EXPLOITING THE CAPACITY OF *LABRYS PORTUCALENSIS* STRAIN F11 FOR BIOTRANSFORMATION OF FLUOROAROMATIC COMPOUNDS

Thesis submitted to the Universidade Católica Portuguesa to attain

the degree of PhD in Biotechnology – with specialization in Environmental

Science and Engineering

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Irina Susana Sousa Moreira

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Under the supervision of Professor Doctor Paula Maria Lima Castro

Under the co-supervision of Professor Doctor Carlos Afonso and Doctor

Fátima Carvalho

June 2012

To my mother and my brother,

To my grandmother and my aunt

And to Luis

ABSTRACT

Environmental contamination with toxic chemicals of human origin is a threat to ecosystems and public health. Thus, it is extremely important to understand and explore the removal of these contaminants from different environments through biodegradation. The main objective of the work described in this thesis was the exploitation of the potential of *Labrys portucalensis* F11 – a bacterial strain previously isolated by its ability to degrade fluorobenzene (FB) - for biotransformation of fluorinated aromatic compounds of different complexity.

Typically, contaminated environments are not contaminated with a single pollutant but with mixtures of pollutants. In particular the presence of organic compounds and metals in the same ecosystem, or appearing simultaneously in wastewater streams, is very common. In this study, the effect of three metals with different biological importance - iron, copper and silver - on the degradation of FB by strain F11 was evaluated. At a concentration of 1 mM, iron proved to be beneficial for bacterial growth without adversely affecting the biodegradation of up to 2 mM of FB. The presence of 1 mM of copper and silver inhibited the degradation of FB and led to the accumulation of intermediate metabolites, catechol and 4-fluorocatecol suggesting inhibition of the catechol 1,2-dioxygenase, which is a key enzyme of the metabolic pathway of FB.

The degradation of compounds with chemical structures similar to FB, namely chlorobenzene (CB) and difluorobenzenes (DFBS), by *L. portucalensis* was investigated. Strain F11 was able to cometabolise CB in the presence of FB or when previously induced by this compound. Total degradation of 0.5 mM of each substrate was observed when both were added to the culture medium. Strain F11 was capable of degrading CB when the expression of enzymes is induced by FB however CB was not able to induce the enzymes for its own degradation. For DFBS, strain F11 proved to be able to degrade 0.5 mM of 1,3-DFB as sole carbon source, and to degrade 1,4-DFB (0.5 mM) in cometabolism with FB (0.5 mM). Strain F11 was unable to degrade 1,2-DFB and this compound inhibited the degradation of FB. These results reinforce the importance of the nature, number and position of the substituents in the molecule for enzyme expression and subsequently conversion of the target compounds.

Fluoxetine (FLX) is a fluorinated chiral drug, which contamination, toxicity and persistence in the environment have been well documented in the past years. *L. portucalensis* F11

showed to be able to degrade both enantiomers of this compound as the sole carbon source (up to 9 µM) and in the presence of a conventional carbon source, sodium acetate (up to 89 µM of FLX). Degradation extents of at least 80% of total FLX were obtained. At the lowest FLX concentration tested (2 µM) as single carbon source, degradation was complete and fluoride release was stoichiometric. The degradation was shown to be enantioselective, with preferential degradation of *R*-FLX in relation to *S*-FLX. The transient formation of norfluoxetine (NFLX) as an intermediary metabolite was detected.

With the objective of finding the genes responsible for the expression of FB dioxygenase, a genomic library consisting of 960 clones was constructed from the DNA of *L. portucalensis* F11. This library can be used for future work, such as the generation and confirmation of sequencing data, for comparative genomic studies or to search for other genes of interest. It is important to note that this strain has shown extraordinary capabilities of degradation of toxic compounds, and as such it would be very interesting to further study the genes that confer these capabilities.

A partial nucleotide sequence of the gene cluster involved in FB degradation was determined. Sequencing results revealed the presence of four open reading frames, namely the gene coding for 1,2-catechol dioxygenase, and three genes encoding a ring-hydroxylating dioxygenase (alpha and beta subunit of the dioxygenase component and the oxidoreductase component). Alignment of the deduced aminoacid sequences with sequences of others ring-hydoxylating dioxygenases revealed a high degree of similarity (≥80% identity) to the components of (halo)benzoate dioxygenases. The conserved amino acid residues that are involved in cofactor binding were also identified in the protein sequence. Recombinant strains carrying the putative FB dioxygenase genes were tested for expression. The SDS-PAGE analysis revealed that most of the expressed protein was on the pellet fraction and not on the soluble form, which could be due to improper folding of the enzyme components. Decrease of substrate concentration was observed in bioconversion experiments but the product formed was not detected/ identify.

Overall, strain F11 revealed to be capable of degrading a vast range of fluorinated compounds with different complexity and as such can be a potential strain to devise biotechnological solutions for biotransformation processes.

RESUMO

A contaminação do ambiente com produtos químicos de origem humana constitui uma ameaça para os ecossistemas e para a saúde pública. Deste modo, é extremamente importante compreender e explorar a sua biodegradação. O trabalho descrito nesta tese teve como objectivo principal a investigação do potencial da estirpe *Labrys portucalensis* F11 - uma bactéria anteriormente isolada pela sua capacidade de degradar fluorobenzeno (FB) - para a biotransformação de compostos aromáticos fluorados de diferente complexidade.

Tipicamente, a contaminação do ambiente resulta da presença de uma mistura de poluentes e não apenas da presença de um único contaminante. Deste modo, a coexistência de compostos orgânicos e metais no mesmo ecossistema, ou em águas residuais, é muito comum. Neste estudo foi avaliado o efeito de 3 metais – cobre, ferro e prata, com diferente importância biológica na degradação do FB. Na concentração de 1 mM, o ferro foi benéfico para o crescimento bacteriano sem afectar adversamente a biodegradação de FB até à concentração de 2 mM. Na presença de 1 mM de cobre e de prata verificou-se a inibição da degradação do FB levando à acumulação de dois metabolitos intermediários, o catecol e o 4-fluorocatecol, sugerindo a inibição da catecol 1,2-dioxigenase, uma enzima chave da via metabólica do FB.

A degradação de compostos com estruturas químicas semelhantes à do FB, clorobenzeno (CB) e difluorobenzenos (DFBS), foi avaliada. A estirpe F11 foi capaz de cometabolizar o CB na presença do FB, com degradação total de 0,5 mM de cada um dos substratos quando adicionados simultaneamente ou quando previamente induzida pelo FB. No entanto, o CB não induziu as enzimas para a sua própria degradação. Em relação aos DFBs, a estirpe F11 foi eficaz na degradação de 0,5 mM de 1,3-DFB como única fonte de carbono, e na degradação de 1,4-DFB (0,5 mM) em cometabolismo com FB (0,5 mM). No entanto, a estirpe F11 foi ineficaz na degradação do 1,2-DFB, tendo este composto inibido a degradação de FB. Estes resultados reforçam a importância da natureza, número e posição dos substituintes na molécula para a expressão enzimática e consequente conversão de compostos alvo.

A fluoxetina (FLX) é um fármaco quiral fluorado, cuja toxicidade, contaminação e persistência no ambiente têm sido bem documentadas. A estirpe F11 mostrou ser capaz de degradar os dois enantiómeros deste composto como única fonte de carbono (até 9 μM) e

na presença de acetato de sódio (até 89 mM de FLX). Foram obtidas extensões de degradação de pelo menos 80% de FLX total. Na concentração mais baixa de FLX (2 µM), testada como única fonte de carbono, a degradação foi completa e a libertação de fluoreto foi estequiométrica. A degradação revelou ser enantiosselectiva, com a degradação preferencial de *R*-FLX em relação a *S*-FLX, tendo sido detectada a formação de norfluoxetina (NFLX) como metabolito intermediário.

Com o objectivo de pesquisar os genes responsáveis pela expressão da FB dioxigenase, foi construída uma biblioteca genómica, constituída por 960 clones, a partir do DNA da estirpe F11 de *L. portucalensis*. Esta biblioteca poderá ser usada em trabalhos futuros, como a geração e confirmação dos dados da sequenciação, em estudos de genómica comparativa ou na pesquisa de outros genes de interesse. É importante realçar que a estirpe F11 tem demostrado uma capacidade extraordinária na degradação de compostos tóxicos, sendo deste modo, de grande interesse estudar os genes que lhe conferem estas capacidades.

Foi determinada uma sequência parcial dos nucleótidos do operão envolvido na degradação do FB. A sequenciação mostrou a existência de 4 genes, nomeadamente, o gene que codifica a 1,2-catecol dioxigenase, e os 3 genes que codificam as 3 subunidades da FB dioxigenase (alfa e beta da componente dioxigenase e oxidoreductase). O alinhamento das sequências de aminoácidos obtidas com as sequências de outras dioxigenases aromáticas revelou um elevado grau de similaridade (≥80%) com os componentes das (halo)benzoato dioxigenases. A sequenciação também permitiu identificar resíduos de aminoácidos conservados que estão envolvidos na ligação de cofactores. Estirpes recombinantes contendo os genes putativos da FB dioxigenase foram testadas para a expressão da enzima. A análise de SDS-PAGE revelou que a maioria da proteína expressa se encontrava na fracção do pellet e não na fracção solúvel, o que pode estar relacionado com uma conformação incorrecta dos componentes enzimáticos. Nas experiências de bioconversão foi observada diminuição da concentração de substrato mas o produto formado não foi detectado / identificado.

Em geral, a estirpe F11 revelou ser capaz de degradar uma vasta gama de compostos fluorados com diferente complexidade e, como tal, pode ser considerada uma estirpe com potencial para aplicação e desenvolvimento de soluções biotecnológicas em processos de biotransformação.

ACKNOWLEDGMENTS

LIST OF ABBREVIATIONS

1,2-DFB	1,2-Difluorobenzene
1,3-DFB	1,3-Difluorobenzene
1,4-DFB	1,4-Difluorobenzene
4-FCat	4-Fluorocatechol
ATP	Adenosine-5'-triphosphate
BAC	Bacterial Artificial Chromosome
BLAST	Basic Local Alignment Search Tool
BlastP	Protein Blast
bp	Base pairs
BTX	Benzene, toluene, xylene
Cat	Catechol
catA	Catechol 1,2-dioxygenase genes
CB	Chlorobenzene
DFB	Difluorobenzene
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
EF	Enantiomer Fraction
EPA	Environmental Protection Agency
FAD	Flavin Adenine Dinucleotide
FB	Fluorobenzene

Fbd	Fluorobenzene dioxygenase
fbdABC	Fluorobenzene dioxygenase genes
FLX	Fluoxetine
FMN	Flavin mononucleotide
IPTG	Isopropyl-β-D-thiogalactopyranoside
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NFLX	Nor-fluoxetine
ORF	Open Reading Frame
PAC	P1 Artificial Chromosome
PCR	Polymerase Chain Reaction
PMSF	Phenyl Methane Sulfonyl Fluoride
RNA	Ribonucleic acid
RNAse	Ribonuclease
RO	Rieske type non-heme iron oxygenases
SA	Sodium acetate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TE	Tris-EDTA buffer
tRNA	transfer RNA

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GENERAL INTRODUCTION

1.1. *HALOGENATED AROMATIC COMPOUNDS*

1.1.1. *Environmental problematic*

Halogenated aromatic hydrocarbons are molecules containing one or more atoms of a halogen (chloride, fluoride, bromide or iodide) and one or more benzene rings. Due to their widespread use and persistence in the environment, these compounds constitute environmental pollutants of great concern. Halogenation of the aromatic nucleus leads to an increase of molecular stability, which makes these compounds very attractive for several industrial uses. However, halogenation also increases molecular toxicity and resistance to biotic and abiotic degradation which, consequently, leads to the environmental accumulation of this class of compounds (Commandeur and Parsons, 1990). These compounds can be introduced into the environment from industrial effluents that are discharged directly into soils or wastewater treatment facilities, eventually contaminating water resources (Field and Sierra-Alvarez, 2004; Ren, 2004). Aromatic pollutants can cause various toxic effects to humans, such as carcinogenic, mutagenic and teratogenic effects (Cao et al., 2009). There are also reports that relate these compounds to skin diseases (Yamamoto and Tokura, 2003).

Research on the environmental fate of halogenated compounds has been focused mainly on chlorinated and brominated organics (Chaudry and Chapalamadugu, 1991). Chlorinated aromatic compounds are produced widely through human activities, in large quantities and for various purposes. Vast amounts of these compounds have been deliberately or accidently released into the biosphere, which has caused public concern over their adverse effects in the health of humans and wildlife, since they tend to be persistent in the environment and to accumulate in food chains due to their low degradability (Kunze et al., 2009; Ziagova and Liakopoulou-Kyriakides, 2007). Chlorinated aromatic compounds are considered among the most problematic categories of environmental pollutants, some being non-degradable or slowly degradable by microorganisms (van der Meer, 1997). They are widespread persistent contaminants in soils and sediments (Fennell et al., 2004). These compounds are chemically stable in nature; their photochemical degradation and hydrolysis does not play an important role in soil and aquatic environments (Monferrán et al., 2005).

1.1.2. Fluorinated aromatic compounds

The environmental fate of fluorinated organics has been subject of less attention comparing to chlorinated and brominated compounds, despite the fact that, in general, fluorinated chemicals are prominent xenobiotics and have low biodegradability (Iwai et al., 2009).

Although fluorine is the 13th most abundant element in the earth's crust, and the most abundant halogen, it plays a minor role in biology, because most of the fluorine in the environment is present in an insoluble form (e.g. as calcium fluoride), being biologically unavailable (Murphy et al., 2009). On the other hand, the synthetic production and use of fluorinated substances has increased enormously in the last few decades (Murphy et al., 2008; Cociglio et al., 1996). Fluorinated organic molecules currently account for up to 40% of all agrochemicals and 20% of all pharmaceuticals on the market (Grushin, 2010). These compounds are also used as propellants, surfactants, adhesives, refrigerants and fire retardants (Chaojie et al., 2007).

The incorporation of fluorine into organic compounds can dramatically alter their properties such as stability, lipophilicity and biological activity. These biological and/or physical effects are due to the unique properties of fluorine, such as small van der Waals radius (1.47 Å), strong electronegativity (4.0) and high strength of the carbon-fluorine bond (485 KJ mol^{-1}), which strongly contribute to the high stability of the fluorinated materials (Iwai et al., 2009).

Due to its increasing use and high stability, organofluorine compounds are becoming ubiquitous environmental contaminants (Key et al., 1997; Chaojie et al., 2007). Their apparent stability, bioactivity and potential for accumulation in the environment stress the importance of understanding the environmental fate and biodegradation mechanisms of these compounds.

1.1.3. Fluorinated pharmaceuticals

Organofluorine molecules exhibit significant biological effects as inhibitors of enzymes, cell-cell communication, membrane transport and processes for energy generation (Key et al., 1997). The effects of fluorine on biological properties of molecules have had a marked impact on various fields such as pharmacology (Iwai et al., 2009). As a result, the number of fluorinated drugs is continually increasing, and around a fifth of all drugs have at least one fluorinated substituent, including three of the current top ten selling medicines: Lipitor, Prevacid and Seretide (Isanbor and O'Hagan, 2006). Fluorine is often considered the favorite substituent to modulate unfavorable pharmacokinetic drug properties. Depending on the position of atom incorporation, it can improve metabolic stability, bioavailability and interactions with the biological target (Purser et al., 2008). Selective aromatic fluorine substitution can increase the affinity of a molecule for a macromolecular recognition site through non-covalent interactions (DiMagno and Sun, 2006). It is estimated that up to 20% of pharmaceuticals prescribed or administered in the clinic contain a fluorine atom and 30% of the leading 30 blockbuster drugs sales contain fluorine (O'Hagan, 2010).

Pharmaceuticals are considered emerging pollutants due to their widespread occurrence in the environment, recalcitrance nature and extensive use (Wu et al., 2010; Daugthon and Ternes, 1999). The presence of pharmaceutically active compounds (PhACs) in the environment can pose significant human and wildlife health threats. As with other anthropogenic substances, aquatic systems become the final recipient of PhACs, being found in sewage treatment plant effluents (Zorita et al., 2009), surface waters (Kolpin et al., 2002) and even in drinking waters (Benotti et al., 2009).

Generally, drugs are not completely metabolized in the human body and, as a consequence, a significant fraction of the original substances are excreted into the sewage, the same happening with active metabolites (Vasskog et al., 2009). Another pollution source is throwing expired medicines into the toilets or into landfills together with other solid urban wastes. Finally, some contribution is expected to come from manufacture plants (Suaréz et al., 2005). PhACs and their metabolites continuously enter the environment largely through effluents from wastewater treatment plants (WWTPs) due to incomplete elimination or sporadic direct wastewater discharge (Carballa et al., 2004; Wu et al., 2010).

Even low concentrations of these substances may lead to unwanted effects in aquatic systems, since they are conceived to have a biological effect at low concentrations (Daughton and Ternes, 1999; Fent et al., 2006). Although pharmaceuticals are designed as bioactive molecules to treat diseases, they can affect non-target organisms with harmful effects (Foran et al., 2004). These compounds have been found to persist in the environment and to exhibit bioaccumulative and endocrine disruptive activities (Caliman et al., 2009). Therefore, understanding their environmental behavior and impact is a topic of major concern.

1.2. BIODEGRADATION

1.2.1. Biodegradation of haloaromatic compounds

In the 1980s, the rapidly increasing of environmental contamination raised concerns about the adverse effects of pollutants to the ecosystems and human health, which was accompanied by a greater research in biological methods for pollution cleanup (Martínková et al., 2009). Biodegradation is the ability of microorganisms to transform or mineralize organic contaminants into less harmful, non-hazardous substances, which are then integrated into natural biogeochemical cycles (Margesin and Schinner, 2001). Biodegradation can lead to complete and cost-effective elimination of pollutants, playing an important role in environmental clean-up (Cao et al., 2009).

About 25% of the Earth's biomass is composed of compounds that have a benzene ring as the main structural constituent. In addition to natural sources, anthropogenic activities have added a plethora of new aromatic chemicals to the environment. Their novel structures pose major challenges to the microbial communities that are the major recyclers of natural products (Gibson and Harwood, 2002). Despite the fact that naturally occurring halogenated aromatic compounds are not common, many bacteria have been isolated which can degrade such chemicals (Commandeur and Parsons, 1990; Seo et al., 2009). When microorganisms face a new organic chemical in their environment, the catabolic genes needed for its degradation can be obtained through conjugational or

transformational events from other microorganisms, or microorganisms can suffer an adaptive process through selection and mutation events leading to the modification of existing genes (Chaudhry and Chapalamadugu, 1991).

The pathways by which halogenated aromatic compounds are degraded by microorganisms are similar to those for the degradation of aromatic compounds in general. Two major strategies can contribute to the removal of aromatic pollutants from the environment, depending on the presence or absence of oxygen. The anaerobic catabolism is based on reductive reactions to attack the aromatic ring (Carmona et al., 2009). In aerobic degradation, well-defined channels within the biodegradation pathways have evolved for most commonly encountered aromatic compounds. Structurally diverse pollutants are first transformed into a few intermediates through peripheral pathways, which are then further channeled, via a few central pathways, to the central metabolism. Most peripheral pathways carry out oxygenation reactions catalyzed by mono- or dioxygenases that convert the aromatic pollutants to dihydroxy aromatic intermediates, which are then cleaved by intradiol (*ortho*-cleavage) or extradiol dioxygenases (*meta*-cleavage). The generated metabolites are then channeled to subsequent central pathways leading to the formation of Krebs cycle intermediates. The ring-cleavage enzymes from various bacteria display significant functional similarities. Peripheral enzymes, which recognize and convert different aromatic pollutants into several central metabolites, play a significant role in the degradation of different pollutants (Cao et al., 2009).

Since halogen substituents are to a large extent responsible for the properties of halogenated aromatic compounds, removal of these substituents constitutes a key step in the biodegradation pathway of these compounds. In most cases, dehalogenation occurs after the cleavage of the aromatic ring. However, direct dehalogenation without loss of aromaticity has been also demonstrated (Commandeur and Parsons, 1990). Eight dehalogenation mechanisms were described for the metabolism of halogenated compounds: hydrolytic dehalogenation, thiolytic dehalogenation, intramolecular substitution, dehydrohalogenation, dehalogenation by hydration, dehalogenation by methyltransfer, oxidative dehalogenation and reductive dehalogenation (Van Pée and Unversucht, 2003). As a general rule, the resistance to enzymatic cleavage of the carbon-halogen bond increases with the increasing of substituent's electronegativity, being also

dependent on the specificity of the enzymes catalyzing the cleavage (Fetzner and Lingens, 1994).

Microbial transformations are gaining increased relevance. In particular, *in-situ* processes for the removal of polluting aromatic compounds can benefit of the capabilities of bacterial enzymatic systems to perform fast and gentle treatment conditions, reducing the environmental pollution at a low cost (Randazzo et al., 2004). Furthermore, the environmental benign procedures utilizing such enzymes may have also interesting applications for the synthesis of useful chemicals (Gibson and Parales, 2000; Hudlicky et al., 1999; Boyd et al., 2001).

Biodegradation of halogenated aromatic compounds has been focused especially on chlorinated compounds, such as chlorobenzoic acids (Kozlovsky et al., 1993), chloroanilines (Hongsawat and Vangnai, 2011) and chlorinated benzenes (Rapp et al., 2001; Rehfuss and Urban, 2005; Guerin, 2008; Monferrán et al., 2005; Zhang et al., 2011). Several reviews on this subject are available in the literature (Field and Sierra-Alvarez, 2004; Field and Sierra-Alvarez, 2008; Chaudry and Chapalamadugu, 1991; Häggblom, 1992). Less attention has been given to fluoroaromatics, probably because of the misconception that, due to their characteristics, fluorinated compounds should be inert and, thus, not pose an environmental threat. The high energy of the C-F bond is one of the highest energies in nature, which contributes to the high recalcitrance to microbial degradation. As a consequence of the low biodegradability of these compounds, their accumulation in the environment has become a worldwide issue. Nevertheless, reports on the biodegradation of fluorinated compounds are increasing in the literature and some of them are listed in Table 1.1. In some studies the compounds are reported to be degraded as single carbon source while other studies describe cometabolic processes.

Table 1.1. Examples of fluorinated aromatic compounds for which biodegradation was reported

Compound	Degrading microorganism	Reference
Fluorobenzene	*Labrys portucalensis* F11	Carvalho et al. 2006a
2-Fluorobenzoate	*Pseudomonas* spp.	Vora et al. 1988
2-Fluorobenzoate	*Alcaligenes eutrophus* B9 and *Pseudomonas* sp. B13	Engesser et al. 1980
2-Fluorobenzoate	Pseudomonad	Milne 1968
3-Fluorobenzoate	*Sphingomonas* sp. HB-1	Boersma et al. 2004
4-Fluorobenzoate	*Pseudomonas knackmussii* B13	Misiak et al. 2011
4-Fluorobenzoate	*Aureobacterium* sp. RHO25	Oltmanns et al. 1989
4-Fluorobenzoate	*Alcalines eutrophus and Pseudomonas cepacia*	Schlömann et al. 1990a
2- and 4-Fluorobenzoate	*Pseudomonas stutzeri*	Vargas et al. 2000
2- and 4-Fluorobenzoate	*Proteobacteria*	Song et al. 2000
2-, 3- and 4-Fluorobenzoate	*Pseudomonas* sp. B13	Schreiber et al. 1980
2-Fluorophenol	*Rhodococcus* sp.	Duque et al. 2012
4-Fluorophenol	*Arthrobacter* sp. IF1	Ferreira et al. 2008
Monofluorophenols	*Rhodococcus opacus* 1cp	Finkelstein et al. 2000
2-, 3- and 4-Fluorophenol	Acclimated activated sludge	Chaojie et al. 2007
Difluorophenols	*Penicillium frequentans* Bi 7/2	Wunderwald et al. 1998
Fluorophenols	*Exophiala jeanselmei*	Boersma et al. 1998
Fluorophenols	*Rhodococcus* sp.	Bondar et al. 1998
2-Trifluoromethylphenol	*Bacillus thermoleovorans* A2	Reinscheid et al. 1998
2- and 4-Fluorobiphenyl	*Pseudomonas pseudoalcaligenes* KF707	Murphy et al. 2008
3,4-Difluoroaniline	*Pseudomonas fluorescens* 26-K	Travkin et al. 2003
4-Fluorocinnamic acid	*Arthrobacter* sp. G1 + *Ralstonia* sp. H1	Hasan et al. 2012

1.2.2. Effects of co-contamination in biodegradation

1.2.2.1. Co-contamination with heavy metals

According to the U.S. Environmental Protection Agency (EPA), 40% of the hazardous waste sites included in the national priority list (NPL) is co-contaminated with organic and heavy metal pollutants that are hazardous both to humans and wildlife (http://www.epa.gov/superfund/sites/query/queryhtm/nplprop.htm).

Heavy metals are metals with a density above 5 g/cm^3. Most heavy metals are transition elements with incomplete filled orbitals, which confer the ability to form complex compounds. Some heavy metal cations play an important role as oligoelements in

biochemical reactions. However, at high concentrations, they form unspecific compounds in the cells, leading to toxic effects. This is the case of oligoelements such like Zn^{2+}, Ni^{2+} and especially Cu^{2+}. Other heavy metals cations, such as Hg^{2+}, Cd^{2+} and Ag^+, form strong toxic complexes, becoming too dangerous for any physiological function. Other heavy metals are important trace elements with low toxicity, as is the special case of iron, the only macro-bioelement known within the heavy metals (Nies, 1999).

Many studies indicate that contamination with metal ions may inhibit microorganisms by decreasing bacterial growth and biodegradation rates (Lin et al., 2006; Riis et al., 2002; Utgikar et al., 2003; Sokhn et al., 2001). The level of inhibition may depend on the nature, concentration and availability of the heavy metals (Amor et al., 2001; Hoffman et al., 2005). Heavy metals inhibit microorganisms by blocking essential functional groups or by interfering with essential metal ions incorporation into biological molecules (Doelman et al., 1994; Gadd and Griffiths, 1978). Heavy metals cations, especially those with high atomic numbers, as Hg^{2+}, Cd^{2+} and Ag^+, tend to bind to SH groups, inhibiting the activity of sensitive enzymes (Nies, 1999).

Some metals, such as Ag^+ and Cu^{2+}, are reported to be inhibitors of catechol 1,2-dioxygenases, which are key enzymes on the biodegradation of aromatic compounds (Kim et al., 2001; Wang et al., 2006; Matsumura et al., 2004). Iron is, on the other hand, a cofactor of various enzymes involved in the degradation of aromatic compounds, like the Rieske-type non-heme iron oxygenases and the ring-cleavage dioxygenases (Gibson and Parales, 2000; Wackett, 2002; Bugg and Lin, 2001).

1.2.2.2. Co-contamination with other organic pollutants

Contaminated ecosystems typically contain heterogeneous mixtures of organic compounds, which is an important problem because the removal or degradation of one component can be impacted by other components of the mixture. This happens not only for mixtures of toxic chemicals but also for mixtures of pollutants with easily degradable compounds, such as sugars. Different effects have been observed during the degradation of mixtures. The positive effect on the degradation of a second compound in a mixture has been demonstrated in experiments showing an increased growth at low substrates levels (Delgadillo-Mirquez et al., 2011) or the induction of suitable degrading enzymes (Alvarez

and Vogel, 1991). Negative interactions may also occur and are attributed to competitive and or non-competitive inhibition (Tsai and Juang, 2006), toxicity (Reardon et al., 2000), formation of toxic intermediates by non-specific enzymes (Bartels et al., 1984) and synergistic inhibition of bacterial growth (Smith et al., 1991).

Among interactions between substrates during biodegradation, special attention has been paid to the mechanism of cometabolism. Cometabolism is a mechanism in which a non-growth substrate is transformed in the presence of a growth substrate or another transformable compound that is capable to induce enzymes or produce cofactors and/or metabolites required for the transformation and/or to support cell growth by generating energy and carbon polymers. The growth substrate can be an analogue of the toxic compound, often leading to a competitive inhibition between growth and non-growth substrates, when they share the same enzymatic pathway, or a more conventional carbon source as yeast extract, sugars or acids (Ziagova et al., 2007). Several publications have shown cometabolism as a successful mechanism for the transformation of various recalcitrant contaminants (Elango et al., 2011; Zhong et al., 2010; Jechorek et al., 2003; Zhang and Bajpai, 2000).

1.2.3. Enantioselectivity in biodegradation

A significant number of the organic chemicals regulated by the U.S. EPA are chiral, possessing at least one element of asymmetry and leading to the existence of two or more stereoisomers called enantiomers, and are released to the environment as racemates (mixtures of equal amounts of enantiomers) (http://water.epa.gov/scitech/methods/ cwa/ pollutants.cfm). About 25% of all agrochemicals are chiral (Williams, 1996). Also, many pharmaceuticals found in the aquatic environment are chiral chemicals, from which some are dispensed and consumed as mixtures of enantiomers, while others are dispensed as relatively pure single enantiomers (Murakami, 2007).

Enantiomers of chiral compounds have similar physical-chemical properties but they are known to selectively interact with biological systems, which are usually enantioselective. This may result in enantioselective toxicity, mutagenicity, carcinogenicity, endocrine

disruptive activity (Liu et al., 2005; Lewis et al., 1999; Wong, 2006). In the environment, abiotic transformations of chiral compounds are mostly non-enantioselective whereas biological degradation usually proceeds with high enantioselectivity (Wong, 2006; Müller and Kohler, 2004). Enantioselective biodegradation implies that the enzymes involved in the conversion of such compounds are able to differentiate between the enantiomers. The biodegradation of enantiomers of chiral pollutants may proceed through different pathways, by two enantioselective enzymes or by one enzyme that degrades both enantiomers simultaneously, but at different rates, or degrades the enantiomers sequentially (Müller and Kohler, 2004).

In some enzymes, with overall conserved folding, a few amino acids residues determine stereoselectivity. This is the case of ring-hydroxylating dioxygenases, which are responsible for the initial attack of the benzene ring of aromatic compounds, converting aquiral substrates to chiral products (*cis*-dihydrodiols) by a regio- and enantioselective process, from which results enantiomerically pure products (Hudlicky et al., 1999). In the model naphthalene dioxygenase, phenylalanine residue 253 has been shown to play an important role in controlling regio- and enantioselectivity (Parales et al., 2000).

Other enzymes express opposite stereospecificity and have completely different folds and surprisingly similar active sites that are mirror-images. Theoretically, two enzymes with identical sequences but built from enantiomeric amino acids, one built with D-amino acids and other built with L-amino acids, should have opposite stereospecificity for chiral substances (Müller and Kohler, 2004).

1.3. RIESKE NON-HEME IRON OXYGENASES

1.3.1. Significance for aerobic biotransformation

The initial step in the bacterial degradation of aromatic compounds is usually the dihydroxylation of the aromatic ring of the compound. Hydroxylations belong to the oxygen transfer reactions leading to the introduction of the hydroxyl group (-OH) into organic molecules, primarily via the substitution of functional groups or hydrogen atoms (Ullrich

and Hofrichter, 2007). Addition reactions leading to dearomatisation of benzene ring are generally very slow due to the significant resonance energy deficit involved and thermodynamic drive to preserve the aromatic sextet (Boyd and Bugg, 2006). The direct and selective introduction of the hydroxyl group into aromatic rings is one of the most challenging fields in chemical synthesis. However, a remarkable family of biocatalysts able to regulate the activity of dioxygen overcomes this problem - aromatic ring-dihydroxylating dioxygenases. As these enzymes have been more recently found to catalyze both monooxygenase and dioxygenase reactions, they are more accurately identified as Rieske type non-heme iron oxygenases (ROs) (Gibson and Parales, 2000).

ROs are interesting enzymes not only for the development of biodegradation technologies but also due to their potential for industrial applications in biocatalysis. ROs catalyze stereo- and regiospecific reactions. Their specificity allows the selective oxygenation of organic molecules under environmental friendly conditions; they are a source of enantiopure arene *cis*-diols that are not attainable by conventional chemical synthesis (Ullrich and Hofrichter, 2007). Chiral *cis*-1,2-dihydrodiols are important intermediates for the production of fine chemicals, pharmaceuticals and a wide variety of bioactive natural products (Boyd et al., 2001; Hudlicky et al., 1999; Sello and Orsini, 2004; Gibson and Parales, 2000; Wackett, 2002).

In general, the clustering of oxygenases into families correlates with the native substrates oxidized by the members and sequence alignments. Gibson and Parales (2000) distinguished four families of arene dioxygenases: the toluene/biphenyl, naphthalene, benzoate and phthalate families. However, there are several arene dioxygenases that do not cluster with any of these families. ROs resemble cytochrome P450 in its ability to catalyze monohydroxylation, sulfoxidation, desaturation, dehydrogenation, and *O-* and *N-*dealkilation reactions (Gibson et al., 1995; Resnick et al., 1996). However, P450 does not catalyze the enantiospecific *cis*-hydroxylation of arenes and ROs do not catalyze alkenes to epoxides.

1.3.2. Structure and mechanism

ROs are multicomponent enzyme systems consisting of two separate functional units, comprising one or two electron transport proteins which precede the oxygenase component. Oxygenase components are either homo (α_n) or hetero-oligomers ($\alpha_n\beta_n$). The α-subunit contains two conserved regions, a Rieske [2Fe-2S] center and a non-heme mononuclear iron. The α-subunit is the catalytic component involved in the transfer of electrons to the oxygen molecules (Kweon et al., 2008). The electron transport chain consists of either a flavoprotein reductase or a separate flavoprotein reductase and a ferredoxin. The multicomponent ROs use reduced pyridine nucleotide as the initial source of two electrons for dioxygen activation. The electrons are passed to the protein component containing the flavin cofactor that can act as two to one electron redox switch. The single electrons are then handed off to a [2Fe-2S] center, either contained at the flavoprotein reductase or on the separate ferredoxin. Ultimately, electrons are transferred to the Rieske dioxygenase component (Wackett, 2002).
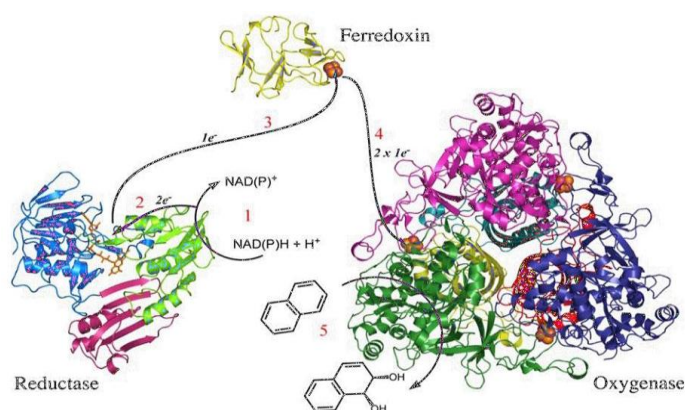
Figure 1.1. Example of a Rieske oxygenase system constituted by three components. (1) The reductase oxidizes NAD(P)H to $NADP^+$ at the NAD(P)H binding site, capturing 2 electrons. (2) The electrons are stored on the flavin until (3) the reductase completes a 1 electron reduction of the ferredoxin component. (4) The ferredoxin shuttles the electron received from the reductase to the oxygenase Rieske cluster. This step occurs twice for (5) each molecule of product formed at the mononuclear iron site. The flavin is shown as a stick representation, the Rieske cluster and the mononuclear iron are shown as spheres (from Ferraro et al., 2005).

