role of accumulation of intermediates in decomposition of phenolic compounds (phenol, *p*-cresol, benzoate, *p*-hydroxybenzoate, dimethylphenols) in mixed substrate cultivations of pseudomonads possessing different catabolic types of degradation of phenol and *p*-cresol.
2. To evaluate the biodegradation efficiency of genetically different phenol/*p*-cresol degrading pseudomonads by kinetic parameters.
3. To investigate the effectiveness of different phenol/*p*-cresol degrading indigenous bacterial strains in microcosms experiments simulating bioaugmentation performances.

4. STUDIED PHENOL/*p*-CRESOL DEGRADING BACTERIAL STRAINS

Bacterial strains used in this thesis are listed in Table 1.The strains were isolated in mid nineties from water samples of channels surrounding semicoke mounds, and from the Kohtla and Purtse Rivers continuously polluted with phenolic compounds (Heinaru *et al*., 2000). Identification by BIOLOG GN and REP-PCR fingerprint analysis demonstrated both the predominance and also heterogeneity of *Pseudomonas* strains within isolated phenol/*p*-cresol degrading bacteria. Based on determination of catabolic genes three almost evenly distributed catabolic types of degradation of phenol and *p*-cresol were revealed among the studied 39 bacterial strains: 1) *meta-meta* type (both phenol and *p*-cresol are degraded through catechol *meta* pathway); 2) *ortho-ortho*^{prot} type (phenol and *p*-cresol are metabolised via catechol *ortho* and protocatechuate *ortho* pathways, respectively); and 3) *meta-ortho*^{prot} type (catechol *meta* and protocatechuate *ortho* pathways are induced by phenol and *p*-cresol, respectively) (Heinaru *et al*., 2000).

The multicomponent phenol hydroxylase (mPH) containing bacterial strains were more frequent as compared with single component PHs (sPH) possessing strains. All sPH-possessing strains (from which PC20, PC24 and PC31 were used in this study) the synthesis of sPH and C12O was determined by the plasmid-borne *pheBA* operon (Heinaru *et al*., 2000). The analysis of the sequences of the large subunit of mPH (LmPH) and C23O revealed four species- and biotype-specific phylogenetic groups (Fig. 1 in Merimaa *et al*., 2006). The strains degrading phenol and *p*-cresol through the catechol *meta* pathway (PC1) formed two clusters (group IIa and IIb) which were both closely related to reference strains having the Dmp family LmPHs. The strains of *meta-ortho*^{prot} catabolic type (PC18) formed separate cluster (group I) on the phylogenetic trees. These strains were also specific in *p*-cresol degradation because *p*-cresol methylhydroxylase (PCMH), the first enzyme of degradation of *p*-cresol through protocatechuate *ortho* pathway, is induced by phenol and *p*-cresol (Heinaru *et al*., 2000). Strains of *ortho-ortho*^{prot} catabolic type (PC17, PC30, PC16, P69) constitute another LmPH cluster (group IIIa and IIIb) and were described as the first pseudomonads possessing the Mop-type LmPH (mPH linked to C12O). Co-presence of the genes for sPH and mPH was demonstrated in strains PC16 and P69 (Merimaa *et al*., 2006).

Table 1. Description of studied *Pseudomonas* strains

Strain	Catabolic type of phenol – *p*-cresol degradation	Type of phenol hydroxylase (relevant catabolic genotype)	Additional information
P. mendocina PC1	*meta-meta*	mPH (IIa[3], 98% similarity to DmpN)	Heinaru *et al*., 2000
P. fluorescens B PC18	*meta-ortho*^{prot}	mPH (I[3], 97% s. to DmpN)	Heinaru *et al*., 2000; phenol induces PCMH
P. fluorescens F PC20	*ortho*[1]*-meta*	sPH (*pheBA*)	Heinaru *et al*., 2000; contains PHE and NAH plasmids
P. fluorescens C PC24	*ortho-ortho*^{prot}	sPH (*pheBA*)	Heinaru *et al*., 2000
P. fluorescens C PC31	*ortho-ortho*^{prot}	sPH (*pheBA*)	Heinaru *et al*., 2000
P. fluorescens F PC17	*ortho-ortho*^{prot}	mPH (IIIb[3], 95% s. to MopN)	Heinaru *et al*., 2000
P. putida B PC30	*ortho-ortho*^{prot}	mPH (IIIa[3], 95% s. to MopN)	Heinaru *et al*., 2000
P. putida B PC16	*ortho-ortho*^{prot}	sPH (*pheBA*) + mPH (IIIa[3], 95% s. to MopN)	Heinaru *et al*., 2000
P. fluorescens F P69	*ortho*[2]	sPH (*pheBA*) + mPH (IIIb[3], 95% s. to MopN)	Merimaa *et al*., 2006
P. putida EST1412	*ortho*-ND	sPH (*pheBA*)	Kivisaar *et al*., 1990
P. sp. CF600	*meta-meta*	mPH (*dmpN*)	Shingler *et al*., 1989

[1] in this strain after the catechol *ortho* pathway also the catechol *meta* pathway is induced;
[2] the strain does not degrade *p*-cresol;
[3] phylogenetic groups of the large subunit of multicomponent phenol hydroxylase determined by Merimaa *et al*. (2006).
sPH – single component phenol hydroxylase (PH);
mPH – multicomponent PH;
ND – not determined.

5. RESULTS AND DISCUSSION

5.1. THE ROLE OF ACCUMULATION OF INTERMEDIATES IN DECOMPOSITION OF SUBSTRATE MIXTURES. SIMULTANEOUS VERSUS SEQUENTIAL CONSUMPTION

Microorganisms degrade substrates from their mixtures either simultaneously or sequentially. Simultaneous utilization is usually observed when the pathways involved in the degradation of both substrates are almost identical or at least similar, when the enzyme induction system is non-specific or catabolic pathways converge (Hutchinson and Robinson, 1988). On the other hand, catabolic repression of expression of enzymes involved in catabolism of aromatic compounds by other substrates (organic acids, carbohydrates or even by other aromatic compounds) can be revealed (Hollender *et al*., 1994; Ampe *et al*., 1998). Also, accumulation of (dead-end) intermediates determines the rise of sequential degradation of compounds (Gaines *et al*., 1996).

Two-substrate cultivations were carried out in experiments described in Paper I. We found that if the substrates (phenol in combination with *p*-cresol or benzoate) were degraded from mixtures only via the catechol *meta* pathway (strain PC1 on a mixture of phenol and *p*-cresol), via the catechol *ortho* and protocatechuate *ortho* pathways (strain PC24 on a mixture of phenol and *p*-cresol), or only via the catechol *ortho* pathway (strain PC24 on a mixture of phenol and benzoate), then both substrates were consumed simultaneously (Fig. 1). In these cases no significant accumulation of intermediates was found, except during simultaneous degradation of both benzoate and phenol via the catechol *ortho* pathway by the strain PC24, where irreversible accumulation of *cis,cis*-muconate (CCM) in the growth medium was detected (Fig. 1C). However, accumulation of CCM did not inhibit degradation of phenol in the presence of benzoate.

Sequential consumption of substrates and diauxic growth occurred when two substrates were consumed via *meta* and *ortho* pathways due to metabolic conflict between the alternative pathways, and catabolic pathway used second is repressed by reversible accumulation of the intermediate (*p*-hydroxybenzoate, POB or catechol, CAT) of the first pathway (Fig 2; Fig. 9B of Paper I). Therefore we suppose that inability of the strain PC18 to degrade *p*-cresol in combination with phenol or benzoate simultaneously may be caused by transient accumulation of POB (Fig. 2AC), and because induction of the second catabolic pathway (catechol *meta* or *ortho* pathway, respectively) occurs only after the exhaustion of protocatechuate *ortho* pathway intermediate (Table 1 in Paper I). The preferred consumption of *p*-cresol via protocatechuate *ortho*

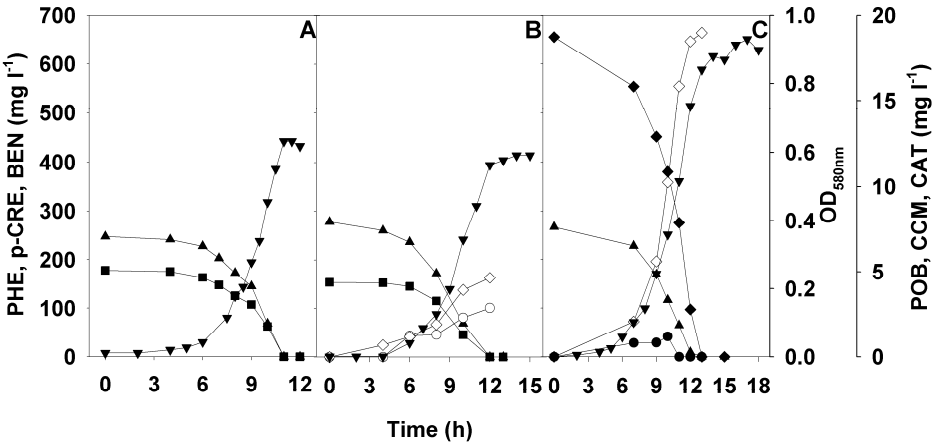


Figure 1 (Paper I, Figs. 4AC and 7A). Non-diauxic growth, simultaneous consumption of substrates and accumulation of metabolites in strains PC1 (A) and PC24 (B, C) cultivated on mixtures of phenol and *p*-cresol (A, B) or phenol and benzoate (C). Growth (▼) and concentrations of phenol (▲), *p*-cresol (■), benzoate (◆), *p*-hydroxybenzoate (○), *cis,cis*-muconate (◇) and catechol (●).

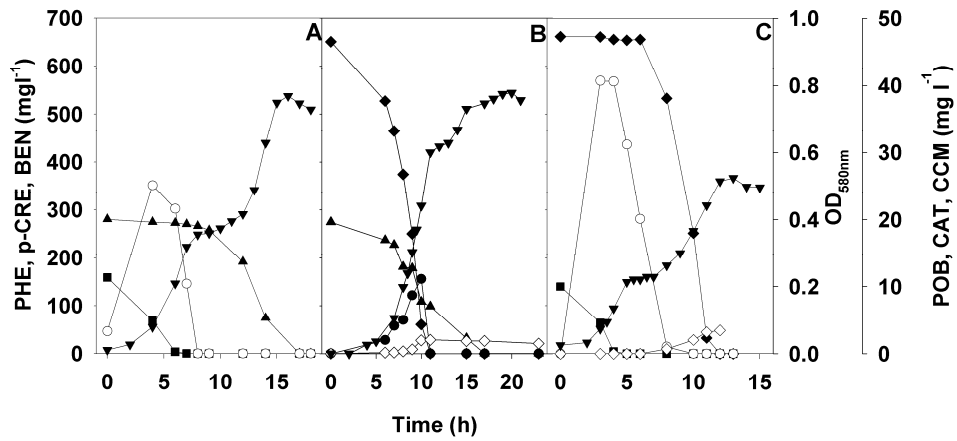


Figure 2 (Paper I, Figs. 4B, 7D and 9A). Diauxic growth, sequential consumption of substrates and accumulation of metabolites in strains PC18 (A, C) and PC1 (B) cultivated on mixtures of phenol and *p*-cresol (A), phenol and benzoate (B) and *p*-cresol and benzoate (C). Growth (▼) and concentrations of phenol (▲), *p*-cresol (■), benzoate (◆), *p*-hydroxybenzoate (○), *cis,cis*-muconate (◇) and catechol (●).

pathway in strain PC18 may be caused also by a high level of induction of enzymes of this pathway by phenol and benzoate and due to a low level of induction of catechol pathway enzymes (Table 2 in Heinaru *et al*. 2000). At the same time, in the strain PC24 the enzymes of both branches of *ortho* pathways are induced (Table 1 in Paper I) and phenol and *p*-cresol (or POB) are degraded simultaneously (Fig. 1 B; Fig 5B in Paper I). The opposite consumption order of the two substrates was obtained in strains *P. putida* (Nichols and Harwood, 1995) and *Acinetobacter calcoaceticus* (Gaines *et al*., 1996) in which benzoate or its descendants repressed the utilization of POB. Thus, it seems that there are no inherent properties of these compounds that dictate the order of their consumption (Brzostowicz *et al*. 2003); rather the genetic background of the strain is important.

Replacement of sequential degradation with simultaneous one at low substrate concentrations is quite usual in batch cultures (Kovárová-Kovar and Egli, 1998). In our experiments lowering of initial concentrations of POB and phenol or *p*-cresol 200-400 times decreased the antagonistic effect of POB on the degradation of phenol or *p*-cresol (Figs. 5AC and 6 in Paper I) in strain PC18, and simultaneous utilization of substrates was observed.

Transient accumulation of catechol during the consumption of benzoate via catechol *ortho* pathway repressed decomposition of both phenol (Fig. 2B) and *p*-cresol (Fig. 9B in Paper I) via catechol *meta* pathway in strain PC1. Though after the *ortho* cleavage of catechol the accumulation of CCM into the growth medium was detected (Fig. 2B; Fig. 9B in Paper I), we found no evidence of its effect on catechol *meta* pathway as was indicated for POB utilisation by *A. calcoaceticus* (Gaines *et al*., 1996).

Biodegradation of 2,4- and 3,4-dimethylphenol (DMP) in combination with phenol and/or *p*-cresol confirmed the assumption that decomposition pattern of substrates depends on induction of corresponding catabolic pathways and on transient accumulation of intermediates (Paper II). Representatives of different catabolic types of phenol and *p*-cresol degradation metabolised DMPs via different pathways. Namely, in strains PC18 and PC24, 2,4- and 3,4-DMP were converted into dead-end metabolites 4-hydroxy-3-methylbenzoic acid (4H3MBA) and 4-hydroxy-2-methylbenzoic acid (4H2MBA), respectively, after the attack of *p*-cresol methylhydroxylase, PCMH (Fig. 3AD, BE). The third transiently accumulating metabolite, identified as 4-hydroxy-2-methylbenzaldehyde (4H2MB), is the precursor of 4H2MBA in cometabolic degradation of 3,4-DMP by strains PC18 and PC24 (Fig. 3AD, BE).