Iron-sulfur clusters are frequent in electron transport proteins, being involved in the storage of electrons. There are multiple types of iron-sulfur clusters, including two general types of [2Fe-2S] clusters, classified according to their coordinating residues. The plant-type cluster is coordinated to its protein by four cysteine residues. The Riske-type [2Fe-2S] cluster is coordinated by two cysteine and two histidine residues (Ferraro et al., 2005).

Reductase enzymes are classified into two separate structural families: the ferredoxin-NADP reductase (FMN), which contains a FAD or FMN-binding domain, a NADH-binding domain and a plant type [2Fe-2S] cluster domain (Karlsson et al., 2002), and the glutathione reductase, which contains a FAD-binding domain, a NADH-binding domain and a C-terminal domain (Lee et al., 2005).

The ferredoxin component is only present in the three component RO systems and carries an electron from the reductase to the terminal oxygenase. There are small proteins containing either a plant-type [2Fe-2S] or a Rieske-type [2Fe-2S] cluster (Nam et al., 2005).

The α-subunit of the oxygenase enzyme can be divided into the Rieske [2Fe-2S] cluster domain and the mononuclear iron-containing catalytic domain. The Rieske cluster accepts electrons from the reductase of ferredoxin and passes them to the mononuclear iron for catalysis. The mononuclear iron is part of the predominantly hydrophobic active site and comprised of the C-terminal portion of the protein and the first ≈40 residues of the N-terminal sequence (Dong et al., 2005).

Some oxygenase enzymes contain an α- and β-subunit. The function attributed to the β-subunit is only structural (Kauppi et al., 1998), despite some reports suggesting that it can influence substrate specificity (Hurtubise et al., 1998).

Studies on the structures of oxygenase enzymes have revealed an α_3 or $\alpha_3\beta_3$ mushroom-shaped quaternary structure with three-fold symmetry. The active site consists of a Rieske [2Fe-2S] cluster from one α-subunit juxtaposed within 12 Å of a mononuclear iron center from an adjacent α-subunit (Ferraro et al., 2005).

1.3.3. *Biotechnological applications*

ROs are promising biocatalysts for biotechnological applications due to their versatility and to regio- and stereospecificity of the catalyzed reactions. However, because of their complexity and the requirement for NAD(P)H, the focus remains on whole-cell biotransformations. In addition to the microbial production of valuable molecules by wild-strains possessing these enzymes, recent developments in molecular biology and genetics have led to the construction of engineered strains, which increase the yield of the target product by gene amplification, elimination of undesired side reactions and transfer of bioconversion potential to well-known and easier to handle microorganisms. In this context, process parameters of whole-cell biotransformations are optimized using special bioreactors and enzyme properties are improved by genetic engineering and directed evolution (Di Gennaro et al., 2006; Zhang et al., 2000; Boyd and Bugg, 2006).

cis-Dihydrodiols formed by ROs have been demonstrated to have some important applications as, for example, as chiral precursors for the synthesis of drugs (Buckland et al., 1999; Reddy et al., 1999; Zhang et al., 2000). These enzyme systems are also promising for bioremediation, on the basis of the more than 200 substrates they can act upon (Hudlicky et al., 1999).

1.4. TARGET HALOGENATED AROMATIC COMPOUNDS

1.4.1. *Fluorobenzene (FB)*

Fluorobenzene is a fluoroaromatic compound mainly used as a reagent for plastic and resin polymers production (Maruta and Nishimiya, 1991) and for the production of herbicides (Hirai et al., 1995) and pharmaceuticals (Whitehead and Traverse, 1972). Its physical-chemical properties and environmental fate characteristics indicate that FB may be persistent in air, moderately persistent in terrestrial compartments, and is not expected to readily biodegrade (http://www.epa.gov/hpv/pubs/summaries/flurbenz/c14602rt.pdf). Its biodegradation, by a bacterial strain identified as *Labrys portucalensis* F11, was first

reported by Carvalho et al. (2005). Figure 1.2 shows the molecular structure of this compound.
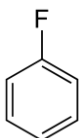
Figure 1.2. Molecular structure of FB.

1.4.2. *Chlorobenzene (CB) - the chlorinated analogue of fluorobenzene*

Chlorobenzene (Figure 1.3) is an environmental pollutant of great concern due to its toxicity, persistence and accumulation in the food chain (Monferrán et al., 2005), being identified as a priority pollutant by the U.S. EPA (http://water.epa.gov/scitech/methods/cwa/pollutants.cfm).
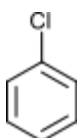
Figure 1.3. Molecular structure of CB.

CB is an important industrial intermediate used in the production of phenol and nitrochlorobenzene (*ortho-* and *para*-isomers), in the formulation of herbicides, in the production of additional chlorobenzenes and as a solvent in the manufacture of adhesives, paints, resins, dyestuffs, drugs and of the chemicals diphenyl oxide, phenylphenol, silicone resin and other halogenated organics (Malcolm et al., 2004). Its widespread use has resulted in broad environmental contamination (Field and Sierra-Alvarez, 2008). CB is released to the environment during its manufacture and the production of other chemicals and during the disposal of CB containing products, such as from incinerators and hazardous waste sites. It is also released directly to the environment due to its use as a pesticide

carrier, through its use in deodorizers, fumigants, degreasers, insecticides, herbicides and defoliants (Guerin, 2008).

Levels of CB in groundwater, sediments and soils are generally in the range of ng kg^{-1} to μg kg^{-1}, although levels in the range of mg kg^{-1} have been reported in samples from industrial areas (Guerin, 2008; Bittkau et al., 2004). While the amount of CB can be high in polluted soils and sediments, the concentration in the aqueous phase is rather low, due to the poor water solubility (484 mg l^{-1}) of this compound and adsorption to either colloid particles in surface water or to organic matrix of sediments (van der Meer, 1997). As a consequence, CB is found worldwide adsorbed to sediments (Meharg et al., 2000; Nikolaou et al., 2002). Due to the high vapour pressure of CB (1.58 kPa), this compound tends to become volatilized and so atmospheric emissions of this compound are important (Field and Sierra-Alvarez, 2008). CB environmental emissions to air in the U.S. were reported to be 314 tonnes in 2001, compared to 0.3 tonnes found in surface waters and 0.01 tonnes in land (Malcolm et al., 2004).

CB is chemically stable and its chemical abiotic degradation, such as hydrolysis or photolysis, in the environment is limited (Malcom et al., 2004). However, some bacterial strains have evolved degradative pathways for degrading this compound (Kunze et al., 2009; Zhang et al., 2011; Jiang et al., 2009; Göbel et al., 2004; Nishino et al., 1992; Reineke and Knackmuss, 1984). Strains enriched in this compound have been shown to be able to degrade several related compounds, except fluorinated ones (Zaitsev et al., 1995; Freitas dos Santos et al., 1999) due to the characteristics of the C-F bond, which strongly contributes to the high stability of the fluorinated molecules. On the other hand, whether strains isolated on a fluorinated compound are capable to evolve strategies to degrade the chlorinated analogue is a possibility yet unexplored.

1.4.3. Difluorobenzenes (DFBs)

Difluorobenzenes (Figure 1.4) are employed as chemical intermediates in a variety of applications within the pharmaceutical, agricultural (Nalelwajek and van der Puy, 1989) and electronic fields (Kawakami et al., 2009). For example, 1,3-DFB is used in the preparation of

Diflunisal (trademark), an anti-inflammatory agent, in Difluobenzuron (trademark), a potential insecticide, in Rufinamide (trademark), an anticonvulsant (Kankan et al., 2011), in adhesive polyimide resin and in heat resistant polymers (Tomoshige et al., 2003). Benzodiazepinones are prepared by reacting 1,2- or 1,4-DFB in a multistep procedure to yield compounds which exhibit sedative and/or anticonvulsant activity (Nalelwajek and van der Puy, 1989).
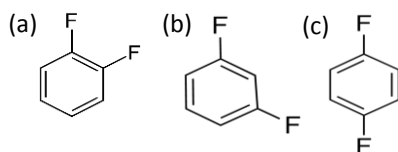
Figure 1.4. Molecular structure of (a) 1,2-DFB, (b) 1,3-DFB and (c) 1,4-DFB.

There are no particular reports about environmental contamination with DFBs. However, the use of these compounds in agriculture leads to their direct release or to the release of their derivatives into the environment. Moreover, the fact that DFBs are used as precursors of pharmaceuticals confirms their biological effects, which can be dramatic to other organisms. In contrast to several studies on dichlorobenzenes biodegradation (Adebusoye et al., 2007; Sommer and Görisch, 1997; Spiess et al., 1995; Haigler et al., 1988), little attention has being given to their fluorinated counterparts (Renganathan, 1989; Renganathan and Johnston, 1989; Rapp and Gabriel-Jürgens, 2003). Studying the biodegradation of DFBs is important not only because of the recalcitrance and consequent persistence of these compounds, but also because it allows assessing the effect of the number and position of the substituents in the biodegradability of fluorinated aromatics.

1.4.4. Fluoxetine (FLX)

The antidepressant fluoxetine (FLX) (*N*-methyl-*γ*-[4-(trifluoromethyl)phenoxy]benzene-propanamine), sold as the racemate and more commonly known as Prozac® (Eli Lilly), is a molecule featuring a trifluoromethyl group on one of its aryl rings (Figure 1.5). It was

approved by the Food and Drug Administration (FDA) in December 1987, and grew to become the most prescribed antidepressant drug worldwide, achieving annual sales of ca. one billion U.S. dollars. In 1994, the FDA approved the drug for use in the treatment of both obsessive-compulsive disorder and bulimia. Studies have shown that depression is linked to low levels of the neurotransmitter 5-hydroxytryptamine (5-HT), also known as serotonin. Fluoxetine acts by selectively inhibiting the reuptake of serotonin, allowing the neurotransmitter to activate its specific receptor (Purser et al., 2008). Structure - activity relation studies showed that the inclusion of a trifluoromethyl group in the *para*-position of the phenolic ring increased the potency for inhibiting 5-HT uptake by 6-fold, compared to the nonfluorinated parent compound (Wong et al., 1995). It is believed that the steric bulk of the trifluoromethyl group at this position allows the phenoxy ring to adopt a conformation which favours binding to the serotonin transporter (Roman et al., 2003)
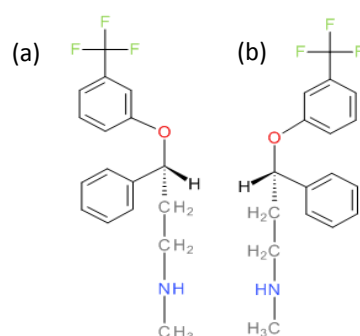
Figure 1.5. Molecular structure of (a) *R*-FLX and (b) *S*-FLX.

FLX is metabolized by Cytochrome P-450 isoenzymes to norfluoxetine (NFLX), its active metabolite, and ca. 10% of the unchanged parent compound is excreted in the urine (Hiemke and Härtter, 2000). Significant concentrations of FLX have been detected in environmental samples, including effluents of wastewater treatment plants, rivers, streams and even in drinking water (Table 1.2). Due to the importance of serotonin in numerous physiological processes, contamination with FLX can impact these processes and have severe consequences to organisms, as reported by many authors (Brooks et al., 2003; Henry and Black, 2008; Winder et al., 2009; Sánchez-Argüello et al., 2009). FLX is relatively recalcitrant to hydrolysis, photolysis and microbial degradation and is rapidly removed from

surface waters by adsorption to sediments, where it appears to be persistent (Kwon and Armbrust, 2006)

Table 1.2. Occurrence of Fluoxetine in the environment

Environmental sample	Range or maximum concentration of FX (ng l^{-1}) (ng kg^{-1})	Reference
Household sewage	34.4 ± 14.5	Zorita et al. 2009
Hospital sewage	16.8	Zorita et al. 2009
Sewage treatment plant	11.0 ± 6.0	Zorita et al. 2009
River system (seasonal)	18.0 - 66.1	Fernández et al. 2010
Wastewater treatment plant - raw sewage	3.5 ± 0.3	Lajeunesse et al. 2008
Wastewater treatment plant - effuent	3.7 ± 0.1	Lajeunesse et al. 2008
Receiving waters	1.3 ± 0.1	Lajeunesse et al. 2008
Sewage treatment plant - effluents	38.0 - 99.0	Metcalfe et al. 2003
Surface waters	13.0 - 46.0	Metcalfe et al. 2003
Wastewater raw influent	18.0 ± 2.0	MacLeod et al. 2007
Wastewater treated effluent	14.0 ± 0.1	MacLeod et al. 2007
Surface water	12.0	Kolpin et al. 2002
Sewage treatment plant	3.9 - 10.1	Conley et al. 2008
Surface waters	12.0 - 20.0	Schultz and Furlong 2008
Water facility effluent (seasonal)	1.23 - 5.40	Kinney et al. 2006a
Soils	6.15 - 8.80	Kinney et al. 2006a
Biosolids	212 - 1501	Wu et al. 2010
Soils receiving biosolids	3.3 - 6.7	Wu et al. 2010
Drink water - source	3.0	Benotti et al. 2009
Drink water - finished	0.82	Benotti et al. 2009
Drink water - distribution	0.64	Benotti et al. 2009
Wastewater treatment plant - influent	18.7	Vasskog et al. 2008
Wastewater treatment plant - effluent	0.6 - 8.5	Vasskog et al. 2008
Wastewater treatment plant - influent	0.4 - 2.4	Vasskog et al. 2006
Wastewater treatment plant - effluent	1.3	Vasskog et al. 2006

There are only few studies concerning FLX biodegradation (Borges et al., 2009; Redshaw et al., 2008; Suarez et al., 2010; Vasskog et al., 2009). The widespread contamination and toxicity potential of this compound stresses the importance to study its biodegradation and understand the enantioselective mechanisms of its biological behaviour.

1.5. SCOPE AND OBJECTIVES OF THIS THESIS

Organofluorine compounds have been widespread used in the agrochemical, pharmaceutical and plastic industries, resulting in significant environmental contamination by these compounds. However, while much attention has been paid to the microbial degradation of chlorinated organic compounds, our understanding of the biodegradation of organofluorine compounds is still scarce. Because of their apparent stability, bioactivity and potential for accumulation in the environment, it is important to understand their environmental fate and their biodegradation mechanisms.

At ESB there is wide experience in devising solutions for the environmental problematic of organofluorine compounds. Under this scope several bacteria have been isolated for the ability to degrade these recalcitrant compounds, such as: *Labrys portucalensis* F11, used in this work, able to degrade FB (Carvalho et al., 2005); *Burkholderia cepacia* FB2, able to degrade fluorobenzoates (Emanuelsson, 2008); and, *Rhodococcus* sp. FP1, able to degrade 2-fluorophenol (Duque et al., 2012). The metabolic pathways of degradation of these compounds have been elucidated (Carvalho et al. 2006a; Duque et al., 2012) and, the establishment and operation of bioreactors for biodegradation, inoculated with these strains, have been performed (Emanuelsson et al., 2006; Carvalho et al., 2006b; Duque et al., 2011a; Duque et al., 2011b).

The work described in this thesis focuses on exploring fluoroaromatics biotransformation potential of *Labrys portucalensis* F11, a strain isolated for its ability to degrade FB as sole carbon and energy source, and on cloning and sequencing its aromatic ring-hydroxylating dioxygenase, an enzyme with a central role in the biodegradation of xenobiotic aromatic compounds. Strain F11 was isolated from an industrially contaminated sediment located at Estarreja, in northern Portugal (Carvalho et al., 2005). Taxonomic studies revealed that this strain belongs to subgroup 2 of the class *Alphaproteobacteria* (Woese et al., 1984) and falls within the order *Rhizobiales*. A more detailed classification allowed to conclude that strain F11 represents a novel species within the genus *Labrys*, for which the name *Labrys portucalensis* sp. nov. was proposed (Carvalho et al., 2008).

The metabolic pathway for FB degradation by *L. portucalensis* F11 was found to start with a dioxygenase attack to the aromatic ring, leading to the production of two different

fluorinated dihydrodiols: 4-fluoro-*cis*-benzene-1,2-dihydrodiol and 1-fluoro-*cis*-benzene-1,2-dihydrodiol. The first intermediate is subjected to a rearomatization reaction, catalysed by a dihydrodiol dehydrogenase, resulting in the production of 4-fluorocatechol as the predominant central metabolite, while 1-fluoro-*cis*-benzene-1,2-dihydrodiol is spontaneously defluorinated in a non-enzymatic reaction, leading to the production of catechol, the minor product of the initial dioxygenase reaction. Further metabolism of the resulting catechol intermediates proceeds via an *ortho*-cleavage pathway, in which 4-fluorocatechol and catechol are respectively converted to 3-fluoro-*cis,cis*-muconate and *cis,cis*-muconate, through the action of a catechol 1,2-dioxygenase. The fluorinated muconate is then expected to be converted, with concomitant defluorination, into maleylacetate, which is then channelled into the tricarboxylic acid cycle via 3-oxoadipate, while *cis,cis*-muconate is proposed to be converted to the lactone derivative being then also channeled into the tricarboxylic acid cycle (Carvalho et al., 2006a).

Metabolic versatility studies revealed that strain F11 grew very well in aromatic compounds with a chemical structure similar to FB, such as benzoate, benzene, phenol, 4-fluorobenzoate and 4-fluorophenol, with the latest three compounds being completely defluorinated. Chloro-, bromo-, and iodobenzenes, 3-chloro-4-fluoroaniline, 4-chlorobenzoate and 4-chlorophenol did not serve as growth substrates for strain F11 at the time the strain was isolated (Carvalho et al., 2005).

With the aim to further explore the metabolic capabilities of *L. portucalensis* F11, the objectives of this thesis were:

- To evaluate the effect of co-contamination with metals, specifically iron, copper and silver, in the biodegradation of FB by *L. portucalensis* F11;

- To evaluate the ability of *L. portucalensis* F11 to degrade other halogenated compounds, such as chlorobenzene, difluorobenzenes and fluoxetine (*N*-methyl-*γ*-[4-(trifluoromethyl)phenoxy]benzene-propanamine), and investigate the effect of the nature, number and position of the halogen on the aromatic ring;

- To assess the enantioselectivity of biodegradation of chiral compounds by *L. portucalensis* F11;

- To clone and sequence the aromatic ring hydroxylating dioxygenase of strain *L. portucalensis* F11 for further biocatalytic studies.

1.6.   OUTLINE OF THE THESIS

This thesis comprises eight chapters.

In the current chapter (**Chapter 1**), a literature review concerning the environmental problematic of halogenated aromatic compounds, focusing the fluorinated compounds, is presented. The importance of Rieske non-heme iron dioxygenases for the biodegradation of xenobiotic compounds and for the production of valuable chiral building blocks for industry is also described.

The first experimental part of this thesis (**Chapter 2)** describes the effect of three metals, namely iron, copper and silver, on fluorobenzene (FB) biodegradation by growing and resting cells of *L. portucalensis* F11. Metabolites accumulated due to metal inhibition were identified.

Chapter 3 evaluates the biodegradation of chlorobenzene by *L. portucalensis* F11 as sole carbon source and in cometabolism with FB by non-induced cells and by FB induced cells.

The biodegradation of difluorobenzenes by *L. portucalensis* F11 is reported in **Chapter 4**. The biodegradation was evaluated using the tested compounds as sole carbon source and in the presence of FB. The effect of inoculum density and substrate concentration on the biodegradation of the target compounds was assessed.

Chapter 5 reports the degradation of fluoxetine by *L. portucalensis* F11 as sole carbon source and in the presence of a supplementary carbon source. The enantioselectivity and kinetics of biodegradation was assessed. The major intermediary metabolite produced during biodegradation was identified.

In **Chapter 6** the construction of a genomic library of *L. portucalensis* F11 and the screening for the genes encoding fluorobenzene dioxygenase is reported.

The cloning, nucleotide sequence and expression of fluorobenzene dioxygenase is described in **Chapter 7**. Blast and phylogenetic analysis were performed and amino acid deduced sequence was analyzed for the presence of conserved putative co-factor binding sites.

In the last chapter, **Chapter 8**, the main findings, highlighting the major conclusions, are summarized and suggestions for further research are presented.

CHAPTER 2

Effect of the metals iron, copper and silver on fluorobenzene biodegradation by *Labrys portucalensis* F11

Abstract

Organic and metallic pollutants are ubiquitous in the environment. Many metals are reported to be toxic to microorganisms and to inhibit biodegradation. The effect of the metals iron, copper and silver on the metabolism of Labrys portucalensis F11 and on fluorobenzene (FB) biodegradation was examined. The results indicate that the addition of 1 mM of Fe^{2+} to the culture medium has a positive effect on bacterial growth and has no impact in the biodegradation of 1 and 2 mM of FB. The presence of 1 mM of Cu^{2+} was found to strongly inhibit the growth of F11 cultures and to reduce the biodegradation of 1 and 2 mM of FB to ca. 50%, with 80% of stoichiometrically expected fluoride released. In the experiments with resting cells, the FB degraded (from 2 mM supplied) was reduced ca. 20% whereas the fluoride released was reduced to 45% of that stoichiometrically expected. Ag^+ was the most potent inhibitor of FB degradation. In experiments with growing cells, the addition of 1 mM of Ag^+ to the culture medium containing 1 and 2 mM of FB resulted in no fluoride release, whereas FB degradation was only one third of that observed in control cultures. In the experiments with resting cells, the addition of Ag^+ resulted in 25% reduction in substrate degradation and fluoride release was only 20% of that stoichiometrically expected. The accumulation of catechol and 4-fluorocatechol in cultures supplemented with Cu^{2+} or Ag^+ suggest inhibition of the key enzyme of FB metabolism - catechol 1,2-dioxygenase.

2.1. INTRODUCTION

Fluorine-containing organic compounds are very important in a range of life-science industries. The use of fluorine in the manufacture of many commercially important products, such as pharmaceuticals and agrochemicals, has increased enormously (Murphy et al., 2008; Cociglio et al., 1996). The replacement of hydrogen atoms by fluorine substituents in organic compounds is of great interest in synthetic chemistry since it highly increases their electronegativity without distortion of the geometry of the molecule (Nyffeler et al., 2005). Although fluorine atoms are small and cause little steric hindrance, they can profoundly alter the properties of organic compounds, such as molecular stability, lipophilicity and biological activity. The bio-physical-chemical effects arise from the unique properties of fluorine: strong electronegativity, small size and strong bonding with carbon (Iwai et al., 2009).

Fluorinated compounds are becoming ubiquitous environmental contaminants and because of their apparent stability, bioactivity and potential for accumulation in the environment, investigating the biodegradation mechanisms of these compounds is essential to understand their environmental fate (Chaojie et al., 2007; Key et al., 1997). Among other factors, the bacterial degradation of organic pollutants may be affected by the presence of metals. Several studies in the literature report that the presence of metals at a contaminated site may inhibit microorganisms causing a decrease in bacterial growth and biodegradation rates (Hong et al., 2007; Lin et al., 2006; Said and Lewis, 1991; Hoffman et al., 2005).

According to the U.S. Environmental Protection Agency (EPA), 40% of the hazardous waste sites included in the national priority list (NPL) is co-contaminated with organic and heavy metal pollutants that are hazardous both to humans and wildlife (http://www.epa.gov/superfund/sites/query/queryhtm/nplprop.htm). From the contaminants that integrate the EPA's priority list of pollutants, the metals copper and silver represent metal contaminants of major concern (http://water.epa.gov/scitech/methods/cwa/pollutants.cfm). Simultaneous contamination with iron and aromatic hydrocarbons has been reported (D'Annibale et al., 2007).

It is known that copper plays an important role as a trace element in several biochemical reactions. However, when present at high concentrations this metal forms unspecific complex compounds in the cell, leading to toxic effects. Silver is a metal highly dangerous for any physiological function, forming strong toxic complexes within the cells (Nies, 1999). These metals are reported as inhibitors of catechol 1,2-dioxygenases (Kim et al., 2001; Wang et al., 2006; Matsumura et al., 2004). These enzymes are non-heme iron-containing enzymes essential for the microbial degradation of aromatic compounds, catalyzing the oxidative cleavage of *ortho*-dihydroxy substituted aromatic rings through the incorporation of two atoms of molecular oxygen. The catechol 1,2-dioxygenases convert catechols to *cis,cis*-muconates which can be further degraded to central carbon metabolites (Ridder et al., 1998). Iron is, on the other hand, the most important metal to living organisms (Nies, 1999) and is required for optimal growth of microorganisms (Neilands, 1995). This metal is also a cofactor of various enzymes involved in the degradation of aromatic compounds, like the Rieske-type non-heme iron oxygenases and the ring-cleavage dioxygenases (Gibson and Parales, 2000; Wackett, 2000; Bugg and Lin, 2001). The expression of iron-containing oxygenases during the bacterial degradation of aromatic compounds may increase the bacterial requirement of iron and, thus, the presence of this metal may play a positive role in the biodegradation of aromatic pollutants.

This work focused in understanding if metal contamination can interfere with the biodegradation of fluorinated organic pollutants and in investigating the effects of this type of contamination at the metabolic level. With this purpose, the effect of the metals Cu^{2+}, Ag^+ and Fe^{2+}, which are reported as environmental contaminants, and have the potential to interfere with degradative enzymes, on the biodegradation of fluorobenzene (FB) by the microorganism *Labrys portucalensis* F11, a bacterial strain which has the capacity to degrade FB as sole carbon and energy source (Carvalho et al., 2005), was assessed.

2.2. MATERIAL AND METHODS

2.2.1. Cultivation conditions

L. portucalensis strain F11 (Carvalho et al., 2005) was grown in sealed flasks containing a sterile minimal salts medium (MM) (composition per litre: $Na_2HPO_4.2H_2O$, 2.7 g; KH_2PO_4, 1.4 g; $(NH_4)_2SO_4$, 0.5 g; $MgSO_4.7H_2O$, 0.1 g; and, 10 ml trace elements solution of the following final composition per litre: $Na_2EDTA.2H_2O$, 12.0 g; NaOH, 2.0 g; $MnSO_4.4H_2O$, 0.4 g; $ZnSO_4.7H_2O$, 0.4 g; H_2SO_4, 0.5 ml; Na_2SO_4, 10.0 g; $Na_2MoO_4.2H_2O$, 0.1 g; $FeSO_4.7H_2O$, 2.0 g; $CuSO_4.5H_2O$, 0.1 g; $CaCl_2$ 1.0 g). FB (1 mM) was supplied as sole carbon and energy source. Cultures were incubated on a rotary shaker (130 rpm) at 25ºC. Growth was monitored by measuring the optical density at 600 nm (OD_{600}) using a spectrophotometer (Helios Gamma, Unicam Instruments, UK).

2.2.2. Biodegradation experiments

Cells of *L. portucalensis* F11 grown in FB (1 mM) were harvested by centrifugation (10 000 rpm for 15 min at 4ºC), washed with KH_2PO_4-Na_2HPO_4 buffer (25mM, pH 7.2), and ressuspended in MM/phosphate buffer (for growing/ resting cells experiments, respectively), containing 1 mM of ascorbic acid, to an OD_{600} of 0.05/0.5 (for growing/ resting cells experiments, respectively), in sealed flasks. These cells suspensions were then used to examine the effect of the metals Fe^{2+}, Cu^{2+} and Ag^+ (under the forms of $FeSO_4$, $CuSO_4$ and $AgNO_3$, respectively, and at 1 mM concentration) on FB biodegradation. FB concentrations of 1 and 2 mM were used on the growing cells experiments and the resting cells experiments were performed with 2 mM of FB. Control assays were also performed under similar conditions but without supplementation of the target metals. The cultures were incubated at 25ºC on a rotary shaker (130 rpm). Samples were taken at regular intervals to determine cell growth and FB biodegradation. The experiments were repeated in triplicate under sterile conditions.

2.2.3. Analytical methods

2.2.3.1. Fluoride analysis

Biomass was removed from culture samples by centrifugation at 8000 rpm for 10 min. The concentration of fluoride ions in the supernatant was measured with an ion-selective combination electrode (model Orion 96-09, Thermo Electron Corporation, Beverly, MA), which was calibrated with NaF (0.01 to 5 mM) in MM. The ionic strength of the standards and of the samples was adjusted with a buffer solution (total ionic strength adjustment solution – TISAB). The composition of the TISAB solution was: NaCl 1 M, CH_3COOH 0.25 M, $NaCH_3COO$ 0.75 M and sodium citrate 0.002 M.

2.2.3.2. GC analysis

FB was analysed by gas chromatography using a gas chromatograph Varian CP-3800 (Agilent Technologies, California, USA) and a CP-Wasc 52 CP capillary column (Chrompack International B.V., Middelburg, The Netherlands), using a temperature program starting at 50ºC for 2 min, increasing to 150ºC at a rate of 25ºC min^{-1} and reaching the final temperature of 250ºC at a rate of 50ºC min^{-1}. Injector and detector temperatures were 250ºC. Culture samples (4.5 ml) were extracted with 2 ml of diethyl ether, containing mesitylene as internal standard, by vortexing the extraction tube 1 min at maximum speed. The ether layer was analysed by split injection of 1 µl samples. The concentration of FB was calculated using a calibration curve.

2.2.3.3. HPLC-DAD analysis

Biomass was removed from culture samples by centrifugation at 8000 rpm for 10 min and acidified with 0.1M of HCl. The presence of FB metabolites was verified by high-performance liquid chromatography, on a System Gold 126 (Beckman Coulter, Fullerton, USA) using a reversed-phase C18 column (Nucleosil 150 mm x 2.1 mm, 5 µm particle size, Grace, Lokeren, Belgium). Elution was carried out at a flow rate of 0.2 ml min^{-1} with methanol/water containing 1% of trifluoroacetic acid (10:90). The effluent was monitored at 278 nm by the use of a diode array detector (DAD). Samples of 20 µl of supernatant were analysed. The concentrations of catechol and 4-fluorocatechol were calculated using previously established calibration curves.

2.2.3.4. LC-MS analysis

In order to elucidate the structure of the metabolites, supernatant of the culture media was analysed by liquid chromatography – mass spectrometry (LC-MS) analysis. LC analysis was carried out on a Prostar 210 LC pump (Varian, CA, USA). Elution was carried out with methanol/water with 0.1% formic acid (50:50). Solvents were filtered prior to use through an FA 0.22 μm filter (Millipore). The mobile phase was prepared daily, degassed using an in-line degasser (MetaChem) and delivered at a flow rate of 0.3 ml min^{-1} at isocratic mode. Samples were injected on a reversed-phase C18 column (Nucleosil 150 mm x 2.1 mm, 5 μm particle size, Grace, Lokeren, Belgium), used for chromatographic separation using a 20 μl loop.

Mass Spectra Acquisition – A Varian 1200 triple quadrupole mass spectrometer was used with electrospray ionization in positive and negative modes. The operating parameters of the atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) source, including nebulizing gas pressure, drying gas pressure, drying gas temperature, housing temperature, needle voltage and shield voltage, as well as optimal Collision Energy on Q2 for MSMS breakdown were all optimized with regard to maximum signal intensity of pseudomolecular ion by flow injection of 1000 μL samples with 0.1 and 0.5 μl min^{-1} for ESI and APCI sources, respectively.

2.2.4. Chemicals

4-Fluorocatechol was purchased from TCI Europe NV (Antwerp, Belgium). All other chemicals used were analytical-grade (Sigma-Aldrich Chemie, Steinheim, Germany; Merck, Darmstadt, Germany).

2.2.5. Statistical analysis

Data were analysed using one-way analysis of variance (ANOVA; SPSS 17.0, Chicago, IL, USA), and means compared using Duncan's multiple range test ($P < 0.05$).

2.3. RESULTS

2.3.1. Experiments with growing cells

Fluoride and GC analysis were used to follow the effect of the metals Ag^+, Cu^{2+} and Fe^{2+} (at 1 mM concentration) on the biodegradation of FB. Growing cultures of *L. portucalensis* strain F11 were used at two FB concentrations (1 and 2 mM). Control assays consisting of F11 cultures fed with 1 or 2 mM of FB and with no metals addition revealed that in these conditions FB concentration did not have a significant effect on growth rate (Table 2.1). However, the FB degradation rate obtained in these cultures was significantly higher in the cultures fed with 2 mM of FB (Table 2.1). During the time course of the experiment, stoichiometric liberation of fluoride occurred at both concentrations (Figures 2.1a and 2.1b).

When the culture medium was supplemented with $FeSO_4$, F11 cultures exhibited the highest growth rate at both FB concentrations. With the addition of Fe^{2+}, the growth rate was not negatively affected by the increase of FB concentration, as was observed in the control assay, being approximately the same for both FB concentrations (Table 2.1). Also, the addition of Fe^{2+} did not affect FB degradation extent by F11 cells (Figures 2.1c and 2.1d). FB degradation rates obtained were also very similar to the control cultures (Table 2.1). In these conditions, a stoichiometric fluoride release was also observed (Table 2.1).

The addition of $CuSO_4$ to F11 cultures resulted in the inhibition of growth both in the cultures fed with 1 or 2 mM of FB (Figures 2.1e and 2.1f). The presence of Cu^{2+} was found to negatively affect FB degradation rates by 2-3 fold compared to the controls and, the degradation rate obtained with 2 mM of FB was significantly higher than with 1 mM of the compound (Table 2.1). FB degradation extent was reduced by approximately 50% when the cultures were supplemented with $CuSO_4$. Moreover, in these cultures fluoride liberation was only 80% of that expected from stoichiometry (Figures 2.1e and 2.1f).

The presence of $AgNO_3$ in the culture medium was found to greatly affect FB degradation by F11 cultures, with a 65% reduction on the degradation of FB in relation to the control cultures (Figures 2.1g and 2.1h). Also, no fluoride release was observed in those cultures and the lowest FB degradation rate was observed, with a 3.6-3.8 fold reduction compared

to control cultures (Table 2.1). The degradation rate observed at 1 mM of FB was similar to that obtained in the presence of Cu^{2+}, while at 2 mM Ag^+ caused a significantly higher reduction in the degradation rate of the compound (Table 2.1). The effect of this metal on biomass growth was not assessed as it interfered with the measurement of the optical density of the cultures.

The results obtained in the control cultures as well as in the cultures supplemented with the three target metals also showed that FB degradation rates were always significantly higher at 2 mM of FB than at 1 mM of this substrate (Table 2.1). On the contrary, the growth yield, which was determined by calculating the quotient between the biomass dry weight increment and the substrate degraded during the experiment, was significantly higher in the cultures fed with 1 mM of FB than in the cultures fed with 2 mM of this substrate, both in the controls and in the flasks supplemented with $FeSO_4$ (Table 2.1).

Table 2.1. Kinetic parameteres of *L. portucalensis* F11 cultures fed with FB

	Heavy metal			
	Cu^{2+}	Ag^+	Fe^{2+}	Control
Growth rate (h^{-1})[a]				
1 mM FB	N.D.	N.D.	0.120 ± 0.002 [a]	0.044 ± 0.003 [b]
2 mM FB	N.D.	N.D.	0.126 ± 0.006 [a]	0.036 ± 0.005 [b]
		F=375.1 (***)		
Growth yield (g mol^{-1})[a]				
1 mM FB	N.D.	N.D.	85.5 ± 2.36 [a]	82.0 ± 1.96 [a]
2 mM FB	N.D.	N.D.	71.0 ± 1.37 [b]	41.4 ± 6.11 [c]
		F=99.0 (***)		
FB degradation rate ($\mu mol\ L^{-1}\ h^{-1}$)[a]				
Growing cells				
1 mM FB	6.50 ± 0.03 [a]	5.35 ± 0.01 [a]	20.8 ± 0.02 [e]	20.3 ± 0.04 [e]
2 mM FB	16.0 ± 0.05 [b]	8.62 ± 0.06 [d]	30.6 ± 0.02 [g]	31.2 ± 0.03 [g]
Resting cells				
2 mM FB	13.6 ± 0.02 [c]	11.9 ± 0.08 [c]	22.5 ± 0.05 [f]	21.2 ± 0.12 [ef]
		F=463 (***)		
Fluoride released/ FB degraded (%)				
Growing cells				
1 mM FB	80 [b]	0 [a]	100 [c]	100 [c]
2 mM FB	80 [b]	0 [a]	100 [c]	100 [c]
Resting cells				
2 mM FB	45 [e]	20 [d]	76 [f]	76 [f]
		F=2137.5 (***)		

Results are expressed as means ± SD ; N.D. Not Determined

Means with different letters in the same column differed significantly according to Duncan's Multiple Range test at *** P<0.001
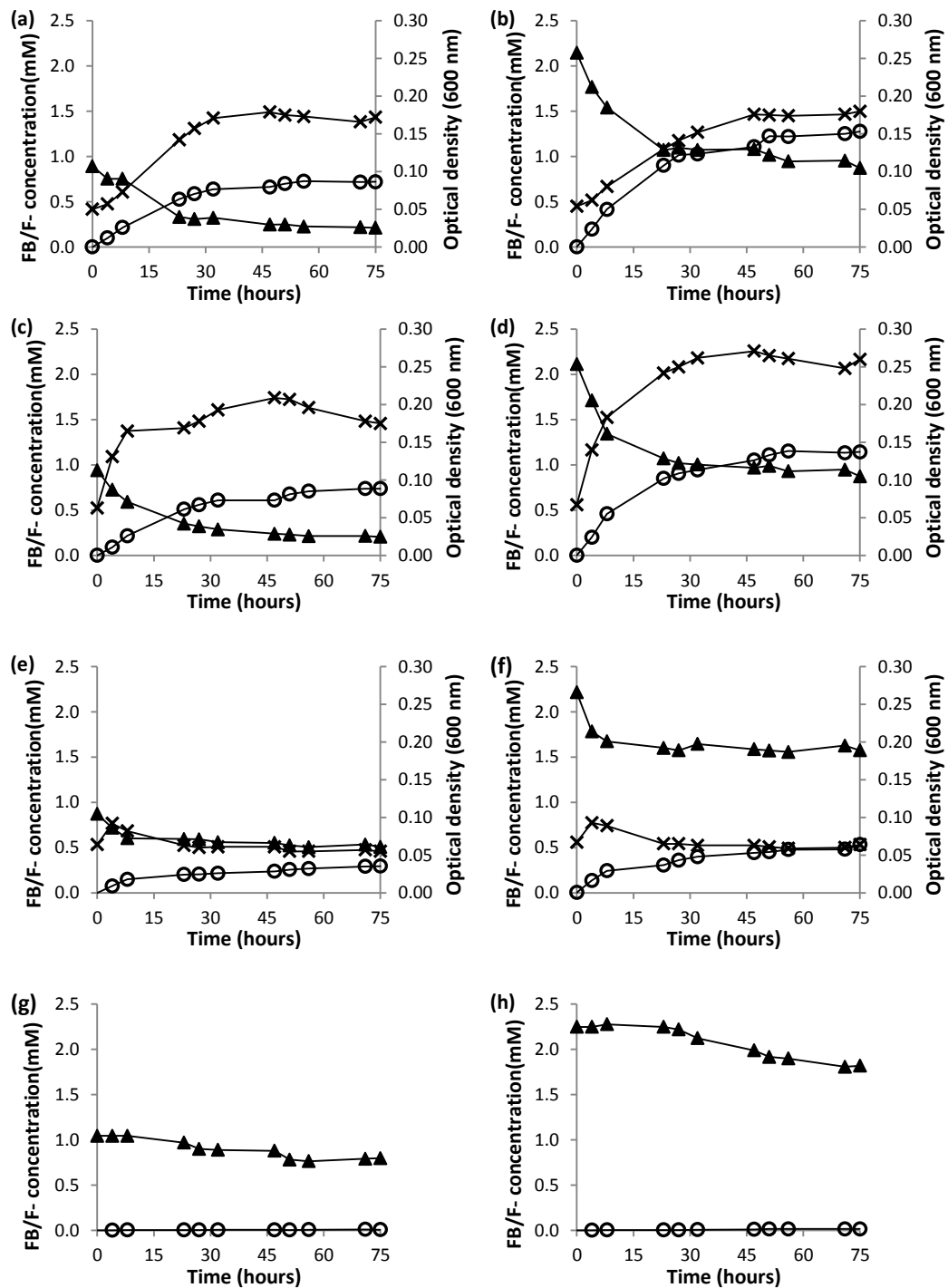
Figure 2.1. Typical FB degradation by growing cultures of *L. portucalensis* strain F11 when supplemented in batch mode with (a) 1 mM of FB, (b) 2 mM of FB, (c) 1 mM of FB and 1 mM of $FeSO_4$, (d) 2 mM of FB and 1 mM of $FeSO_4$, (e) 1mM of FB and 1 mM of $CuSO_4$, (f) 2 mM of FB and 1 mM of $CuSO_4$, (g) 1 mM of FB and 1 mM of $AgNO_3$ and (h) 2 mM of FB and 1 mM of $AgNO_3$. The experiment was performed in triplicate. Optical density (**x**), FB concentration in the culture medium (▲) and fluoride release (**o**) are indicated. It was not possible to evaluate the effect of $AgNO_3$ on biomass growth as it was found to interfere with the measurement of the optical density.

2.3.2. *Experiments with resting cells*

In order to detect the accumulation of possible FB metabolites, experiments with resting cells were conducted. For this, washed cells of strain F11 were resuspended in phosphate buffer, at an initial OD_{600} of 0.5. Microbial growth was not observed under these conditions (data not shown). The FB concentration used in these experiments was 2 mM, as higher degradation rates were obtained in the growing cells experiments at this concentration. The results showed that in the control flasks, where no metals were added, 76% of the fluoride stoichiometrically expected from FB degraded was released (Figure 2.2a). In general, the effect of the target metals on the degradation of FB by resting cells of F11 followed a pattern similar to that observed in the experiments with growing cells. The addition of $FeSO_4$ to resting cells of F11 had no effect on the biodegradation of FB, with approximately the same amount of the supplied FB being degraded and 76% of the fluoride expected from the degraded compound being released (Figure 2.2b, Table 2.1), although FB degradation and fluoride release were still occurring when the experiment was finished at 32 hours (Figures 2.2a and 2.2b). The FB degradation rates were also very similar in the control flasks and in the flasks supplemented with Fe^{2+} (Table 2.1).

In the presence of $CuSO_4$, the FB degraded by F11 cells was decreased by 23% in relation to the control (Figure 2.2c) and only 45% of fluoride was released from the degraded FB (Table 2.1). The supplementation of $AgNO_3$ to resting cells of F11 caused a similar effect, with the results showing a 15% decrease on FB degradation in these cultures (Figure 2.2d) and a release of 20% of the fluoride (Table 2.1). In Ag^+ and Cu^{2+} supplemented cultures, fluoride liberation ceased after 6 hours, with the accumulation of intermediates (Figures 2.2c and 2.2d). Thus, both Cu^{2+} and Ag^+ revealed to partially inhibit FB biodegradation by strain F11, as also evidenced by the much lower substrate degradation rates obtained in the presence of these compounds (Table 2.1). Comparing the results obtained with growing and resting cells of strain F11, it is possible to observe that the impact of these metals in FB degradation was less pronounced in the resting cells cultures, which may be due to the higher cell density of the cultures.

Samples of the culture medium were also analysed by HPLC-DAD to detect possible FB metabolites. Metabolites identification was carried out through comparison of retention times and ultraviolet spectra with those of authentic standards and confirmed by LC-MS. In

the control and in the $FeSO_4$ supplemented experiments, no FB metabolites were observed in the HPLC chromatograms. In the presence of $AgNO_3$ and $CuSO_4$, the HPLC analysis revealed the accumulation of two dead-end metabolites (Figures 2.2c and 2.2d). One metabolite (I) co-eluted with a catechol (Cat) standard when analysed by HPLC and showed the same absorbance spectrum of this standard. The identification of the other metabolite (II) - 4-fluorocatechol (4-FCat) - was achieved with LC-MS MS. The experimental result from LC-MS MS shows that the 4-FCat was easily deprotonated at the electrospray ion source with -60 V under negative mode to form the negative molecular ions $[M-H]^-$ of 127 Da. The collision energy to MS MS breakdown was set to 21 V, originating 124 mz as a product ion. Figure 2.3 shows the chromatograms in the select ion monitor (SIM) mode for the MSMS product ion 124 m/z for a 4-FCat standard and samples of cultures incubated in the presence of Ag^+ and Cu^{2+}. The results show that the concentration of 4-FCat on the analysed samples was above 0.1 mM in both treatments, which is in accordance with the concentration calculated from preliminary HPLC analysis (Figures 2.2c and 2.2d).
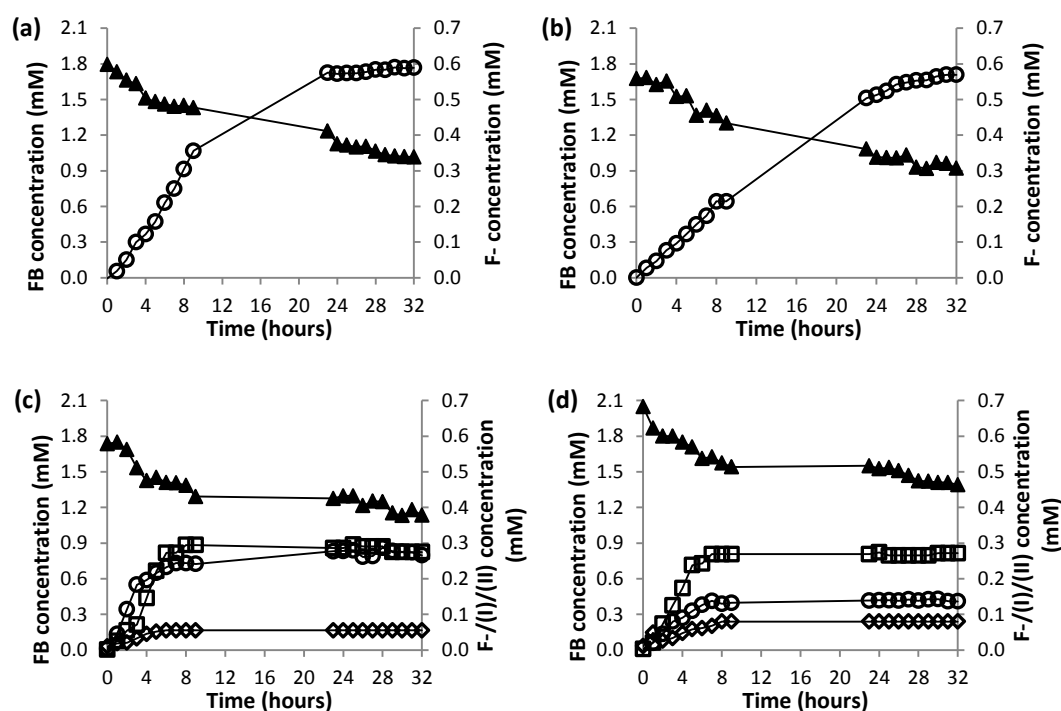
Figure 2.2. Typical FB degradation by *L. portucalensis* strain F11 resting cells when supplemented in batch mode with (a) 2 mM of FB, (b) 2 mM of FB and 1 mM of $FeSO_4$, (c) 2 mM of FB and 1 mM of $CuSO_4$ and (d) 2 mM of FB and 1 mM of $AgNO_3$. The experiment was performed in triplicate. FB (▲) Cat (◊) and 4-FCat (□) concentrations in the culture medium and fluoride release (o) are indicated.
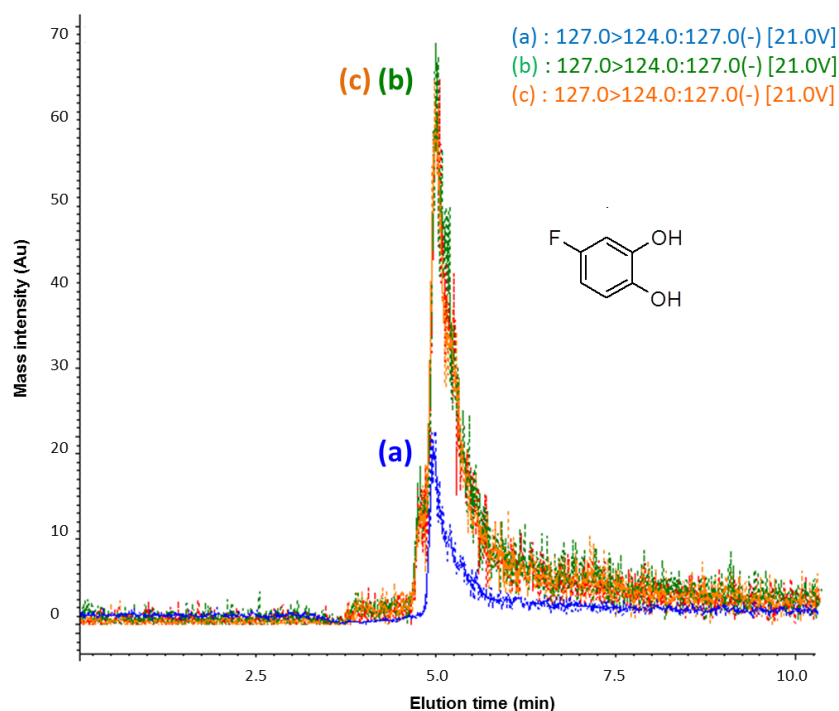
Figure 2.3. LC-MS chromatograms in MSMS mode for a standard of 4-FCat and culture samples obtained after incubation, in batch mode, of resting cells of *L. portucalensis* strain F11 with (a) 2 mM of FB and 1 mM of CuSO$_4$ and (b) 2 mM of FB and 1 mM of AgNO$_3$.

2.4. DISCUSSION

The presence of metals can be stimulatory, inhibitory or even toxic in biochemical reactions depending on the metal concentration and speciation, the state of microbial growth and the biomass concentration (Wang et al., 2010). The aerobic degradation of FB by *L. portucalensis* strain F11 starts with the attack of the aromatic ring performed by a Rieske-type non-heme iron oxygenase - FB dioxygenase - leading to the production of two different fluorinated dihydrodiols that are further metabolised to 4-fluorocatechol and catechol (Carvalho et al., 2006a). The metabolism of these intermediates proceeds by an *ortho*-cleavage pathway and involves the action of the aromatic ring cleavage enzyme - catechol 1,2-dioxygenase. The generated metabolites are then subjected to a few more degrading steps before being channeled into the tricarboxylic acid cycle (Carvalho et al.,

2006a). Both oxygenases families of this metabolic pathway require iron as a cofactor (Gibson and Parales, 2000; Wackett, 2002; Bugg and Winfield, 1998; Bugg and Lin, 2001). Iron is also important as a cofactor in various other proteins, including Krebs cycle enzymes and proteins of the respiratory pathway (Hubbard et al., 1986). It is also known that microorganisms growing under aerobic conditions need iron for a variety of functions, including reduction of oxygen for synthesis of ATP, reduction of ribotide precursors of DNA, formation of heme and for other essential purposes (Neilands, 1995). Thus, the fact that extra supplementation with soluble iron (1 mM) to the culture medium (containing 0.07 mM of Fe^{2+}) or to phosphate buffer (containing no Fe^{2+}) improved microbial growth and had no negative effect on FB biodegradation is not surprising. Other authors have also reported a positive effect of a soluble form of iron on cell growth and biodegradation of aromatic compounds (Aldric et al., 2003; Santos et al., 2008; Husain, 2008; Simarro et al., 2011). In addition, iron-limited conditions were reported to reduce growth yield and toluene degradation by *P. putida* strains mt2 and WCS358 (Dinkla et al., 2001).

An inhibition of FB biodegradation by *L. portucalensis* F11 was observed in this study when the bacterial cultures were supplemented with Cu^{2+} or Ag^{+}. Inhibition of pollutants biodegradation by metals has been reported for several bacterial genera tested under pure culture conditions (Springael et al., 1993; Hong et al., 2007; Lin et al., 2006; Amor et al., 2001). Metals are described to inhibit pollutants biodegradation either by interacting with enzymes directly involved in the biodegradation process (e.g., pollutant-specific oxygenases) or with enzymes involved in the general cellular metabolism (Sandrin and Maier, 2003). In either case, inhibition is mediated by the ionic form of the metal (Angle and Chaney, 1989).

Copper is an essential element for all living organisms (Burgess et al., 1999). However, high concentrations of this metal inhibit cell metabolism. The inhibitory effect of copper on bacterial growth and degradation of organic compounds has been previously reported by other authors. Hong et al. (2007) reported inhibition of growth of *S. wittichii* RW1 and of dibenzofuran degradation by this strain with the addition of 0.1 mM of copper. Similar results were observed by Lin et al. (2006) in the degradation of methyl *tert*-butyl ether. Copper had also toxic and inhibitory effects on sulphate-reducing bacteria at 0.025 mM (Utgikar et al., 2002). Degradation of diesel fuel in a contaminated soil was slower in the

presence of 0.1 and 0.2 mM of copper, and 0.5 mM of this metal caused oxygen consumption below the endogenous respiration of the soil microbial community (Riis et al., 2002). Higher copper concentrations (4.4 and 44 mM) were found to reduce phenanthrene biodegradation and microbial respiration, which was not observed at a copper concentration of 0.4 mM (Sokhn et al., 2001). In the present study the toxic and inhibitory effect of copper was noticed at a concentration of 1mM. According to Said and Lewis (1991), copper may affect organic pollutants biodegradation due to its accumulation in the microbial cells. An interesting aspect in copper toxicity is that this transition metal negatively affects Fe–S enzymes (Grass et al., 2011), such as ring-hydroxylating dioxygenases and intradiol cleavage dioxygenases, which are enzymes involved in the metabolic pathway of FB (Carvalho et al., 2006a).

To the best of our knowledge, there are no studies in the literature reporting the influence of silver on the biodegradation of xenobiotic compounds. However, silver is referenced in the EPA's priority list of pollutants and it is possible that co-contamination of this metal with organic xenobiotics is occurring. Studies reporting the inhibitory and antimicrobial properties of this metal can be found in the literature (Durán et al., 2010; Yamanaka et al., 2005; Silver, 2003). Silver affects the function of membrane-bound enzymes, such as those in the respiratory chain, by binding to thiol groups (Flemming et al., 1990; Bragg and Rainnie, 1974). This metal also interacts with DNA, resulting in a marked enhancement of pyrimidine dimerization by photodynamic reaction and in the possible prevention of DNA replication (Fox and Modak, 1974).

The accumulation of catechol and 4-fluorocatechol observed in resting cells cultures of strain F11 in the presence of copper and silver (which was not observed nor in the control or in the presence of iron) indicates inhibition of the key enzyme of FB metabolism – catechol 1,2-dioxygenase. Activity assays with purified catechol 1,2-dioxygenase have previously shown that this enzyme is significantly inhibited by these metals (Wang et al., 2001; Wang et al., 2006; Patel et al., 1976). In these experiments, as well as in the present study, the inhibition was found to be more prominent with silver than with copper, whereas iron showed less or none inhibition. This higher toxic effect of silver can be explained by the fact that metal cations with high atomic numbers, like Ag^+, tend to bind to

enzyme SH groups, inhibiting the activity of enzymes, as intradiol dioxygenases (Nies, 1999).

In summary, the results obtained in this study indicate that the metals Ag^+ and Cu^{2+} affect FB degradation through inhibition of the central FB metabolism enzyme – catechol 1,2-dioxygenase, with the accumulation of catechol and 4-fluorocatechol, emphasizing the need to consider the interference of metallic species when devising treatment strategies for organic contaminated wastes. In the case of water streams or wastewater co-contaminated with metals and organic contaminants, a pre-removal of the metal contaminants could be necessary to allow biodegradation of the organic fraction by sensitive strains, such as strain F11. Several techniques for metals removal are available (Fu and Wang, 2011).

2.5. CONCLUSIONS

This study focused on the investigation of the effect of three metals, Fe^{2+}, Ag^+ and Cu^{2+}, on the biodegradation of FB by the bacterial strain *L. portucalensis* F11. The main conclusions drawn from this work are:

•   The addition of $FeSO_4$ to F11 cultures showed no effect on FB biodegradation and had a positive effect on bacterial growth;

• $AgNO_3$ and $CuSO_4$ were found to inhibit FB biodegradation both by growing and resting cells of strain F11;

•   Fluoride liberation was not stoichiometric to the FB degraded when the F11 cultures were supplemented with $AgNO_3$ or $CuSO_4$;

•   HPLC/LC-MS analysis revealed the accumulation of catechol and 4-fluorocatechol during FB biodegradation in the presence of $AgNO_3$ or $CuSO_4$, suggesting that these metals inhibit the key enzyme of FB metabolism – catechol 1,2-dioxygenase.

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CHAPTER 3

Co-metabolic degradation of chlorobenzene by the fluorobenzene degrading wild strain *Labrys portucalensis* F11

Abstract

Due to the widespread use of chlorobenzene (CB), environmental contamination with this compound is a major concern. A previously isolated bacterium named Labrys portucalensis (strain F11), that is able to use fluorobenzene (FB) as sole carbon and energy source, was tested for its capability to degrade CB. Strain F11 was able to partially degrade CB only when F11 cells were previously grown in FB. Biodegradation of 0.5 mM of CB was achieved at a rate of 7.95 ± 0.39 µmol l^{-1} day^{-1} with concomitant stoichiometric release of 50% of the chloride, while degradation of 1 mM of this compound resulted in 85% degradation at a rate of 16.9 ± 0.81 µmol l^{-1} day^{-1} and with a 15% chloride release on the basis of the amount of compound biodegraded. Total CB biodegradation and dechlorination was only achieved when FB was also supplied to F11 cultures, suggesting cometabolic transformation. Total degradation of 0.5 mM of CB and 0.5 mM of FB occurred simultaneously at degradation rates of 105 ± 6.07 µmol l^{-1} day^{-1} and 126 ± 16.2 µmol l^{-1} day^{-1} respectively, with stoichiometric halogen release. Growth yield was lower when both substrates were present, suggesting synergistic inhibition. To our knowledge, this is the first time that co-metabolic biodegradation of CB in the presence of the fluorinated analogue is reported.

3.1. INTRODUCTION

Vast amounts of halogenated aromatic compounds have been deliberately or accidentally released into the biosphere due to their extensive use in agricultural and industrial processes, since halogenation of the aromatic nucleus generally leads to increased stability, thus making these compounds very attractive, however, this high stability often results in a higher resistance to chemical and microbial degradation (Häggblom et al., 2006). Among haloaromatic compounds, chlorinated and fluorinated benzenes are of great concern, due to their persistence, toxicity and bioaccumulation potential (Neilson, 1995; Parsek et al., 1995).

Chlorobenzene (CB) is widely used as solvent, degreaser, odorize and intermediate in the synthesis of various pesticides and dyes (Yadav et al., 1995). It has been identified as a priority pollutant by the U.S. Environmental Protection Agency web page (US EPA, 2012). Acute inhalation exposure of animals to CB produces narcosis, restlessness, tremors and muscle spasms and chronic (long-term) exposure of humans to this compound affects the central nervous system. Signs of neurotoxicity in humans include numbness, cyanosis, hyperesthesia (increased sensation) and muscle spasms – U.S. Environmental Protection Agency web page (US EPA, 2000). Studies on chlorobenzene (CB) biodegradation revealed that this compound is generally metabolized through a modified *ortho*-cleavage pathway (Reineke and Knackmuss, 1984; Mars et al., 1999; Sommer and Gorisch, 1997; van der Meer et al., 1992), although the *meta*-cleavage pathway has also been reported in some cases (Mars et al., 1997; Mars et al., 1999). In the *ortho*-cleavage pathway, the aromatic ring of the CB molecule is attacked by a dioxygenase enzyme, producing *cis*-chlorobenzene dihydrodiol which after rearomatization by a dehydrogenase, results in chlorocatechol that undergoes intradiol ring cleavage, catalyzed by catechol 1,2-dioxygenase, with concomitant elimination of chloride.

In the environment, the occurrence of contaminants in mixtures is an important issue because the removal or degradation of one compound can be strongly impacted by the presence of others. Although several studies describing the degradation of substituted benzenes by single bacteria can be found in the literature, including chlorobenzenes

(Monferrán et al., 2005; Adebusoye et al., 2007; Guerin, 2008; Zhang et al., 2011) and fluorobenzenes (Carvalho et al., 2006; Moreira et al., 2012), less is known about the microbial degradation of mixtures of those organic compounds. Some insights into this issue have been gained through previous studies on, for example, substrate interactions of BTX compounds (Alvarez and Vogel, 1991), biodegradation of mixtures of substituted aromatic substrates (Pettigrew et al., 1991; Haigler et al., 1992; Reardon et al., 2000; Stoilova et al., 2007), cometabolism (Dean-Ross et al., 2002; Jechorek et al., 2003; Baggi et al., 2008; Wen et al., 2011) and preexposure to other aromatic hydrocarbons (Goswami et al., 2002). It is also important to stress that in a scenario of contaminants mixture negative interactions may occur, like competitive inhibition (Bielefeldt and Stensel, 1999), toxicity (Haigler et al., 1992), co-metabolic cofactor dependency (Sáez and Rittmann, 1993) and the formation of toxic intermediates by nonspecific enzymes (Klečka and Gibson, 1981; Bartels et al., 1984; Munõz et al., 2007).

To our knowledge, the simultaneous biodegradation of chloro- and fluorobenzene has not been yet reported. Most of the strains reported in the literature with the ability to degrade chlorobenzenes failed to degrade fluorobenzenes (Reineke and Knackmuss, 1984; Sander et al., 1991; Zaitsev et al., 1995). The present study describes the degradation of CB by a FB degrading bacterium – *Labrys portucalensis* strain F11, supplied as a sole carbon source and in mixture with FB.

3.2. MATERIAL AND METHODS

3.2.1. Cultivation conditions

L. portucalensis strain F11 (Carvalho et al., 2005) was grown in sealed flasks containing a sterile minimal salts medium (MM) (composition per litre: $Na_2HPO_4.2H_2O$, 2.7 g; KH_2PO_4, 1.4 g; $(NH_4)_2SO_4$, 0.5 g; $MgSO_4.7H_2O$, 0.1 g; and, 10 ml trace elements solution of the following final composition per litre: $Na_2EDTA.2H_2O$, 12.0 g; NaOH, 2.0 g; $MnSO_4.4H_2O$, 0.4 g; $ZnSO_4.7H_2O$, 0.4 g; H_2SO_4, 0.5 ml; Na_2SO_4, 10.0 g; $Na_2MoO_4.2H_2O$, 0.1 g; $FeSO_4.7H_2O$, 2.0 g; $CuSO_4.5H_2O$, 0.1 g; $CaCl_2$ 1.0 g). FB (1 mM) was supplied as sole carbon and energy source. Cultures were incubated on a rotary shaker (130 rpm) at 25ºC. Growth was monitored by

measuring the optical density at 600 nm (OD_{600}) using a spectrophotometer (Helios Gamma, Unicam Instruments, UK).

3.2.2. Biodegradation experiments

Degradation of CB was tested as a single substrate, at different concentrations, and in a mixture with FB. Experiments were conducted both with cells pre-grown in FB and with non-induced cells obtained by growing *L. portucalensis* strain F11 on Nutrient Agar (NA) plates. Induced cells of *L. portucalensis* strain F11 were obtained after pre-growing the microorganism in MM supplemented with 1 mM of FB. Cultures were then harvested by centrifugation (10 000 rpm for 15 min at 4 °C), washed and resuspended in MM. 500 ml sealed flasks containing MM supplemented with CB or/and FB were inoculated with the prepared suspension to an OD_{600} of ca. 0.05, in a working volume of 200 ml. The cultures were incubated at 25 °C on a rotary shaker (130 rpm). All the experiments were repeated twice and performed in triplicate under sterile conditions. Control assays consisted of cells suspensions, with the same OD and in the same medium with FB. Controls without inoculum addition were also included. Samples were taken at regular intervals to assess growth and degradation of CB and/or FB.

3.2.3. Analytical methods

3.2.3.1. Fluoride analysis

For fluoride analysis, biomass was previously removed from culture samples by centrifugation at 8000 rpm for 10 min. The concentration of fluoride ions in the samples supernatant was measured with an ion-selective combination electrode (model Orion 96-09, Thermo Electron Corporation, Beverly, MA), which was calibrated with NaF (0.01– to 5 mM) in MM. The ionic strength of the standards and of the samples was adjusted with a buffer solution (total ionic strength adjustment solution – TISAB). The composition of the TISAB solution was: NaCl 1 M, CH_3COOH 0.25 M, $NaCH_3COO$ 0.75 M and sodium citrate 0.002 M.

3.2.3.2. Chloride analysis

For chloride analysis, biomass was previously removed by centrifugation at 8000 rpm for 10 min. The chloride concentration in the samples supernatant was determined using the colorimetric method described by Iwasaki et al. (1956).

3.2.3.3. Halobenzenes analysis

CB and FB were analyzed by gas chromatography using a CP-Wasc 52 CP capillary column (Chrompack International B.V., Middelburg, The Netherlands), under a temperature regimen starting at 50ºC for 2 min, increasing to 150ºC at a rate of 25ºC min^{-1} and reaching the final temperature of 250ºC at a rate of 50ºC min^{-1}. Injector and detector temperatures were 250ºC. Culture samples (4.5 ml) were extracted with 2 ml of diethyl ether containing mesitylene as internal standard by vortexing the extraction tube 1 min at maximum speed. The ether layer was analyzed by split injection of 1 µl samples. The concentrations of halobenzenes were calculated using previously established calibration curves.

3.2.4. Chemicals

All chemicals were analytical-grade (Sigma–Aldrich Chemie, Steinheim, Germany; Merck, Darmstadt, Germany).

3.2.5. Statistical analysis

Data were analysed using one-way analysis of variance (ANOVA; SPSS 17.0, Chicago, IL, USA), and means compared using Duncan's multiple range test ($P < 0.05$).

3.3. RESULTS AND DISCUSSION

3.3.1. Biodegradation of CB by non-induced cells

To investigate if non-induced cells of *L. portucalensis* strain F11 were able to metabolize CB when supplied alone or if they can only co-metabolize it when supplied together with FB, cells previously grown on NA plates were inoculated into 200 ml of MM supplemented with 0.5 mM of CB or with 0.5 mM of each substrate. Cultures supplied with 0.5 mM of FB were used as control. As shown in Figure 3.1a, control cultures completely defluorinated FB during a 7 days period. The results presented in Figure 3.1b show that when CB was supplied to F11 cultures as the sole carbon and energy source, the compound was not degraded. However, when FB was added together with CB to the culture medium, complete depletion of both substrates was observed in 10 days, with concomitant halogen release (Figure 3.1c). The FB and CB depletion rates observed in these cultures were very similar and although lower than the value obtained in the control cultures supplemented only with FB they were not statistically different (Table 3.1). The growth rate obtained in the cultures supplemented with CB and FB was the same as that observed in the control experiments, however, the cultures with the mixture of the two substrates had a lag phase of ca. 4 days, while in the control cultures no lag phase was observed. Growth yield, calculated as the dry weight increment and on the basis of total substrate utilized was approximately 54% of that obtained in the control cultures (Table 3.1). These results suggest that CB was co-metabolized in the presence of FB but the mixture of both substrates had an inhibitory effect on the growth yield of strain F11. The positive effect observed in CB degradation by strain F11 when the medium was supplemented with FB is characteristic of a co-metabolic process and corroborates the importance that the presence of an inducible substrate may have on the biodegradation of other haloaromatic compounds. In the non-inoculated control assays, both removal of CB/FB and chloride/fluoride release were not observed (data not shown), indicating that substrate losses due to evaporation or chemical decomposition did not occur under the tested conditions.