During growth of the strain PC18 on 3,4-DMP and phenol containing mixtures transient accumulation of 4H2MB caused the repression of phenol degradation and diauxic growth (Fig. 3BD in Paper II), as in case of growth on *p*-cresol and phenol mixtures in which POB accumulated (Fig. 2A). On the contrary, simultaneous degradation of phenolic compounds was observed in strain PC24 if catechol and protocatechuate *ortho* pathways were used (Fig. 3BE). Third strain, PC1, possessed only catechol *meta* pathway for degradation of phenolic compounds and data

presented in Fig. 3C revealed simultaneous degradation of 3,4-DMP, phenol and *p*-cresol without accumulation of intermediates and confirmed its inability to degrade 2,4-DMP. We suppose that in this case DMP was oxidised into dimethylcatechol and cleaved by C23O. The oxidation of DMPs and (*ortho* or *meta*) cleavage of substituted catechols depends on the position of methyl groups on an aromatic ring (Pieper *et al*., 1995). Usually *ortho* cleavage of dimethylcatechols results in formation of dead-end products dimethylmuconolactones, whereas *meta* cleavages leads to TCA cycle (Pieper *et al*., 1995).

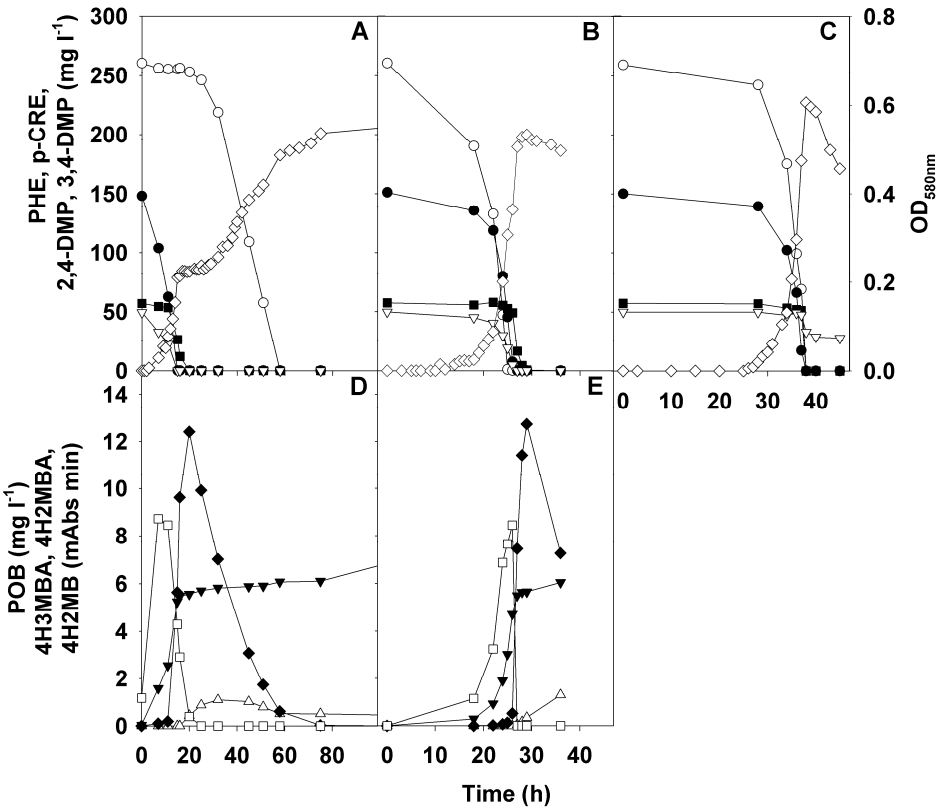


Figure 3 (Paper II, Figs. 6AC and 8). Growth, substrate consumption and accumulation of metabolites by strains PC18 (A, D), PC24 (B, E), PC1 (C) on mixture of 2,4-DMP, 3,4-DMP, phenol and *p*-cresol. Growth (◇) and concentrations of phenol (○), *p*-cresol (●), 2,4-DMP (▽), 3,4-DMP (■), POB (□), 4H2MB (◆), 4H2MBA (△) and 4H3MBA (▼).

It is generally accepted that inherent properties of aromatic compounds do not dictate the order of their consumption (Brzostowicz *et al*., 2003). The scientific

literature tends to support the idea that growth rate has a major influence on substrate preference, namely substrates that support comparable specific growth rates are degraded simultaneously in case of mixed substrates (Kovárová-Kovar and Egli, 1998). But Narang and Pilyugin (2007) have concluded after comparison of different studies that similar growth rates are neither necessary nor sufficient for simultaneous consumption of substrates and saturation concentration for induction must also be considered. Evaluation of growth rates of three strains growing on phenol, *p*-cresol or on their mixtures (Table 2) revealed that growth rates of strains PC1 and PC24 on single substrates are similar and the two substrates were degraded simultaneously from phenol-*p*-cresol mixture (Fig. 1AB). The strain PC18, which had higher growth rate on *p*-cresol than on phenol utilized *p*-cresol preferentially from the mixture of two substrates (Fig. 2A).

Table 2. Comparison of growth rates[a] of strains growing on phenol, *p*-cresol or their mixtures

Strain	μ (h^{-1})		
	phenol	*p*-cresol	phenol + *p*-cresol
PC1	0.69 ± 0.08^{i}	0.68 ± 0.08^{i}	0.57^{i}
PC24	0.50 ± 0.02^{i}	0.52 ± 0.17^{i}	0.47^{i}
PC18	0.26 ± 0.05^{i}	0.63 ± 0.09^{i}	0.49^{ii} 0.20^{iii}

[a] Growth rates were calculated from exponential growth phase (i) or in the case of diauxic growth from the first (ii) and the second (iii) exponential growth phases. Concentrations of substrates: phenol 250 mg l^{-1} and *p*-cresol 170 mg l^{-1}.

In summary, the following conclusions can be made from above described experiments:

1) when substrates are degraded using either catechol *meta*, catechol *ortho* or catechol *ortho* and protocatechuate *ortho* pathways, they are catabolised from mixtures simultaneously (nondiauxic growth) without the accumulation of intermediates;
2) when substrates are degraded through *meta* and *ortho* pathways, they are catabolised from mixtures sequentially (diauxic growth) with reversible accumulation of intermediates (*p*-hydroxybenzoate, catechol or 4-hydroxy-2-methylbenzaldehyde).

5.2. EVALUATION OF BIODEGRADATION EFFICIENCY OF GENETICALLY DIFFERENT PSEUDOMONADS BY KINETIC PARAMETERS

The calculations of substrate-oxygenating activities at different substrate concentrations from the data of oxygen consumption allow determination of kinetic parameters of degradation of phenolic compounds. Watanabe *et al*. (1998, 2002) showed that phylogenetic grouping of bacteria on the basis of the DNA sequences encoding LmPH was in good correlation with physiological grouping based on kinetics of phenol-oxygenating activity of whole cells. Works of Kiesel and Müller (2002) and Jiang *et al*., (2006) have shown that phenol-degrading strains with catechol *ortho* or *meta* cleavage pathways hold different potential for practical use in bioaugmentation. In carbon limited chemostat cultures it has been shown that strains possessing catechol *meta* pathway have higher growth rate values than strains with catechol *ortho* pathway (Kiesel and Müller, 2002). However, K_S values for phenol according to phenol-oxygenating activity are similar for C12O- and C23O-possessing strains (Jiang *et al*., 2006).

Degradative bacterial strains described first by Heinaru *et al*. (2000) and later by Merimaa *et al*. (2006) can be divided into groups not only by catabolic types of phenol and *p*-cresol degradation but also according to types of phenol hydoxylases (PH) and combined catechol dioxygenases (C23O, C12O) involved in degradation of phenol. So, there are three different PH groups: sPH- (PC24, PC31, PC20), mPH- (PC1, PC18, PC30, PC17) and sPH+mPH-possessing strains (PC16, P69). While all sPH-possessing strains cleave catechol via *ortho* pathway, in mPH-strains cleavage of catechol is catalysed by C23O (PC1, PC18) or C12O (PC17, PC30) (Table 1). As respective kinetic parameters of genetically different phenol-degrading strains have not been investigated systematically, we evaluated genetically diverse collection of phenol degrading pseudomonads by kinetic parameters (Paper IV).

The evaluation of different PH possessing strains by kinetic parameters of phenol-oxygenating activity (Table 3) revealed that the values of half-saturation constant for phenol-oxygenating activity (K_S), were by almost one order of magnitude lower in the strains harbouring mPH (PC1, PC18, PC17, PC30 and the reference strain CF600) compared to the strains having sPH (PC24, PC31, PC20 and the reference strain EST1412). The type of catechol cleavage pathway did not affect the kinetics of phenol oxidation – the K_S and V_{max}/K_S values were similar for mPH-harbouring strains regardless to whether catechol was subjected to *ortho* (PC17, PC30) or *meta* (PC1, PC18, CF600) cleavage pathway (Table 3).

Table 3 (Paper IV, Table 2). Comparison of kinetic parameters[a] for specific phenol-oxygenating activity and growth of the strains studied

Strain	Apparent kinetic constants in Michaelis-Menten equation[1] for specific phenol-oxygenating activity		Apparent kinetic constants in Aiba-Edwards equation[2] for growth		Yield factor
	K_S (μM)	V_{max}/K_S (U g^{-1} DW μM^{-1})	μ_{max} (h^{-1})	K_I (mM)	$Y_{X/S}$[b] (g DW g^{-1} PHE)
EST1412	12.8±1.5	7.2±0.9	0.931±0.080	7.57±0.25	0.545
PC24	21.4±5.1	3.9±1.0	0.231±0.010	7.44±0.30	0.469
PC31	12.8±5.3	10.2±4.5	0.377±0.017	4.31±0.22	0.726
PC20	13.1±2.5	24.5±4.9	0.699±0.027	3.44±0.10	0.591
PC1	1.7±0.2	105±13	0.596±0.042	9.41±1.20	0.669
CF600	4.9±1.2	33.2±8.4	0.857±0.015	13.15±0.88	ND
PC18	1.4±0.5	134±49	0.535±0.075	2.52±0.18	0.700
PC17	1.9±0.5	200±54	0.689±0.009	3.56±0.05	0.790
PC30	1.3±0.3	43.1±10	0.789±0.240	4.40±1.30	0.849
PC16	2.4±0.7	31.3±9.6	0.914±0.084	3.84±0.17	0.843
P69	27.1±10.1	3.9±1.6	0.381±0.012	1.99±0.10	0.570

[a] Values of kinetic constants were determined using a non-linear regression method (SigmaPlot 2001 Enzyme Kinetic Module, SYSTAT) and are expressed as means of three independent experiments ± standard deviations of the mean.

[b] The standard deviation for $Y_{X/S}$ was about 10%.

U - μmol of oxygen consumed per minute; DW – dry weight; ND – not determined.

[1] $V = \frac{V_{\max} S}{K_S + S}$ where K_S is half saturation constant, V_{max} is maximum specific activity and S is concentration of phenol.

[2] $\mu = \frac{\mu_{\max} S}{K_{SG} + S} \exp\left(-\frac{S}{K_I}\right)$ where μ_{max} is maximum specific growth rate, S is concentration of phenol, K_{SG} is half-saturation constant and K_I is inhibition constant.

Comparison of maximum specific growth rate (μ_{max}) values showed that the mPH-strains (including PC16) had significantly higher μ_{max} values than sPH-strains PC24, PC31 and P69, whereas the sPH-strains PC20 and EST1412 were exceptional as they also had high μ_{max} values (Table 3). A particular genetic background and genetic regulation in these strains can cause such a difference. Namely, the strain PC20 has two cleavage pathways for catechol: besides C12O activity, it also reveals constitutive expression of C23O and the laboratory-constructed strain EST1412 has two sets of genes for C12O, i.e. the chromosomally encoded *cat*A gene and the plasmid-borne *pheB* gene of the *pheBA* operon (Kivisaar *et al*., 1990). Evaluation of the μ_{max} values of the mPH-strains possessing either *ortho* (PC17 and PC30) or *meta* (PC1, PC18 and CF600) cleavage type did not exhibit relevant differences. However, about 1.5 to 2 times lower μ_{max} values have been obtained for strains degrading phenol through catechol *ortho* pathway (batch cultivation: Polymenakou and Stephanou, 2005; chemostat cultivation: Müller and Babel, 1996) than for strains degrading phenol through catechol *meta* pathway (batch cultivation: Chen *et al*., 2004; chemostat cultivation: Müller and Babel, 1996).

The strains PC16 and P69, possessing both sPH and mPH genes, had K_S as well as μ_{max} values similar to those of the strains possessing only mPH or sPH, respectively (Table 3). These results allow us to speculate that only mPH is active in the strain PC16 while in the strain P69, only sPH is functional. This assumption is in good concordance with our previous finding that *pheA* expression (sPH) in PC16 was by one order of magnitude lower than in P69 (Merimaa *et al*., 2006).

The inhibiting effect of phenol on growth of bacteria was evaluated according to inhibition constant K_I (Table 3) and duration of the lag-time (Fig. 1 in Paper IV). The K_I values revealed strain-specific tolerance to phenol but did not allow differentiation of the strains according to mPH and sPH types (Table 3). The length of the lag-time (λ) increased exponentially with phenol concentration and the inhibitory effect of phenol was much stronger in strains P69, PC20, PC17, PC30 and PC18 with low K_I values, while the strains PC1, PC16, CF600 and EST1412 were highly tolerant (Fig.1 in Paper IV). The mPH+C12O and mPH+C23O strains (PC17, PC30 and PC1, CF600, respectively), which are the representatives of two different LmPH groups, had similar phenol tolerance. The strain PC18 being a representative of a unique phylogenetic group of LmPH (Merimaa *et al*., 2006) was more sensitive to phenol than the other mPH+C23O-strains. Strains with two PHs, PC16 and P69, differed largely for phenol tolerance.