(a)

(b)

(c)
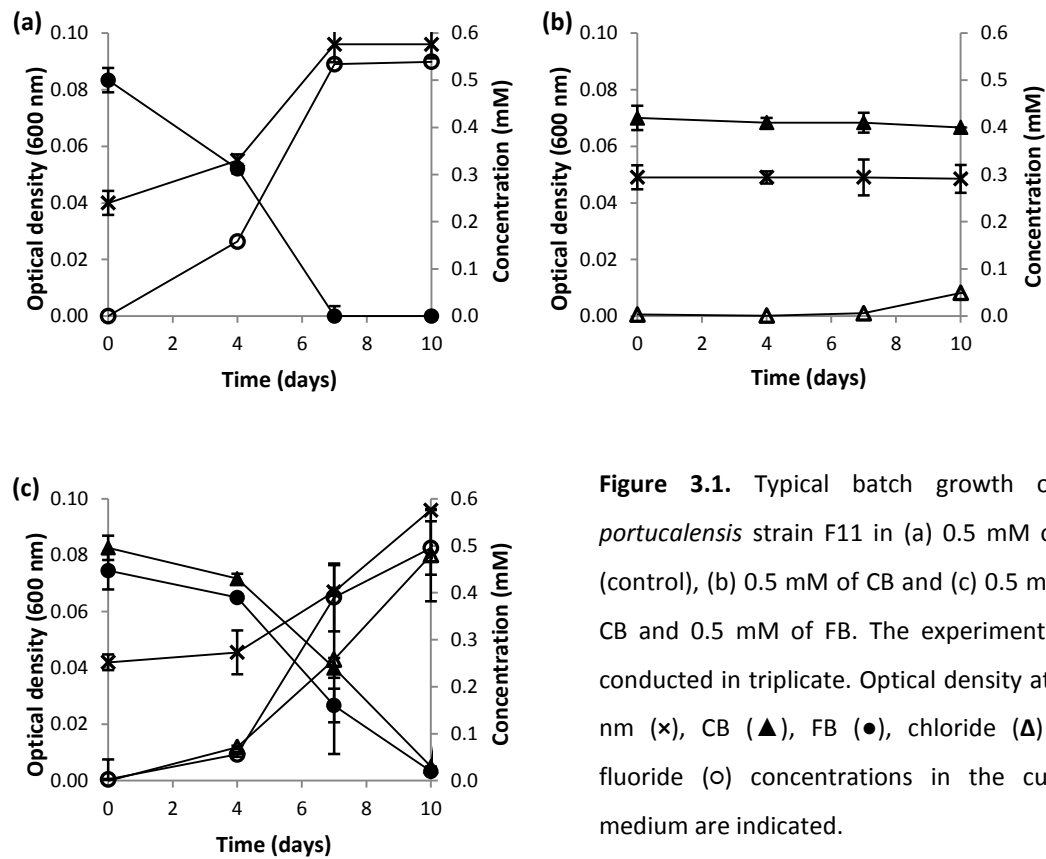
Figure 3.1. Typical batch growth of *L. portucalensis* strain F11 in (a) 0.5 mM of FB (control), (b) 0.5 mM of CB and (c) 0.5 mM of CB and 0.5 mM of FB. The experiment was conducted in triplicate. Optical density at 600 nm (×), CB (▲), FB (●), chloride (Δ) and fluoride (○) concentrations in the culture medium are indicated.

Table 3.1. Selected growth and substrate degradation parameters obtained for *L. portucalensis* strain F11 cultures in CB and /or FB, by non-induced or induced cells

Substrate supplied	CB degradation rate (μmol l^{-1} day^{-1})	FB degradation rate (μmol l^{-1} day^{-1})	Growth rate (day^{-1})	Growth yield (g mol^{-1})
Non-induced cells				
0.5 mM FB	N.A	73.8 ± 1.34 [b]	0.12 ± 0.04 [a]	48.3 ± 1.84 [bc]
0.5 mM CB + 0.5 mM FB	50.2 ± 0.65 [c]	51.2 ± 0.32 [b]	0.12 ± 0.03 [a]	25.9 ± 1.36 [ab]
Induced Cells				
0.5 mM CB	7.95 ± 0.39 [a]	N.A.	0.10 ± 0.06 [a]	51.4 ± 1.65 [c]
1 mM CB	16.9 ± 0.81 [b]	N.A.	0.13 ± 0.03 [a]	55.6 ± 1.75 [c]
0.5 mM FB	N.A.	459 ± 21.0 [d]	0.67 ± 0.01 [d]	49.7 ± 1.32 [bc]
1 mM FB	N.A.	301 ± 32.7 [a]	0.59 ± 0.01 [c]	55.6 ± 6.23 [c]
0.5 mM CB + 0.5 mM FB	105 ± 6.07 [d]	126 ± 16.2 [c]	0.25 ± 0.01 [b]	22.6 ± 5.13 [a]
	F=616.8 (***)	F=656.5 (***)	F=280.8 (***)	F=3.231 (*)

Results are expressed as means ± SD; N.A. Not Applicable

Means with different letters in the same column differed significantly according to Duncan's Multiple Range test at P < 0.05; * P<0.05; *** P<0.001

3.3.2. Biodegradation of CB by induced cells

To investigate if *L. portucalensis* strain F11 was able to use CB as a sole carbon and energy source when cells were previously induced for haloaromatics degradation, cells grown on FB were inoculated into 200 ml of MM supplemented with 0.5 or 1 mM of CB. Cultures supplied with 1 mM of FB were used as a control. The results obtained in the control flasks revealed that fluoride was completely released in 3 days and the cultures did not show a lag phase period (Figure 3.2a). Figure 3.2b shows that *L. portucalensis* F11 was able to biodegrade 0.5 mM of CB in 50 days, but only ca. half of the expected chloride was released. It is interesting to note that chloride was only released when ca. 70% of the supplied CB was already consumed by the cells (Figure 3.2b). Concerning the degradation of 1 mM of CB, the results showed that ca. 85% of the supplied compound was consumed during the time course of the experiment, but only 15% of it was dechlorinated (Figure 3.2c). This incomplete CB consumption may be probably related to a decrease in the levels of the catabolic oxygenases due to the absence of appropriate enzyme-inducing growth substrates (FB) (Alvarez-Cohen and Speitel, 2001). The CB concentration was found to affect the degradation rate, which increased with the increasing of substrate concentration, being much lower in the cultures fed with CB than in the ones fed with FB (Table 3.1). However, when the CB concentration supplied to the F11 cultures increased from 0.5 mM to 1 mM, chloride release decreased to approximately half of the value observed in the cultures fed with 0.5 mM of CB.

During approximately the first 4 days, a decrease in the optical density was observed both in the cultures fed with 0.5 or 1 mM of CB, after which the biomass started to increase (Figures 3.2b and 3.2c). In these cultures the growth rates obtained were similar but were much lower than those observed in the control experiments. Concerning growth yields, calculated as a dry weight increment and on the basis of the substrate utilized, the obtained values were similar both in the cultures supplemented with 0.5 and 1 mM of CB and were not statistically different from the values obtained in the controls (Table 3.1). It is interesting to note that the growth pattern observed in the cultures fed with CB followed the trend of chloride release, suggesting that the obtained growth was supported by the percentage of CB that was degraded (Figure 3.2b and 3.2c). This result indicates that when the F11 enzymatic system is pre-induced for FB degradation, the cells are able to partially

biodegrade CB and, in these conditions, this substrate seems to be capable to support partial growth. In the control flasks without inoculum addition, both removal of CB/FB and chloride/fluoride release were not observed (data not shown).
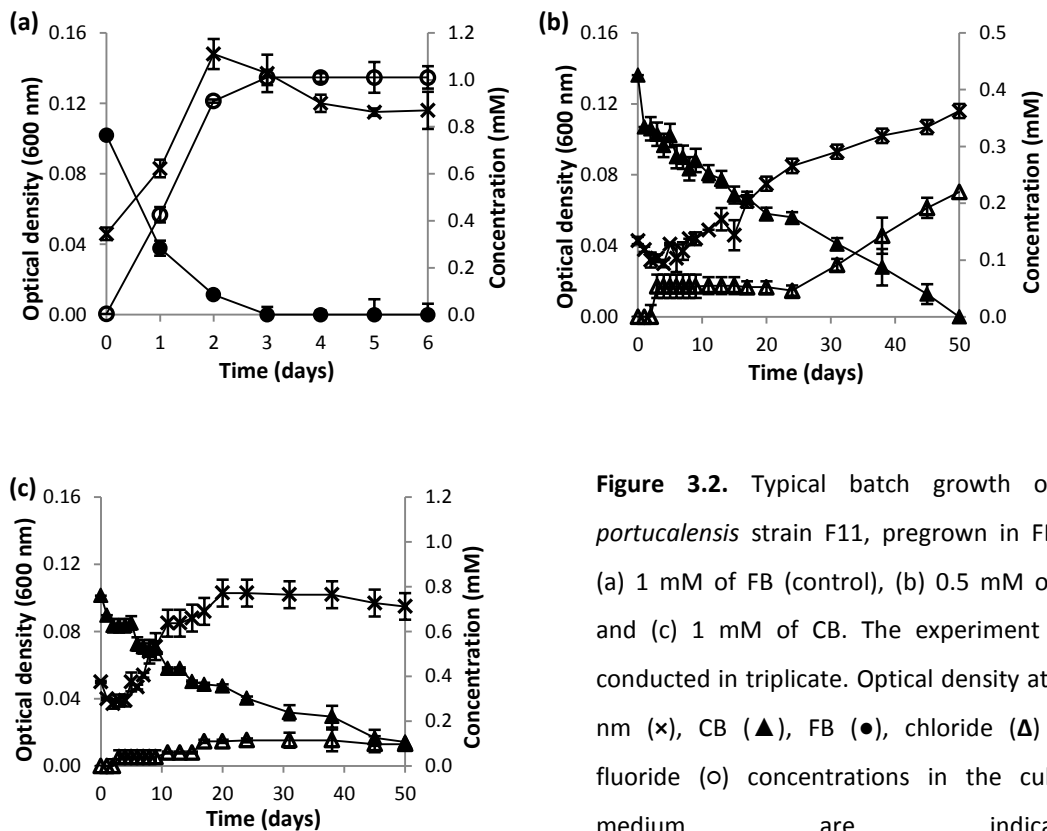
Figure 3.2. Typical batch growth of *L. portucalensis* strain F11, pregrown in FB, in (a) 1 mM of FB (control), (b) 0.5 mM of CB and (c) 1 mM of CB. The experiment was conducted in triplicate. Optical density at 600 nm (×), CB (▲), FB (●), chloride (Δ) and fluoride (○) concentrations in the culture medium are indicated.

3.3.3. Biodegradation of CB in the presence of FB by induced cells

To test if the addition of FB to the culture medium could increase the biodegradation of CB by induced cultures of strain F11, these were fed simultaneously with 0.5 mM of FB and 0.5 mM of CB. Cultures supplied with 0.5 mM of FB were used as control. The results obtained in the control cultures revealed that complete fluoride release was obtained in 1 day and during this period biomass doubled (Figure 3.3a). In the cultures supplemented with both substrates, total consumption of CB and FB was simultaneously achieved in 4 days, with

concomitant halogen release. No lag phase was observed in these cultures (Figure 3.3b). The degradation rate obtained for FB was slightly higher than that obtained for CB, being in both cases much lower than that observed in the control experiments. The lower degradation rates obtained in the cultures supplemented with both substrates compared to the cultures fed only with FB, a result also observed with the non-induced cells, is suggestive of a competitive inhibition mechanism between the primary (in this case FB) and the co-metabolic substrate (CB) (Alvarez-Cohen and Speitel, 2001).
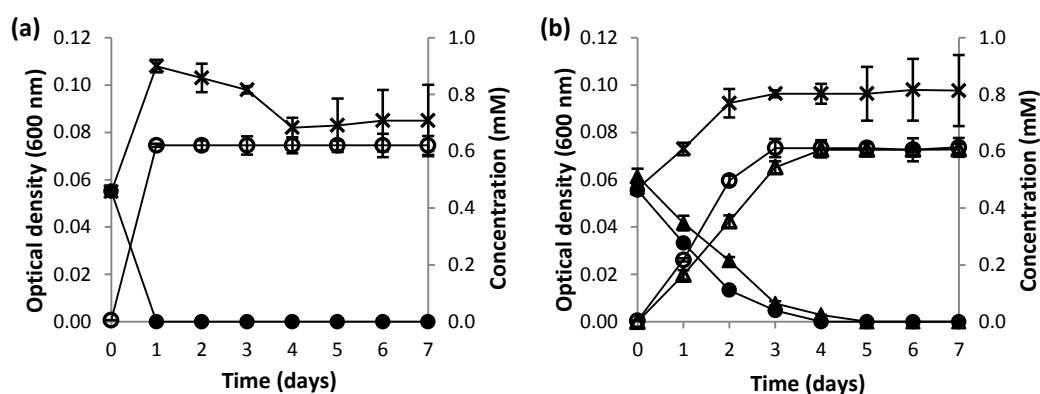
Figure 3.3. Typical batch growth of *L. portucalensis* strain F11, pregrown in FB, in (a) 0.5 mM of FB (control) and (b) 0.5 mM of CB and 0.5 mM of FB. The experiment was conducted in triplicate. Optical density (×), CB (▲), FB (●), chloride (Δ) and fluoride (○) concentrations in the culture medium are indicated.

Growth rate and growth yield were found to be 50% lower than those obtained in the control cultures (Table 3.1). These parameters followed a pattern similar to those obtained with non-induced cells and clearly suggest a growth inhibitory effect due to the simultaneous presence of CB and FB, which was not observed when the CB was supplied alone to the induced cells (Table 3.1). The obtained results suggest an inhibition mechanism similar to the one described by Smith et al. (1991) as synergistic inhibition. In that study, the authors reported that *Pseudomonas* sp. NCIB 10643 was not able to grow on a mixture of ethylbenzene and biphenyl, despite both compounds serving as growth substrates when

supplied separately and being catabolized by converting pathways. The inhibitory effect was also not observed when the substrates were added sequentially.

It is worthy to notice that when FB was present in the culture medium, CB degradation was much faster than when it was present alone, and a stoichiometric chloride release was obtained. The higher rate and extent of CB degradation observed in the presence of FB may be explained by a better induction of the suitable catabolic enzymes and by a more efficient regeneration of the NADH necessary for the activity of the dioxygenase responsible for the initial attack of these compounds, in accordance to what was reported by Alvarez-Cohen and Speitel (2001). In the control flasks without inoculum addition, both removal of CB/FB and chloride/fluoride release were not observed (data not shown).

It has been observed that prior substrate acclimation significantly affects biodegradation pattern. Fluorene induced cells of *Pseudomonas cepacia* F297 were capable to transform a wide variety of other polycyclic aromatic compounds (Grifoll et al., 1995). Goswami et al. (2002) reported an increase in the biodegradation rate of chlorophenol by benzoate induced cells. The results obtained in our study are in agreement with these observations as the experiments using cells previously grown on FB showed that when the cells were induced for FB degradation they were able to metabolize another aromatic compound with a similar structure (CB), despite the difference in the halogen atom. However, CB degradation was much slower than FB degradation, as evidenced by the much lower degradation rates observed in the cultures fed with CB compared to the cultures fed with FB (Table 3.1), and complete dehalogenation of the chloroaromatic compound was not achieved during the time course of the experiment (Figure 3.2).

L. portucalensis strain F11 has the capacity to degrade FB as sole carbon and energy source using an *ortho*-cleavage pathway, with 4-fluorocathecol and catechol as central metabolites (Carvalho et al., 2006a). The fact that the F11 cells pregrown in FB were stimulated to metabolize CB suggests that strain F11, while metabolizing FB through the *ortho*-cleavage pathway, is able to induce the pathway for CB degradation. It is interesting to notice that the *ortho*-cleavage is a typical route reported for the microbial degradation of CB (Reineke and Knackmuss, 1984; Jiang et al., 2009).

The total dechlorination of CB observed in the presence of FB may be considered as a co-metabolic mechanism. Our results illustrate a scenario of simultaneous substrate biodegradation, in which the presence of a growth substrate (FB) in the culture medium allows the microorganism to degrade a second compound (CB), by providing to the cells an energy source and the appropriate co-metabolic enzymes (Alvarez-Cohen and Speitel, 2001).

Several studies on CB biodegradation can be found in the literature (Reineke and Knackmuss, 1984; Nishino et al., 1992; Field and Sierra-Alvarez, 2008; Zhang et al., 2011) and strains enriched in this compound have been shown to be able to degrade several related compounds, except fluorinated ones (Zaitsev et al., 1995; Freitas dos Santos et al., 1999). This microbial inability to degrade fluorinated compounds may be attributed to the characteristics of the C–F bond. Fluorine is extremely electronegative which confers a strong polarity to the C–F bond. This bond has also one of the highest bond energies in nature (Key et al., 1997), strongly contributing to the high stability of the fluorinated molecules. The recalcitrance of halogenated compounds usually increases in the following order C–I < C–Br < C–Cl < C–F (Freitas dos Santos et al., 1999).

The bacterial strain used in this study was obtained through enrichments with a fluorinated compound – FB – used as a sole carbon and energy source (Carvalho et al., 2005) and is able to also use difluorobenzenes (Moreira et al., 2012). According to Cao et al. (2009), microorganisms capable to degrade more recalcitrant compounds can be more versatile in the degradation of less recalcitrant compounds. In this context, it is also important to evaluate the specificity of the catabolic enzymes and the possibility of formation of toxic intermediates during the biodegradation process. The results obtained in this study suggest that the catabolic enzymes involved in FB degradation are also able to degrade CB, as CB was completely dehalogenated when FB was present in the culture medium.

It is important to notice that *L. portucalensis* F11 is only able to consume CB if FB is present in the culture medium or if the F11 cells are previously grown in FB. Possibly, FB is a better inducer of the suitable metabolic enzymes than CB. For an effective aerobic cometabolism, the dioxygenase that initiates the degradation must be present in abundance. The induction of oxygenase synthesis responds to the presence of an inducer, which is typically the substrate. When the inducer is present, the transcription of the structural genes coding

for the oxygenase is up-regulated resulting in a significant increase in the synthesis of the oxygenase and/or of other enzymes involved in the metabolic pathway (Arp et al., 2001).

Pollutants are usually released into the environment as mixtures and, thus, studies investigating the biodegradation of such mixtures are important for evaluating their fate. The ability of several bacteria to degrade simple mixtures of compounds has been reported in the literature. Haigler et al. (1992) investigated the biodegradation of mixtures of substituted benzenes and found that some compounds could only be metabolized under these conditions, not serving as a growth substrate when provided alone. Alvarez and Vogel (1991) reported an enhanced degradation of benzene and *p*-xylene caused by the presence of toluene in *Pseudomonas* sp. strain CFS-215 cultures, as well as a benzene-dependent degradation of toluene and *p*-xylene by *Arthrobacter* sp. strain HCB. These findings demonstrate that isolated strains can degrade mixtures of compounds provided that a suitable inducing substrate, that can activate the appropriate metabolic enzymes, is present.

3.4. CONCLUSIONS

L. portucalensis strain F11 is, to our knowledge, the first microorganism described in the literature that is capable to simultaneously biodegrade CB and FB. The strain cannot use CB as a sole carbon and energy source, but when pre-grown on FB it is able to partially degrade the chlorinated compound. In the presence of FB in the culture medium CB is completely degraded, with stoichiometric chloride release, through a co-metabolic process.

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CHAPTER 4

DEGRADATION OF DIFLUOROBENZENES BY THE WILD STRAIN *LABRYS PORTUCALENSIS* F11

Abstract

This study focuses on the biodegradation of difluorobenzenes (DFBs), compounds commonly used as intermediates in the industrial synthesis of various pharmaceutical and agricultural chemicals. A previously isolated microbial strain (strain F11), identified as Labrys portucalensis, able to degrade fluorobenzene (FB) as sole carbon and energy source, was tested for its capability to degrade 1,2-, 1,3- and 1,4-DFB in batch cultures. Strain F11 could use 1,3-DFB as a sole carbon and energy source, with quantitative release of fluoride, but 1,4-DFB was only degraded and defluorinated when FB was supplied simultaneously. Growth of strain F11 with 0.5 mM of 1,3-DFB led to stoichiometric release of fluoride ion. The same result was obtained in cultures fed with 1 mM of 1,3-DFB or 0.5 mM of 1,4-DFB, in the presence of 1 mM of FB. No growth occurred with 1,2-DFB as substrate, and degradation of FB was inhibited when supplied simultaneously with 1,2-DFB. To our knowledge, this is the first time biodegradation of 1,3-DFB as a sole carbon and energy source, and cometabolic degradation of 1,4-DFB, by a single bacterium, is reported.

4.1. INTRODUCTION

Fluorinated aromatic compounds are industrially produced in increasing amounts for a variety of applications in medicine, agriculture and electronics. These compounds constitute a special class of halogenated compounds due to the unique chemical and physical properties of the fluorine atom. The high electronegativity of fluorine confers a strong polarity to the carbon–fluorine bond. In addition, this bond has also one of the highest energies in nature, which contributes to the high stability (thermal and oxidative) and resistance to hydrolysis, photolysis and microbial degradation of the fluorinated compounds. Due to their vast applications, improper waste disposal and discharge, these compounds have become ubiquitous environmental pollutants. And, their persistence has led to their accumulation in the environment. Thus, information on the biodegradation of these compounds is of great interest (Chaojie et al., 2007; Frank et al., 1996; Key et al., 1997; McCulloch, 2003; Moody and Field, 2000).

Difluorobenzenes constitute a group of fluoroaromatics that are commonly employed as chemical intermediates. For example, 1,3-difluorobenzene (1,3-DFB) is used for the preparation of an anti-inflammatory agent and of an insecticide, whereas 1,2-difluorobenzene (1,2-DFB) and 1,4-difluorobenzene (1,4-DFB) are intermediates in the production of compounds which exhibit sedative and/or anticonvulsant activity (Nalelwajek and van der Puy, 1989).

The biodegradation of a vast range of halogenated aromatic compounds, especially chlorinated compounds, has been described (Haggblom, 1992; Janssen et al., 1994; Adebusoye et al., 2007; Field and Sierra-Alvarez, 2008) but less information is available on the microbial metabolism of fluorinated aromatic compounds. Examples of fluoroaromatic compounds of which biodegradation have been most investigated include fluorobenzoic acids (Boersma et al., 2004; Engesser et al., 1980; Oltmanns et al., 1989; Schlomann et al., 1990; Milne et al., 1968) and fluorophenols (Ferreira et al., 2008; Chaojie et al., 2007; Boersma et al., 1998; Bondar et al., 1998). The biodegradability of these compounds is influenced by the number and position of the fluorine substituents. Among fluoroaromatics, the DFBs have received less attention and little is known about their

biodegradation. The capability of *Rhodococcus opacus* GM-14 to use fluorobenzene (FB) and DFBs as a sole carbon and energy source was tested by Zaitsev et al. (1995), but no biodegradation was observed. Renganathan (1989) has described 36% of defluorination of 1,3-DFB, in cometabolism with glucose, by *Pseudomonas* sp. strain T-12 previously fed with glucose and FB. This strain was also able to cometabolically transform 1,4-DFB into catechol (Renganathan, 1989). A slight growth of *Rhodococcus* sp. strain MS11 in the presence of 1,4-DFB that was added as sole carbon source was reported by Rapp and Gabriel-Jürgens (2003), but the extent of degradation was not quantified.

Complete biodegradation of DFBs and their use as growth substrate have not yet been reported, to the best of our knowledge. Thus, this study aimed at investigating the biodegradation of 1,2-, 1,3- and 1,4-DFB by a previously isolated microbial strain (F11) of *Labrys portucalensis*. This organism has the capacity to degrade FB as a sole carbon and energy source (Carvalho et al., 2005) and, thus, its capacity to degrade DFBs as sole carbon source and in cometabolism with FB was investigated.

4.2. MATERIAL AND METHODS

4.2.1. Cultivation conditions

Labrys portucalensis strain F11 (Carvalho et al., 2005) was grown in sealed flasks containing a sterile minimal salts medium (MM) (Caldeira et al., 1999) and FB (1 mM) supplied as sole carbon and energy source. Cultures were incubated on a rotary shaker (130 rpm) at 25ºC. Growth was monitored by measuring the optical density at 600 nm (OD_{600}).

4.2.2. Biodegradation experiments

After growth, cells of *L. portucalensis* strain F11 were harvested by centrifugation (10 000 rpm for 15 min at 4ºC), washed with MM and resuspended in the same medium. 500 ml sealed flasks containing MM supplemented with DFBs or/and FB were inoculated with the

prepared suspension to an OD_{600} of 0.05, in a working volume of 200 ml. The cultures were incubated at 25ºC on a rotary shaker (130 rpm).

Degradation of DFBs was tested as a single substrate and with the addition of FB. Degradation in the presence of FB was tested using cells at different initial biomass concentration (OD_{600} of ca. 0.05 and 0.2) and with different concentrations of substrates. All experiments were done in triplicate and controls without inoculum were also monitored. Samples were taken with regular intervals to determine growth and degradation of fluorobenzenes.

4.2.3. Analytical methods

4.2.3.1. Fluoride analysis

Biomass was removed from culture samples by centrifugation at 8000 rpm for 10 min. The concentration of fluoride ions in the supernatant was measured with an ion-selective combination electrode (model Orion 96-09, Thermo Electron Corporation, Beverly, MA), which was calibrated with NaF (0.01 to 5 mM) in MM. The ionic strength of the standards and of the samples was adjusted with a buffer solution (total ionic strength adjustment solution – TISAB). The composition of the TISAB solution was: NaCl 1 M, CH_3COOH 0.25 M, $NaCH_3COO$ 0.75 M and sodium citrate 0.002 M.

4.2.3.2. GC analysis

FB and DFBs were analysed by gas chromatography using a gas chromatograph Varian CP-3800 (Agilent Technologies, California, USA) and a CP-Wasc 52 CP capillary column (Chrompack International B.V., Middelburg, The Netherlands), using a temperature program starting at 50ºC for 2 min, increasing to 150ºC at a rate of 25ºC min^{-1} and reaching the final temperature of 250ºC at a rate of 50ºC min^{-1}. Injector and detector temperatures were 250ºC. Culture samples (4.5 ml) were extracted with 2 ml of diethyl ether, containing mesitylene as internal standard, by vortexing the extraction tube 1 min at maximum speed. The ether layer was analysed by split injection of 1 µl samples. The concentrations were calculated using a calibration curve.

4.2.3.3. Chemicals

All chemicals were analytical-grade (Sigma-Aldrich Chemie, Steinheim, Germany; Merck, Darmstadt, Germany).

4.3. RESULTS

4.3.1. Biodegradation of DFBs as a sole carbon source

To investigate if *L. portucalensis* strain F11 is able to use 1,2-, 1,3- and 1,4-DFB as a sole carbon and energy source, cells previously grown on FB were inoculated into 200 ml of MM supplemented with 0.5 mM of the respective DFBs. Cultures supplied with FB (0.5 mM) were used as control. The initial concentration of the fluorinated compounds analysed in the liquid phase was 0.35 mM and not 0.5 mM actually fed to the cultures. This is related to the Henry partition coefficient of DFBs and FB, which determines the distribution of these volatile compounds between the gas and the liquid phases.

The results presented in Figure 4.1 show that 1,3-DFB was completely degraded in 20 days, with stoichiometric liberation of fluoride. Cell growth, as monitored by the increase in OD_{600}, was observed during 1,3-DFB degradation (Figure 4.1a). Interestingly, biomass increase was only observed when ca. 90% of the substrate was already degraded, being not proportional to the gradual 1,3-DFB consumption and fluoride release (Figure 4.1a). *L. portucalensis* F11 did not defluorinate 1,2- and 1,4-DFB during the time course of the experiment, showing no substrate consumption or growth (Figures 4.1b and 4.1c). In the control flasks fed with 0.5 mM of FB, complete fluoride release was obtained after 1 day and the optical density of the cultures doubled in this period (Figure 4.1d). In the control flasks without inoculum addition, both removal of DFBs and fluoride release were not observed (data not shown).
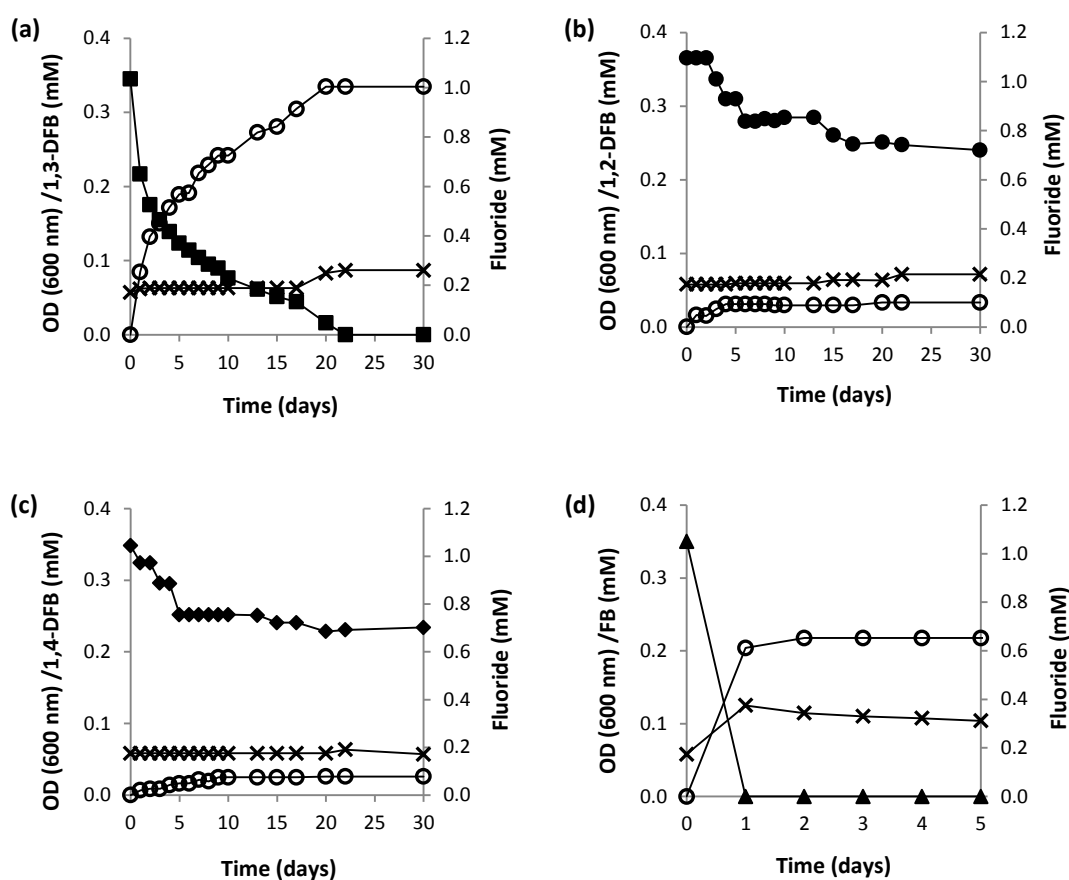
Figure 4.1. Typical growth of *L. portucalensis* strain F11, pregrown on FB, on 0.5 mM of (a) 1,3-DFB, (b) 1,2-DFB, (c) 1,4-DFB or (d) FB, in batch culture. The experiment was conducted in triplicate. Optical density (**x**), 1,3-DFB (**■**), 1,2-DFB (**●**), 1,4-DFB (**♦**) and FB (**▲**) concentrations in the culture medium and fluoride release (**o**) are indicated.

4.3.2. Biodegradation of DFBs in the presence of FB

To test if the addition of FB to the culture medium could stimulate the biodegradation of DFBs, cultures of strain F11 were fed simultaneously with FB and DFBs. Cells previously grown on FB were inoculated in MM supplemented with 0.5 mM of DFBs and 0.5 mM of FB. In these experiments, both 1,3-DFB and FB were completely degraded within 5 days, with a concomitant increase in the biomass and with stoichiometric fluoride release (Figure 4.2a). It is important to notice that when FB was present in the medium, 1,3-DFB degradation was much faster than when it was present as the only carbon source. With the addition of FB,

about half of the 1,4-DFB fed to the cultures was degraded with stoichiometric fluoride release (Figure 4.2b). The biomass increase in these cultures was lower than that observed with 1,3-DFB (Figure 4.2a and 4.2b). The degradation of 1,2-DFB in the presence of FB resulted in the consumption of ca. 30% of each substrate, but no fluoride release or biomass increase were observed (Figure 4.2c).
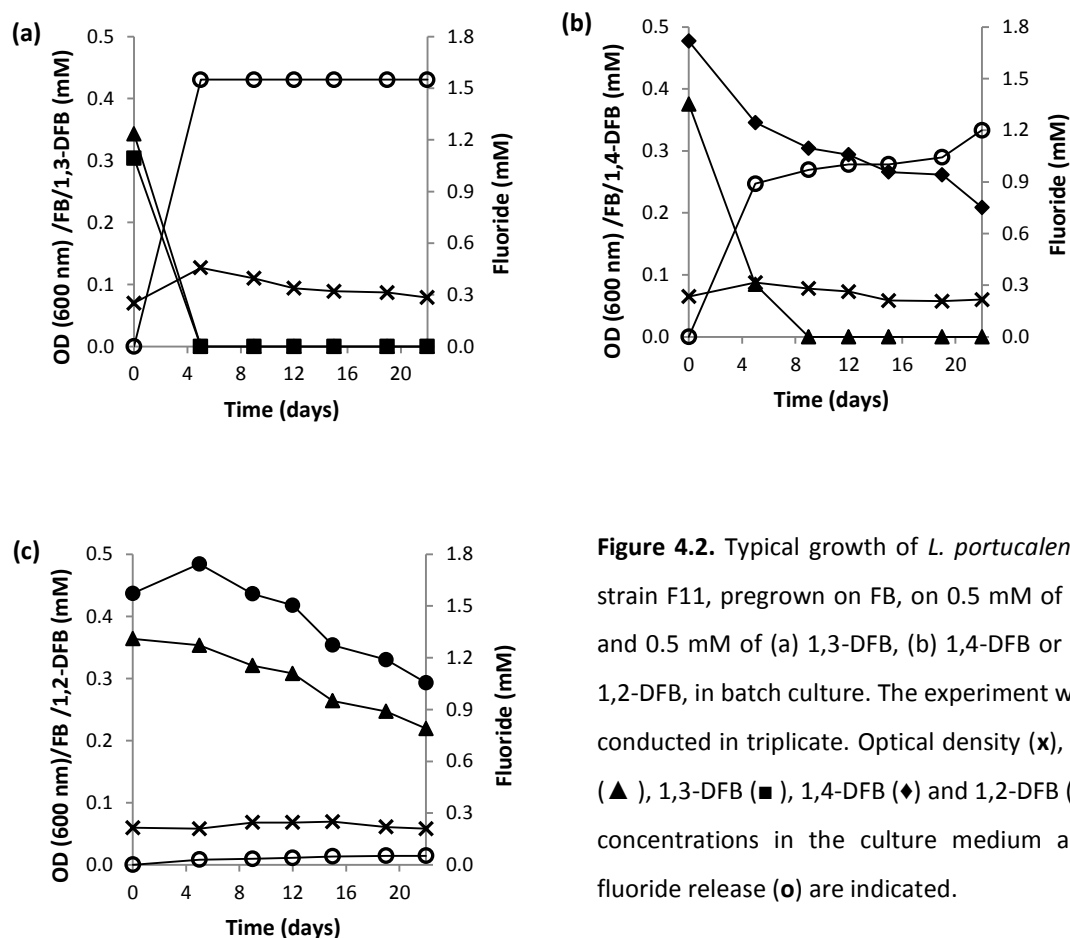
Figure 4.2. Typical growth of *L. portucalensis* strain F11, pregrown on FB, on 0.5 mM of FB and 0.5 mM of (a) 1,3-DFB, (b) 1,4-DFB or (c) 1,2-DFB, in batch culture. The experiment was conducted in triplicate. Optical density (**x**), FB (▲), 1,3-DFB (■), 1,4-DFB (♦) and 1,2-DFB (●) concentrations in the culture medium and fluoride release (**o**) are indicated.