Thus, we can conclude:

1) the values of half-saturation constants for phenol-oxygenating activity (K_S) of strains harbouring mPH are almost by one order of magnitude lower than of strains having sPH;

2) mPH-strains have higher maximum specific growth rate (μ_{max}) values than sPH-possessing strains grown on phenol;
3) the comparison of inhibition constant (K_I) values reveals strain-specific tolerance to phenol.

5.3. RELATIONSHIPS BETWEEN THE YIELD FACTOR VALUES AND THE CONCENTRATIONS OF *cis,cis*-MUCONATE DURING THE GROWTH OF GENETICALLY DIFFERENT PSEUDOMONADS ON PHENOL

The efficiency of energy generation from substrates in microbial cells is reflected in biomass yield factor ($Y_{X/S}$) values. Although high $Y_{X/S}$ values are advantageous to establish a stable biological remediation process, they may cause (unwanted) increased production of excess sludge in a wastewater treatment plant. During degradation of phenol via catechol *ortho* or *meta* pathways accumulation of intermediates, such as catechol and *cis,cis*-muconate (CCM), and 2-hydroxymuconic semialdehyde, in the growth medium has been detected. Accumulation of non-degradable intermediates (for example exogenous CCM) or high concentrations of toxic substrates may result in low $Y_{X/S}$ values due to the loss of part of the substrate and stronger inhibition effects, thereby the amount of energy needed to maintain the cell activity is higher. Both catechol cleavage pathways are represented in strains studied in this work (Table 1), and some of the strains excrete metabolites while degrading aromatic compounds (Paper I). Therefore the yield factor values were determined for the strains growing on 2.7 mM phenol (Table 3). The obtained results revealed that $Y_{X/S}$ values for the sPH-possessing strains were lower compared to the mPH-strains. Among the mPH-strains the $Y_{X/S}$, as well as μ_{max}, did not depend on the type of catechol cleavage pathway of the strain. These data, determined from batch cultivations, contradict those by Kiesel and Müller (2002) obtained in chemostat systems under carbon limitation, according to which the catechol *meta* pathway results in a high growth rate and a low growth yield.

It is known that a cleavage product of catechol, *cis,cis*-muconate, is a critical intermediate of *ortho* decay in *Pseudomonas* strains (Feist and Hegeman, 1969; Ornston, 1971) and exogenous CCM can be used for growth only by permeable mutants (Williams and Shaw, 1997). As the possible overflow of CCM hints to the pathway bottleneck localised at the level of C12O and muconate cycloisomerase (MCI), the ratio between the activities of C12O and MCI in the middle and late log-phases during the growth of the strains expressing the catechol *ortho* pathway on 2.7 mM phenol (Fig. 4) were studied. The experiments revealed that the mPH-strains with catechol *ortho* cleavage pathway (PC17 and PC30), which had a high growth yield, accumulated less exogenous CCM than the sPH-strains (Fig. 4). The ratio between specific

activities of C12O and MCI showed that in sPH-strains the activity of MCI was always lower than that of C12O. The imbalance between the levels of the enzymes determining sequential catabolic reactions may cause intracellular accumulation of CCM as well as its exogenous accumulation in case of free diffusion. The conversion of phenol to CCM and accumulation of the latter in the growth medium due to the increased expression of C12O may be an important advantage for a strain enabling survival at high substrate concentrations. However, a high growth rate (Table 3) and short lag-phase (Fig. 1 in Paper IV) of the sPH-strains PC31 and EST1412 improved the growth yield.

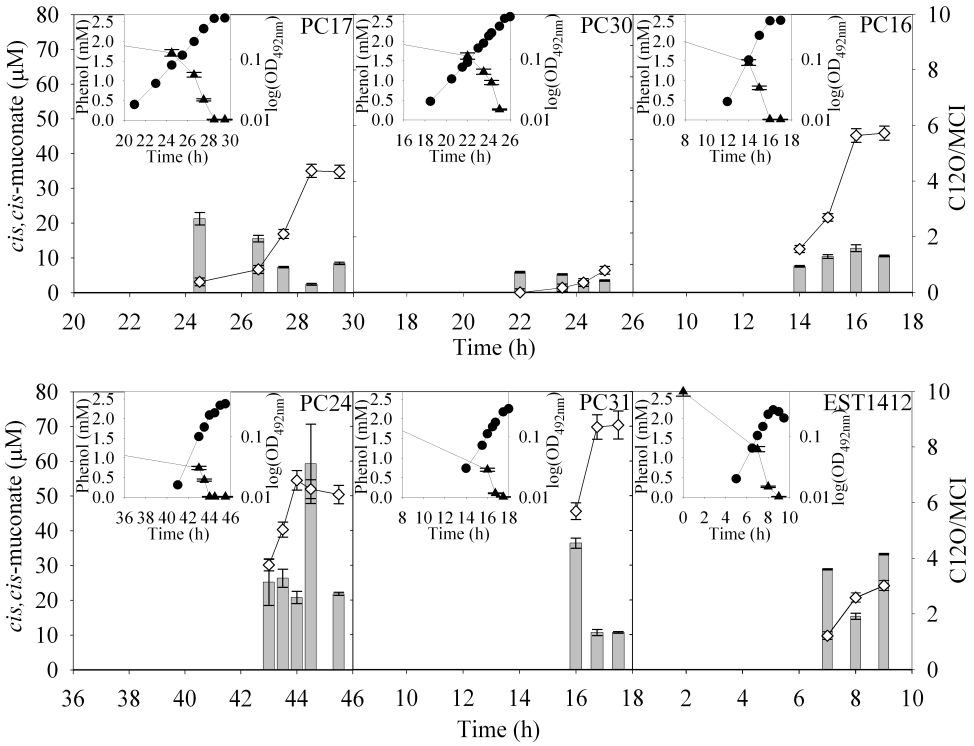


Figure 4 (Paper IV, Fig. 2). Accumulation of *cis,cis*-muconate (◇) in the medium and ratio of specific activities of catechol 1,2-dioxygenase and muconate cycloisomerase (columns) in strains PC17, PC30, PC16, PC24, PC31 and EST1412 growing on 2.7 mM phenol. Insets show biomass production (●) and phenol consumption (▲) as a function of time. Values are the averages of triplicate determinations. Error bars indicate the combined standard uncertainties of phenol and *cis,cis*-muconate concentrations or standard deviations of C12O/MCI data.

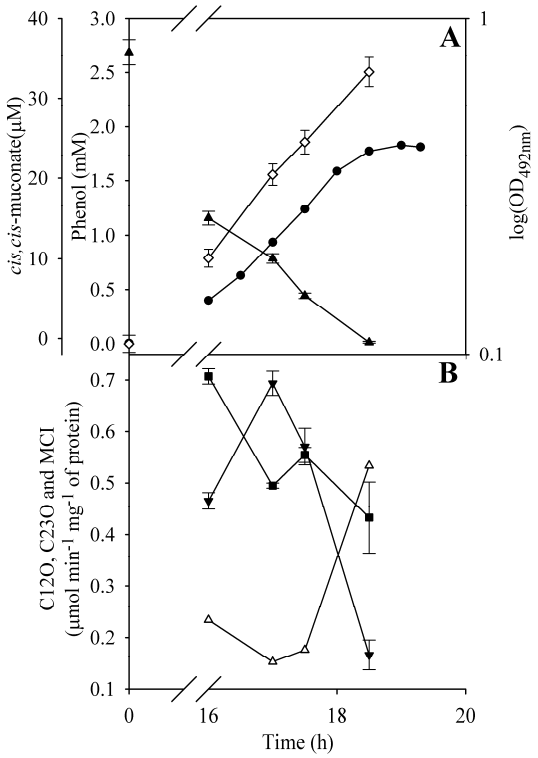


Figure 5 (Paper IV, Fig. 3). Biomass production (●), phenol consumption (▲), accumulation of *cis,cis*-muconate (◇) (A) and specific activities of catechol 1,2-dioxygenase (■), catechol 2,3-dioxygenase (△), and muconate cycloisomerase (▼) as a function of time in strain PC20 (B). Values are the averages of triplicate determinations. Error bars indicate the combined standard uncertainties of phenol and *cis,cis*-muconate concentrations or standard deviations of C12O, C23O and MCI data.

The strain PC20 is exceptional and very specific because it possesses two catabolic plasmids, NAH and PHE, and consequently, two catechol cleavage pathways (Heinaru *et al*., 2000; Paper III). Phenol degradation via sPH and C12O is encoded by the *pheBA* operon induced by phenol, whereas the synthesis of C23O (from *nah* operon) is constitutive (Paper III). Consumption of phenol, accumulation of CCM and the activities of C12O, C23O and MCI in exponentially growing cells of PC20 were measured (Fig. 5). Expectedly, accumulation of CCM in the growth medium was accompanied by a decrease in specific activity of MCI. The inverse correlation was established between the activities of C12O and C23O (Fig. 5), and activation of the catechol *meta* pathway resulted in reversible accumulation of a yellow coloured product,

2-hydroxymuconic semialdehyde (detected visually) in the growth medium. The *ortho* cleavage pathway is the main route of phenol degradation in PC20 and after disappearance of phenol the synthesis of C23O will be derepressed. The co-presence of two cleavage pathways in PC20 led to higher μ_{max} and $Y_{X/S}$ values compared to other sPH-strains. The induction of C23O after C12O during the growth on benzoate in late stationary growth phase was observed also in *Alcaligenes eutrophus* strain 355, but in this case reversible accumulation of catechol (up to 3 mM) was detected. Here, accumulation of the intermediate was explained by limited amount of C12O synthezised and by inhibition effect of catechol on the induction of MCI (Ampe and Lindley, 1996).

In summary, the following conclusions can be made:

1) as a result of low activity of MCI compared to the C12O the exogenous concentrations of *cis,cis*-muconate in catechol *ortho* cleavage possessing sPH-strains is higher and $Y_{X/S}$ values lower compared to respective values of mPH+C12O-strains;
2) higher $Y_{X/S}$ and μ_{max} values are obtained in a sPH-strain possessing both catechol cleavage pathways (PC20) than in sPH-strains having only catechol *ortho* pathway.

5.4. EFFECTIVENESS OF DIFFERENT PHENOL/*p*-CRESOL DEGRADING INDIGENOUS BACTERIAL STRAINS IN MICROCOSM EXPERIMENTS SIMULATING BIOAUGMENTATION PERFORMANCES

The use of microorganisms for bioremediation of polluted soils and waters has become the focus of attention because of the potential for cost-effective restoring or reclaiming such sites compared to physico-chemical remediation methods. The current approach in bioremediation of contaminated waste sites relies primarily on the use of strains indigenous to the site. Until now the initial strain selection step has been dictated by the search for catabolically competent microorganisms, with little or no consideration given to other essential features necessary for functional activity and persistence in target habitats that has often lead to failure of the remediation (Thompson *et al*., 2005). Due to technical advances in molecular biology and analytical chemistry the assessments of the functional diversity and spatial distribution of microbial communities can be made. The use of gene probes is an attractive method used most widely for this purpose. For example, primers have been developed for particular microbial groups degrading one- and two-ringed aromatic contaminants, from which more often the catechol 2,3-dioxygenase (Wikström *et al*., 1996; Mesarch et al., 2000; Junca and Pieper, 2003) and multicomponent phenol hydroxylase (Watanabe *et*

al., 1998; Futamata *et al*., 2001) have been used as the target genes. On the other hand, several microbiological methods, including plating onto selective minimal agar to enumerate viable bacteria and microscopic examinations, are still used for estimation of activity of different microbial strains in mixed culture cultivations.

The previous data of our work have shown that indigenous phenol/*p*-cresol-degrading pseudomonads have different biochemical, physiological and genetic parameters (Heinaru *et al*., 2000, Papers I, II and IV) and can be used in bioaugmentation experiments at semi-coke mounds or areas with similar pollution. So, the next step in our study was to investigate the behaviour and relative abundance of indigenous phenol/*p*-cresol degrading bacterial populations during degradation of phenolic compounds in phenolic leachate- and oil-amended microcosms (Paper III). Microcosm experiments with equal quantities of cell suspensions (~$1x10^7$ CFU ml^{-1}) of strains PC1, PC18, PC20 and PC24 were carried out in flasks containing filter-sterilised leachate or crude shale oil (60 mg/100 ml).

The functional activities and structural fluctuations in bacterial consortia in leachate- and oil-amended microcosms were identified using denaturing gradient gel electrophoresis (DGGE) analysis of the amplified genes encoding 16S rRNA and a large subunit of multicomponent phenol hydroxylase (LmPH) (Paper III). The 16S rRNA gene probe has been used most widely for overall community structure analyses, while LmPH is a catabolic enzyme and only strains possessing this respective gene can be detected and followed. In our study the semi-quantitative analysis of DGGE gel showed that strain PC1 became dominant and strain PC20 disappeared after the incubation of a mixed population in leachate microcosm; at the same time phenol and benzoate were removed during the first day, cresols after 10 days, and from dimethylphenols 3,4-DMP in two days (Fig. 6). In contrast, no dominant population was detected in oil-amended microcosms by DGGE, while naphthalene, cresols and part of dimethylphenols were degraded after 30 days incubation (Fig. 7). The catabolic significance of LmPH-possessing strains (PC1 and PC18) in biodegradation of pollutant mixtures revealed dominance of the strain PC1 in leachate and that of the strain PC18 in oil-amended microcosms (Fig. 2 in Paper III).

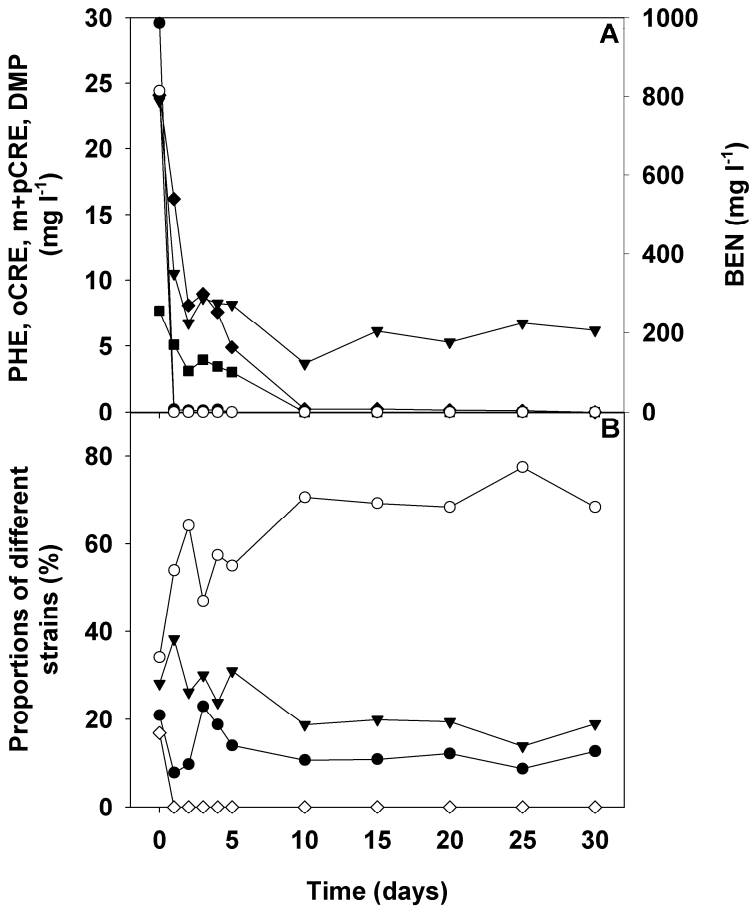


Figure 6. Removal of substrates (phenol ●, *o*-cresol ■, *m*- and *p*-cresol ◆, dimethylphenols ▼, benzoate ○; panel A) and relative percentage of bacterial strains (PC1 ○, PC18 ●, PC20 ◇, PC24 ▼; panel B) in a mixed culture during 30 days of incubation in a leachate microcosm. Values of the relative percentages (%) were calculated from the image of ethidium bromide-stained DGGE gel containing PCR-amplified fragments of 16S rRNA genes using program Quantity One (Bio-Rad).

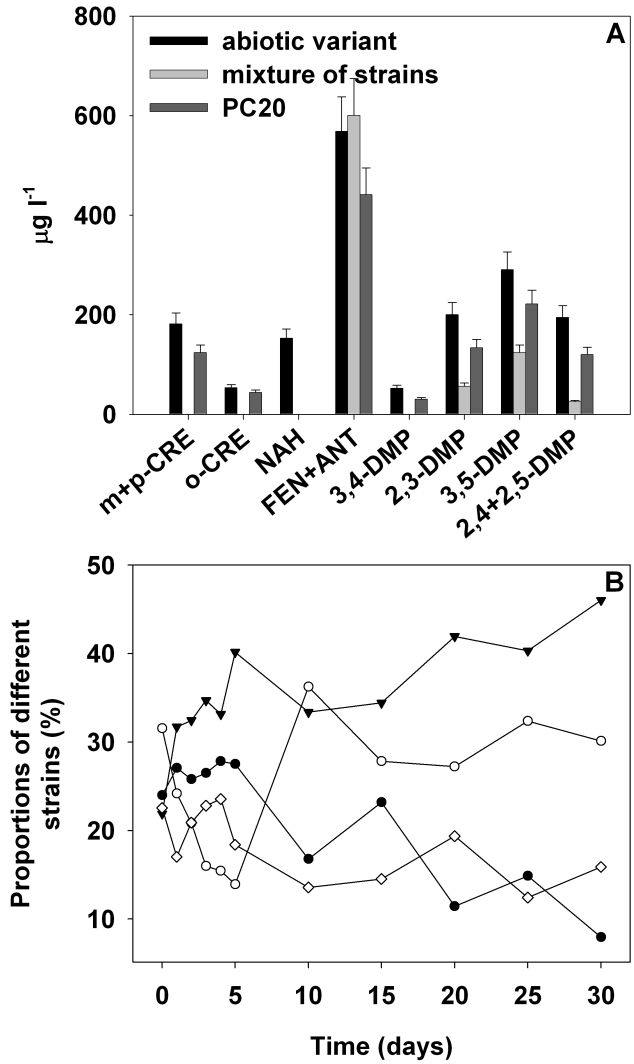


Figure. 7. Comparison of decomposition of substrates (*m*- and *p*-cresol, *o*-cresol, naphthalene, phenanthrene and anthracene, and dimethylphenols) in abiotic control, in mixture of four strains and in single culture of strain PC20 after incubation for 30 days (panel A) and relative percentage of bacterial strains (PC1 ○, PC18 ●, PC20 ◇, PC24 ▼) in mixed culture during 30 days incubation (panel B) in an oil-amended microcosm. Values of relative percentages (%) were calculated from the image of ethidium bromide-stained DGGE gel containing PCR-amplified fragments of 16S rRNA genes using program Quantity One (Bio-Rad). Error bars indicate the combined standard uncertainties of the results.

Disappearance or maintenance of a particular population in the microcosm throughout the entire experiment indicates that changes in bacterial consortia largely depend on chemical composition of growth medium and on catabolic genes possessed by the strains. For example, a phenol-degrading strain PC20 differs from the other used strains by the ability to degrade naphthalene and other PAHs that possibly causes the persistence of this strain in oil-amended microcosm (Fig. 7). The ability to degrade salicylate, an intermediate of naphthalene degradation, may be the reason why the LmPH-possessing strain PC18 dominated over PC1 through the first 10 days in oil-amended microcosms. Additionally, the strain PC18 can decompose dimethylphenols in the presence of phenol and *p*-cresol (Paper II).

The changing concentrations of substrates may cause microbial population densities and distributions to be quite variable (Hosein *et al*., 1997). Jiang *et al*. (2002) and Watanabe *et al*. (1996) showed that high phenol concentrations promoted growth of strains with high half saturations constant (K_S) values, while strains with low-K_S were enriched in case of low phenol concentrations in the medium. Additionally, high concentrations of phenol cause inefficient growth of low-K_S bacteria, which are outcompeted by high-K_S bacteria (Futamata *et al*., 2001). In our experiments the strain PC1 grew rapidly on phenol and its K_S value was almost one order of magnitude lower than that of the strain PC20 (Paper IV). High growth rate and a short lag-time are also essential to outcompete other strains in mixed cultures (Franck-Mokroβ and Schmidt, 1998). The kinetic parameters of growth determined in Paper IV revealed that unlike the strain PC18 the strain PC1 had a high value of both μ_{max} and K_I, and the length of the lag-time showed only minor dependence on initial phenol concentration in the medium. This may be the reason why the strain PC1 dominated in leachate microcosm experiments.

Alongside with microcosm experiments, the other members of our laboratory carried out bioaugmentation field experiments on semi-coke mounds using selected degradative bacterial strains PC1, PC18 and PC24 (Truu et al., 2003). The obtained data revealed that already within three months of study period the plant roots biomass and length increased, the concentration of residual shale oil decreased and the number of phenol-degrading bacteria increased by one order of magnitude, while the number of heterotrophic bacteria remained at the same level compared to the untreated plot. After two years of combined phytoremediation and bioaugmentation experiments, the diversity and activity of the microbial community in the test sites increased.

The following conclusion result from microcosm experiments imitating bioaugmentation:

1) the specific bacterial populations are selected during mixed culture cultivations in phenolic leachate and oil-amended microcosms.

6. CONCLUSIONS

The continually growing pollution of the environment with anthropogenic compounds has lead to development of many remediation technologies. A favoured approach for enhancement of bioremediation applications is the use of indigenous biodegradative bacterial strains in bioaugmentation. Different pseudomonads, earlier isolated from the river water continuously polluted with phenolic compounds, were studied in this thesis with intention to characterize their biochemical parameters in relation to both treatment efficiency and community structure/function.

Based on the results presented in the thesis, following conclusions are made concerning three main subjects studied:

1. Mixed substrate (combining phenol, *p*-cresol, benzoate, *p*-hydroxybenzoate, dimethylphenols) batch cultivations of single strains (*Pseudomonas mendocina* PC1, *Pseudomonas fluorescens* PC18 or *P. fluorescens* PC24) revealed that:
   1.1. if either catechol *meta*, catechol *ortho* or catechol *ortho* and protocatechuate *ortho* pathways are used for the degradation of substrates, accumulation of intermediates is not detected and simultaneous consumption of substrates (nondiauxic growth) is observed;
   1.2. if *meta* and *ortho* pathways are used for degradation of substrates, reversible accumulation of intermediates (*p*-hydroxybenzoate, catechol or 4-hydroxy-2-methylbenzaldehyde) and sequential consumption of the substrates (diauxic growth) is observed.
2. Evaluation of biodegradation efficiency of genetically different phenol hydroxylase-possessing pseudomonads (11 strains) by kinetic parameters of phenol-oxygenation and growth on phenol showed that:
   2.1. strains possessing single- (sPH) or multicomponent phenol hydroxylase (mPH) are distributed between separate groups:
      2.1.1. the values of half-saturation constants for phenol-oxygenating activity (K_S) of strains harbouring mPH are almost by one order of magnitude lower than of strains having sPH, whereas mPH-strains have higher maximum specific growth rate (μ_{max}) and yield factor ($Y_{X/S}$) values than sPH-possessing strains;
      2.1.2. the lower $Y_{X/S}$ values of sPH+C12O-strains are considered to be caused by low specific activity of muconate cycloisomerase compared to the catechol 1,2-dioxygenase (C12O) that may result in accumulation of higher exogenous concentrations of *cis,cis*-muconate compared to mPH+C12O-strains;
   2.2. the tolerance to elevated phenol concentrations is strain-specific.
3. The microcosm experiments using mixed cultures in phenolic leachate and oil-amended microcosms indicated that:

3.1. specific bacterial populations are selected in both studied microcosms, and strains possessing different pathways for degradation of aromatic compounds hold different potential for bioremediation.

Consequently, the data presented in the thesis show that genetically different catabolic genes possessing pseudomonads have wide biodegradation potential and can be used for bioaugmentation of various polluted areas.

7. REFERENCES

Ampe F, Leonard D, Lindley ND. 1998. Repression of phenol catabolism by organic acids in *Ralstonia eutropha*. Appl. Environ. Microbiol. 64(1), 1–6.

Ampe F, Lindley ND. 1995. Acetate utilization is inhibited by benzoate in *Alcaligenes eutrophus*: evidence for transcriptional control of the expression of *acoE* coding for acetyl coenzyme A synthetase. J. Bacteriol. 177(20), 5826–5833.

Ampe F, Lindley ND. 1996. Flux limitations in the *ortho* pathway of benzoate degradation of *Alcaligenes eutrophus*: metabolite overflow and induction of the *meta* pathway at high substrate concentrations. Microbiology 142(7), 1807–1817.

Aranda-Olmedo I, Marin P, Ramos JL, Marques S. 2006. Role of the *ptsN* gene product in catabolite repression of the *Pseudomonas putida* TOL toluene degradation pathway in chemostat cultures. Appl. Environ. Microbiol. 72(11), 7418–7421.

Assinder SJ, Williams PA. 1990. The TOL plasmids: determinants of the catabolism of toluene and the xylenes. Adv. Microb. Physiol. 31, 1–69.

Baranyi J, Roberts TA. 1994. A dynamic approach to predicting bacterial growth in food. Int. J. Food Microbiol. 23, 277–294.

Basu A, Apte SK, Phale PS. 2006. Preferential utilization of aromatic compounds over glucose by *Pseudomonas putida* CSV86. Appl. Environ. Microbiol. 72(3), 2226–2230.

Bayly RC, Chapman PJ, Dagley S, Di Bernardino D. 1980. Purification and some properties of maleylpyruvate hydrolase and fumarylpyruvate hydrolase from *Pseudomonas alcaligenes*. J. Bacteriol. 143, 70–77.

Bayly RC, Dagley S, Gibson DT. 1966. The metabolism of cresols by species of *Pseudomonas*. Biochem. J. 101(2), 101–113.

Bielefeldt AR, Stensel HD. 1999. Biodegradation of aromatic compounds and TCE by a filamentous bacteria-dominated consortium. Biodegradation 10(1), 1–13.

Boopathy R. 2000. Factors limiting bioremediation technologies. Bioresource Technol. 74(1), 63–67.

Brzostowicz PC, Reams AB, Clark TJ, Neidle EL. 2003. Transcriptional cross-regulation of the catechol and protocatechuate branches of the beta-ketoadipate pathway contributes to carbon source-dependent expression of the *Acinetobacter* sp. strain ADP1 *pobA* gene. Appl. Environ. Microbiol. 69(3), 1598–1606.

Buchanan RL, Cygnarowicz ML. 1990. A mathematical approach toward defining and calculating the duration of the lag phase. Food Microbiology 7, 237–240.

Cafaro V, Izzo V, Scognamiglio R, Notamista E, Capasso P, Casbarra A, Pucci P, Di Donato A. 2004. Phenol hydroxylase and toluene/*o*-xylene monooxygenase from *Pseudomonas stutzeri* OX1: Interplay between two enzymes. Appl. Environ. Microbiol. 70(4), 2211–2219.