Degradation of DFBs was also investigated with a higher initial cell density (OD_{600} of ca. 0.2), and in this experiment cultures were fed with 0.5 mM of DFBs and 0.5 mM of FB. The results presented in Figure 4.3a show that in these conditions 1,4-DFB and FB were

completely degraded in 4 days, with stoichiometric fluoride release. In the cultures fed simultaneously with 1,2-DFB and FB, 30% consumption of each compound was observed and fluoride release was obtained being, although, half of that expected (Figure 4.3b). Nevertheless, this fluoride release was higher than the one obtained in the low density cultures (Figure 4.2c). No differences were observed in the degradation of 1,3-DFB in the cultures with a higher initial cell density (Figure 4.2a and 4.2c). Control flasks without inoculum addition showed no consumption of DFBs and FB and no fluoride release (data not shown).
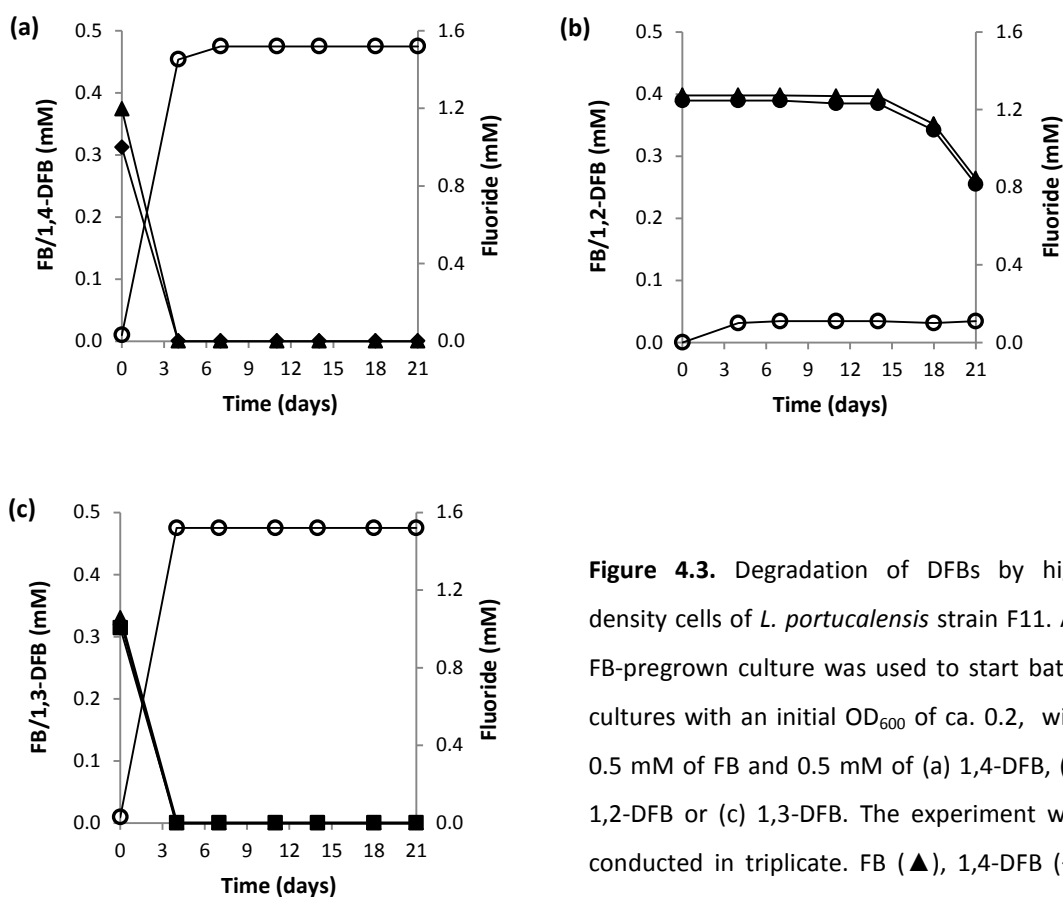
Figure 4.3. Degradation of DFBs by high density cells of *L. portucalensis* strain F11. An FB-pregrown culture was used to start batch cultures with an initial OD_{600} of ca. 0.2, with 0.5 mM of FB and 0.5 mM of (a) 1,4-DFB, (b) 1,2-DFB or (c) 1,3-DFB. The experiment was conducted in triplicate. FB (▲), 1,4-DFB (♦), 1,2-DFB (●) and 1,3-DFB (■) concentrations in the culture medium and fluoride release (**o**) are indicated

The effect of substrate concentration on the degradation of 1,3- and 1,4-DFB, in the presence of FB, was also investigated. Three different concentrations of 1,3- and 1,4-DFB were tested: 0.5, 1 and 2 mM, while FB was added at a concentration of 1 mM. Cultures fed with 0.5 mM of 1,3-DFB and 1 mM of FB had the highest growth (Figure 4.4a), with strain F11 being capable to degrade these compounds in less than 3 days. *L. portucalensis* F11 was also able to degrade 1 mM of 1,3-DFB and 1 mM of FB in 5 days, with a stoichiometric fluoride release (Figure 4.4c). F11 cultures fed with 2 mM of 1,3-DFB and 1 mM of FB were not able to completely degrade the substrates supplied during the time course of the experiment. In these experiments, a 30% decrease for each substrate was observed with a stoichiometric fluoride release (Figure 4.4e).

Concerning the degradation of 1,4-DFB, the cultures fed with 0.5 mM of this compound and with 1 mM of FB were capable of degrading both compounds in a 7 days period, with concomitant fluoride release (Figure 4.4b). F11 cultures fed with the highest concentrations of 1,4-DFB and with 1 mM of FB, did not completely degrade these compounds during the time course of the experiment. In the cultures fed with 1 mM of 1,4-DFB and 1 mM of FB, 20% of 1,4-DFB and 40% of FB were degraded by strain F11, while in the cultures fed with 2 mM of 1,4-DFB and 1 mM of FB, a 10% degradation of each compound was obtained. Fluoride release was always concomitant with substrate depletion (Figures 4.4d and f).

4.4. DISCUSSION

Biodegradation experiments with all three isomeric difluorobenzenes showed that *L. portucalensis* strain F11 is able to use 1,3-DFB as sole carbon and energy source, with stoichiometric fluoride release. To our knowledge, this is the first time that complete defluorination of a difluorobenzene by a bacterial culture is reported. Partial defluorination of this compound was reported previously by Renganathan (1989) using cells of *Pseudomonas* sp. strain T-12, which were found to metabolize and partially defluorinate 1,3-DFB that was added together with glucose. *L. portucalensis* strain F11 could not grow on 1,2- and 1,4-DFB when present as sole carbon and energy source.
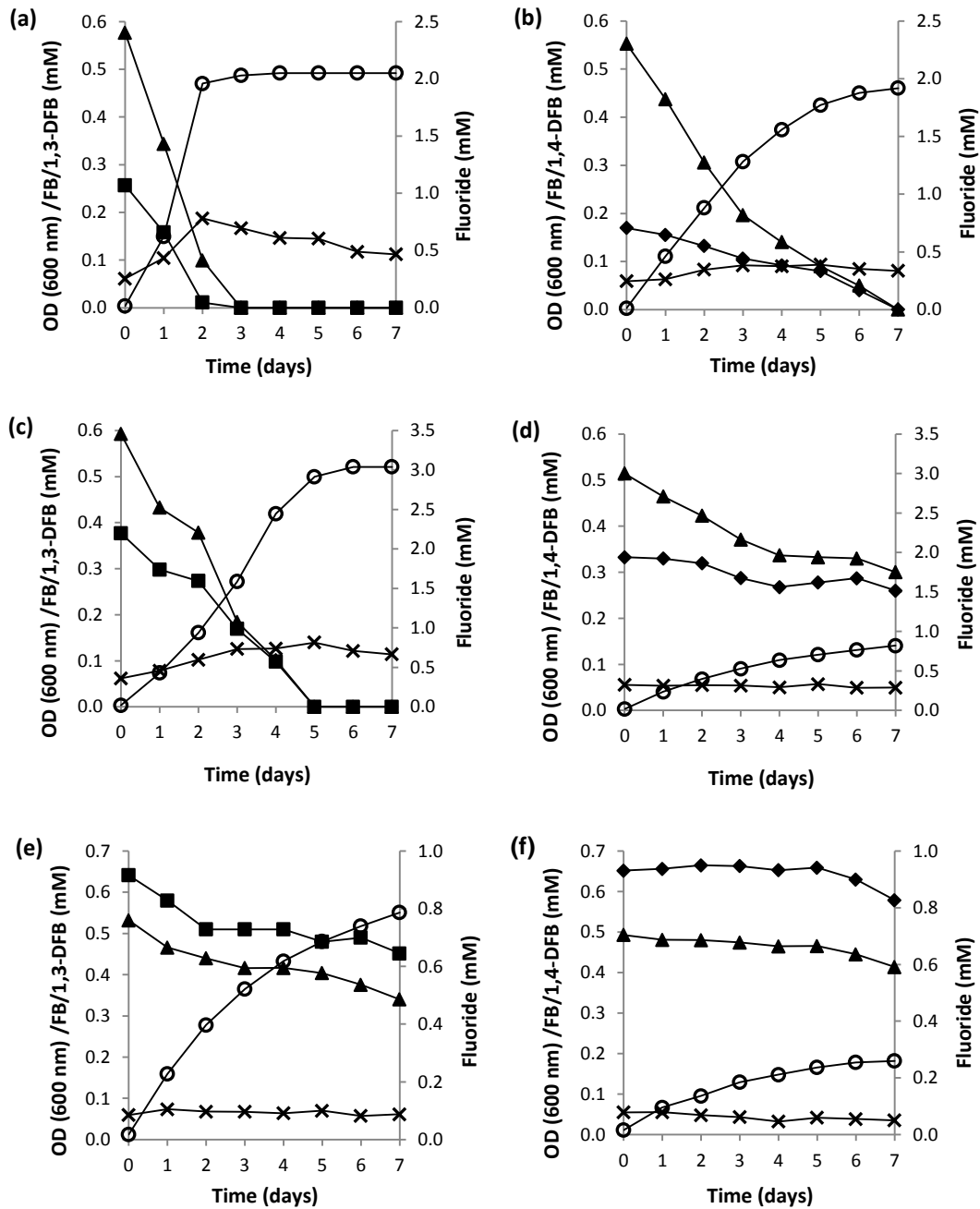
Figure 4.4. Effect of concentration on the degradation of 1,3- and 1,4-DFB in the presence of FB. Cultures of *L. portucalensis* strain F11, pregrown on FB, were incubated in batch mode with 1mM of FB and (a) 0.5 mM of 1,3-DFB, (b) 0.5 mM of 1,4 DFB, (c) 1 mM of 1,3-DFB, (d) 1 mM of 1,4-DFB, (e) 2 mM of 1,3-DFB or (f) 2 mM of 1,4-DFB. The experiment was conducted in triplicate. Optical density (**x**), FB (▲), 1,3-DFB (■) and 1,4-DFB (♦) and concentrations in the culture medium and fluoride release (**o**) are indicated.

Data on bacterial growth with 1,2-DFB could not be found in the literature, whereas it was found only one study reporting a slight growth of a *Rhodococcus* sp. in a medium containing 5 mM of 1,4-DFB present as sole carbon source (Rapp and Gabriel-Jürgens, 2003).

Zaitsev et al. (1995) described the utilization of halogenated benzenes by *Rhococcus opacus* GM-14, which was selected on chlorobenzene. The organism did not grow on (di)fluorobenzenes, but 1,3-dichlorobenzene (1,3-DCB), 1,4-dichlorobenzene (1,4-DCB) and the corresponding dibromobenzenes were good growth substrates, whereas 1,2-dihalogenated benzenes were not used. This preference for the 1,3-dihalogenated regioisomers was shared with *L. portucalensis* which completely metabolized 1,3-DFB, whereas 1,4-DFB was degraded only in the presence of FB and 1,2-DFB was not mineralized at all under any of the conditions tested. Similar results were reported for *Alcaligenes* sp. strain OBB65 (deBont et al., 1986), *Alcaligenes* sp. strain A175 (Schraa et al., 1986) and *Xanthobacter flavus* 14p1 (Spiess et al., 1995). These strains were enriched on 1,3-DCB or 1,4-DCB but none was capable of degrading 1,2-DCB. On the other hand, this degradation pattern is not a strict rule since *Pseudomonas* sp. strain JS100, enriched from sewage with 1,2-DCB as growth substrate, was able to grow on CB and 1,2-DCB but not on 1,3-DCB or 1,4-DCB (Haigler et al., 1988). There are also reports of bacterial strains capable of utilizing CB and all three DCB isomers, like *Pseudomonas* sp. strains PS12 and PS14, which were enriched with 1,2,4-trichlorobenzene and 1,2,4,5-tetrachlorobenzene, respectively (Sander et al., 1991), a *Rhodococcus* sp. enriched with 1,2,4-trichlorobenzene (Rapp and Gabriel-Jürgens, 2003) and *Acidovorax avenae* enriched with 1,2-DCB (Monferrán et al., 2005).

The results obtained in this study suggest that 1,3-DFB is the only difluorinated benzene that is able to induce the appropriate enzymes for its degradation by strain F11. Although strain F11 can produce a set of enzymes required for conversion and defluorination of 1,3-DFB, the associated biomass increase was low when compared to growth with the same amount of FB. This could be due to higher toxicity or slower metabolism as a result of the additional carbon-fluorine bond in the aromatic ring. The aerobic biodegradability of difluorinated benzenes by *L. portucalensis* F11 decreased in the order: 1,3-DFB, 1,4-DFB and 1,2-DFB. The poor degradability of 1,2- and 1,4-DFB by strain F11 might be due to (1) lack of induction of the appropriate catabolic enzymes; (2) lack of catalytic activity of enzymes of haloaromatic metabolism with 1,2- and 1,4-difluorine substituted substrates; (3) production

of toxic intermediates during the metabolism of the recalcitrant difluorinated substrates. In case of *Pseudomonas* sp. strain T-12, 1,4-DFB was suggested to induce the enzymes involved in the toluene metabolic pathway, but the catechol produced was found to inactivate catechol 2,3-dioxygenase, leading to the accumulation of this compound (Renganathan and Johnston, 1989). Also, Munõz et al. (2007) confirmed the inhibitory effect of catechol accumulation during the biodegradation of benzene by cultures of *Pseudomonas putida* F1. The toxicity of chlorophenol for microorganisms was profoundly affected by the position of the chlorine substituents in the phenol molecule (Liu et al., 1982). A similar effect caused by position of the fluorine in the molecule might explain the incapacity of *L. portucalensis* F11 to degrade 1,2- and 1,4-DFB as sole carbon sources.

The effect of the addition of a second carbon source (FB) and of different substrate concentrations on the biodegradation of DFBs was also investigated. The presence of FB in the medium accelerated the biodegradation of 1,3-DFB, suggesting that this compound is metabolized by the same enzymes as FB. Possibly, FB is a better inducer of the DFB-degradation enzymes than 1,3-DFB or FB allows more rapid formation of active biomass. The results also showed that when FB was present, growth of *L. portucalensis* was stimulated, unless the concentration of DFB became too high. High concentrations of toxic organic compounds can induce inhibitory effects and the accumulation of high amounts of toxic intermediates (Christen et al., 2002; Halsey et al., 2005). The initial degradation of FB yields a mixture of catechol and 4-fluorocatechol (Carvalho et al., 2006a), and it is well possible that metabolism of 1,3-DFB also yields a mixture of catechols due to the relaxed specificity of the initial dioxygenase. Of such a mixture, not all components may be good substrates for complete conversion and productive metabolism.

The relative recalcitrance and toxicity of 1,4-DFB and 1,2-DFB was observed both with the pure substrates and in the mixed-substrate cultures. Whereas degradation of 1,3-DFB and FB occurred simultaneously, cultures containing both 1,4-DFB and FB first metabolized FB. In these cultures FB was removed earlier than 1,4-DFB, and biomass increase was lower in the cultures fed with both FB and 1,4-DFB than in the cultures fed only with FB. The fact that F11 cells were able to degrade 1,4-DFB only in the presence of FB suggests that 1,4-DFB is not capable by itself to induce the appropriate catabolic enzymes. In these mixed-

substrate cultures the consumption and defluorination of both substrates was complete, indicating no formation of suicidal intermediates.

The complete defluorination of the substrate consumed (1,4-DFB), in the presence of a growth substrate (FB), is different from the classical definition of cometabolism (Horvath, 1972), whereby the organism is unable to further metabolize a dead-end product. The complete metabolism of compounds that could not serve as growth substrates when provided alone was also reported by Haigler et al. (1992).

Labrys portucalensis F11 did not mineralize 1,2-DFB even in the presence of FB. Also, FB was not fully mineralized when fed simultaneously with 1,2-DFB, indicating that 1,2-DFB inhibits the degradation of FB. A partial uptake of both substrates was observed but no fluoride was released. This result suggests that the enzymes involved in the metabolism of FB are able to also attack 1,2-DFB, but the metabolites generated are toxic, thus inhibiting further degradation of both substrates. Similar inhibitory effects have been reported by other researchers during the degradation of mixtures of polycyclic aromatic hydrocarbons (Dean-Ross et al., 2002; Stringfellow and Aitken, 1995). Reasons for negative interactions include competitive inhibition and toxicity as the case of BTEX mixtures (Reardon et al., 2000), the formation of toxic intermediates by nonspecific enzymes as the case of chlorinated phenolic mixtures (Bartels et al., 1984; Klecka and Gibson, 1981), as well as cometabolic cofactor dependency (Sáez and Rittmann, 1993). It is known that in many cases cometabolic reactions are responsible for the generation of metabolites that are more toxic than the parent compound. Wigmore and Ribbons (1980) have previously stated that cooxidation of halogenated aromatic compounds may not proceed, even if a potential exists, due to the formation of inhibitory products.

The improvement on 1,3- and 1,4-DFB degradation observed in the presence of FB suggests that these compounds are productively metabolized by the same enzymes involved in the metabolic pathway of FB, described in Carvalho et al. (2006a), while the attack of these enzymes on 1,2-DFB may lead to the formation of toxic products. In fact, the metabolic pathway for FB degradation by *L. portucalensis* F11 starts with a dioxygenase attack to the aromatic ring, leading to the production of two different fluorinated dihydrodiols: 4-fluoro-*cis*-benzene-1,2-dihydrodiol and 1-fluoro-*cis*-benzene-1,2-dihydrodiol. The first intermediate is subjected to a rearomatization reaction, catalyzed by a dihydrodiol

dehydrogenase, resulting in the production of 4-fluorocatechol as the predominant central metabolite, while 1-fluoro-*cis*-benzene-1,2-dihydrodiol is spontaneously defluorinated in a non-enzymatic reaction, leading to the production of catechol, the minor product of the initial dioxygenase reaction. Further metabolism of the resulting catechol intermediates proceeds via an *ortho*-cleavage pathway, in which 4-fluorocatechol and catechol are respectively converted to 3-fluoro-*cis,cis*-muconate and *cis,cis*-muconate, through the action of a (fluoro)catechol dioxygenase. The fluorinated muconate is then expected to be converted, with concomitant defluorination, into maleylacetate, which is then channelled into the tricarboxylic acid cycle via 3-oxoadipate, while *cis,cis*-muconate is proposed to be converted to the lactone derivative being then also channelled into the tricarboxylic acid cycle (Carvalho et al., 2006a).

An increase in the biodegradation of the DFBs using higher initial cell density cultures was seen, which may be due to several reasons: (i) as the cells were not growing, higher amounts of energy could be channelled to the catalytic processes; (ii) due to the higher cell density of the cultures, the interactions between cell and substrate increased leading to a higher degradation rate; (iii) the higher cell density of the cultures may have also contributed to dilute the cellular toxic effects of the DFBs.

4.5. CONCLUSIONS

L. portucalensis strain F11 is, to our knowledge, the first microorganism described that is capable to mineralize 1,3-DFB and 1,4-DFB. The strain can use 1,3-DFB as a sole carbon and energy source. The presence of FB in the culture medium was found to accelerate the degradation of 1,3-DFB and to allow the mineralization of 1,4-DFB, revealing that the addition of a cometabolic substrate structurally analogous to the DFBs is beneficial for the degradation of these compounds. Strain F11 does not have the ability to degrade 1,2-DFB and the presence of this compound in the culture medium was found to inhibit the degradation of FB. The results indicate that biodegradability of DFBs is strongly affected by the presence of a second carbon source and the by the position of the fluoride atoms in the molecule.

CHAPTER 5

ENANTIOSELECTIVE BIODEGRADATION OF FLUOXETINE BY THE WILD STRAIN *LABRYS PORTUCALENSIS* F11

Abstract

Fluoxetine (FLX) is a chiral fluorinated pharmaceutical indicated mainly for treatment of depression and is one of the most dispensed drugs in the world. There is clear evidence of environmental contamination with this drug and its active metabolite norfluoxetine (NFLX). In this study the enantioselective biodegradation of FLX by L. portucalensis strain F11 was assessed. When 2 µM of racemic FLX was supplemented as sole carbon source, complete biodegradation of both enantiomers, with stoichiometric liberation of fluoride, was achieved in 30 days. For racemic FLX concentration of 4 and 9 µM, partial degradation of the enantiomers was obtained. In the presence of an additional carbon source, sodium acetate (SA), at 4, 9 and 21 µM of racemic FLX complete degradation of the two enantiomers occurred. At 45 and 89 µM of racemic FLX, partial degradation was achieved. Preferential degradation of the R-enantiomer was observed in all experiments. A single enantiomer of NFLX was identified as intermediary metabolite during FLX biodegradation. To our knowledge, this is the first time that complete and enantioselective biodegradation of FLX by a single bacterium is reported.

5.1. INTRODUCTION

The occurrence of pharmaceuticals in the environment has been recognized as an issue of major concern due to their recalcitrance nature and ecotoxicity effects (Fent et al., 2006). Pharmaceuticals may not be completely metabolized in the human body and can enter the municipal sewage system as the pharmaceutical itself and as their biologically active metabolites (Vasskog et al., 2009). These compounds cannot be easily removed at wastewater treatment plants (WWTPs) (Carballa et al., 2004). Consequently, they are easily introduced into the environment via effluent discharge into waterways and by the land application of treated sewage sludge in agricultural settings (Wu et al., 2010).

Fluoxetine (*N*-methyl-*γ*-[4-(trifluoromethyl)phenoxy]benzene-propanamine) is one of the most frequently prescribed antidepressants (marketed with diverse trade names such as its original brand Prozac®). Fluoxetine (FLX) belongs to a group of medicines known as selective serotonin reuptake inhibitors (SSRIs). FLX is a chiral pharmaceutical that is commercialized as a racemic mixture (Figure 5.1), whereby the *S*-enantiomer is approximately 1.5 more potent than the *R*-enantiomer in the inhibition of serotonin reuptake. In the human body FLX is metabolized to norfluoxetine (NFLX), its active metabolite, and is primarily excreted as less than 10% as unchanged parent compound in urine (Hiemke and Härtter, 2000).
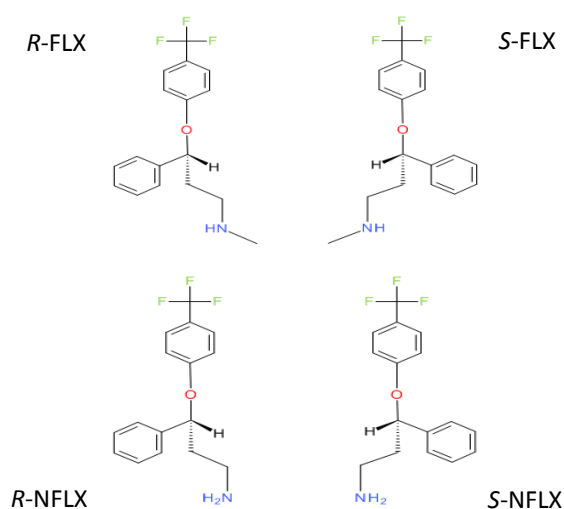
Figure 5.1. Molecular structure of FLX enantiomers and their chiral active metabolite NFLX.

FLX and its major metabolite (NFLX) have been detected in a number of environmental water samples including wastewater effluents, rivers and streams in concentrations ranging from ng l^{-1} to µg l^{-1} (Lajeunesse et al., 2008; Ternes, 1998; Kolpin et al., 2002). FLX has also been detected in potable water before and after treatment at maximum concentrations of 3.0 ng l^{-1} and 0.82 ng l^{-1}, respectively (Benotti et al., 2009). Furthermore, FLX and NFLX have been detected in fish samples obtained from effluent-dominated streams, indicating its bioaccumulation potential (Nakamura et al., 2008). Since these molecules often act by modulating the effects of the neurotransmissor serotonin, which regulates a wide range of physiological systems, they can have tremendous effects on organisms. Several authors reported the high toxicity of FLX to aquatic organisms (Fent et al., 2006; Nakamura et al., 2008). In laboratory scale experiments Kwon and Armbrust (2006) have shown that FLX is relatively recalcitrant to hydrolysis, photolysis, and microbial degradation and is rapidly removed from surface waters by adsorption to sediments, where it appears to be persistent. High amounts of FLX were found in biosolids produced by WWTP, in the range of 100-4700 µg kg^{-1} organic carbon (Kinney et al., 2006).

Several studies have revealed different enantiomeric fraction of chiral compounds in the environment (Liu et al., 2005; Wang et al., 2009). The enantioselective in pharmacokinetic and pharmacodynamic is undeniable, therefore the enantiomers biological degradation, uptake, accumulation and toxicological effects are also expected to present selective behavior (Liu et al., 2005; Wang et al., 2009; Ribeiro et al., 2012). Enantioselective microbial degradation of chiral pesticides have been frequently reported, as examples *Xanthobacter flavus* PA1 degrades enantioselectively 2-phenylbutyric acid (Liu et al., 2011), Zipper et al. (1998) reported that *Sphingomonas herbicidovorans* MH was able to completely degrade both enantiomers of the chiral herbicide dichlorprop [(*RS*)-2-(2,4-dichlorophenoxy)propanoic acid], with preferential degradation of the *S*-enantiomer. Similar results were reported for the chiral herbicide mecoprop [(*RS*)-2-(4-chloro-2-methylphenoxy)propionic acid] (Zipper et al., 1996); Garbe et al. (2006) reported preferential attack of the *S*-configured ether-linked carbons in bis-(1-chloro-2-propyl)ether Q2 by *Rhodococcus* sp. strain DTB. Regarding enantioselective biodegradation of pharmaceuticals there are only few examples, such as the degradation of warfarin in soils (Lao and Gan, 2012).

In this study, we investigated the degradation of chiral FLX by *Labrys portucalensis* F11, using enantioselective High Performance Liquid Chromatography (HPLC). Here we describe the enantioselective biodegradation and the formation of one major degradation intermediate during the process.

5.2. MATERIAL AND METHODS

5.2.1. Cultivation conditions

L. portucalensis strain F11 (Carvalho et al., 2005) was grown in sealed flasks containing sterile minimal salts medium (MM) (Caldeira et al., 1999). Cultures were incubated on a rotary shaker (130 rpm) at 25ºC. Growth was monitored by measuring the optical density at 600 nm (OD_{600}) using a spectrophotometer (Helios Gamma, Unicam Instruments, UK).

5.2.2. Biodegradation of FLX

Degradation of FLX was tested as a single substrate and with the addition of a supplementary carbon source, sodium acetate (SA), at different concentrations. Experiments were conducted with cells obtained by growing *L. portucalensis* strain F11 on Nutrient Agar (NA) plates. Cells of *L. portucalensis* strain F11 were inoculate to an OD_{600} of ca. 0.05 into sealed flasks containing MM supplemented with FLX, as sole carbon source or with the addition of SA. FLX concentrations of 2, 4, and 9 µM (0.6, 1.2 and 2.8 mg l^{-1}) were supplied as sole carbon source. When SA was supplied to the cultures, the FLX concentrations used were 4, 9, 21, 45 and 89 µM (1.2, 2.8, 6.5, 13.9 and 27.5 mg l^{-1}). SA was supplied at 5.9 mM. The biodegradation assays were monitored during 56 days.

The capacity of cumulative degradation of FLX was performed in the presence of SA carrying out additions of ca. 45 µM of FLX and 20 mM of SA to the culture medium at the beginning of the experiment, at day 17 and at day 48. The biodegradation was monitored during 55 days.

All the cultures were incubated at 25ºC on a rotary shaker (130 rpm). FLX was supplied as a racemic mixture. Experiments were performed in duplicate under sterile conditions and protected from light. Control assays without inoculation were included. Samples were taken at regular intervals to assess for growth, and degradation of FLX. Validated enantioselective HPLC method was used to follow *R*- and *S*-enantiomer concentrations. Purity of the cultures was evaluated through regular plating on NA plates.

5.2.3. Analytical methods

5.2.3.1. Fluoride analysis

For fluoride analysis, biomass was previously removed from culture samples by centrifugation at 14000 rpm for 10 min at 4ºC. The concentration of fluoride ions in the samples supernatant was measured with an ion-selective combination electrode (model Orion 96-09, Thermo Electron Corporation, Beverly, MA), which was calibrated with NaF (0.005 to 5 mM) in MM. The ionic strength of the standards and of the samples was adjusted with a buffer solution (total ionic strength adjustment solution – TISAB). The composition of the TISAB solution was: NaCl 1 M, CH_3COOH 0.25 M, CH_3COONa 0.75 M and sodium citrate 0.002 M.

5.2.3.2. HPLC analysis

For enantiomeric quantification of FLX and NFLX, biomass was previously removed from culture samples by centrifugation at 14000 rpm for 10 min at 4ºC, after which 20 μL was injected. Chromatographic analysis were performed on a Shimadzu UFLC Prominence System using a vancomycin-based Chiral Stationary Phase (CSP), a Chiral HPLC Astec Chirobiotic™ V, 5 μm (15 x 0.46 cm ID) supplied by SUPELCO Analytical (Sigma-Aldrich, Steinhein, Germany). Elution was carried out in isocratic mode with a flow rate of 1.0 ml min^{-1} with etanol/10 mM aqueous ammonium acetate buffer (92.5/7.5, v/v), pH 6.8 and the column oven set at 20ºC. The effluent was monitored with to a RF-10AXL Fluorescence Detector set at excitation and emission wavelengths of 230 and 290 nm, respectively.

Enantiomeric fraction (EF) was adopted as the standard descriptor, according to equation: EFS = [S] / ([S] + [R]). The EF values can range from 0 to 1 with EF = 0.5 representing a racemic mixture.

The degradation rate constants of the two enantiomers were calculated by assuming first-order kinetic, in which the residual concentration changes with time (t) according to the following relationship: $C = C_0e^{-kt}$, where C_0 is the initial concentration and k is the degradation rate constant. The half-life of biodegradation ($t_{1/2}$) is estimated from k using: $t_{1/2} = \ln2/k$.

5.2.4. Chemicals

All chemicals were analytical-grade (Sigma-Aldrich Chemie, Steinheim, Germany; Merck, Darmstadt, Germany).

5.3.   RESULTS

5.3.1. Biodegradation of FLX as a sole carbon source

To investigate if cells of *L. portucalensis* strain F11 were able to use FLX as a sole carbon and energy source, cells previously grown on NA plates were inoculated into MM supplemented with racemic FLX at concentrations of 2, 4 and 9 µM and monitored during 56 days. The results presented in Figure 5.2a show that, when 2 µM of racemic FLX was supplied, complete degradation of both enantiomers was obtained in 30 days, with stoichiometric liberation of fluoride. For upper FLX concentrations, at 4 µM of racemic FLX, 80% of *S*-FLX and 97% of *R*-FLX were degraded, with liberation of 59% of the stoichiometric value of fluoride in relation to the total amount of consumed substrate, during the time course of the experiment (Figure 5.2b). When 9 µM of racemic FLX was supplied, 67% of *S*-FLX and 89% of *R*-FLX were degraded and the total liberation of fluoride was 56% of the stoichiometric expected value (Figure 5.2c). Production of one intermediate, identified as NFLX, was observed during degradation at concentration levels of 4 and 9 µM of FLX

(Figures 5.2b and 5.2c). The degradability of FLX was inversely correlated with its initial concentration. No growth of the strain was observed at any of the tested concentrations. In the non-inoculated control assays, removal of FLX, fluoride release and formation of NFLX were not observed (data not shown), indicating that chemical degradation did not occur under the tested conditions.
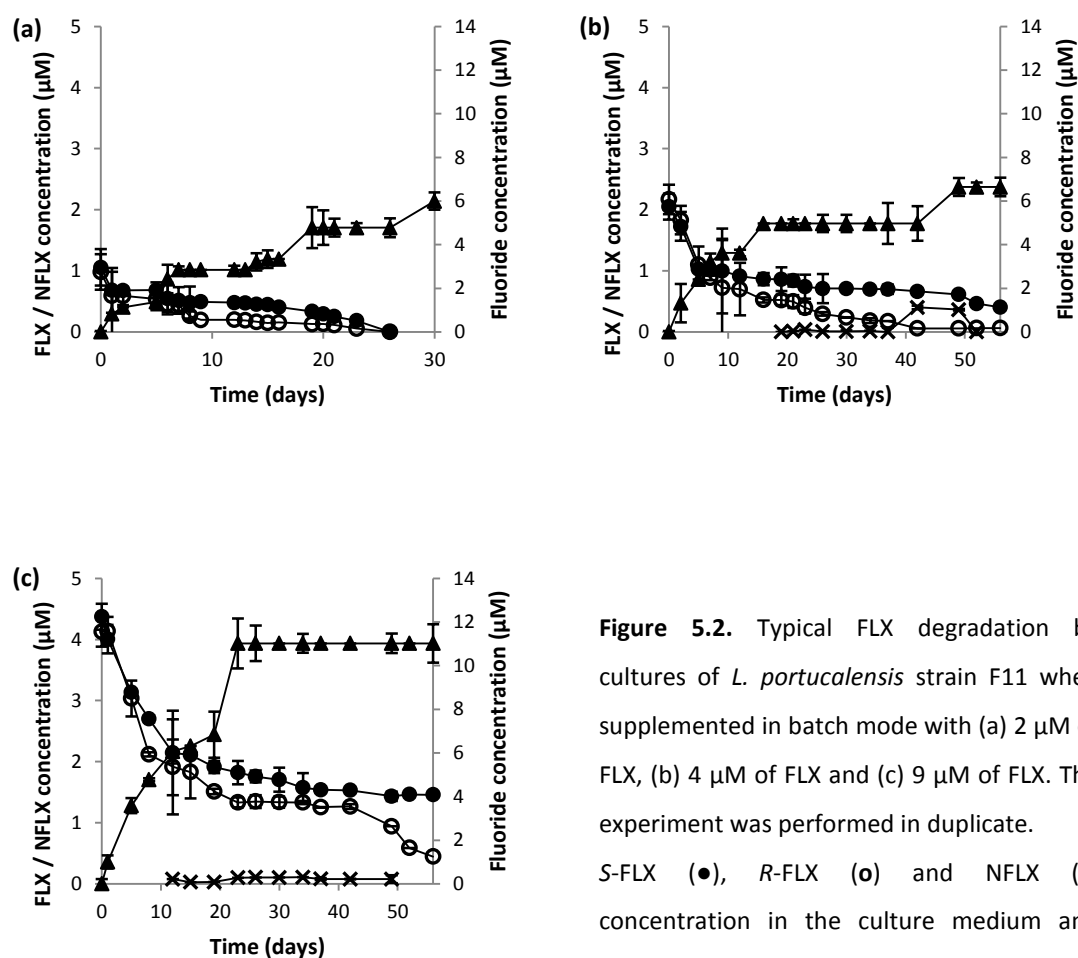
Figure 5.2. Typical FLX degradation by cultures of *L. portucalensis* strain F11 when supplemented in batch mode with (a) 2 µM of FLX, (b) 4 µM of FLX and (c) 9 µM of FLX. The experiment was performed in duplicate.