Cafaro V, Notomista E, Capasso P, Di Donato A. 2005. Regiospecificity of two multicomponent monooxygenases from *Pseudomonas stutzeri* OX1: Molecular basis for catabolic adaptation of this microorganism to methylated aromatic compounds. Appl. Environ. Microbiol. 71(8), 4736–4743.

Chapman PJ, Hopper DJ. 1968. The bacterial metabolism of 2,4-xylenol. Biochem. J. 110, 491–498.

Chen W-M, Chang J-S, Wu C-H, Chang S-C. 2004. Characterization of phenol and trichloroethene degradation by the rhizobium *Ralstonia taiwanensis*. Research Microbiol. 155(8), 672–680.

Cowles CE, Nichols NN, Harwood CS. 2000. BenR, a XylS homologue, regulates three different pathways of aromatic acid degradation in *Pseudomonas putida*. J. Bacteriol. 182(22), 6339–6346.

Crawford RL. 1976. Pathways of 4-hydroxybenzoate degradation among species of *Bacillus*. J. Bacteriol. 127(1), 204–210.

Dagley S, Evans WC, Ribbons DW. 1960. New pathways in the oxidative metabolism of aromatic compounds by micro-organsms. Nature, 188, 560–566.

Dagley S, Patel MD. 1957. Oxidation of *p*-cresol and related compounds by a *Pseudomonas*. Biochem. J. 66(2), 227–233.

Dalgaard P, Koutsoumanis K. 2001. Comparison of maximum specific growth rates and lag times estimated from absorbance and viable count data by different mathematical models. J. Microbiol. Meth. 43, 183–196.

Debarati P, Pandey G, Pandey J, Jain RK. 2005. Accessing microbial diversity for bioremediation and environmental restoration. Trends Biotechnol. 23(3), 135–142.

Dens EJ, Bernaerts K, Standaert AR, van Impe JF. 2005. Cell division theory and individual-based modeling of microbial lag. Part I. The theory of cell division. Int. J. Food Microbiol. 101, 303–318.

Deziel E, Paquette G, Villemur R, Lepine F, Bisaillon J. 1996. Biosurfactant production by a soil *Pseudomonas* strain growing on polycyclic aromatic hydrocarbons. Appl. Environ. Microbiol. 62(6), 1908–1912.

DiMarco AA, Averhoff BA, Kim EE, Ornston LN. 1993. Evolutionary divergence of *pobA*, the structural gene encoding *p*-hydroxybenzoate hydroxylase in an *Acinetobacter calcoaceticus* strain well-suited for genetic analysis. Gene 125(1), 25–33.

Dua M, Singh A, Sethunathan N, Johri AK. 2002. Biotechnology and bioremediation; successes and limitations. Appl. Microbiol. Biotechnol. 59, 143–152.

Duetz WA, Marques S, de Jong C, Ramos JL, van Andel JD. 1994. Inducibility of the TOL catabolic pathway in *Pseudomonas putida* (pWW0) growing on succinate in continuous culture: evidence of carbon catabolite repression control. J. Bacteriol. 176(8), 2354–2361.

Duetz WA, Marques S, Wind B, Ramos JL, van Andel JG. 1996. Catabolite repression of the toluene degradation pathway in *Pseudomonas putida* harbouring pWW0 under various conditions of nutrient limitation in chemostat culture. Appl. Environ. Microbiol. 62(2), 601–606.

Ehrt S, Schirmer F, Hillen W. 1995. Genetic organization, nucleotide sequence and regulation of expression of genes encoding phenol hydroxylase and catechol 1,2-dioxygenase in *Acinetobacter calcoaceticus* NCIB8250. Mol. Microbiol. 18(1), 13–20.

Erb RW, Eichner CA, Wagner-Döbler I, Timmis KN. 1997. Bioprotection of microbial communities from toxic phenol mixtures by a genetically designed pseudomonad. Nature Biotechnol. 15, 378–382.

Feist CF, Hegeman GD. 1969. Phenol and benzoate metabolism by *Pseudomonas putida*: regulation of tangential pathways. J. Bacteriol. 100(2), 869–877.

Fialová AM, Boschke E, Bley T. 2004. Rapid monitoring of the biodegradation of phenol-like compounds by the yeast *Candida maltosa* using BOD measurement. Int. Biodeter. Biodegr. 54, 69–76.

Franck-Mokroβ AC, Shmidt E. 1998. Simultaneous degradation of chloro- and methyl-substituted aromatic compounds: competition between *Pseudomonas* strains using the *ortho* and *meta* pathway or the *ortho* pathway exclusively. Appl. Microbiol. Biotechnol. 50, 233–240.

Fujita M, Ike M, Hioki JI, Kataoka K, Takeo M. 1995. Trichloroethylene degradation by genetically engineered bacteria carrying cloned phenol catabolic genes. J. Ferment. Bioeng. 79, 100–106.

Futamata H, Harayama S, Watanabe K. 2001. Group-specific monitoring of phenol hydroxylase genes for a functional assessment of phenol-stimulated trichloroethylene bioremediation. Appl. Environ. Microbiol. 67(10), 4671–4677.

Gaines III GL. Smith L, Neidle EL. 1996. Novel nuclear magnetic resonance spectroscopy methods demonstrate preferential carbon source utilization by *Acinetobacter calcoaceticus*. J. Bacteriol. 178(23), 6833–6841.

Gogarten JP, Townsend JP. 2005. Horizontal gene transfer, genome innovation and evolution. Nat. Rev. Microbiol. 3, 679–682.

Grady Jr CPL, Smets BF, Barbeau DS. 1996. Variability in kinetic parameter estimates: a review of possible causes and proposed terminology. Wat. Res. 30(3), 742–748.

Guha S, Peters CA, Jaffe PR, 1999. Multisubstrate biodegradation kinetics of naphthalene, phenanthrene, and pyrene mixtures. Biotechnol. Bioeng. 65 (5), 491–499.

Harayama S, Kok M, Neidle EL. 1992. Functional and evolutionary relationships among diverse oxygenases. Annu. Rev. Microbiol. 46, 565–601.

Harayama S, Mermod N, Rekik M, Lehrbach PR, Timmis KN. 1987. Roles of the divergent branches of the *meta*-cleavage pathway in the degradation of benzoate and substituted benzoates. J. Bacteriol. 169(2), 558–564.

Harayama S, Rekik M. 1989. Bacterial aromatic ring-cleavage enzymes are classified into two different gene families. J. Biol. Chem. 264(26), 15328–15333.

Harayama S, Rekik M. 1990. The *meta* cleavage of TOL degradative plasmid pWW0 comprises 13 genes. Mol. Gen. Genet. 221(1), 113–120.

Harpel RM, Lipscomb JD. 1990. Gentisate 1,2-dioxygenase from *Pseudomonas*. Substrate coordination to active site Fe^{2+} and mechanism of turnover. J. Biol. Chem. 265, 22187–22196.

Harwood CS, Parales RE. 1996. The *β*–ketoadipate pathway and the biology of self-identity. Annu. Rev. Microbiol. 50, 553–590.

Heinaru E, Truu J, Stottmeister U, Heinaru A. 2000. Three types of phenol and *p*-cresol-degrading bacteria isolated from river water continuously polluted with phenolic compounds. FEMS Microbiol. Ecol. 31, 195–205.

Hester KL, Lehman J, Najar F, Song L, Roe BA, MacGregor CH, Hager PW, Phibbs Jr PV, Sokatch JR. 2000. Crc is involved in catabolite repression control of the *bkd* operons of *Pseudomonas putida* and *Pseudomonas aeruginosa*. J. Bacteriol. 182(4), 1144–1149.

Hino S, Watanabe K, Takahashi N. 1998. Phenol hydroxylase cloned from *Ralstonia eutropha* strain E2 exhibits novel kinetic properties. Microbiology 144, 1765–1772.

Hollender J, Dott W, Hopp J. 1994. Regulation of chloro- and methylphenol degradation in *Comamonas testosteroni* JH5. Appl. Environ. Microbiol. 60(7), 2330–2338.

Holtel A, Marques S, Möhler I, Jakubzik U, Timmis KN. 1994. Carbon source-dependent inhibition of *xyl* operon expression of the *Pseudomonas putida* TOL plasmid. J. Bacteriol., 176(6), 1773–1776.

Hopper DJ. 1976. The hydroxylation of *p*-cresol and its conversion to *p*-hydroxybenzaldehyde in *Pseudomonas putida*. Biochem. Biophys. Res. Commun. 69(2), 462–468.

Hopper DJ, Chapman PJ. 1971. Gentisic acid and its 3- and 4-methyl-substituted homologues as intermediates in the bacterial degradation of *m*-cresol, 3,5-xylenol and 2,5-xylenol. Biochem. J. 122, 19–28.

Hopper DJ, Chapman PJ, Dagley S. 1971. The enzymic degradation of alkyl-substituted gentisates, maleates and malates. Biochem. J. 122, 29–40.

Hopper DJ, Jones MR, Causer MJ. 1985. Periplasmic location of *p*-cresol methylhydroxylase in *Pseudomonas putida*. FEBS Lett. 182(2), 485–488.

Hopper DJ, Kemp PD. 1980. Regulation of enzymes of the 3,5-xylenol-degradative pathways in *Pseudomonas putida*: evidence for a plasmid. J. Bacteriol. 142(1), 21–26.

Hopper DJ, Taylor DG. 1977. The purification and properties of *p*-cresol-(acceptor) oxidoreductase (hydroxylating), a flavocytochrome from *Pseudomonas putida*. Biochem. J. 167(1), 155–162.

Hosein SG, Millette D, Butler BJ, Greer CW. 1997. Catabolic gene probe analysis of an aquifer microbial community degrading creosote-related polycyclic aromatic and heterocyclic compounds. Microb. Ecol. 34(2), 81–89.

Hutchinson DH, Robinson CW. 1988. Kinetics of the simultaneous batch degradation of *p*-cresol and phenol by *Pseudomonas putida*. Appl. Microbiol. Biotechnol. 29, 599–604.

Ideon T. 2007. Tööstusjäätmete ja poolkoksi ladestuspaikade sulgemise ettevalmistus Kohtla-Järvel ja Kiviõlis (2003/EE/16/P/PA/012), keskkonnamõju hindamise aruanne. Tallinn, AS Maves. http://www.envir.ee/346855

Jahnke M, Lehmann F, Schoebel A, Auling G. 1993. Trasposition of the TOL catabolic genes (Tn4651) into the degradative plasmid pSAH of *Alcaligenes* sp. O–1 ensures simultaneous mineralization of sulfo-substituted and methyl-substituted aromatics. J. Gen. Microbiol. 139(9), 1959–1966.

Jannasch HW, Egli T. 1993. Microbial growth kinetics: a historical perspective. Antonie van Leeuwenhoek 63(3–4), 213–224.

Jiang H-L, Tay JH, Tay ST-L. 2002. Aggregation of immobilized activated sludge cells into aerobically grown microbial granules for the aerobic biodegradation of phenol. Lett. Appl. Microbiol. 35, 439–445.

Jiang H-L, Tay ST-L, Maszenan AM, Tay J-H. 2006. Physiological traits of bacterial strains isolated from phenol-degading aerobic granules. FEMS Microbiol. Ecol. 57, 182–191.

Johnson BF, Stanier RY. 1971. Regulation of the β-ketoadipate pathway in *Alcaligenes eutrophus*. J. Bacteriol. 107, 476–485.

Junca H, Pieper DH. 2003. Amplified functional DNA restriction analysis to determine catechol 2,3-dioxygenase gene diversity in soil bacteria. J. Microbiol. Meth. 55, 697–708.

Kemp MB, Hegeman GD. 1968. Genetic control of the 3-ketoadipate pathway in *Pseudomonas aeruginosa*. J. Bacteriol. 96, 1488–1499.

Kiesel B, Müller RH. 2002. The *meta* pathway as a potential energy-generating sequence and its effects on the growth rate during the utilisation of aromatics. Acta Biotechol. 22(3–4), 221–134.

Kim J, Fuller JH, Cecchini G, McIntire WS. 1994. Cloning, sequencing, and expression of the structural genes for the cytochrome and flavoprotein subunits of *p*-cresol methylhydroxylase from two strains of *Pseudomonas putida*. J. Bacteriol. 176(20), 6349–6361.

Kivisaar M, Horak R, Kasak L, Heinaru A, Habicht J. 1990. Selection of independent plasmids determining phenol degradation in *Pseudomonas putida* and the cloning and expression of genes encoding phenol monooxygenase and catechol 1,2-dioxygenase. Plasmid 24(1), 25–36.

Kivisaar M, Kasak L, Nurk A. 1991. Sequence of the plasmid-encoded catechol 1,2-dioxygenase-expressing gene, *pheB*, of phenol-degrading *Pseudomonas* sp. strain EST1001. Gene 98(1), 15–20.

Kovárová-Kovar K, Egli T. 1998. Growth kinetics of suspended microbial cells: from single-substrate-controlled growth to mixed-substrate kinetics. Microbiol. Mol. Biol. Rev. 62(3), 646–666.

Kowalchuk GA, Hartnett GB, Benson A, Houghton JE, Ngai K-L, Ornston LN. 1994. Contrasting patterns of evolutionary divergence within the *Acinetobacter calco-aceticus pca* operon. Gene 146(1), 23–30.

Kukor JJ, Olsen RH. 1990. Molecular cloning, characterization, and regulation of a *Pseudomonas pickettii* PKO1 gene encoding phenol hydroxylase and expression of the gene in *Pseudomonas aeruginosa* PAO1c. J. Bacteriol. 172, 4624–4630.

Kukor JJ, Olsen RH. 1991. Genetic organization and regulation of a *meta* cleavage pathway for catechols produced from catabolism of toluene, benzene, phenol, and cresols by *Pseudomonas pickettii* PKO1. J. Bacteriol. 173(15), 4587–4594.