S-FLX (●), *R*-FLX (o) and NFLX (x) concentration in the culture medium and fluoride release (▲) are indicated.

5.3.2. Biodegradation of FLX in the presence of a supplementary carbon source

To assess the effect of a supplementary carbon source on the biodegradation of FLX by cultures of strain F11, these were fed simultaneously with 5.9 mM of SA and 4, 9, 21, 45

and 89 µM of racemic FLX. In the cultures supplemented with 4 and 9 µM of racemic FLX, total consumption of both enantiomers was achieved in 10 and 22 days, respectively, with liberation of approximately 50% of the stoichiometric fluoride. The liberation of fluoride continued for both cultures, reaching 70 and 65% respectively, after which the liberation ceased (Figures 5.3a and 5.3b). The supplementation with 21 µM of racemic FLX resulted in total disappearance of both enantiomers of the substrate in 31 days, with liberation of fluoride reaching 50% of the expected value at the end of the experiment (Figure 5.3c). In the experiments supplemented with 45 µM of racemic FLX, degradation of 81% of *S*-FLX and 92% of *R*-FLX was observed during the time course of the experiment, with 46% of stoichiometric fluoride release in relation to the total amount of consumed substrate (Figure 5.3d). When 89 µM of racemic FLX was supplied, 81% of *S*-FLX and 86% of *R*-FLX were degraded and 35% of the stoichiometric expected value of fluoride was released (Figure 5.3e). Growth on acetate was similar at all FX concentrations. The typical growth pattern observed, presented as average of the OD_{600} observed at all FLX concentrations tested, is presented in Figure 5.3f. The growth rate obtained was 1.004 ± 0.002 day^{-1}. The formation of NFLX as intermediate was observed. In the non-inoculated control assays, removal of FLX, fluoride release and formation of NFLX were not observed (data not shown), indicating that chemical degradation did not occur under the tested conditions.

An example of the HPLC chromatograms obtained, at the beginning of the experiments and after 30 days, for assays supplied with 9 µM of racemic FLX, is given in Figure 5.4. At the beginning of the experiment the concentration of both enantiomers was similar (EF_S = 0.49). After 30 days, the concentration of the *S*-enantiomer was much higher than the concentration of the *R*-enantiomer (EF_S = 0.70). At this sampling point, a single enantiomer of the metabolite NFLX was observed.

The comparison of the results obtained in the cultures supplemented with 4 and 9 µM of FLX with and without supplementation with SA, reveals that there is a positive effect of the addition of a second carbon and energy source to the FLX degradation, suggesting co-metabolic degradation. This is in accordance to the fact that the biodegradation of this compound by *L. portucalensis* F11 was not able to support growth at the concentrations tested.
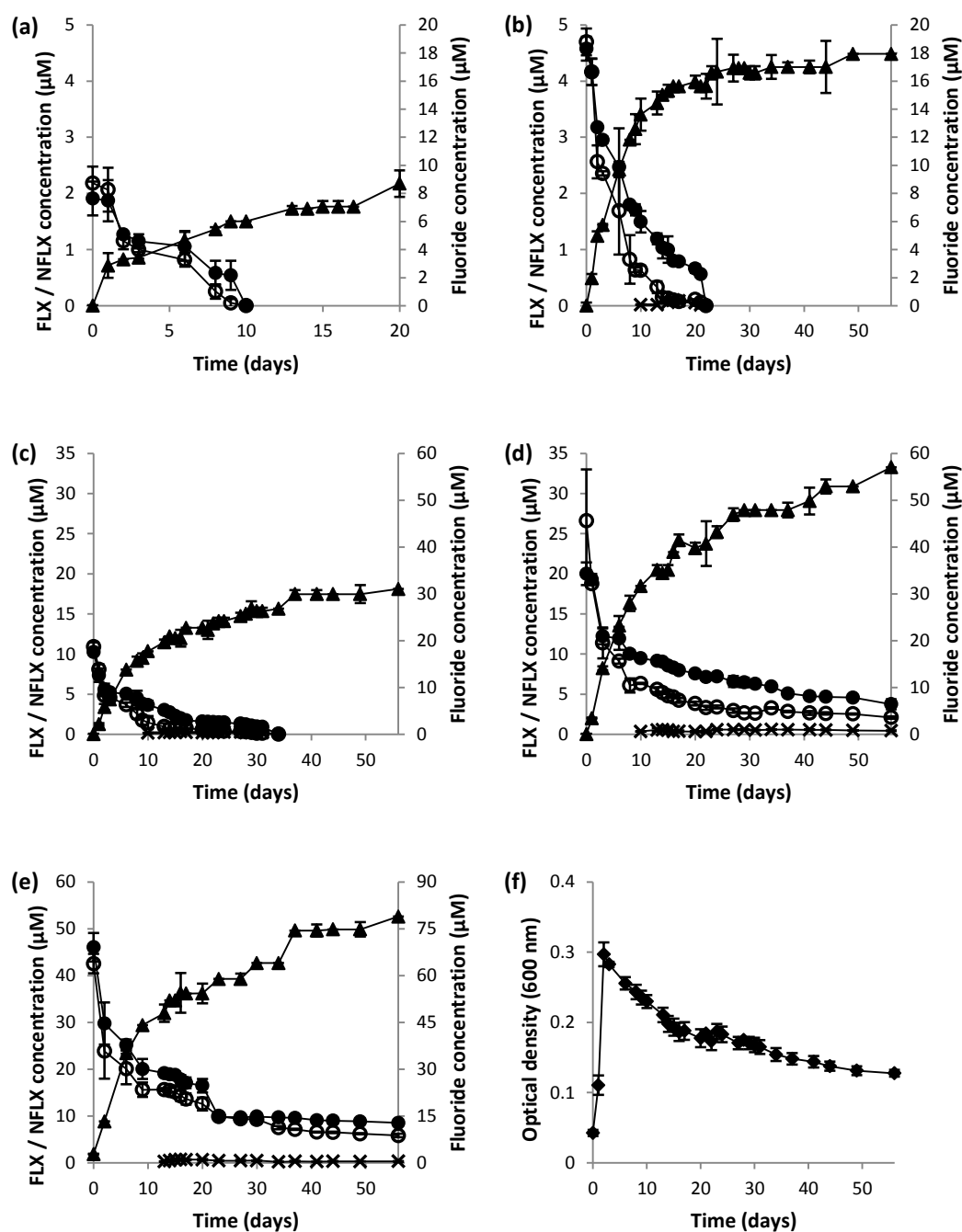
Figure 5.3. Typical FLX degradation by cultures of *L. portucalensis* strain F11 when supplemented in batch mode with 5.9 mM of SA and (a) 4 µM of FLX, (b) 9 µM of FLX, (c) 21 µM of FLX, (d) 45 µM of FLX and (e) 89 µM of FLX. (f) Optical density presented as average of all experiments. The experiment was performed in duplicate. *S*-FLX (●), *R*-FLX (o) and NFLX (x) concentration in the culture medium and fluoride release (▲) are indicated.
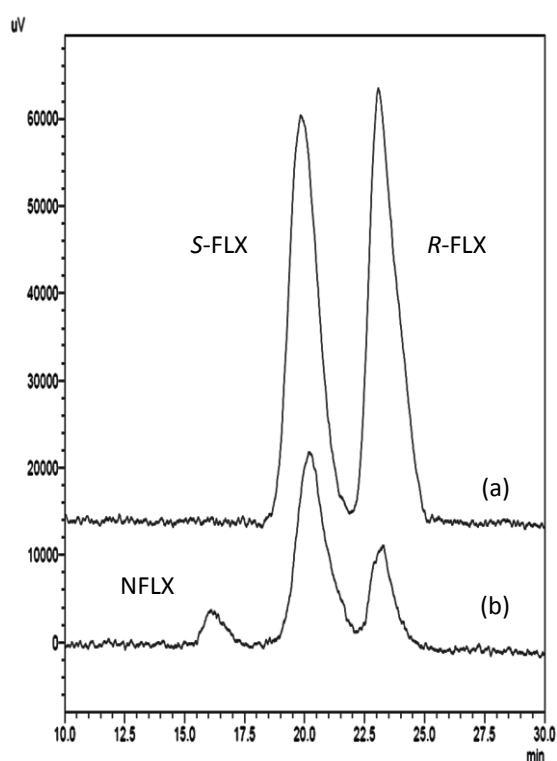
Figure 5.4. HPLC chromatograms of FLX enantiomers, showing the degradation by cultures of *L. portucalensis* strain F11 when supplemented in batch mode with 5.9 mM of SA and 9 µM of FLX (a) at the beginning of the experiment and (b) after 30 days.

5.3.3. Cumulative biodegradation of FLX in the presence of a supplementary carbon source

In order to test if *L. portucalensis* F11 cells could tolerate and degrade successive additions of FLX, cultures were fed simultaneously with 45µM of racemic FLX and 20 mM of sodium acetate (SA), at the beginning of the experiment and at days 17 and 48. A higher amount of SA was added to assess if this could improve FLX biodegradation. First supplementation resulted in the degradation of 71% of *S*-FLX and 93% of *R*-FLX with liberation of 93% of the stoichiometric fluoride in relation to the total amount of substrate supplied, in 17 days. This represents an increase in the extent of FLX biodegradation comparing to experiment with lower SA concentration, especially from the point of view of fluoride release, which increased by 42%. This result indicates that the higher availability of a second carbon source to supply energy to the cells may be a critical factor for biodegradation. The second

supplementation with FX and SA resulted in the degradation of 28% of *S*-FLX and 48% of *R*-FLX with the liberation of 34% of the expected fluoride in 6 days, after which the decrease in FLX concentration ceased but the liberation of fluoride continued - stoichiometric release in relation to the consumed substrate was achieved at day 48. The third supplementation with FLX and SA resulted in the degradation of 36% of *S*-FLX and 46% of *R*-FLX with release of 53% of the stoichiometric fluoride after 11 days, after which the experiment was finished (Figure 5.5). The fact that the decrease in FLX ceased after 6 days of the second supplementation, while the fluoride liberation continued till achieving stoichiometric values, suggests that some factor was limiting the initial steps of the degradative pathway but not its further degradation. When the third supplementation was performed, FLX degradation was resumed.
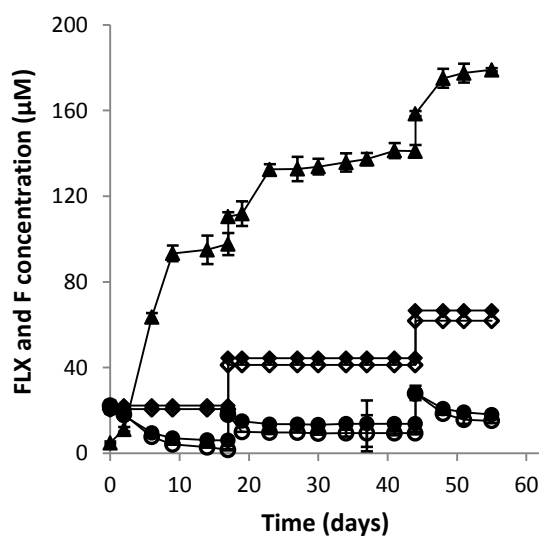
Figure 5.5. Biodegradation of FLX during a fed-batch culture. Variation on *S*-FLX (●) and *R*-FLX concentration (○), cumulative supply of *S*-FLX (♦) and *R*-FLX (◊) and cumulative fluoride release (▲) are indicated.

5.3.4. *Enantioselectivity and kinetics of FLX biodegradation*

FLX was supplied in all experiments as a racemic mixture. Degradation of FLX in an enantioselective manner by *L. portucalensis* F11 was observed. The decline of *R*-enantiomer was much faster than the *S*-enantiomer, at all conditions tested.

Enantioselectivity in biodegradation was characterized by evaluating the changes in enantiomeric composition of FLX. In an early stage of incubation, the concentrations of the two enantiomers were approximately equal but at later stages, the concentration of *S*-enantiomer was higher than *R*-enantiomer. The EF_S values increased gradually from 0.5 tending to 1, showing the preferential degradation of *R*-enantiomer by strain F11. Despite the same tendency, the net increase in EF_S was not equal at all FLX concentrations. When FLX was the only carbon source, the higher EF value (0.92) was reached at 4 µM FLX; whereas at 2 and 9 µM of FLX, the maximum EF value was 0.76. A similar trend was observed in the presence of SA, in which the higher EF_S was 0.92 observed at 9 and 21 µM of FLX, followed by 0.72 observed at 4 and 45 µM of FLX; whereas at 89 µM of FLX, the EF_S only reached 0.59. The variation in EF_S throughout the experiments is exemplified in Figures 5.6a and 5.6b. In the abiotic control assays, there was no significant variation in the EF_S during the experiments.
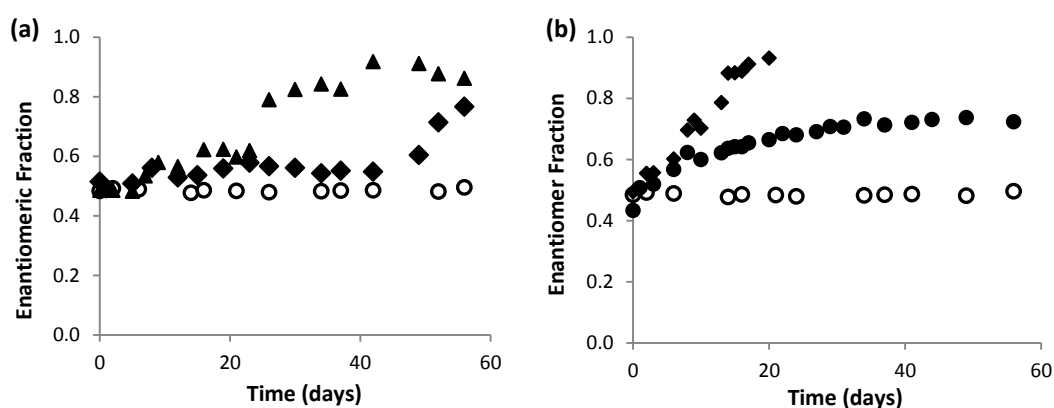
Figure 5.6. Changes in enantiomeric fraction (EF) of FLX during biodegradation by *L. portucalensis* F11 (a) as sole carbon and energy source and (b) in the presence of SA. EF of supplementations with 4 µM of FLX (▲), 9 µM of FLX (♦),45 µM of FLX (●) and abiotic control with 45 µM of FLX (o).

The degradation rate constants of the two enantiomers were calculated by assuming first-order kinetics (Table 5.1). The *k* value was always higher for the *R*-enantiomer than for the *S*-form, whereas the half-life revealed obviously an inversed trend. In relation to the initial concentration, the *k* value decreased with increasing FLX concentrations. The addition of SA to the culture medium increased the rate of the reaction, being this increase more pronounced for the *S*- than for the *R*-enantiomer. In the first supplementation of the cumulative degradation experiment, the higher amount of SA resulted in increased *k* values at 45 µM of FLX.

Table 5.1. First-order rate constant (*k*) and half-life (*t1/2*) for degradation of FLX enantiomers by *L. portucalensis* F11

Supplied FLX concentration (µM)	*S*-FLX		*R*-FLX	
	k (d^{-1})	*t1/2* (d)	*k* (d^{-1})	*t1/2* (d)
sole carbon source				
2	0.074	9.37	0.113	6.13
4	0.069	10.05	0.090	7.70
9	0.039	17.77	0.051	13.59
FLX + 5.9 mM SA				
4	0.106	6.54	0.293	2.37
9	0.103	6.73	0.204	3.40
21	0.083	8.35	0.174	3.98
45	0.086	8.06	0.142	4.88
89	0.083	8.35	0.098	7.07
FLX + 20 mM SA				
45	0.092	7.53	0.155	4.47

5.3.5. *Identification of the intermediate produced during FLX degradation*

The formation of one major degradation intermediate was detected on the culture supernatants by HPLC analysis and was identified as one enantiomer of the nor-fluoxetine (NFLX) (Figures 5.2, 5.3 and 5.4). The formation of only one enantiomer of NFLX corroborates with the enantioselective biologically mediated process. The concentration of metabolite detected was dependent on FLX initial concentration; never exceeding 0.73 µM

and being transiently accumulated. This is indicative of further transformation of this metabolite, which is in agreement with the observed liberation of fluoride.

5.4. DISCUSSION

Due to their physicochemical properties, FLX is very persistent and significant concentrations of this pharmaceutical have been detected in environmental samples, including effluents of wastewater treatment plants, rivers, streams and even in drinking water (Benotti et al., 2009; Zorita et al., 2009; Fernández et al., 2010; Schultz and Furlong, 2008). This is a particular motif of concern due to its high toxicity and tendency to bioaccumulate (Nakamura et al., 2008; Nałęcz-Jawecki, 2007; Brooks et al., 2005). Despite the fact that this compound is used in large amounts, there are few studies concerning its biodegradation and reports on enantioselective biodegradation are rare (Ribeiro et al., 2012). Borges et al. (2009) reported the failure of biotransformation of FLX to NFLX by endophytic fungi whereas Rodarte-Morales et al. (2011) reported 23 to 46% degradation of 1 mg l^{-1} (3.2 µM) of FLX in two weeks by white-rot fungi. Redshaw et al. (2008) found FLX and NFLX resistant to degradation in both soil or liquid cultures containing microbial populations representative of sewage sludge-amended soils. On the other hand, Suarez et al. (2010) indicated transformations of >75% under aerobic and >65% under anoxic conditions of FLX fed at 20 µg l^{-1} (0.6 µM) in reactors inoculated with biomass collected from a conventional activated sludge pilot plant. Vasskog et al. (2009) reported depletion of FLX, supplied to a final concentration of 7.2 mg kg^{-1} during sewage sludge composting but it was not clear in their work if it was due to chemical and/or biological degradation.

In this study, *L. portucalensis* F11 was shown to be able to complete biodegrade the two enantiomers of 2 µM of racemic FX supplied as sole carbon source. Overall biodegradation of FLX was achieved at various extents according to initial FLX concentration, in the range of 4 to 89 µM, but was always higher than 80% with at least 35% of the expected fluoride being released. The FLX concentrations tested as single carbon source could not support the growth of bacteria. The supplementation with SA, a conventional carbon source, resulted in cells growth and increased biodegradation rate constants. These results suggest

co-metabolism, i. e., transformation of a non-growth substrate in the presence of a growth substrate, which supports cell growth by generating energy and carbon polymers (Ziagova and Liakopoulou-Kyriakides, 2007). This is a common mechanism approach for the biodegradation of micropollutants (Delgadillo-Mirquez et al., 2011). In the presence of SA, the half-life of the *S*-enantiomer varied between 6.5 and 8.4 days and the half-life of the *R*-enantiomer varied between 2.4 and 7.1 days (for 4 - 89 µM of racemic FLX) whereas when FLX was supplied as single carbon source higher values, between 9.4 and 17.8 days for the *S*-FLX and between 6.1 and 13.6 days for *R*-FLX (for 2 - 9 µM of racemic FLX), were obtained.

It was not observed any evidence of toxic effect of FLX on *L. portucalensis* F11 cells. The growth pattern observed in co-metabolism was almost equal at all FLX concentrations tested, showing that FLX did not affect growth even at higher concentrations. Moreover, the cells started degrading the compound from the beginning of the experiments, without any pre-induction needed, showing no acclimation period, and maintained actively degrading the compound even after 50 days at higher concentrations. However, the *k* values decreased with the increase in FLX concentration, both when the cells were cultivated with FLX as single carbon source and in co-metabolism with supplementary carbon source. For the same FLX concentration, *k* was higher when SA was added. In the experiments supplemented with SA, the transformation of FLX accompanied the decrease in the optical density (OD_{600}) of the culture, suggesting that the decrease in biodegradation rate is probably due to energy limitations for cells growth and maintenance. This hypothesis is also supported by the results obtained in the cumulative degradation experiments, in which the degradation after the first supplementation with FLX and SA was higher than that obtained for the same concentration of FLX with lower SA concentration. Additionally, the FLX biodegradation that had ceased 6 days after the second supplementation, restarted with the third supplementation with FLX and SA.

Enantioselectivity is normally observed in biological systems (Liu et al., 2005). In this study, FLX was degraded in an enantioselective manner by *L. portucalensis* F11, with preferential degradation of the *R*-enantiomer. Enantioselective degradation implies that the enzymes involved in the transformation process discriminate the enantiomers. There are no reports on enantioselective biodegradation of FLX by a single bacterium in the literature. A study of FLX in wastewater collected from a WTTP revealed that its influent was more enriched in *R*-

enantiomer than the effluent, suggesting the preferential degradation of this enantiomer in wastewater (MacLeod et al., 2007). However, it is necessary to take in account that influent and effluent samples do not necessarily represent the same plug of water and that the EF in the influent does not necessarily remain constant.

During FLX biodegradation by *L. portucalensis* F11, one enantiomer of NFLX was detected as intermediary metabolite, which was further degraded, as indicated by the stoichiometric liberation of fluoride. NFLX was also identified as intermediary of FLX degradation by Vasskog et al. (2009) however the enantioselectivity was not accessed in this study. NFLX is the metabolite produced in the human body during the metabolism FLX and it is also active as serotonin reuptake inhibitor (Hiemke and Härtter, 2000). NFLX is highly toxic, reported to be 50% more toxic than the parent compound (Nałęcz-Jawecki, 2007). It is thus of major importance that NFLX is further metabolized by strain F11 and did not accumulate during FLX degradation.

According to Sanchez and Hytell (1999), in mammals FLX is metabolized to NFLX by cytochrome P450. It is interesting to notice that an enantioselectivity similar to that evidenced by strain F11 was observed by one isoform of human cytochrome P450 (CYP2C9) that preferentially catalyses *R*-FLX demethylation (McDonagh et al., 2011). *L. portucalensis* F11 is able to degrade aromatic compounds through dihydroxylation of the aromatic ring by a Rieske non-heme iron oxygenase (Carvalho et al., 2006a). Enzymes from this family resemble cytochrome P450 in its ability to catalyze monohydroxylation, sulfoxidation, desaturation, dehydrogenation, and *O*- and *N*-dealkylation reactions (Gibson et al., 1995; Resnick et al., 1996). This suggests that *N*-demethylation of FLX to NFLX by strain F11 could be performed by its Rieske non-heme iron oxygenase.

5.5. CONCLUSIONS

In this study, biotransformation of FLX was complete, without accumulation of the intermediate NFLX, as indicate by the stoichiometric fluoride release achieved at the lower substrate concentration. This feature makes *L. portucalensis* F11 a potential candidate for devising biodegradation technologies able to deal with contamination by this

pharmaceutical. The observed changes in EF gave conclusive evidence of the enantioselective biodegradation, in which the *R*-enantiomer was preferentially degraded over the corresponding *S*-enantiomer. To our knowledge, this is the first time that complete and enantioselective biodegradation of FLX by a single bacterium is reported.

CHAPTER 6

CONSTRUCTION OF A GENOMIC LIBRARY OF

LABRYS PORTUCALENSIS F11

Abstract

Labrys portucalensis F11 is a Gram-negative bacterium with the ability to biodegrade a wide range of aromatic compounds. This strain possesses an aromatic dioxygenase enzyme that dihydroxylates the aromatic ring of fluorobenzene (FB) yielding two different fluorinated dihydrodiols. Aromatic dioxygenases are enzymes with particular interest for its central role in the biodegradation of xenobiotic compounds and for the production of valuable chiral building blocks for industry. Using the vector pLAFR3, a genomic library consisting of 960 cosmid clones was prepared in Escherichia coli VCS 257 with DNA extracted from L. portucalensis F11. Screening for FB dioxygenase genes was conducted using two different approaches: a PCR-based method and a colorimetric method based on activity. From the screening results it was assumed that the FB dioxygenase genes were not present in the constructed cosmid library. However, the F11 cosmid library is a useful tool for the generation and confirmation of sequence data as well as for comparative genomics and other postgenomic applications. It represents a resource for present and future L. portucalensis research projects.

6.1. INTRODUCTION

Labrys portucalensis strain F11 was isolated from a sediment sample collected from an industrially polluted site in northern Portugal, due to its capacity to degrade fluorobenzene (FB) as sole carbon and energy source (Carvalho et al., 2005). Metabolic versatility studies demonstrated that strain F11 is also able to degrade benzoate, benzene, phenol, 2-fluorobenzoate, 4-fluorobenzoate, and 4-fluorophenol, with the last two compounds being completely defluorinated (Carvalho et al., 2005). Further studies reported the ability of this strain to degrade difluorobenzenes (Moreira et al., 2012, Chapter 4 of this thesis) and chlorobenzene (Chapter 3 of this thesis), apparently using the same degradative enzymes used in the metabolism of FB.

The metabolic pathway for FB degradation by *L. portucalensis* F11 starts by the dihydroxylation of the aromatic ring by an aromatic dioxygenase (FB dioxygenase), leading to the production of two different fluorinated dihydrodiols: 4-fluoro-*cis*-benzene-1,2-dihydrodiol and 1-fluoro-*cis*-benzene-1,2-dihydrodiol (Carvalho et al., 2006a). Aromatic dioxygenases are enzymes of particular interest for two reasons. First, aromatic compounds are common environmental contaminants for which removal by microorganisms represents a potential solution (Gibson and Parales, 2000). Dioxygenase-containing bacterial strains have been used for the transformation of aromatic environmental pollutants to nonaromatic compounds (Cavalca et al., 2004; Parales and Haddock, 2004). Second, the literature has highlighted the importance of *cis*-dihydrodiols as potential chiral building blocks for a large number of applications, such as fine chemicals, pharmaceuticals and a wide variety of bioactive natural products (Boyd et al., 2001; Gibson et al., 1995; Arthurs et al., 2007; Gibson and Parales, 2000; Ouyang et al., 2007; Ward et al., 2004). Bacterial dioxygenases are able to incorporate two hydroxyl groups into the aromatic substrates under mild conditions simply using molecular oxygen, allowing a convenient production of *cis*-diols via a feasible biotransformation process (Wahbi et al., 1996). This is in contrast to the difficulties in synthesizing these compounds by purely chemical reactions (Reddy et al., 1999; Quintana and Dalton, 1999).

Genomic libraries are a powerful resource for genetic studies of bacteria; there are various examples of successful application of this technology in sequencing projects, for the map-based cloning of target genes or genomic regions, physical mapping, and comparative genomics (Dorella et al., 2006; Wu et al., 2012; Tauch et al., 2002; Feng et al., 2005). Presently, many cloning vectors are available for constructing a genome library. Cosmids allow cloning and maintenance of DNA fragments of about 40 kb in bacteria using the conventional bacteriophage-based transfection method to deliver the constructs into bacterial cells (Zhang and Wu, 2001; Collins and Hohn, 1978). For some purposes a cosmid vector is the best choice. Although the insert size is smaller compared to the frequently used BAC or PAC clone, it is easier to handle and has fewer restriction fragments making analyses or modifications easier. Therefore, a cosmid clone is useful for detailed analyses of a restricted region (Ohtsuka et al., 2002).

This study describes the construction of a *L. portucalensis* F11 cosmid library in pLAFR3 (Staskawicz et al., 1987) and its screening for the FB dioxygenase gene through a sequence-based Polymerase Chain Reaction (PCR) strategy and using a colorimetric method to detect dioxygenase activity (Joern et al., 2001).

6.2. MATERIAL AND METHODS

6.2.1. Bacterial strains and plasmids

Labrys portucalensis strain F11, a wild-type strain isolated for its ability to grow on FB (Carvalho et al., 2005), was the source of the total genomic DNA. *E. coli* VCS 257 (Stratagene, USA) was used as host strain for transduction. The cosmid vector pLAFR3 (Staskawicz et al., 1987) was used as cloning vector for F11-derived genomic DNA fragments.

6.2.2. *Media and growth conditions*

Luria-Bertani medium (LB) was used for the cultivation of bacteria except where noted otherwise. Cultures of *L. portucalensis* F11 and *E. coli* VCS 257 were carried out at 30ºC and 37ºC respectively, on horizontal shakers at 200 rpm. When appropriate, tetracycline was added in concentrations of 15-25 μg ml^{-1}.

6.2.3. *Genomic DNA preparation*

For isolation of total genomic DNA from wild-type *L. portucalensis* F11, cells were resuspended in Lysis buffer (10 mM NaCl, 20 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.5% SDS, 100 μg/ml Proteinase K and 10 mg/ml RNAse) and incubated at 55ºC overnight. DNA was purified by phenol-chloroform extraction and isopropanol precipitation. Precipitated DNA was ressuspended in TE buffer pH 7.4 (10 mM Tris, 1 mM EDTA). DNA concentration was determined by measuring the absorbance at 260 nm. Genomic DNA was partially digested with restriction enzyme *Sau*3A1 (0.03 U μg^{-1} DNA; New England BioLabs, UK) at 37ºC for 30 minutes. Digested DNA was again purified by phenol-chloroform extraction and isopropanol precipitation.

6.2.4. *Cosmid vector preparation*

Extraction of cosmid vector DNA from *E. coli* HB101 was performed using the QIAfilter Plasmid Maxi Kit (Qiagen, Germany) according to the manufacturer's instructions. Afterwards, 20 μg of pLAFR3 was digested with 40 U of restriction enzyme *Bam*HI (New England BioLabs, UK) for 2 hours at 37ºC, and desphosphorylated with 1 U of alkaline phosphatase (New England BioLabs, UK) at 37ºC for 30 minutes. Cosmid vector DNA was purified by phenol-chloroform extraction and isopropanol precipitation and analysed by agarose gel electrophoresis.

6.2.5. Library construction

Ligation of pLAFR3, cut with *Bam*HI, with *Sau*3A1 digested genomic DNA was performed by T4 DNA ligase (New England BioLabs, UK) overnight at 16ºC. Packaging of ligation reactions in bacteriophage λ particles was performed using the Packagene Lambda DNA Packaging System (Promega, USA) strictly following the manufacturer's protocol. *E. coli* VCS 257 (Stratagene, USA) was used as host strain for titration of the cosmid packaging reaction following the manufacturer's instructions. Cosmid clones were plated on LB agar plates containing 15 µg ml^{-1} of tetracycline.

Analysis of the generated cosmid library was performed by cosmid digestion with *Eco*RI (New England BioLabs, UK). Recombinant cosmid DNA from *E. coli* clones was isolated by the alkaline lysis miniprep method as described in Sambrook and Russel (2001). Recombinant clones were picked into 96-well microtiter plates (MTPs) containing LB supplemented with 25 µg ml^{-1} tetracycline. The plates were incubated at 37ºC for 24 hours. Sterile glycerol to a final concentration of 15% was then added. The library was stored at -80ºC.

6.2.6. Screening of the genomic library for FB dioxygenase genes

6.2.6.1. Sequence-based screening by PCR

Sequence-based screening of the genomic library was performed by PCR using primers designed based on a 300 bp DNA fragment, previously identified using degenerate primers for conserved regions of dioxygenase genes (Ferreira, 2007). A first PCR was carried out using primers F11DO-FW (5'-GTTGTTATCATGGTTGGACCTTCTCC-3') or F11DO-FW2 (5'-GGCTCCTGAAGGTGAAGGACG-3') in combination with F11DO-RV (5'-CCGTCATAGATGTAGGAGGAATTGC-3') and a second one using primers F11DO-FW3 (5'-GGTGAAGGACGAAAAGACCA-3') and F11DO-RV2 (5'-CTTCCTTGAGACTGCCGAAC-3') designed for an internal sequence part. A pool of each MTP with *E. coli* library clones was used to isolate recombinant cosmid DNA by the alkaline lysis miniprep method as described in Sambrook and Russel (2001). These cosmid DNA pools served as templates for initial PCR reactions. Positive pools were broken down into rows and then in columns of the MTPs to

determine a set of addresses corresponding to potential clones, which were subsequently used in the second PCR reaction on individual clone-level. DNA amplification was performed with Taq DNA Polymerase (New England BioLabs, UK), Phusion High-Fidelity DNA Polymerase (FINNZYMES, Finland) and Go Taq Flexi DNA Polymerase (Promega, USA) as recommended by the respective manufacturers.

6.2.6.2. Activity-based screening by a colorimetric method

Solid-phase screening for dioxygenase activity was performed by a modification of the method described by Joern et al. (2001). Plates containing *E. coli* clones of the F11 genomic library were incubated at 37ºC overnight. Colonies were lifted with a nitrocellulose membrane and transferred to M9 media plates (Sambrook et al., 1989) containing 2% agar, 25 μg ml^{-1} tetracycline, 2% glycerol and 80 mg l^{-1} FeSO$_4$.7H$_2$O. The colonies were then incubated for 1-7 h at 30ºC under a FB atmosphere. Afterwards, the membrane was transferred to a 2% agarose plate also containing 0.025% Gibbs reagent (2,6-dichloro-*p*-benzoquinone), added as a 2% solution in ethanol.

6.2.7. Chemicals

All chemicals used were of the highest available purity (Sigma-Aldrich Chemie, Steinheim, Germany; Merck, Darmstat, Germany).

6.3. RESULTS AND DISCUSSION

The initial strategy to obtain the complete sequence of the FB dioxygenase genes was the construction of a cosmid library prepared from genomic DNA of *L. portucalensis* F11. It was constructed by using the vector pLAFR3 and partial *Sau*3A-digested genomic DNA fragments. The ligation mixture was packaged in lambda phages and introduced into *E. coli* VCS 257 by transfection. The obtained library consisted of 960 clones which were stored in 10 microtiter plates at -80ºC. Restriction endonuclease analysis (*Eco*RI) of 10 randomly chosen clones showed that 90% of the plasmids carried an insert.

Generally, two types of strategies are used to identify clones carrying desired genes from a genomic library: function-based screening and sequence-based screening. In order to find the desired FB dioxygenase genes, screening of the genomic library was first conducted using PCR. The necessary primers were designed based on a 300 bp fragment of a dioxygenase gene from F11, previously identified by Ferreira et al. (2007), showing 79% nucleotide sequence identity to the Rieske domain of the α subunit of a chlorobenzoate dioxygenase from *Burkholderia* sp.. Optimal conditions for PCR employing three different polymerases were first established using the genomic DNA of strain F11. The same conditions were applied afterwards in the sequence-based screening of the cosmid library of F11 in order to identify clones containing the dioxygenase gene. However, it was not possible to obtain amplification of a DNA fragment of correct size with any of the pLAFR3 clones.

In the functional screening approach, the library is subjected to activity-based assays. A screening methodology based on the activity of the FB dioxygenase was tested using a colorimetric assay as described by Joern et al. (2001). In this assay, the arene *cis*-diol products formed by the dioxygenase are either converted to phenolic compounds through acidification or converted to catecholic compounds through subsequent reaction with *cis*-dihydrodiol dehydrogenase, which is co-expressed with the dioxygenase. This step is then followed by colorimetric detection of the products after reaction with 2,6-dichloro-*p*-benzoquinone (Gibbs reagent) giving blue-colored colonies. Since there was the possibility that the DNA fragments generated from total DNA of strain F11 could contain the dioxygenase gene coupled to the *cis*-dihydrodiol dehydrogenase gene, the assay for screening of the F11 cosmid library was performed with and without acidification. However, also in this case no color development was obtained for any of the genomic library clones, indicating that there was no dioxygenase activity present. This could be due to the lack of dioxygenase genes in the library or the lack of their recombinant expression in *E. coli*. In fact, the major drawback of the function-based screening is that in order to work efficiently, the genes of interest must comply with the expression machinery provided by the heterologous screening host with respect to transcription, translation and protein folding (Gabor et al., 2004). The minimal set of requirements for gene expression in a heterologous host cell include the presence of functional cis-acting DNA sequences (promoter, ribosome binding site) and of trans-acting factors that need to be provided by

the host organism such as special transcription factors, tRNAs for rare codons, chaperones, cofactors or protein-modifying enzymes (Angelov et al., 2009).

The failure in identifying FB dioxygenase genes can be due to the absence of these genes in the constructed cosmid library, which would mean that not the whole F11 genome sequence is covered by the library. In spite of the fact that cosmid libraries have played a crucial role in genome sequencing projects (Kunst et al., 1997; Brosch et al., 1998), it was reported before that some regions of the respective chromosomes were apparently not represented in the library. For example, a cosmid library carrying chromosomal DNA of *Streptomyces coelicolor* represented the entire genome with the exception of three short gaps (Redenbach et al., 1996). Furthermore, the cosmid library of the *C. glutamicum* chromosome was very biased such that 18 regions of the genome with a total size of 436 kb (13.3%) were not covered despite 28-fold genome coverage of the library (Tauch et al., 2002). It is also possible that the desired dioxygenase sequence is present in the library but for some reason the applied screening methods did not work owing to problems with PCR amplification and/or with expression of the FB dioxygenase genes in *E.coli*.

6.4. CONCLUSIONS

A genomic library of *L. portucalensis* F11 was constructed. Despite the failure in identifying the FB dioxygenase genes of F11, the generated genomic library can be very useful for future work, e.g., for the generation and confirmation of sequencing data, comparative genomics or, when searching for other genes of interest since strain F11 exhibits extraordinary biodegradation capabilities.

CHAPTER 7

CLONING, NUCLEOTIDE SEQUENCE AND EXPRESSION OF FLUOROBENZENE DIOXYGENASE FROM *LABRYS PORTUCALENSIS* F11

Abstract

Labrys portucalensis strain F11 is able to grow aerobically with fluorobenzene as the sole source of carbon and energy. Fluorobenzene degradation proceeds via ortho-cleavage pathway with formation of 4-fluoro-cis-benzene-1,2-dihydrodiol by fluorobenzene dioxygenase in the first step. A partial nucleotide sequence of the putative gene cluster involved in fluorobenzene degradation was determined. Sequencing results revealed the presence of four open reading frames, namely the gene coding for 1,2-catechol dioxygenase and three genes encoding a ring-hydroxylating dioxygenase (alpha and beta subunit of the dioxygenase component and the oxidoreductase component). Blastp revealed a high degree of similarity (≥80% identity) to the components of benzoate dioxygenase. The conserved amino acid residues that are involved in cofactor binding were also identified in the protein sequence. The putative fluorobenzene dioxygenase genes were cloned into different vectors and transformed into several expression strains, resulting in different recombinants, which were tested for expression of the dioxygenase by SDS-PAGE analysis and for substrate conversion under different conditions.

7.1. INTRODUCTION

Rieske non-heme iron oxygenases (ROs) comprise a large class of aromatic ring-hydroxylating dioxygenases that are responsible for the generation of *cis*-dihydroxylated metabolites in the first step of the bacterial degradation of many aromatic compounds (Ferraro et al., 2005). The composition and the subunit characteristics of this dioxygenase system vary considerably. However, all of them consist of a hydroxylase and an electron transfer component. The hydroxylase component itself is composed of two subunits: an alpha subunit of about 50 kDa and a beta subunit of about 20 kDa. The oxygenase alpha subunit contains an N-terminal Rieske domain with a [2Fe-2S] cluster and a C-terminal catalytic domain with a mononuclear Fe(II) binding site. Conserved Cys and His residues in the N-terminal region may provide 2Fe-2S ligands, while conserved His and Tyr residues may coordinate the iron. The Rieske [2Fe-2S] cluster accepts electrons from the electron transfer component and transfers them to the mononuclear iron for catalysis (Harayama et al., 1992). The electron transfer component is composed of two subunits, a ferredoxin and a ferredoxin reductase, or of a single bifunctional ferredoxin/reductase subunit. The reductase transfers two electrons from NAD(P)H to the oxygenase resulting in the activation of a dioxygen for substrate dihydroxylation (Senda et al., 2007). No functional role except for a structural one has been attributed to the beta subunit of the oxygenase component (Kauppi et al., 1998).

The study of ROs is important for two reasons. First, in the development of bioremediation technologies, since these enzyme systems catalyze essential reactions in bacterial degradation pathways of many aromatic compounds considered environmental pollutants. In addition, many of these enzymes have broad substrate ranges and many of the obtainable products are enantiomerically pure compounds that are difficult to synthesize by standard chemical methods (Parales, 2003). Furthermore, these molecules are interesting as potential chiral building blocks for various applications.

Labrys portucalensis F11 is able to grow aerobically with fluorobenzene (FB) as the sole source of carbon and energy. FB degradation proceeds via *ortho*-cleavage pathway with formation of 4-fluoro-*cis*-benzene-1,2-dihydrodiol by fluorobenzene dioxygenase (FBD) in

the first step (Carvalho et al., 2006a) (Figure 7.1). In the present study, the genes encoding the enzyme system that catalyzes this conversion have been presumably identified; they were given the distinct name *fbdABC*. The three genes encoding the two subunits of the terminal oxygenase and the NADH: acceptor reductase component, were cloned and sequenced. Putative cofactor-binding domains were identified in the deduced amino acid sequences of FbdABC. Furthermore, protein expression and substrate conversion by recombinant strains were tested at different conditions.
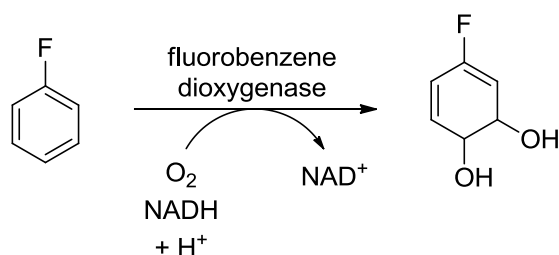
Figure 7.1. Reaction catalyzed by fluorobenzene dioxygenase from *L. portucalensis* F11, with fluorobenzene as a substrate.

7.2. MATERIAL AND METHODS

7.2.1. Bacterial strains and plasmids

L. portucalensis F11, which had been isolated by selective enrichment on fluorobenzene as the sole source of carbon and energy, has been described previously (Carvalho et al., 2005). *E. coli* BL21 (DE3) (Promega, USA), *E. coli* C41 (DE3) (Lucigen, USA), *E. coli* C43 (DE3) (Lucigen, USA), *E. coli* Top10 (Invitrogen, USA) and *E. coli* JM109 (Promega, USA) were used as host strains (Table 7.1). *Pseudomonas putida* and *Pseudomonas fluorescens* used as host strains were a kind gift from Prof. Patrizia Di Gennaro from Department of Genetics and Biology of Microorganism, University of Milan, Italy.

Cloning vectors pGEM-T Easy (Promega, USA), TOPO (Invitrogen, USA) and pZero-2 (Invitrogen, USA); expression vectors pET-28a(+) (Novagen, Germany), pIT2 (Schallmey et al., 2011), pBAD*N* (Jin et al., 2011), and pACYCDuet™-1 (Novagen, Germany); as well as

vectors pKJE7, pGKJE8, pGTf2 and pGro7 (Takara, Japan), encoding chaperone proteins have been described previously (Table 7.1).

Table 7.1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Bacterial strains		
L. portucalensis F11	FB$^+$	Carvalho et al., 2005
E. coli BL21 (DE3)	F$^-$, ompT, hsdSB (rB$^-$, mB$^-$), dcm, gal, λ(DE3), pLysS, Cmr, T7	Promega, USA
E. coli C41 (DE3)	F$^-$, ompT, hsdSB (rB$^-$ mB$^-$), gal, dcm (DE3), T7	Lucigen, USA
E. coli C43 (DE3)	F$^-$, ompT, hsdSB (rB$^-$ mB$^-$), gal, dcm (DE3), T7	Lucigen, USA
E. coli Top10	F$^-$, mcrA, Δ(mrr-hsdRMS-mcrBC), Φ80lacZΔM15, ΔlacX74, recA1, araD139, Δ(araleu),7697 galU, galK, rpsL (StrR), endA1, nupG	Invitrogen, USA
E. coli JM109	endA1, recA1, gyrA96, thi, hsdR17 (rk$^-$, mk$^+$), relA1, supE44, Δ(lac-proAB), [F′, traD36, proAB, laqIqZΔM15]	Promega, USA
P. putida	FB$^-$	Prof. Di Gennaro
P. fluorescens	FB$^-$	Prof. Di Gennaro
Plasmids		
pGEM-T Easy	Amp^r, T7	Promega, USA
TOPO	Amp^r, Kn^r	Invitrogen, USA
pZero-2	Kn^r, T7	Invitrogen, USA
pET-28a(+)	Kn^r, T7	Novagen, Germany
pIT2	Tc^r	Schalmey et al., 2011
pBAD*N*	Amp^r	Jin et al., 2008
pACYCDuet™-1	Cm^r, T7	Novagen, Germany
pKJE7	dnaK-dnaJ-grpE, Cm^r	Takara, Japan
pGKJE8	dnaK-dnaJ-grpE groES-GroEL, Cm^r	Takara, Japan
pGTf2	groES-groEL-tig, Cm^r	Takara, Japan
pGro7	groES-groEL, Cm^r	Takara, Japan
pABC1	pBAD*N* carrying *fbdABC*, Amp^r	This work
pABC2	pIT2 carrying *fbdABC*, Tc^r	This work
pA1	pIT2 carrying *fbdA*, Tc^r	This work
pA2	pET-28a (+) carrying *fbdA*, Kn^r	This work
pBC	pAcycDuet-1 carrying *fbdBC*, Cm^r	This work

Abbreviations: FB+, degradation of FB; r, resistance; Amp, ampiciline; Kn, kanamycine; Tc, tetracycline; Cm, chloramphenicol.

7.2.2. Media and growth conditions

For genomic DNA isolation, an overnight culture (30ºC) of *L. portucalensis* F11 in Luria-Bertani (LB) medium (Sambrook et al., 1989) was used. Expression experiments for SDS-PAGE and for substrate bioconversion were performed using recombinant clones at 25, 30 or 37ºC in Terrific Broth (TB) medium (Sambrook et al., 1989) or LB medium supplemented with 20 µM FeCl$_3$; in mineral salts medium (M9) supplemented with glucose or glycerol (0.4% final concentration) as carbon source; or, in a minimal medium with higher amounts of iron (MME) (Endoma et al., 2002). Where appropriate, tetracycline, ampicillin, chloramphenicol and kanamycin were added to the media at final concentrations of 10, 100, 50 and 30 µg ml^{-1}, respectively. In relation to inducers, IPTG (isopropyl-β-D-thiogalactopyranoside) was supplied at concentrations of 0.4 mM (*E. coli*) or 0.5-5 mM (*Pseudomonas*). L-arabinose was supplied at a concentration of 0.02 – 0.4 % (w/v).

7.2.3. DNA isolation, PCR amplification and nucleotide sequencing

For isolation of total genomic DNA from wild-type *L. portucalensis* F11, cells were resuspended in lysis buffer (10 mM NaCl, 20 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.5% SDS, 100 µg ml^{-1} Proteinase K and 10 mg ml^{-1} RNAse) and incubated at 55ºC overnight. DNA was purified by phenol-chloroform extraction and isopropanol precipitation.

The 5'- and 3'-ends of the fluorobenzene dioxygenase (Fbd) genes were cloned by cassette ligation-mediated PCR with the TaKaRa LA PCR in vitro cloning kit (Takara Bio Inc., Japan) as follows. Two oligonucleotide pairs of primers S1 and S2, complementary to the known region of the dioxygenase gene previously identified (Ferreira, 2007), were used: S1-FW (5'-CCTGAAGGTGAAGGACGAAAAGACCAC-3'); S1-RV (5'-CACGTCTTCCTTGAGACTGCCGAAC-3'); S2-FW (5'-GTTCGGCAGTCTCAAGGAAGACGTG-3'); and, S2-RV (5'-GTGGTCTTTTCGTCCTTCACCTTCAGG-3').

Two separately complete digestions of F11 genomic DNA were performed, one with *Hind*III (New England BioLabs, UK) and other with *Pst*I (New England BioLabs, UK) restriction enzymes, at 37ºC overnight. The digested DNA was then ligated to the cassettes with the corresponding restriction sites following the manufacturer's instructions. The two cassettes

were used to perform two sets of PCR − each in the forward and in the reverse direction; one first PCR using cassette primer C1 and primer S1 of the known region and, a second PCR using cassette primer C2 and primer S2 complementary to inner sequences of the known region. After the second PCR, one amplified fragment in the forward direction, obtained from *Hind*III cassette and another amplified fragment in the reverse direction, obtained with *Pst*I cassette, were extracted from the agarose gel and purified by Gel Purification (Qiagen, Germany). The *Hind*III fragment was ligated to the TOPO vector, using the TOPO TA Cloning Kit for sequencing (Invitrogen, USA), and transformed in *E. coli* Top 10 chemically competent cells (Invitrogen, USA). The *Pst*I fragment was used to set up a ligation with *Eco*RV (New England BioLabs, UK) digested pZero-2 vector (Invitrogen, USA), and transformed in electrocompetent Top10 cells (Invitrogen, USA), following the manufacturer's instructions.

Obtained transformants were individually inoculated in microtiter plates for colony PCR screening. Two positives transformants from each transformation were used for plasmid isolation using the QIAprep Spin Miniprep Kit (Qiagen, Germany), in order to confirm the presence of an insert of the right size by PCR and restriction digest. Two plasmids from each transformation were sent for sequencing (GATC, Germany).

7.2.4. Nucleotide sequence analysis

Sequence alignments were performed using BioEdit software (Abbott Laboratories, USA). Open Reading Frames (ORFs) were analysed using the ORF finder program (NCBI, USA) and searches for nucleotide and amino acid sequence similarities were done using the BLAST program (NCBI, USA).

7.2.5. Cloning of FB Dioxygenase genes

Primers, containing restriction sites for cloning, were designed based on sequencing results of *fbdABC*: alpha subunit, DO-*Nde*I-FW (5'-AAATGTATC<u>CATATG</u>TCCGCAATCATCGACAAGG-3'), DO-*Hind*III-RV (5'-ATTATTTG<u>AAGCTT</u>ATGCGACCACTCCCTTC-3'); beta subunit, B-*Nde*I-FW

(5'-AAATAA<u>CATATG</u>CCGGCCTGGGACGAC-3'), B-*Xho*I-RV (5'-TATATT<u>CTCGAG</u>TTAGATGTGGTAGACGTCGATGACCTGG-3'); reductase, R-*Nco*I-FW (5'-TATTAA<u>CCATGG</u>CCTTCTACACGATCGCATTG-3'), , R-*Bam*HI-RV (5'-ATTATT<u>GGATCC</u>TTATAGGGAGAAGCTTGCGGGC-3'); fragment containing the three genes, DO-*Nde*I-FW and DOR-*Sac*I-RV (5'-AATATT<u>GAGCTC</u>TTATAGGGAGAAGCTTGCGGG-3'). Amplification was performed using F11 genomic DNA as template and Phusion High-Fidelity DNA Polymerase (FINNZYMES, Finland). Amplified fragments were purified using Gel Purification (Qiagen, Germany), digested with the respective restriction enzymes and purified by PCR Purification (Qiagen, Germany). Cloning vectors were digested with respective restriction enzymes and purified by Gel Purification (Qiagen, Germany). The ligation reactions, using T4 DNA ligase (New England BioLabs, UK), were performed as follows: DNA encoding the alpha subunit was ligated with pIT2, yielding vector pA1, and with pET-28a(+), yielding vector pA2; DNA encoding the beta subunit and DNA encoding the oxidoreductase were ligated to two different cloning sites of pACYCDuet™-1, yielding vector pBC; one fragment containing all the three genes was ligated with vector pBADN, yielding pABC1 , and vector pIT2, yielding pABC2 (Table 7.1).

Ligation products were transformed into chemically competent *E. coli* Top10 cells. Transformants were screened by colony PCR using 10 random clones. The presence of an insert of the right size was confirmed, after plasmid extraction using QIAprep Spin Miniprep Kit (Qiagen, Germany), by additional PCR, using extracted plasmid as template and restriction digestion. One positive plasmid per transformation was sent for sequencing to confirm the identity of the genes.

7.2.6. Recombinant expression of FbdABC

Chemically competent *E. coli* cells were prepared following the rubidium chloride method according to Hanahan (1983). Vectors pA1 and pA2, each in combination with vector pBC, were individually transformed into the chemically competent host strains *E. coli* BL21 (DE3), *E. coli* C41 (DE3) and *E. coli* C43 (DE3) (Miroux and Walker, 1992). Vectors pABC1 and pABC2 were individually transformed into the chemically competent host strains *E. coli* BL21 (DE3), *E. coli* C41 (DE3), *E. coli* C43 (DE3), *E. coli* Top10 and *E. coli* JM109, resulting in

16 different vector-strain combinations. Chemically competent host strain *E. coli* BL21 (DE3) was also transformed with vectors for chaperone expression (pKJE7, pG-KJE8, pG-Tf2 and pGro7), individually combined with vector pABC1.

Chemically competent *Pseudomonas* cells were prepared following the protocol described by Chuanchuen et al. (2002). Transformation was performed according to Gallie (2010). Chemically competent *Pseudomonas* strains were transformed with vector pABC2.

Recombinant expression of FbdABC was performed in 50 ml medium, containing the necessary antibiotic, and inoculated 1:100 with an overnight culture of the respective recombinant strain. After growth at 37ºC (*E. coli*) or 25ºC (*Pseudomonas*), the appropriate inducer, (L-arabinose for pBADN plasmid and IPTG (Isopropyl β-D-1-thiogalactopyranoside) for the others, was added when OD_{600} reached 1 (*E. coli*) or 0.5-0.7 (*Pseudomonas*). The cultures were further incubated at 25, 30 or 37ºC. Different amounts of inducer were tested. Addition of 0.5 mM of sorbitol was also tested in TB medium. Samples were taken at different time points to check the expression on Tris-Tricine SDS-PAGE gels. The samples were treated with B-PER Bacterial Protein Extraction Reagent (Thermo Scientific, USA), following the manufacturer's instructions.

7.2.7. Tris-Tricine SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Schägger and von Jagow (1987), using a 10% T–3% C separating gel with a 5% T–3% C stacking gel (T denotes the total percentage concentration of both monomers [acrylamide and bisacrylamide] and C denotes the percentage concentration of the cross-linker relative to the total concentration T). Protein bands were stained with Coomassie brilliant blue R-250 dissolved in aqueous 45% (v/v) methanol-10% (v/v) acetic acid and, destained in 45% (v/v) methanol-10% (v/v) acetic acid.

7.2.8. Substrate bioconversion by recombinant cells

7.2.8.1. Substrate bioconversion by E. coli growing cells

Growing cells of *E. coli* recombinant strains were tested for substrate conversion in 50 ml medium containing the appropriate antibiotic and inoculated 1:100 with an overnight culture of the respective recombinant strain. After growth at 37ºC, the respective inducer was added when OD_{600} reached 1 and FB (1 or 3 mM) was added as substrate. The cultures were further incubated at 25, 30 or 37°C. Supplemental addition of oxygen or air during experiments was also tested. Samples were taken at different time points and analysed by GC, TLC and Gibbs' reaction to check the conversion. Benzoic acid and 2-chlorobenzoic acid (each 1 mM) were also tested as substrates in MME. Samples were taken at different time points and conversion was analysed by HPLC. Controls with *E. coli* cells harbouring the respective empty vectors as well as controls without substrate addition were performed under the same culture conditions.

7.2.8.2. Substrate bioconversion by Pseudomonas growing cells

Growing cells of *Pseudomonas* recombinant strains were tested for FB conversion in 50 ml M9 medium containing the appropriate antibiotic and inoculated 1:100 with an overnight culture of the respective strains. After growth at 25ºC, 1.5 mM of IPTG was added when OD_{600} reached 0.5-0.7. FB was supplied at the same time at 1 mM final concentration. The cultures were further incubated at 25ºC. Samples were taken at different time points and analysed by GC to check the conversion.

7.2.8.3. Substrate bioconversion by E. coli resting cells

In order to test the conversion by resting cells, the recombinant strains were incubated in 50 ml LB medium containing the necessary antibiotic and inoculated 1:100 with an overnight culture of the respective *E. coli* strain. After growth at 37ºC, the appropriate inducer was added when OD_{600} reached 1. The cultures were further incubated for 24h at 37ºC. Cells were harvested by centrifugation at 4000 rpm for 15 min at 4ºC, washed twice with 50 mM potassium phosphate buffer (pH 7.4) and resuspended in a final volume of 50 ml M9 medium. FB was added as substrate at 3 mM final concentration. Samples were taken for analysis by GC, TLC and Gibbs' reaction. Controls with *E. coli* cells harbouring the

respective empty vectors as well as controls without substrate addition were performed under the same culture conditions.

7.2.8.4. Substrate bioconversion by E. coli cell free extracts

For preparation of cell free extracts, cultures obtained by expression at 30 and 37ºC in TB medium were harvested by centrifugation at 4000 rpm for 15 min at 4ºC. Cells were washed twice with 50 mM potassium phosphate buffer (pH 7.4) and resuspended in a final volume of 3 ml of the same buffer, with addition of 0.2 mM PMSF (Phenyl Methane Sulfonyl Fluoride). Cells were disrupted by sonication on ice (15 cycles with 15 s on – 30 s off, at 60% amplitude) (Vibracell VCX130) and centrifuged at 4600 rpm for 25 min, at 4°C, yielding the cell free extracts. The bioconversion reaction was performed using 30 mM FB, with addition of 0.05 mM NADH, 0.57 U ml^{-1} FDH (formate dehydrogenase) and 0.15 M sodium formate, in a reaction volume of 1 ml, at 30ºC for 22h.

7.2.9. Analytical methods

7.2.9.1. GC analysis

FB was analysed by gas chromatography using a gas chromatograph Varian CP-3800 (Agilent Technologies, California, USA) and a CP-Wasc 52 CP capillary column (Chrompack International B.V., Middelburg, The Netherlands), with a temperature programme starting at 50ºC for 3 min, increasing to 165ºC at a rate of 20ºC min^{-1} and reaching the final temperature of 265ºC at a rate of 50ºC min^{-1}. Injector and detector temperatures were set to 250ºC. Bioconversion samples (each 1 ml) were extracted with 0.5 ml of ethyl acetate by vortexing the extraction tube for 1 min at maximum speed, followed by centrifugation at 13500 rpm for 5 min at 4ºC. The organic layer was analysed by split injection of 1 μl samples. The concentration of FB was calculated using previously established calibration curves.

7.2.9.2. HPLC analysis

Biomass was previously removed from culture samples by centrifugation at 13200 rpm for 5 min at 4ºC. Benzoic and 2-chlorobenzoic acid were analysed by high-performance liquid

chromatography, on a System Gold 126 (Beckman Coulter, Fullerton, USA) using a reversed-phase C18 column (Nucleosil 150 mm x 2.1 mm, 5 μm particle size, Grace, Lokeren, Belgium). Elution was carried out at a flow rate of 0.8 ml/min with acetonitrile/water containing 1 mM H_3PO_4 (50:50). Substrates and products were monitored at 230 nm by the use of a diode array detector (DAD). Samples of 20 μl were analysed.

7.2.9.3. TLC analysis

Bioconversion samples were, acidified with 0.5 M HCl, extracted with 0.5 equivalent volume of ethyl acetate for three times and dried with Na_2SO_4. Organic phases were collected and evaporated to a residue of about 100 μl. For thin-layer chromatography (TLC) analyses, pre-coated plastic Polygram SilG/UV254 sheets were used (Macherey & Nagel, Düren, Germany). The mobile phase consisted of heptane:ethyl acetate (1:1, vol/vol). For the detection of products, the TLC sheets were stained with phosphomolybdic acid, *p*-anisaldehyde or a solution of 2% Gibbs reagent (2,6-dichloro-*p*-benzoquinone) in methanol.

7.2.9.4. Gibbs' reaction

Biomass was previously removed from culture samples by centrifugation at 13200 rpm for 5 min at 4ºC. The test for dioxygenase activity was performed by a modification of the method described by Joern et al. (2001). An equivalent volume of 0.1 M HCl was added to 420 μl of sample supernatant. This mixture was incubated at 37ºC for 30 min, and then 84 mM of Tris-HCl (pH 8.5) was added to raise the pH. At this point, 150 μl of 0.4% Gibbs reagent in ethanol was added. The absorbance at 652 nm was measured after incubation for 40 min at room temperature.

7.2.9.5. Fluoride analysis

Biomass was previously removed from culture samples by centrifugation at 8000 rpm for 10 min. The concentration of fluoride ions in the sample supernatant was measured with an ion-selective combination electrode (model Orion 96-09, Thermo Electron Corporation, Beverly, MA), which was calibrated with NaF (0.01 to 5 mM) in MM. The ionic strength of the standards and of the samples was adjusted with a buffer solution (total ionic strength adjustment solution – TISAB). The composition of the TISAB solution was: 1 M NaCl, 0.25 M CH_3COOH, 0.75 M $NaCH_3COO$ and 0.002 M sodium citrate.

7.2.10. Chemicals

All chemicals were obtained from Sigma-Aldrich (Steinheim, Germany) and Merck (Darmstadt, Germany) in the highest available purity.

7.3. RESULTS

7.3.1. PCR amplification and cloning of FBD gene sequence

In order to obtain the complete sequence of the genes encoding FBD, specific primers (S1 and S2) complementary to the 300 bp fragment obtained by Ferreira (2007) were designed following the guidelines described in the TaKaRa LA PCR in vitro cloning kit. In the following PCR, a DNA fragment was amplified in the forward direction, using the ligation product of *Hind*III-digested genomic DNA of *L. portucalensis* F11 and the *Hind*III cassette linker as template. This fragment was then inserted into the TOPO vector and sequenced. In the reverse direction a DNA fragment was amplified using the ligation product of *Pst*I-digested genomic DNA and the *Pst*I cassette linker as template. This fragment was then inserted into the pZero-2 vector and sequenced.

The alignment of the sequencing results, obtained in forward and reverse direction with the 300 bp fragment obtained previously was performed using BioEdit software in order to obtain a complete nucleotide sequence of 4425 bp (Figure 7.2). This sequence revealed the presence of four open reading frames (ORFs), which were oriented in the same direction (Figure 7.3). The function of each ORF was deduced from sequence homology to genes in the GenBank database (NCBI, USA) identified by Blast searches. Likewise, the translated protein sequences were compared with sequences in the non-redundant (nr) protein sequence database (NCBI, USA). The reactions putatively catalyzed by these proteins during FB metabolism are shown in Figure 7.4.

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AAATAACTACTATAGGGCGATTGGGCCCTCTAGATGCATGCTCGAGCGGCCGCCAGTGTGATGGATAATACGACTCACTATAGGGAGACTGCAGCGCAGC      100

GGCAAGTGGCGGCATATCTGGCGTATTGCCGGTGCCTACGCGAACTACAGCGTGTTCGATGTGAAGGACAATGCGGAACTGCACGAAATCCTTTCGGGTC      200

TGCCGCTGTTCAAGTTCATACGTATCGAGGTGGCGCCGCTGCTGCGGCACCCCTCCTCGATCCGCGACGGTGATGGCTGACATCACCGCGCCCGGGGCGG      300
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<div align="center">SD   *catA*</div>

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CCATGATGACGAGGCCCCCATAAATTCAAAAGCAAAAAATGGAGGATTGAAATGAGCGTGAAGATCTTCGCAAGGCCGGACATCCAGGATTTCCTGAAGG      400

TCCTGAGCGGCCTCGACAAGGACGGTGGCAATCCGCGCGTCAAGCAAGTCGTCCATCGGATCATGTCGGACCTGTTCAAGGCGATCGACGATCTCGATAT      500

CACCCCGGACGAATACTGGACAGCCATCGCCTGGCTCAACGACATCGGCGCGGCCGGACAAGCGGGCCTGATTTCCCCCGGCCTCGGCCTCGATCACTTC      600

CTCGACGAACGCCTCGACGCGATCGACGCAGAGCTCGGCATCGACAACCCGACACCACGCACGATCGAGGGACCGCTCTATGTCGCCGGAGCGCCGGTCC      700

CACATGGCTTCGCCCGCCTCGACGACGGCACGGATACGAACGGCCATACGCTGATCATGCACGGCACCGTCCGCGGCGCCGACGGCCGGCCGCTGCCGGG      800

GGCGACCGTCGAGGTCTGGCACTGCGACACCCGCGGCTTCTACTCGCATTTCGATCCGACCGGAAAGCAGGCGCCGTTCAACATGCGCCGCACCATCATT      900

GCCGACGGCGAGGGCCGCTACAAATTCCGCAGCATCGTGCCGAACGGCTATGGCGTACCGCCGGGCAGCCCGACGGAAAAGCTGCTCTCCGCCCTTGGCC      1000

GCCATGGCCAGCGCCCGGCCCATATCCACCTCTTCATCAGCGGCGAAGGCCATCGCAAGCTGACGACTCAGATCAACATCGAGGGTGATCCGCTGGTCAA      1100

TGACGACTTCGCCTATGCCACCCGCGACGGCCTCGTTCCAGCCGTCGTCGAGCGGACGGACGCGGCCAGCATCGCGGCCAATGGGCTGGAGGGGCCCTTC      1200
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GCCGAGATCGTGTTCGACATCCATCTGACCGCACTGGTCGACGGCGTCGACAACCAGATCAACGACCAGCGCAAGCGCGCCGTAGCTTGATTGTTACTGG      1300