Kurkela S, Lehväslaiho H, Palva ET, Teeri TH. 1988. Cloning, nucleotide sequence and characterization of genes encoding naphthalene dioxygenase of *Pseudomonas putida* strain NCIB9816. Gene, 73, 355–362.

Lack L. 1959. The enzymic oxidation of gentisic acid. Biochim. Biophys. Acta 34, 117–123.

Lack L. 1961. Enzymic *cis-trans* isomerization of maleylpyruvic acid. J. Biol. Chem. 236, 2835–2840.

Lahtvee V. 2007. Põlevkivi kasutamise riiklik arengukava 2007–2015 keskkonnamõjude strateegiline hindamine. Tallinn, SA Säästva Eesti Instituut, lk 47. http://www.envir.ee/232764

Lange CC, Wackett LP, Minton KW, Daly MJ. 1998. Engineering a recombinant *Deinococcus radiodurans* for organopollutant degradation in radioactive mixed waste environments. Nat. Biotechnol. 16, 929–933.

Leahy JG, Batchelor PJ, Morcomb SM. 2003. Evolution of the soluble diiron mono-oxygenases. FEMS Microbiol. Rev. 27, 449–479.

Leahy JG, Colwell RR. 1990. Microbial degradation of hydrocarbons in the environment. Microbiol. Rev. 54(3), 305–315.
Lendemann U, Snozzi M, Egli T. 1996. Kinetics of the simultaneous utilization of sugar mixtures by *Escherichia coli* in continuous culture. Appl. Environ. Microbiol. 62(5), 1493–1499.
Léonard D, Lindley ND. 1999. Growth of *Ralstonia eutropha* on inhibitory concentrations of phenol: diminished growth can be attributed to hydrophobic perturbation of phenol hydroxylase activity. Enzyme Microb. Tech. 25, 271–277.
MacGregor CH, Arora SK, Hager PW, Dail MB, Phibbs Jr PV. 1996. The nucleotide sequence of the *Pseudomonas aeruginosa pyrE-crc-rph* region and the purification of the *crc* gene product. J. Bacteriol. 178, 5627–5635.
Matsumura E, Sakai M, Haayashi K, Murakami S, Takenaka S, Aoki K. 2006. Constitutive expression of *catABC* genes in the aniline-assimilating bacterium *Rhodococcus* species AN–22: production, purification, characterization and gene analysis of CatA, CatB and CatC. Biochem. J. 393, 219–226.
Merimaa M, Heinaru E, Liivak M, Vedler E, Heinaru A. 2006. Grouping of phenol hydroxylase and catechol 2,3-dioxygenase genes among phenol- and *p*-cresol-degrading *Pseudomonas* species and biotypes. Arch. Microbiol. 186, 287–296.
Mesarch MB, Nakatsu CH, Nies L. 2000. Development of catechol 2,3-dioxygenase-specific primers for monitoring bioremediation by competitive quantitative PCR. Appl. Environ. Microbiol. 66, 678–683.
Mishra V, Lal R, Srinivasan. 2001. Enzymes and operons mediating xenobiotic degradation in bacteria. Crit. Rev. Microbiol. 27 (2), 133–166.
Moonen MJH, Fraaije MW, Rietjens IMCM, Laane C, van Berkel WJH. 2003. Flavoenzyme-catalyzed oxygenations and oxidations of phenolic compounds. Adv. Synth. Catal. 344(10), 1023–1035.
Müller RH, Babel W. 1996. Growth rate-dependent expression of phenol-assimilation pathways in *Alcaligenes eutrophus* JMP 134 – the influence of formate as an auxiliary energy source on phenol conversion characteristics. Appl. Microbiol. Biotechnol. 46, 156–162.
Müller C, Petruschka L, Cuypers H, Burchhardt G, Herrmann H. 1996. Carbon catabolite repression of phenol degradation in *Pseudomonas putida* is mediated by the inhibition of the activator protein PhlR. J. Bacteriol. 178(7), 2030–2036.
Nakai C, Uyeyama H, Kagamiyama H, Nakazawa T, Inouye S, Kishi F, Nakazawa A, Nozaki M. 1995. Cloning, DNA sequencing, and amino acid sequencing of catechol 1,2-dioxygenases (pyrocatechase) from *Pseudomonas putida* mt-2 and *Pseudomonas arvilla* C–1. Arch. Biochem. Biophys. 321(2), 353–362.
Nam J-W, Nojiri H, Yoshida T, Habe H, Yamane H, Omori T. 2001. New classification for oxygenase components involved in ring-hydroxylating oxygenations. Biosci. Biotechnol. Biochem. 65, 254–263.
Narang A, Pilyugin SS. 2007. Bacterial gene regulation in diauxic and non-diauxic growth. J. Theor. Biol. 244(2), 326–348.
Narusk M, Nittim M. 2003. 2002. aasta Eesti veemajanduse ülevaade aruande VEE-KASUTUS alusel. Keskkonnaministeeriumi Info- ja Tehnokeskus, 21.
Neidle EL, Hartnett C, Ornston LN, Bairoch A, Rekik M, Harayama S. 1991. Nucleotide sequences of the *Acinetobacter calcoaceticus benABC* genes for

benzoate 1,2-dioxygenase reveal evolutionary relationships among multicomponent oxygenases. J. Bacteriol. 173(17), 5385–5395.
Newman LM, Wackett LP. 1995. Purification and characterization of toluene 2-monooxygenase from *Burkholderia cepacia* G4. Biochemistry 34, 14066–14076.
Nichols NN, Harwood CS. 1995. Repression of 4-hydroxybenzoate transport and degradation by benzoate – a new layer of regulatory control in the *Pseudomonas putida* beta-ketoadipate pathway. J. Bacteriol. 177(24), 7033–7040.
Nichols NN, Harwood CS. 1997. PcaK, a high-affinity permease for the aromatic compounds 4-hydroxybenzoate and protocatechuate from *Pseudomonas putida*. J Bacteriol. 179(16), 5056–5061.
Ng LC, Shingler V, Sze CC, Poh CL. 1994. Cloning and sequences of the first eight genes of the chromosomally encoded (methyl)phenol degradation pathway from *Pseudomonas putida* P35X. Gene 151(1–2), 29–36.
Noda Y, Nishikawa S, Shiozuka K, Kadokura H, Nakajima H, Yoda K, Katayama Y, Morohoshi N, Haraguchi T, Yamasaki M. 1990. Molecular cloning of the protocatechuate 4,5-dioxygenase genes of *Pseudomonas paucimobilis*. J. Bacteriol. 172(5), 2704–2709.
Nurk A, Kasak L, Kivisaar M. 1991. Sequence of the gene (*pheA*) encoding phenol monooxygenase from *Pseudomonas* sp. EST1001: Expression in *Escherichia coli* and *Pseudomonas putida*. Gene 102(1), 13–18.
Ornston LN. 1966. The conversion of catechol and protocatechuate to β-ketoadipate by *Pseudomonas putida*. IV. Regulation. J. Biol. Chem. 241(16), 3800–3810.
Ornston LN. 1971. Regulation of catabolic pathways in *Pseudomonas*. Bacteriol. Rev. 35(2), 87–116.
Ornston LN, Stanier RY. 1966. The conversion of catechol and protocatechuate to *beta*-ketoadipate by *Pseudomonas putida*. I. Biochemistry. J. Biol. Chem. 241(16), 3776–3786.
Orser CS, Lange CC, Xun L, Zahrt TC, Schneider BJ. 1993. Cloning, sequence analysis, and expression of the *Flavobacterium* pentachlorophenol-4-monooxygenase gene in *Eschericia coli*. J. Bacteriol. 175(2), 411–416.
Ostonen I, Lõhmus K, Alama S, Truu J, Kaar E, Vares A, Uri V, Kurvits V. 2006. Fine roots in Scots pine (*Pinus sylvestris* L.) silver birch (*Betula pendula* Roth.) and black alder (*Alnus glutinosa* (L.) Gaertn.) stands in recultivated areas of oil shale mining and semicoke hills. Oil Shale 23(2), 187–202.
O'Toole GA, Gibbs KA, Hager PW, Phibbs Jr PV, Kolter R. 2000. The global carbon metabolism regulator Crc is a component of a signal transduction pathway required for biofilm development by *Pseudomonas aeruginosa.* J. Bacteriol. 182(2), 425–431.
Pandey G, Jain RK. 2002. Bacterial chemotaxis toward environmental pollutants: role in bioremediation. Appl. Environ. Microbiol. 68(12), 5789–5795.
Parales RE, Resnick SM. 2006. Aromatic ring hydroxylating dioxygenases, 287–340. In Ramos J-L and Levesque RC (eds.) *Pseudomonas*, vol 4. Springer, Netherlands.
Paul D, Pandey G, Jain RK. 2005. Suicidal genetically engineered microorganisms for bioremediation: need and perspectives. Bioessays 27(5), 563–573.
Perkins EJ, Gordon MP, Caceres O, Lurguin PF. 1990. Organization and sequence analysis of the 2,4-dichlorophenol hydroxylase and dichlorocatechol oxidative operons of plasmid pJP4. J. Bacteriol. 172(5), 2351–2359.

Pessione E, Divari S, Griva E, Cavaletto M, Rossi GL, Gilardi G, Gilunta C. 1999. Phenol hydroxylase from *Acinetobacter radioresistens* is a multicomponent enzyme. Purification and characterization of the reductase moiety. Eur. J. Biochem. 265(2), 549–555.

Peters M, Heinaru E, Talpsep E, Wand H, Stottmeister U, Heinaru A, Nurk A. 1997. Acquisition of a deliberately introduced phenol degradation operon, *pheBA*, by different indigenous *Pseudomonas* species. Appl. Environ. Microbiol. 63(12) 4899–4906.

Pieper DH, Reineke W. 2000. Engineering bacteria for bioremediation. Curr. Opin. Biotechnol. 11(3) 262–270.

Pieper DH, Stadler-Fritzsche K, Knackmuss H-J, Timmis KN. 1995. Formation of dimethylmuconolactones from dimethylphenols by *Alcaligenes eutrophus* JMP134. Appl. Environ. Microbiol. 61(6), 2159–2165.

Polymenakou PN, Stephanou EG. 2005. Effect of temperature and additional carbon sources on phenol degradation by an indigenous soil *Pseudomonad*. Biodegradation 16, 403–413.

Reardon KF, Mosteller DC, Bull Rogers JD. 2000. Biodegradation kinetics of benzene, toluene, and phenol as single and mixed substrates for *Pseudomonas putida* F1. Biotechnol. Bioeng. 69(4), 385–399.

Ruiz-Manzano A, Yuste L, Roho F. 2005. Levels and activity of the *Pseudomonas putida* global regulatory protein Crc vary according to growth conditions. J. Bacteriol. 187(11), 3678–3686.

Salu M. 2004. Ohtlike jääkreostuskollete kontroll ja uuringud. Tallinn, AS Maves. http://www.maves.ee/Projektid/2004/OJRK_aruanne_nr_3116.pdf

Sanchez JL, Kamp B, Onysko KA, Budman H, Robinson CW. 1998. Double inhibition model for degradation of phenol by *Pseudomonas putida* Q5. Biotechnol. Bioeng. 60(5), 560–567.

Sayler GS, Ripp S. 2000. Field applications of genetically engineered microorganisms for bioremediation processes. Curr. Opin. Biotechnol. 11(3), 286–289.

Shingler V, Franklin CH, Tsuda M, Holroyd D, Bagdasarian M. 1989. Molecular analysis of a plasmid-encoded phenol hydroxylase from *Pseudomonas* CF600. J. Gen. Microbiol., 135, 1083–1092.

Shingler V, Powlowski J, Marklund U. 1992. Nucleotide sequence and functional analysis of the complete phenol/3,4-dimethylphenol catabolic pathway of *Pseudomonas* sp. strain CF600. J. Bacteriol. 174(3), 711–724.

Sikkema J, de Bont JAM, Poolman B. 1994a. Interactions of cyclic hydrocarbons with biological membranes. J. Biol. Chem. 269, 8022–8028.

Sikkema J, de Bont JAM, Poolman B. 1994b. Mechanism of membrane toxicity of hydrocarbons. Microbiol. Rev. 59, 201–222.

Smith MR, Ewing M, Ratled GEC. 1991. The interactions of various aromatic substrates degraded by *Pseudomonas* sp. NCIB10643 – synergistic inhibition of growth by 2 compounds that serve as growth substrates. Appl. Microbiol. Biotechnol. 34(4), 536–538.

Sugimoto K, Senda T, Aoshima H, Masai E, Fukuda M, Mitsui Y. 1999. Crystal structure of an aromatic ring opening dioxygenase LigAB, a protocatechuate 4,5-dioxygenase, under aerobic conditions. Structure, 7(8), 953–965.

Swinnen IAM, Bernaerts K, Dens EJJ, Geeraerd AH, Van Impe JF. 2004. Predictive modelling of the microbial lag phase: a review. Int. J. Food Microbiol. 94, 137–159.
Takeo M, Maeda Y, Okada H, Miyama K, Mori K, Ike M, Fujita M. 1995. Molecular cloning and sequencing of the phenol hydroxylase gene from *Pseudomonas putida* BH. J. Ferement. Bioeng. 79(5), 485–488.
Tallur PN, Megadi VB, Kamanavalli CM, Ninnekar HZ. 2006. Biodegradation of *p*-cresol by *Bacillus* sp. strain PHN 1. Curr. Microbiol. 53, 529–533.
Thompson IP, van der Gast CJ, Ciric L, Singer AC. 2005. Bioaugmentation for bioremediation: the challenge of strain selection. Environ. Microbiol. 7(7), 909–915.
Timmis KN, Pieper DH. 1999. Bacteria designed for bioremediation. Trends. Biotechnol. 17(5), 200–204.
Truu J, Talpsep E, Vedler E, Heinaru E, Heinaru A. 2003. Enhanced biodegradation of oil shale chemical industry solid wastes by phytoremediation and bioaugmentation. Oil Shale, 20(3) Special, 421–428.
van der Meer JR. 1997. Evolution of novel metabolic pathways for the degradation of chloroaromatic compounds. Antonie van Leeuwenhoek 71(1–2), 159–178.
van der Meer JR. 2006. Environmental pollution promotes selection of microbial degradation pathways. Front. Ecol. Environ. 4(1), 35–42.
van der Meer JR, Roelofsen W, Schraa G, Zehnder AJB. 1987. Degradation of low concentrations of dichlorobenzenes and 1,2,4-trichlorobenzenes by *Pseudomonas* sp strain P51 in nonsterile soil columns. FEMS Microbiol. Ecol. 45, 333–341.
van der Meer JR, de Vos WM, Harayama S, Zehnder AJB. 1992. Molecular mechanisms of genetic adaptation to xenobiotic compounds. Microbiol. Rev. 56(4), 677–694.
van der Meer JR, Werlen C, Nishino SF, Spain JC. 1998. Evolution of a pathway for chlorobenzene metabolism leads to natural attenuation in contaminated groundwater. Appl. Environ. Microbiol. 64(11), 4185–7193.
Vollmer MD, Hoier H, Hecht HJ, Schell U, Groning J, Goldman A, Schlomann M. 1998. Substrate specificity of and product formation by muconate cycloisomerases: an analysis of wild-type enzymes and engineered variants. Appl. Environ. Microbiol. 64(9), 3290–3299.
Watanabe K, Futamata H, Harayama S. 2002. Understanding the diversity in catabolic potential of microorganisms for the development of bioremediation strategies. Antonie van Leeuwenhoek 81(1–4), 655–663.
Watanabe K, Hino S, Takahashi N. 1996. Responses of activated sludge to an increase in phenol loading. J. Ferment. Bioeng. 82(5), 522–524.
Watanabe K, Teramoto M, Futamata H, Harayama S. 1998. Molecular detection, isolation, and physiological characterization of functionally dominant phenol-degrading bacteria in activated sludge. Appl. Environ. Microbiol. 64(11), 4396–4402.
Wikström P, Wiklund A, Andersson AC, Forsman M. 1996. DNA recovery and PCR quantification of catechol 2,3-dioxygenase genes from different soil types. J. Biotechnol. 52(2), 107–120.
Williams PA, Shaw LE. 1997. *mucK*, a gene in *Acinetobacter calcoaceticus* ADP1 (BD413), encodes the ability to grow on exogenous *cis,cis*-muconate as the sole carbon source. J. Bacteriol. 179(18), 5935–5942.

You IS, Ghosal D, Gunsalus IC. 1991. Nucleotide sequence analysis of the *Pseudomonas putida* PpG7 salicylate hydroxylase gene (*nahG*) and its 3'-flanking region. Biochemistry 30(6), 1635–1641.

Zhou N-Y, Fuenmayor SL, Williams PA. 2001. *nag* genes of *Ralstonia* (formerly *Pseudomonas*) sp. strain U2 encoding enzymes for gentisate catabolism. J. Bacteriol. 183(2), 700–708.

Zwietering MH, Jongenburger I, Rombouts FM, Van't Riet K. 1990. Modelling of the bacterial growth curve. Appl. Environ. Microbiol. 56(6), 1875–1881.

Zylstra GJ, McCombie WR, Gibson DT, Finette BA. 1988. Toluene degradation by *Pseudomonas putida* F1: genetic organization of the *tod* operon. Appl. Environ. Microbiol. 54(6), 1498–1503.

Zylstra GJ, Olsen RH, Ballou DP. 1989. Cloning, expression, and regulation of the *Pseudomonas cepacia* protocatechuate 3,4-dioxygenase genes. J. Bacteriol. 171(11), 5907–5914.

8. SUMMARY IN ESTONIAN

Geneetiliselt erinevate pseudomonaadide biokeemiliste parameetrite osa fenoolsete ühendite lagundamisel

Keskkonna üha suurem saastumine antropogeense päritoluga ühenditega on ülemaailmne probleem. Eestis on põhjustanud mitmesuguseid keskkonnaprobleeme põlevkivi kaevandamine ning põlevkivikeemia- ja energiatööstused (Salu, 2004). Nimelt sisaldavad jõgedesse juhitavad kaevandusveed kõrges kontsentratsioonis sulfaate, raskemetalle, õliprodukte ja aromaatseid ühendeid. Aastakümnete pikkuse põlevkivi termilise töötlemise tagajärjel on piirkonnas tekkinud suured poolkoksiladestud (umbes 100 miljonit tonni), millelt sadening uhtevee tulemusel tekkiv nõrgvesi on rikas nii orgaaniliste (fenoolid, polütsüklilised aromaatsed süsivesinikud, õliproduktid, bitumoidid) kui ka anorgaaniliste ühendite (sulfiidid) poolest. Reostunud tööstus- ja nõrgvett küll osaliselt puhastatakse, kuid suur osa sellest satub kontrollimatu heitmena pinnasesse, põhja- ja pinnavette (Ideon, 2007). Poolkoksiladestutel tekkiv nõrgvesi sisaldab 1- ja 2-aluselisi fenoole (summaarne fenoolide kontsentratsioon 100 mg/l), kusjuures fenooli ja *p*-kresooli kontsentratsioonid on enamasti kõrgemad kui toksilisematel dimetüülfenoolidel, resortsinoolidel ja polütsüklilistel aromaatsetel süsivesinikel (Ideon, 2007).

Saastunud alade bakteripopulatsioonide biokeemiline, füsioloogiline ja geneetiline iseloomustamine on oluline mikroobiökoloogias, eriti kataboolselt aktiivsete tüvede efektiivsuse ja koosluse struktuuri/funktsioneerimise uurimisel. Uuritud biodegradatiivseid tüvesid on võimalik kasutada sarnase saastusega alade bioremediatsiooni kiirendamiseks.

Käesoleva töö eesmärkideks oli uurida pideva fenoolse reostusega veest eraldatud geneetiliselt erinevate pseudomonaadide võimet ja efektiivsust lagundada fenoolsete ühendite segusid üksik- ja segakultuuridena, määrata geneetiliselt erinevaid fenooli hüdroksülaase omavate tüvede fenooli lagundamise kineetilisi parameetreid ning selgitada fenoolseid ühendeid lagundavate tüvede käitumine ja osakaal poolkoksiladestute nõrgvett või põlevkiviõli sisaldavates mikrokosmikatsetes.

Töös esitatud tulemused võib kokku võtta järgnevalt:

1. Tüvede (*Pseudomonas mendocina* PC1, *Pseudomonas fluorescens* PC18 või *P. fluorescens* PC24) kasvatamisel substraatide segudel (kombineerides omavahel fenooli, *p*-kresooli, bensoaati, *p*-hüdroksübensoaati ja dimetüülfenoole) leiti, et:
   1.1. kui substraatide lagundamiseks kasutatakse katehhooli *meta*, katehhooli *orto* või katehhooli *orto* ja protokatehhuaadi *orto* rada siis kataboolsete vaheühendite kogunemist ei täheldatud ning substraadid kasutati üheaegselt (diauksiata kasv);

1.2. kui substraatide lagundamiseks kasutati *meta* ja *orto* rada, täheldati vaheühendite (*p*-hüdroksübensoaat, katehhool või 4-hüdroksü-2-metüülbensaldehüüd) pöörduvat kogunemist ning substraatide järjestikulist kasutamist (diauksiaga kasv).

2. Geneetiliselt erinevaid fenooli hüdroksülaase omavate pseudomonaadide (11 tüve) biodegradatsiooni efektiivsuse analüüs fenooli oksüdeerimise ja fenoolil kasvu kineetiliste parameetrite alusel näitas, et:
   2.1. ühe- (sPH) või mitmekomponendilist (mPH) fenooli hüdroksülaasi omavad tüved grupeeruvad erinevatesse rühmadesse;
      2.1.1. mPH-tüvede fenooli poolküllastuskonstandid (K_S) on ühe suurusjärgu võrra madalamad kui sPH-tüvedel ning mPH-tüvedel on kõrgemad maksimaalse kasvukiiruse (μ_{max}) ja saagise koefitsendi ($Y_{X/S}$) väärtused kui sPH-tüvedel;
      2.1.2. sPH+C12O-tüvede madalamad $Y_{X/S}$ väärtused on ilmselt põhjustatud mukonaadi tsükloisomeraasi madalamatest eriaktiivsuste väärtustest võrreldes katehhooli 1,2-dioksügenaasi (C12O) vastavate väärtustega, mistõttu ka eksogeensed *cis,cis*-mukonaadi kontsentratsioonid on kõrgemad kui mPH+C12O-tüvedel;
   2.2. tüvede fenoolitaluvus on inhibitsioonikonstandi (K_I) väärtuste alusel tüvespetsiifiline.

3. Fenoolset nõrgvett või põlevkivi toorõli sisaldavad bioaugmentatsiooni imitatsioonkatsed segakultuuridega osutasid, et:
   3.1. mõlemas mikrokosmis kujunevad välja spetsiifilised bakteripopulatsionid ning erinevaid aromaatsete ühendite lagundamisradasid omavatel tüvedel on erinev degradatiivne potentsiaal bioremediatsioonil.

Kokkuvõtvalt võib öelda, et erinevaid kataboolseid geene omavad pseudomonaadid on sobilikud kasutamiseks mitmesuguste aromaatsete ühenditega saastunud alade ja reovete bioremediatsioonil. Efektiivsuse suurendamiseks tuleks kombineerida erinevaid kineetilisi parameetreid omavaid tüvesid, arvestades saasteainete hulka.

ACKNOWLEDGEMENTS

This study was mostly carried out in the Institute of Molecular and Cell Biology (IMCB, University of Tartu). Some experiments were performed in the Institute of Chemistry (IC, University of Tartu). The financial support of Estonian Science Foundation (grants 4344, 5475 and 5682) and of IMCB is gratefully acknowledged.

I would like to express my deepest gratitude to:
- Professor Ain Heinaru, my supervisor from IMCB, and Professor Toomas Tenno, my supervisor from IC, for support and inspiration during the work
- Eeva Heinaru for introducing me to the world of microbes, assistance and scientific guidance through the years of our collaboration
- The IMCB offering opportunity to use laboratory facilities for the work
- Professor Ivo Leito for assistance in performing some chemical analysis
- Dr. Jyrki Loponen from University of Turku for collaboration in identification of catabolic intermediates
- Dr. Tiina Alamäe for improving the English in the thesis and papers
- My colleagues from the Department of Genetics of IMCB and from the Department of Environmental and Colloidal Chemistry of IC for support and help
- My parents and family for love, support and patience through the university years.

This thesis would have never been completed without your firm support. Thank you!

PUBLICATIONS

I

Reprinted from FEMS Microbiology Ecology, Vol 37 (1),
Heinaru E, Viggor S, Vedler E, Truu J, Merimaa M, Heinaru A.
Reversible accumulation of *p*-hydroxybenzoate and catechol determines
the sequential decomposition of phenolic compounds
in mixed substrate cultivations in pseudomonads,
pp 79–89, copyright © 2001, with permission from Elsevier Science B.V.
DOI: 10.1016/S0168-6496(01)00147-7

II

Reprinted from Environmental Science and Pollution Research, Vol 9, Special Issue 1,
Viggor S, Heinaru E, Loponen J, Merimaa M, Tenno T, Heinaru A.
Biodegradation of dimethylphenols by bacteria
with different ring-cleavage pathways of phenolic compounds,
pp 19–26, copyright © 2002, with permission from ecomed publishers.
DOI: http://dx.doi.org/10.1065/espr2002.04.115.7

III

Reprinted from FEMS Microbiology Ecology, Vol 51 (3),
Heinaru E, Merimaa M, Viggor S, Lehiste M, Leito I, Truu J, Heinaru A.
Biodegradation efficiency of functionally important population selected
for bioaugmentation in phenol- and oil-polluted area,
pp 363–373, copyright © 2005, with permission from Elsevier Science B.V.
DOI: 10.1016/j.femsec.2004.09.009

IV

Reprinted from Biodegradation (in press),
Viggor S, Heinaru E, Künnapas A, Heinaru A.
Evaluation of different phenol hydroxylase-possessing
phenol-degrading pseudomonads by kinetic parameters,
pp 1–11, copyright © 2008, with permission from Springer.
DOI:10.1007/s10532-008-9180-8.

CURRICULUM VITAE

Signe Viggor

Date of birth	21.02.1975, Põltsamaa, Estonia
Citizenship	Estonian
Address	University of Tartu (UT), Institute of Molecular and Cell Biology, 23 Riia Str, Tartu 51010, Estonia
Phone	+372 737 6038
E-post	signev@ut.ee

Education

1993–1997	Student, Department of Chemistry, UT, BSc 1997
1997–2000	Post graduate student, Department of Chemistry, UT, MSc 2000
2000–	PhD student, Department of Chemistry, UT, doctoral advisors Prof. Ain Heinaru and Prof. Toomas Tenno

Professional employment

1999–2002	UT, Institute of Technology; specialist
2003–	UT, Institute of Molecular and Cell Biology; researcher

Main scientific publications

Heinaru E, **Viggor S**, Vedler E, Truu J, Merimaa M, Heinaru A. 2001. Reversible accumulation of *p*-hydroxybenzoate and catechol determines the sequential decomposition of phenolic compounds in mixed substrate cultivations in pseudomonads. FEMS Microbiol. Ecol. 37 (1), 79–89.

Viggor S, Heinaru E, Loponen J, Merimaa M, Tenno T, Heinaru A. 2002. Biodegradation of dimethylphenols by bacteria with different ring-cleavage pathways of phenolic compounds. Environ. Sci. Pollut. Res. 9 (Special Issue 1), 19–26.

Lepik R, Orupõld K, **Viggor S**, Tenno T. 2003. Study of biodegradability of methyl- and hydroxyphenols by activated sludge. Oil Shale 20(2), 99–112.

Heinaru E, Merimaa M, **Viggor S**, Lehiste M, Leito I, Truu J, Heinaru A. 2005. Biodegradation efficiency of functionally important population selected for bioaugmentation in phenol- and oil-polluted area. FEMS Microbiol. Ecol. 51 (3), 363–373.

Viggor S, Heinaru E, Künnapas A, Heinaru A. 2008. Evaluation of different phenol hydroxylase-possessing phenol-degrading pseudomonads by kinetic parameters. Biodegradation. In press. DOI:10.1007/s10532-008-9180-8.

ELULOOKIRJELDUS

Signe Viggor

Sünniaeg	21.02.1975, Põltsamaa, Eesti
Kodakondsus	Eesti
Aadress	Tartu Ülikool (TÜ), Molekulaar- ja rakubioloogia instituut, Riia 23, Tartu 51010
Telefon	+372 737 6038
E-post	signev@ut.ee

Haridus

1993–1997	TÜ keemiaosakonna üliõpilane; BSc (keemia) 1997
1997–2000	TÜ keemiaosakonna magistrant; MSc (keemia) 2000
2000–	TÜ keemiaosakonna doktorant, juhendajad Prof. Ain Heinaru ja Prof. Toomas Tenno

Teenistuskäik

1999–2002	TÜ, Tehnoloogia instituut; tehnoloogiaspetsialist
2003–	TÜ, Molekulaar- ja rakubioloogia instituut, Geneetika õppetool; teadur

Tähtsamad teaduspublikatsioonid

Heinaru E, **Viggor S**, Vedler E, Truu J, Merimaa M, Heinaru A. 2001. Reversible accumulation of *p*-hydroxybenzoate and catechol determines the sequential decomposition of phenolic compounds in mixed substrate cultivations in pseudomonads. FEMS Microbiol. Ecol. 37 (1), 79–89.

Viggor S, Heinaru E, Loponen J, Merimaa M, Tenno T, Heinaru A. 2002. Biodegradation of dimethylphenols by bacteria with different ring-cleavage pathways of phenolic compounds. Environ. Sci. Pollut. Res. 9 (Special Issue 1), 19–26.

Lepik R, Orupõld K, **Viggor S**, Tenno T. 2003. Study of biodegradability of methyl- and hydroxyphenols by activated sludge. Oil Shale 20(2), 99–112.

Heinaru E, Merimaa M, **Viggor S**, Lehiste M, Leito I, Truu J, Heinaru A. 2005. Biodegradation efficiency of functionally important population selected for bioaugmentation in phenol- and oil-polluted area. FEMS Microbiol. Ecol. 51 (3), 363–373.

Viggor S, Heinaru E, Künnapas A, Heinaru A. 2008. Evaluation of different phenol hydroxylase-possessing phenol-degrading pseudomonads by kinetic parameters. Biodegradation. In press. DOI:10.1007/s10532-008-9180-8.

DISSERTATIONES CHIMICAE UNIVERSITATIS TARTUENSIS

1. **Toomas Tamm.** Quantum-chemical simulation of solvent effects. Tartu, 1993, 110 p.
2. **Peeter Burk.** Theoretical study of gas-phase acid-base equilibria. Tartu, 1994, 96 p.
3. **Victor Lobanov.** Quantitative structure-property relationships in large descriptor spaces. Tartu, 1995, 135 p.
4. **Vahur Mäemets.** The ^{17}O and ^{1}H nuclear magnetic resonance study of H_2O in individual solvents and its charged clusters in aqueous solutions of electrolytes. Tartu, 1997, 140 p.
5. **Andrus Metsala.** Microcanonical rate constant in nonequilibrium distribution of vibrational energy and in restricted intramolecular vibrational energy redistribution on the basis of slater's theory of unimolecular reactions. Tartu, 1997, 150 p.
6. **Uko Maran.** Quantum-mechanical study of potential energy surfaces in different environments. Tartu, 1997, 137 p.
7. **Alar Jänes.** Adsorption of organic compounds on antimony, bismuth and cadmium electrodes. Tartu, 1998, 219 p.
8. **Kaido Tammeveski.** Oxygen electroreduction on thin platinum films and the electrochemical detection of superoxide anion. Tartu, 1998, 139 p.
9. **Ivo Leito.** Studies of Brønsted acid-base equilibria in water and non-aqueous media. Tartu, 1998, 101 p.
10. **Jaan Leis.** Conformational dynamics and equilibria in amides. Tartu, 1998, 131 p.
11. **Toonika Rinken.** The modelling of amperometric biosensors based on oxidoreductases. Tartu, 2000, 108 p.
12. **Dmitri Panov.** Partially solvated Grignard reagents. Tartu, 2000, 64 p.
13. **Kaja Orupõld.** Treatment and analysis of phenolic wastewater with microorganisms. Tartu, 2000, 123 p.
14. **Jüri Ivask.** Ion Chromatographic determination of major anions and cations in polar ice core. Tartu, 2000, 85 p.
15. **Lauri Vares.** Stereoselective Synthesis of Tetrahydrofuran and Tetrahydropyran Derivatives by Use of Asymmetric Horner-Wadsworth-Emmons and Ring Closure Reactions. Tartu, 2000, 184 p.
16. **Martin Lepiku.** Kinetic aspects of dopamine D_2 receptor interactions with specific ligands. Tartu, 2000, 81 p.
17. **Katrin Sak.** Some aspects of ligand specificity of P2Y receptors. Tartu, 2000, 106 p.
18. **Vello Pällin.** The role of solvation in the formation of iotsitch complexes. Tartu, 2001, 95 p.

19. **Katrin Kollist.** Interactions between polycyclic aromatic compounds and humic substances. Tartu, 2001, 93 p.
20. **Ivar Koppel.** Quantum chemical study of acidity of strong and superstrong Brønsted acids. Tartu, 2001, 104 p.
21. **Viljar Pihl.** The study of the substituent and solvent effects on the acidity of OH and CH acids. Tartu, 2001, 132 p.
22. **Natalia Palm.** Specification of the minimum, sufficient and significant set of descriptors for general description of solvent effects. Tartu, 2001, 134 p.
23. **Sulev Sild.** QSPR/QSAR approaches for complex molecular systems. Tartu, 2001, 134 p.
24. **Ruslan Petrukhin.** Industrial applications of the quantitative structure-property relationships. Tartu, 2001, 162 p.
25. **Boris V. Rogovoy.** Synthesis of (benzotriazolyl)carboximidamides and their application in relations with *N*- and *S*-nucleophyles. Tartu, 2002, 84 p.
26. **Koit Herodes.** Solvent effects on UV-vis absorption spectra of some solvatochromic substances in binary solvent mixtures: the preferential solvation model. Tartu, 2002, 102 p.
27. **Anti Perkson.** Synthesis and characterisation of nanostructured carbon. Tartu, 2002, 152 p.
28. **Ivari Kaljurand.** Self-consistent acidity scales of neutral and cationic Brønsted acids in acetonitrile and tetrahydrofuran. Tartu, 2003, 108 p.
29. **Karmen Lust.** Adsorption of anions on bismuth single crystal electrodes. Tartu, 2003, 128 p.
30. **Mare Piirsalu.** Substituent, temperature and solvent effects on the alkaline hydrolysis of substituted phenyl and alkyl esters of benzoic acid. Tartu, 2003, 156 p.
31. **Meeri Sassian.** Reactions of partially solvated Grignard reagents. Tartu, 2003, 78 p.
32. **Tarmo Tamm.** Quantum chemical modelling of polypyrrole. Tartu, 2003. 100 p.
33. **Erik Teinemaa.** The environmental fate of the particulate matter and organic pollutants from an oil shale power plant. Tartu, 2003. 102 p.
34. **Jaana Tammiku-Taul.** Quantum chemical study of the properties of Grignard reagents. Tartu, 2003. 120 p.
35. **Andre Lomaka.** Biomedical applications of predictive computational chemistry. Tartu, 2003. 132 p.
36. **Kostyantyn Kirichenko.** Benzotriazole — Mediated Carbon–Carbon Bond Formation. Tartu, 2003. 132 p.
37. **Gunnar Nurk.** Adsorption kinetics of some organic compounds on bismuth single crystal electrodes. Tartu, 2003, 170 p.
38. **Mati Arulepp.** Electrochemical characteristics of porous carbon materials and electrical double layer capacitors. Tartu, 2003, 196 p.

39. **Dan Cornel Fara.** QSPR modeling of complexation and distribution of organic compounds. Tartu, 2004, 126 p.
40. **Riina Mahlapuu.** Signalling of galanin and amyloid precursor protein through adenylate cyclase. Tartu, 2004, 124 p.
41. **Mihkel Kerikmäe.** Some luminescent materials for dosimetric applications and physical research. Tartu, 2004, 143 p.
42. **Jaanus Kruusma.** Determination of some important trace metal ions in human blood. Tartu, 2004, 115 p.
43. **Urmas Johanson.** Investigations of the electrochemical properties of polypyrrole modified electrodes. Tartu, 2004, 91 p.
44. **Kaido Sillar.** Computational study of the acid sites in zeolite ZSM-5. Tartu, 2004, 80 p.
45. **Aldo Oras.** Kinetic aspects of dATPαS interaction with $P2Y_1$ receptor. Tartu, 2004, 75 p.
46. **Erik Mölder.** Measurement of the oxygen mass transfer through the air-water interface. Tartu, 2005, 73 p.
47. **Thomas Thomberg.** The kinetics of electroreduction of peroxodisulfate anion on cadmium (0001) single crystal electrode. Tartu, 2005, 95 p.
48. **Olavi Loog.** Aspects of condensations of carbonyl compounds and their imine analogues. Tartu, 2005, 83 p.
49. **Siim Salmar.** Effect of ultrasound on ester hydrolysis in aqueous ethanol. Tartu, 2006, 73 p.
50. **Ain Uustare.** Modulation of signal transduction of heptahelical receptors by other receptors and G proteins. Tartu, 2006, 121 p.
51. **Sergei Yurchenko.** Determination of some carcinogenic contaminants in food. Tartu, 2006, 143 p.
52. **Kaido Tämm.** QSPR modeling of some properties of organic compounds. Tartu, 2006, 67 p.
53. **Olga Tšubrik.** New methods in the synthesis of multisubstituted hydrazines. Tartu. 2006, 183 p.
54. **Lilli Sooväli.** Spectrophotometric measurements and their uncertainty in chemical analysis and dissociation constant measurements. Tartu, 2006, 125 p.
55. **Eve Koort.** Uncertainty estimation of potentiometrically measured ph and pK_a values. Tartu, 2006, 139 p.
56. **Sergei Kopanchuk.** Regulation of ligand binding to melanocortin receptor subtypes. Tartu, 2006, 119 p.
57. **Silvar Kallip.** Surface structure of some bismuth and antimony single crystal electrodes. Tartu, 2006, 107 p.
58. **Kristjan Saal.** Surface silanization and its application in biomolecule coupling. Tartu, 2006, 77 p.
59. **Tanel Tätte.** High viscosity $Sn(OBu)_4$ oligomeric concentrates and their applications in technology. Tartu, 2006, 91 p.

60. **Dimitar Atanasov Dobchev**. Robust QSAR methods for the prediction of properties from molecular structure. Tartu, 2006, 118 p.
61. **Hannes Hagu**. Impact of ultrasound on hydrophobic interactions in solutions. Tartu, 2007, 81 p.
62. **Rutha Jäger.** Electroreduction of peroxodisulfate anion on bismuth electrodes. Tartu, 2007, 142 p.
63. **Kaido Viht.** Immobilizable bisubstrate-analogue inhibitors of basophilic protein kinases: development and application in biosensors. Tartu, 2007, 88 p.
64. **Eva-Ingrid Rõõm.** Acid-base equilibria in nonpolar media. Tartu, 2007, 156 p.
65. **Sven Tamp.** DFT study of the cesium cation containing complexes relevant to the cesium cation binding by the humic acids. Tartu, 2007, 102 p.
66. **Jaak Nerut.** Electroreduction of hexacyanoferrate(III) anion on Cadmium (0001) single crystal electrode. Tartu, 2007, 180 p.
67. **Lauri Jalukse.** Measurement uncertainty estimation in amperometric dissolved oxygen concentration measurement. Tartu, 2007, 112 p.
68. **Aime Lust.** Charge state of dopants and ordered clusters formation in CaF_2:Mn and CaF_2:Eu luminophors. Tartu, 2007, 100 p.
69. **Iiris Kahn**. Quantitative Structure-Activity Relationships of environmentally relevant properties. Tartu, 2007, 98 p.
70. **Mari Reinik.** Nitrates, nitrites, N-nitrosamines and polycyclic aromatic hydrocarbons in food: analytical methods, occurrence and dietary intake. Tartu, 2007, 172 p.
71. **Heili Kasuk.** Thermodynamic parameters and adsorption kinetics of organic compounds forming the compact adsorption layer at Bi single crystal electrodes. Tartu, 2007, 212 p.
72. **Erki Enkvist.** Synthesis of adenosine-peptide conjugates for biological applications. Tartu, 2007, 114 p.
73. **Svetoslav Hristov Slavov**. Biomedical applications of the QSAR approach. Tartu, 2007, 146 p.
74. **Eneli Härk.** Electroreduction of complex cations on electrochemically polished Bi(*hkl*) single crystal electrodes. Tartu, 2008, 158 p.
75. **Priit Möller.** Electrochemical characteristics of some cathodes for medium temperature solid oxide fuel cells, synthesized by solid state reaction technique. Tartu, 2008, 90 p.