GCAGCGCGACCTCAACGGCACGGTGAGCAAAGTCTGCCTCGAAGACATCGCACTCTGCTCCAGTCTGTACAGACACCGGCGGCCCTGAGGGCCGTCCCAC      1400
```

<div align="center">SD   *fdbA*</div>

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CCCGATCATCGACATCGTTGCAAGGGCCGCGCCGACCGCCGTCCCCTGCGGGAGAGAAGTCATGTCCGCAATCATCGACAAGGCCCGTGATCTCGATCAT      1500

CTGCTCACCACTGCCGTGCAGGATGATCACGAGGCCAGCGCGTTTCGCTGCCGCCGCGACATCTTCACCGACGCTGACCTGTTCGCGCTGGAGATGAAGC      1600

ACATCTTCGAGAGCAATTGGGTCTATCTGGCCCATGAAAGCCAGATCCCCGACAACAATGACTATTACACGACCACAATCGGCCGCCAGCCGGTGGTGAT      1700

CACCCGCGACAAGACCGGCACGCTCAACGCCGTCACCAATGCCTGCGCGCATCGCGGGGCGATGCTCTGCCGGCGCAAGCACGGCAACAAGGGCAGCTTC      1800

ACCTGCCCGTTCCACGGCTGGACCTTTTCCAACACCGGCAGGCTCCTGAAGGTGAAGGACGAAAAGACCACCCAATATCCCGAGCAGTTCGCCAAGAATG      1900

GCTCGCATGATCTGACCCGCGTTGCGCGCTTCGAGAATTATCGCGGCTTCCTGTTCGGCAGTCTCAAGGAAGACGTGGCACCGCTGACCGACTTCCTCGG      2000

GCAGGCGCGGATCATCATCGACCAGATCGTGGACCAGGCGCCGGACAGGCTGGAGGTCCTGCGGGGCAATTCCTCCTACATCTATGACGGCAACTGGAAG      2100

CTCCAGATGGAGAATGGCTGCGACGGCTACCATGTCTCCTCCGTCCACTGGAACTATGCCGCCACCATGGGCCGCCGCAGCGAGGAAGGCACCAAGGCGG      2200

TCGACGCCAATTCGTGGAGCAAATCGGTCGCCGGCGTCTACGGCTTCGAGAACGGCCATATCCTGCTATGGACCAACACGATGAATCCGGAGGTCCGCCC      2300

CGTCTACGGCCAGCGCGAGAAGATCCGCGAGCGCGTCGGCGCCGACAAGGCCGATTTCATCATCAACCAGACCCGCAATCTGTGCATCTATCCCAACCTC      2400

TTCCTGATGGATCAGTTCAGCACGCAGATCCGCGTGACGCGCCCCATCAGCGTCGACAAGACGGAGGTCAGCATCTTCTGCTTCGCCCCGAAGGGCGAAG      2500
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GTGCGGAAGACCGGGCGCTGCGCATCCGCCAATACGAGGATTTCTTCAACGTCTCCGGCATGGGCACGGCCGACGATCTCGAGGAGTTCCGCGCCTGCCA   2600

GTCCGGCTATGCCGGCACGGCAGCGCTGTGGAACGACCTGTCACGCGGCGCGCCGCTCTGGATCGACGGTCCCGACGACAATGCCAGGCGGATGGGCCTC   2700

GAGCCGCTGCTCTCCGGCGAGCGCAGCGAGGACGAGGGGCTGTTCGTCCGCCAGCACGAATATTGGGCGACGGTGATGCGCCAGGCGCTCGCTGCCGAGA   2800

    SD   ***   *fbdB*
GGAAG<u>GGAG</u>TGGTCGCA<u>TGA</u>GC**CTG**TCCTACGATACCGTTCGCGCATTCCTGTTCCGCGAGGCGCGCCTTCTCGACGATCGCCAATGGGACGAATGGCTG   2900

ACCTGCTATGCGCCCGACGTCATCTATTGGATGCCGGCCTGGGACGACGATGATGCGATCACCGAGGATCCGCATGCGCAAATCTCGCTGATCTACTATC   3000

CGAGCCGGGAAGGCCTGGAGGACCGCGTCTTCCGCATCAAGACCGAGCGGTCCGGCGCTTCGACGCCGGAGCCGTGCACCAGCCACAGCGTCATCAATGT   3100

CGAGGTGACGGCCGACCGGGGTGGCGAAGTGGACGTGCGCTACAATTTCCACACGCTCAACCACCGCTACAAGGTCACCGACCAGTTCTTCGGCACCATG   3200

TTCGTGACGCTGCGCCAGGATGGCGACAAGCTGCTGATATCGAACAAGAAAATCGTCCTGAAGAACGACTATATCCGCCAGGTCATCGACGTCTACCACA   3300

   ***   SD   *fbdC*
TC<u>TGA</u>CGGCACCGGCCGGACAGGAACGAAGA<u>GGAGG</u>GTTCC**<u>ATG</u>**ACCTTCTACACGATCGCATTGAACTTCGAGGATGGGGTCACCCGTTTCGTCGATTG   3400

CAAGCAGGGCGAGAAGGTTCTCGATGCCGCCTTCCGCAACAAGATCAACCTGCCGATGGACTGCTCCGACGGCGTGTGCGGCACCTGCAAGTGCCGCGCC   3500

GAAAGCGGCGCCTACGATCTCGGCAGTGATTTCATCGAGGATGCCCTGACCGAGGACGAGGCGGCGGAGGGCCTGGTGCTGACCTGCCAGATGGTGCCTT   3600

CGAGCGATTGCGTCCTCGCGGTGCCGACGACTTCACTCGCCTGCAAGACCGGCCACCAGAAATTCGCCGCCACGGTGGCGGCCGTGACACAGCATCGCGA   3700

CGCCGCGATCGTGCTGGAACTGGAGGTGGATGCCGCCGCCGCGCCCGTCTTCCTGCCCGGCCAATATGTCAATATCGATGTGCCGGGCAGCGGCCAGAAC   3800

CGCTCCTATTCCTTCTCCTCGGCGCCAGGCGATCGGCGTCTCGGCTTCCTGATCAAGAAGGTCCCCGATGGGGTGATGAGCGGATGGCTCGCCGGGGCCA   3900

AGCCCGGCGACAGGCTCGACCTCACCGGCCCGCTGGGCAGCTTCTATCTGAGGGACGTGAAGCGTCCTCTCCTCTTCCTTGCCGGTGGCACGGGGCTCGC   4000

GCCCTTCCTCTCGATGCTGGAGGTCCTTGCCCGCACGAGGTCAGAGCAGGTGGTCCACCTGATCTATGGCGTCACCCGCGATCTCGATTTGGTTCTCGCC   4100

GACGAAATCGCGGCCTATGCCGCTCGCCTGCCGAACTTCACCTTTACCACCGTGGTGGCCGAGGAAGCCTCCAGCCATCCGCGCAAGGGCTGGGTCACGC   4200

AGCACATGCCCGCCGAGCTTCTGCATGGCGGCGATGTCGACGTCTATCTCTGCGGCCCGCCGCCGATGGTCGATGCGGTGCGCCGGCATTTCGACGAGAA   4300

                        ***
TGGCGTGAAGCCCGCAAGCTTCTCCCTATAG<u>TGA</u>GTCGTATTACGCGTTCTAACGACAATATGTACGCTTCTCCCTATAGTGAGTCGTATTACGCGTTCT   4400

AACGAAGGGCGAATTCGCGGCCGCT   4425

Figure 7.2. Nucleotide sequence of the *catA* and *fbdABC* structural genes and flanking regions from *L. portucalensis* F11. Potential ribosomal binding sites (SD; underlined) are shown. Putative stop codons (***), and initiation codons of *catA* and *fbdABC* (underlined) are indicated.
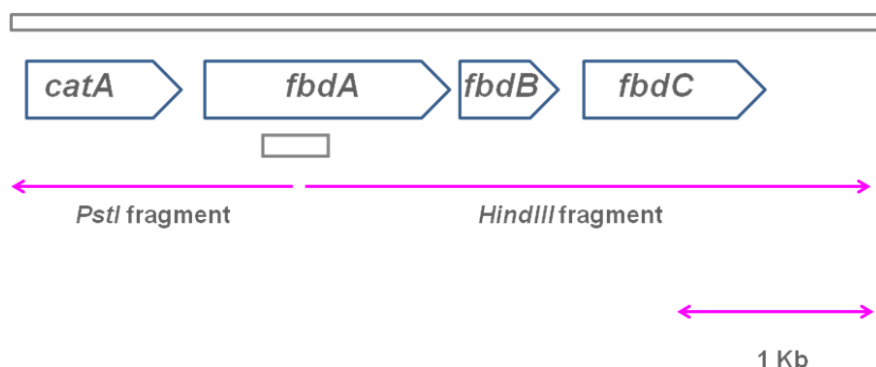
Figure 7.3. Organization of *catA* and *fbdABC* genes from *L. portucalensis* F11. Shown are the locations of the genes and below the DNA fragments that have been cloned for sequencing. Arrows indicate the direction of transcription. Gene products are predicted to be: catechol dioxygenase (*catA*); the α (*fbdA*) and β (*fbdB*) subunits of a terminal dioxygenase and the reductase component (*fbdC*).
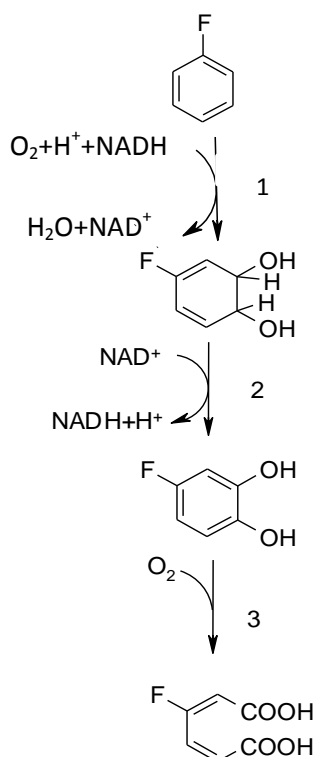
Figure 7.4. Initial reactions of fluorobenzene metabolism by strain F11. The enzyme activities are denoted as follows: 1, fluorobenzene dioxygenase; 2, fluorobenzene dihydrodiol dehydrogenase; 3, fluorocatechol 1,2-dioxygenase

The first ORF, named *catA,* starts at nucleotide 352 (938 bp). The ATG translational start codon of the first ORF is preceded by a putative ribosome binding sequence, 5'-GGAGG-3' (Shine and Dalgarno, 1974). BlastP analysis revealed that CatA is closely related (88% sequence identity) to catechol 1,2-dioxygenase from *Sinorhizobium fredii* HH103 (accession no. YP0051908). Translation would yield a protein with deduced molecular mass of 32.8 KDa, which is in agreement with the size of catechol 1,2-dioxygenase

The second ORF, named *fbdA* starts at nucleotide 1462 (1358 bp). The ATG translational start codon is preceded by the putative ribosome binding sequence 5'-GGAG-3' (Shine and Dalgarno, 1974). The deduced molecular mass of *fbdA*, 51.2 kDa, shows homology to the large subunit of aromatic-ring-hydroxylating dioxygenase systems. Analysis of the translated sequence of *fbdA* showed consensus sequences for the Rieske-type [2Fe-2S] cluster binding region, $CXHX_{17}CX_2H$ (aa 95-118) (Figure 7.5a), and for the catalytic non-heme iron, DX_2HX_4H (aa 221-229) and A372 (Figure 7.5b), which are well conserved among Rieske nonheme iron dioxygenases (Kauppi et al., 1998).

a)

```
        95 97                   115 118
FbdA    VTNACAHRGAMLCRRKHGNKGSFTCPFHGWTFSNTGRL

CbdA    VINACSHRGAELCRRKQGNRSTFTCQFHGWTFSNTGKL

BenA    MINACSHRGAQLCRHKRGNKTTYTCPFHGWTFNNSGKL
```

b)

```
        221 224  229                        273
FbdA    MENGCDGYHVSSVHWNYAATMG     YEDFFNVSGMGTADDLEEFRACQ

CbdA    IENGADGYHVGSVHWNYVATIG     YEDFFNVSGMGTPDDLEEFRACQ

BenA    AENGADGYHVSAVHWNYAATTQ     YEDFFNASGMATPDDLEEFRACQ
```

Figure 7.5. Alignment of the deduced protein sequences of FbdA with the published sequences of CbdA (Haak et al. 1995) and BenA (Neidle et al. 1991). Iron binding motifs in the α-subunits of Rieske nonheme iron oxygenases are indicated. a) Alignment of the Rieske center binding-site. b) 2-His-1-carboxylate motif for binding of the mononuclear iron in the active site. Fbd α-subunit numbering is shown.

The third ORF, named *fbdB* (484 bp), starts at nucleotide 2823. The translational initiation codon at this position is preceded by the ribosome binding sequence 5'-GGAG-3' (Shine and Dalgarno, 1974). The deduced molecular mass of the protein (19 kDa) is in agreement with the molecular mass of the small subunit of the oxygenase component.

The fourth ORF, named *fbdC* (989 bp) starts at position 3342. The translational initiation codon is preceded by the putative ribosome binding site 5'-GGAGG-3' (Shine and Dalgarno, 1974). The deduced molecular mass of the encoded protein, 35.3 kDa, is in agreement with the size of the NADH:acceptor reductase component of Rieske non-heme iron dioxygenases. Analysis of the translated sequence of *fbdC* revealed the CX_4CX_2CXC motif (aa 41-51), characteristic of [2Fe-2S]-binding domains conserved in plant-type ferredoxins, in the N-terminus (Neidle et al., 1991). In the C-terminal region of FbdC, sequences homologous to possible FAD- and NAD-binding domains as reported by Neidle et al. (1991) were identified. In Figure 7.6 the amino acid fingerprints involved in flavin and pyridine nucleotide binding are indicated.

The G+C content of *catA*, *fbdA*, *-B* and *-C* genes, 65, 63, 62 and 65%, respectively, is in accordance with the G+C content previously determined for F11 total DNA (63%) (Carvalho et al., 2008).

BlastP analysis revealed that FbdA, FbdB and FbdC are closely related (≥80% sequence identity) to benzoate dioxygenase from *Sinorhizobium fredii* HH103 (accession no. YP0051908), and to 2-chlorobenzoate dioxygenases from *Rhizobium etli* Kim 5 (accession no. ZP035038) and from *Paracoccus denitrificans* PD1222 (accession no. YP9149).

The FbdA protein sequence was aligned with those from other dioxygenases previously characterized. The phylogenetic tree constructed based on this alignment, showed that FbdA forms a distinct cluster with the large subunits of benzoate and halobenzoate dioxygenase enzymes (Figure 7.7).

7.3.2. Recombinant expression of FbdABC

Different vectors and different *E. coli* expression strains were tested to identify the best combination for recombinant expression of FbdABC. To check protein expression by SDS-PAGE, all recombinant *E. coli* clones were incubated in TB medium, containing the necessary antibiotic. Expression was tested at 25, 30 and 37ºC after addition of the appropriate inducer and samples were taken over time. Samples pre-treated with B-Per reagent were loaded on Tris-tricine SDS-PAGE gels. For some recombinants expression of three bands, with molecular masses in accordance with the deduced molecular masses of the three Fbd subunits, could be identified. The most pronounced expression was observed for *E. coli* C41 (DE3) harboring pABC1. However, most of the expressed protein was observed in the pellet fraction and not in the soluble protein fraction. In Figure 7.8 two bands with molecular masses in accordance with beta subunit and NADH:acceptor reductase component are visible on both fractions whereas the band with molecular mass in accordance with the alpha subunit is only visible in the pellet fraction.

Different approaches were tested in order to get soluble protein. Since higher amounts of protein were obtained in expressions using vector pABC1, new transformations of this vector were performed in *E. coli* BL21 (DE3), *E. coli* C41 (DE3), *E. coli* C43 (DE3) and *E. coli* Top10. The newly obtained recombinants were tested for expression as described above, with addition of 0.2% L-arabinose as inducer. Best expression was again obtained with *E. coli* C41 (DE3) (pABC1) at 30ºC, but most of the protein was found in the pellet fraction. A freshly transformed *E. coli* C41 (DE3) (pABC1) was tested again under the same conditions but with a second addition of 0.2% L-arabinose during expression. This second addition was tested to ensure enough amount of inducer in the culture medium, since *E. coli* C41 can use arabinose as carbon source. However, no effect of this second addition on protein expression was observed.

The expression with *E. coli* C41 (DE3) (pABC1) was repeated in TB medium with addition of 0.5 M sorbitol. It was also tested in M9 medium containing 0.4% glucose or glycerol as carbon source and in MME media (Endoma et al., 2002). However, no improvements of soluble protein expression could be observed. *E. coli* C41 (DE3) harboring chaperone vectors and pABC1 was also tested for expression but the expressed proteins were again predominantely observed in the pellet fraction.

```
                        10        20        30        40        50        60
FbdC       ----------MTFYTIALNFEDGVTRFVDCKQGEKVLDAAFRNKINLPMDCSDGVCGTCK
BenC       MSLYLNRIPAMSNHQVALQFEDGVTRFIRIAQGETLSDAAYRQQINIPMDCREGACGTCR
CbdC       ----------MLHSIALRFEDDVTYFITSSEHETVADAAYQHGIRIPLDCRNGVCGTCK
                 : :**.***.** *:    : *.: ***::: *.:*:** :*.****:

                                                               |-[2Fe-2S]-
FbdC       CRAESGAYDLG-SDFIEDALTEDEAAEGLVLTCQMVPSSDCVLAVPTTSLACKTGHQKFA
BenC       AFCESGNYDMPEDNYIEDALTPEEAQQGYVLACQCRPTSDAVFQIQASSEVCKTKIHHFE
CbdC       GFCEHGEYDGG--DYIEDALSADEAREGFVLPCQMQARTDCVVRILASSSACQVKKSTMT
                .* * **    ::*****: :** :* **.**   . :*.*. : ::* .*:.   :

           Binding Region-----------------|
FbdC       ATVAAVTQHRDAAIVLELEVD-AAAAPVFLPGQYVNIDVPGSGQNRSYSFSSAPGDRRLG
BenC       GTLARVENLSDSTITFDIQLDDGQPDIHFLAGQYVNVTLPGTTETRSYSFSSQPGNRLTG
CbdC       GQMTEIDRGSSSTLQFTLAID-PSSKVDFLPGQYAQLRIPGTTESRAYSYSSMPGSSHVT
           . :: : .  .::: : : :*   .   **.***.:: :**: :.*:**:** **.

              |-FAD -ppi-binding region--|           |-FIB--|
FbdC       FLIKKVPDGVMSGWLAG-AKPGDRLDLTGPLGSFYLRDVKRPLLFLAGGTGLAPFLSMLE
BenC       FVVRNVPQGKMSEYLSVQAKAGDKMSFTGPFGSFYLRDVKRPVLMLAGGTGIAPFLSMLQ
CbdC       FLVRDVPNGKMSGYLRNQATITETFTFDGPYGAFYLREPVRPILMLAGGTGLAPFLSMLQ
           *:::.**:* ** :*    *.   : : : ** *:****:   **:*:*****:*******:

               |--NAD-ppi-binding region---|  |--NAD-ribose binding
FbdC       VLA--RTRSEQVVHLIYGVTRDLDLVLADEIAAYAARLPNFTFTTVVAEEASSHPRKGWV
BenC       VLE--QKGSEHPVRLVFGVTQDCDLVALEQLDALQQKLPWFEYRTVVAHAESQHERKGYV
CbdC       YMAGLQRNDLPSVRLVYGVNRDDDLVGLDKLDELATQLSGFSYITTVVDKDSAQLRRGYV
           :    :   .  *:*::**.:* *** :::     :*. * : *.*.. * : *:*:*

           region--|
FbdC       TQHMPAELLHGGDVDVYLCGPPPMVDAVRRHFDENGVKPASFSL---------
BenC       TGHIEYDWLNGGEVDVYLCGPVPMVEAVRSWLDTQGIQPANFLFEKFSAN---
CbdC       TQQITNDDMNGGDVDIYVCGPPPMVEAVRSWLAAEKLNPVNFYFEKFAPTVGN
           * ::  : ::**:**:*:*** ***:***  :  : ::*..* :
```

Figure 7.6. Alignment of the deduced protein sequence of FbdC with the published sequences of BenC (Neidle et al., 1991) and CbdC (Haak et al., 1995). Conserved amino acid residues are indicated (*). The regions of possible cofactor binding domains as reported by Neidle et al. (1991) are shown. FIB, FAD-isoalloxazine ring-binding domain; ppi, polyphosphoinositides.
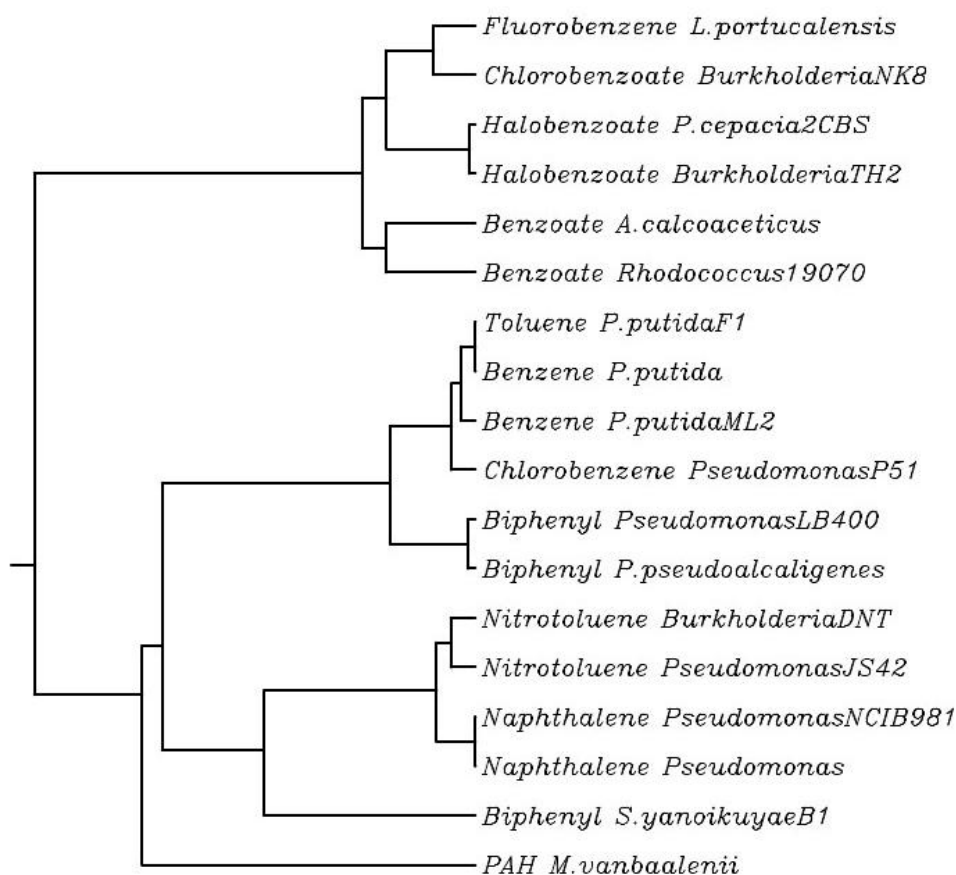
Figure 7.7. Phylogenetic analysis of *L. portucalensis* F11 FbdA and other dioxygenase alpha subunits. The dendrogram was constructed from a ClustalW protein sequence alignment of the different alpha subunits. Gene clusters are associated with the metabolism of the indicated compounds: Chlorobenzoate, *Burkholderia* sp. NK8 (BAB21463.1); Halobenzoate, *P. cepacia* 2CBS (Q51601.3); Halobenzoate, *Burkholderia* sp. (TH2 BAB21584.1); Benzoate, *A. calcoaceticus* (P07769.2); Benzoate, *Rhodococcus* sp. 19070 (AAK58903.1); Toluene, *P. putida* F1 (A5W4F2.1); Benzene, *P. putida* (P0C618.1); Benzene, *P. putida* ML2 (Q07944.1); Chlorobenzene, *Pseudomonas* sp. P51 (AAC43632); Biphenyl, *Pseudomonas* LB400 (P37333.3); Biphenyl, *P. pseudoalcaligenes* (Q52028.1); Nitrotoluene, *Burkholderia* sp. DNT (AAB09766.1); Nitrotoluene, *Pseudomonas* sp. JS42 (AAB40383.1); Naphthalene, *Pseudomonas* sp. NCIB 9816-4 (P0A110.1); Naphthalene, *Pseudomonas* (P0A111.1); Biphenyl, *S. yanoikuyae* B1 (ABM91740.1); Polycyclic Aromatic Hydrocarbon, *M. vanbaalenii* (AAY85176.1). Protein accession numbers are indicated in parentheses.
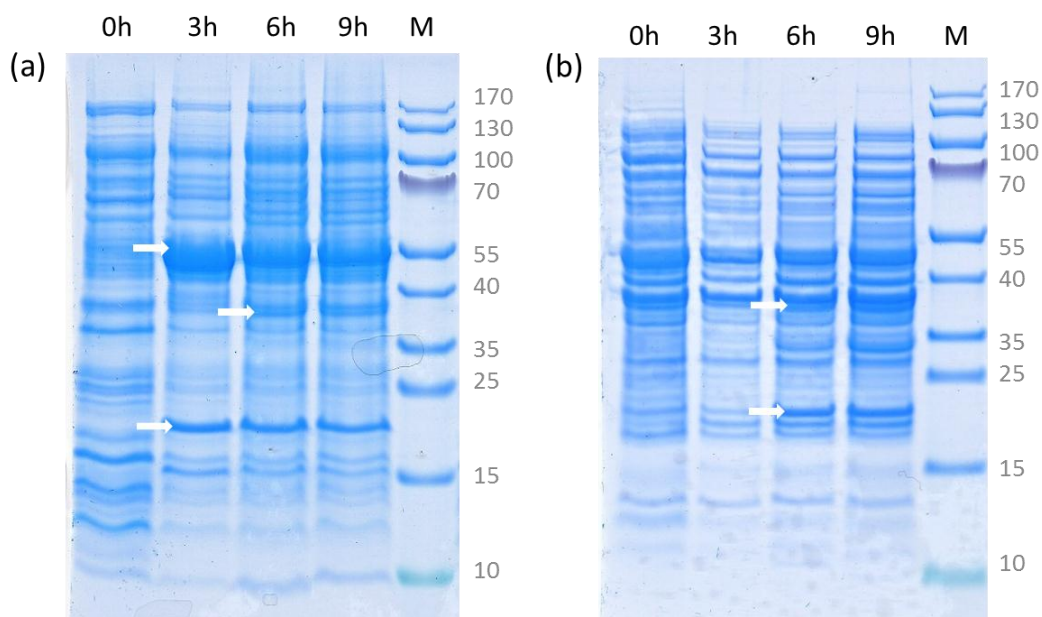
Figure 7.8. SDS-PAGE analysis of recombinant expression of FbdABC in *E. coli* (pABC1) at 30ºC. (a) Pellet and (b) soluble fraction after B-Per treatment. M, molecular weight marker.

In none of these experiments the results obtained were better than those presented in Figure 7.8.

The expression by recombinant *Pseudomonas* strains harboring vector pABC2 was tested by incubating the cells in LB medium, containing the necessary antibiotic, at 25 and 30°C. Different IPTG concentrations (0.5-5 mM) were tested as inducer, however, no expression of FbdABC could be observed on SDS-PAGE gels.

7.3.3. Substrate bioconversion by recombinant strains

Growing cells of *E. coli* C41 (DE3) (pABC1), *E. coli* BL21 (DE3) (pABC1), *E. coli* BL21 (DE3) (pA1+pBC), *E. coli* C43 (DE3) (pABC2) and *E. coli* C43 (DE3) (pA1+pBC) were tested for their ability to convert 1 mM FB in M9 medium, in closed flasks to avoid FB evaporation. Conversion was also tested with freshly transformed *E. coli* strains in M9 and in MME media using 1 and 3 mM FB. Supplementary addition of pure oxygen and air was tested in conversions of FB using *E. coli* C41 (DE3) (pABC1) in M9 medium. Furthermore, resting cells

of *E. coli* C41 (DE3) (pABC1) were tested in conversions with 3 mM FB. Conversion of 3 mM FB by cell free extracts was also tested. Cell free extracts of *E. coli* C41 (DE3) (pABC1), *E. coli* BL21 (DE3) (pA1+pBC) and *E. coli* C43 (DE3) (pA1+pBC) were obtained after expression at 30 and 37ºC.

The results obtained in all these experiments revealed a decrease in FB concentration with time; although different extents were observed depending on conditions tested, the decrease was consistently observed in replicate experiments and not observed in the controls. The higher extent of FB decrease was observed for growing cells *E. coli C41* (DE3) (pABC1) in M9 medium at 30ºC, in which 75% of 1 mM of FB was depleted in 96 hours (Figure 7.9). Fluoride release, indicative of partial or complete degradation of FB, was not observed, suggesting that some fluorinated product or products were formed. However, no intermediate products were detected with the analytical methods used.
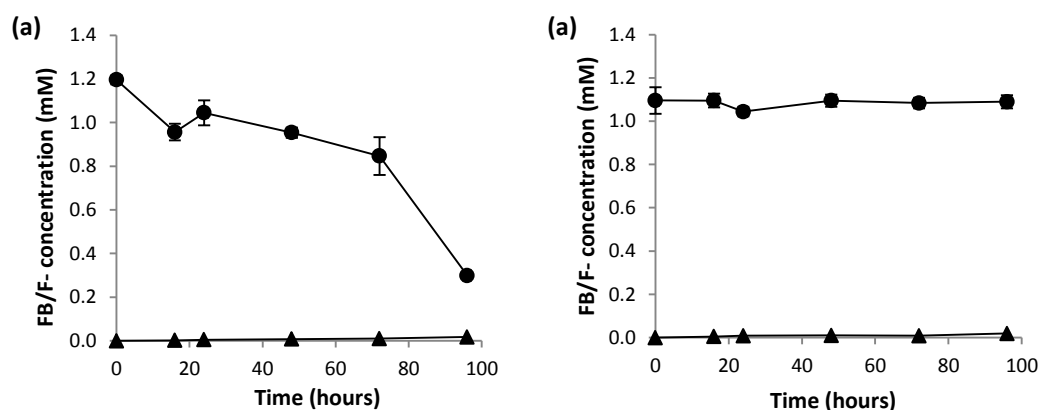
Figure 7.9. Typical FB degradation by growing cultures of (a) *E. coli* C41 (DE3) (pABC1) and (b) *E. coli* C41 (DE3) (pBAD*N*) in M9 medium at 30ºC when supplemented in batch mode with 1 mM of FB. The experiment was performed in triplicate. FB concentration in the culture medium (●) and fluoride release (▲) are indicated.

The genetic similarity of FbdABC to benzoate and 2-chlorobenzoate dioxygenases suggested that the enzyme could prefer the same substrates. For this reason, 1 mM benzoic acid and 2-chlorobenzoic acid were tested in conversions using growing cells of *E. coli* C41 (DE3) (pABC1) in M9 and MME media. No conversion of benzoic acid was observed. However, using 2-chlorobenzoic acid partial substrate depletion was observed and a new peak

(retention time 4.8 min), possibly corresponding to the *cis*-dihydrodiol product, was detected by HPLC. This peak was not observed on the controls. Unfortunately, the identity of this peak could not be confirmed because this compound was not clearly resolved by HPLC under the experimental conditions that were used. GC-MS measurements of the samples indicated the presence of chlorophenol, which could have been formed from the corresponding dihydrodiol by water removal and rearomatization in the GC, due to high temperature.

Bioconversions of FB (1 mM) by recombinant *Pseudomonas* cells harboring pABC2 were tested using growing cultures in M9 medium at 25ºC. During the experiments no FB decrease could be observed.

7.4. DISCUSSION

In this study, the nucleotide sequence of a 4 kb DNA fragment of *L. portucalensis* F11 is reported. This fragment contains the genes *fbdABC* and *catA* putatively encoding the metabolic enzymes for the dihydroxylation of FB and the *ortho*-cleavage of the corresponding catecholic intermediate, respectively. Tight clustering of the structural genes *fbdABC*, which encode the large (FbdA) and small (FbdB) subunits of the oxygenase component and the NADH:acceptor reductase component (FbdC), all transcribed in the same direction, strongly suggest that they belong to a single operon.

Phylogenetic analysis showed that FbdA is closely related to the large subunits of benzoate and halobenzoate dioxygenases, forming a distinct cluster, and is more distantly related to dioxygenases involved in the metabolism of other aromatic compounds. FbdA shares 80% sequence identity on protein level with chlorobenzoate dioxygenase from *Burkholderia* sp. NK8 and 58-60% with other benzoate/halobenzoate dioxygenases. The shared sequence identity to all other dioxygenases included in the analysis is only 18-21% (Figure 7.7).

In order to compare FbdABC with other dioxygenase systems, two dioxygenases that have been investigated thoroughly were chosen based on the high sequence identity found between FbdABC, benzoate and 2-halobenzoate dioxygenase systems. Thus, the deduced

amino acid sequences of the protein components FbdABC were compared with those of the chromosomally-encoded benzoate 1,2-dioxygenase from *A. calcoaceticus* (BenABC) (Neidle et al., 1991) and with those of the conjugative plasmid pBAH1-encoded 2-halobenzoate 1,2-dioxygenase from *Pseudomonas cepacia* 2CBS (CbdABC) (Haak et al., 1995). The overall sequence identity on protein level of FbdABC with BenABC and CbdABC was 53.6% and 54.0%, respectively. Comparisons of the single components FbdA and BenA, FbdA and CbdA, FbdB and BenB, FbdB and CbdB, FbdC and BenC, and FbdC and CbdC revealed amino acid sequence identities of 57.5, 63.0, 52.5, 58.0, 49.8, and 41.0%, respectively. These results suggest that FbdABC, BenABC and CbdABC are related.

The deduced amino acid sequences of the *fbdABC* coding region were analyzed for potential cofactor binding sites. The alpha subunit of ring-hydroxylating dioxygenases consists of two domains, a Rieske domain and a catalytic domain. The Rieske domain contains a Rieske [2Fe-2S] center bound by two cysteine and two histidine residues. In FbdA, the motif $CXHX_{17}CX_2H$ is likely involved in coordinating this Rieske-type iron-sulfur cluster. The motif is conserved among the large subunits of dioxygenases such as 2-halobenzoate 1,2-dioxygenase (Haak et al., 1995), diterpenoid dioxygenase (Martin and Mohn, 1999), naphthalene dioxygenase (Parales, 2003), polycyclic aromatic hydrocarbon dioxygenase (Kim et al., 2006) and biphenyl dioxygenase (Chadhain et al., 2007). Additionally, each alpha subunit also contains one atom of mononuclear Fe^{2+} in the active site, which is coordinated by two histidines, one asparagine residue and a water molecule, in a 2-His-1-Carboxylate facial triad. In FbdA, the motif DX_2HX_4H and Asp 372 may coordinate the mononuclear iron ion, as also observed for polycyclic aromatic hydrocarbon dioxygenase (Kim et al., 2006). Furthermore, Asp 221 may be involved in electron transfer (Parales, 2003). All seven mentioned ligand-binding residues are conserved in Rieske non-heme iron oxygenases of which the sequences are known (Parales, 2003).

The small (β) subunit of ring-hydroxylating dioxygenases does not appear to possess any putative cofactor-binding domains and its role in catalysis is unknown. Reports in the literature have suggested that the beta subunits of toluate 1,2-dioxygenase from *P. putida* mt-2 and benzoate 1,2-dioxygenase from *A. calcoaceticus* may be important for the determination of substrate specificity of the enzyme systems (Harayama et al., 1986; Neidle et al., 1991). However, other studies have indicated that the beta subunit of naphthalene

1,2-dioxygenase does not appear to be directly involved in catalysis or substrate specificity. Its main role is probably a structural one (Kauppi et al., 1998).

The number of electron transfer components of multicomponent aromatic oxygenases varies. In naphthalene, toluene, and benzene 1,2-dioxygenases, two redox proteins are involved in the transfer of electrons from NADH to the hydroxylase component. One is a flavoprotein that has NADH-oxidase activity, and the other one is a ferredoxin, carrying a Rieske-type [2Fe-2S] cluster. In contrast, in benzoate and toluate 1,2-dioxygenases, a single protein contains both flavin and a [2Fe-2S] center for electron transfer (Neidle et al., 1991). The N-terminal region of FbdC was found to resemble the sequences of chloroplast-type ferredoxins, as in benzoate and toluate dioxygenases (Neidle et al., 1991; Haak et al., 1995). Residues important for ferredoxin structure and function are conserved, including binding site for [2Fe-2S] clusters with the motif CX_4CX_2C of (Otaka and Ooi, 1989; Beinert, 1990). Binding sites for [2Fe-2S] clusters with the conserved motif $CX_2GXCGXCX_6GX_{8-13}LX_{8-13}C$ and amino acid residues typical of proposed fingerprints involved in flavin and pyridine nucleotide binding are indicated in Figure 7.6. These residues have also been found in the NADH:acceptor reductases of others oxygenases, such as benzoate and 2-halobenzoate dioxygenase (Neidle et al., 1991; Haak et al., 1995), xylene monoxygenase (Suzuki et al., 1991) and polycyclic aromatic hydrocarbon dioxygenase (Takizawa et al., 1994).

7.5. CONCLUSIONS

In this study, depletion of FB by recombinant E. coli strains carrying FbdABC genes was consistently observed suggesting conversion of FB, although formation of another product could not be proven as no intermediary product was detected. Furthermore, the obtained Blast and alignment results strongly suggest that the genes *fbdABC* are in fact encoding a ring-hydroxylating dioxygenase. Sequence similarity to the components of benzoate and halobenzoate dioxygenases suggests that Fbd is related to these enzymes. Although the conversion of benzoate was not successful, with 2-chlorobenzoate partial substrate conversion was in fact observed, but the identity of the product formed could not be confirmed.

CHAPTER 8

GENERAL CONCLUSIONS AND FUTURE WORK

Abstract

Labrys portucalensis F11 is a bacterial strain previously isolated for its capability to degrade fluorobenzene as sole carbon and energy source. Since co-contamination with organic and metals pollutants is an environmental issue of major concern, the effect of three on fluorobenzene biodegradation was assessed. The biodegradation capabilities of strain F11 were extended to other xenobiotic compounds, chlorobenzene, difluorobenzenes and fluoxetine. A genomic library of strain F11 was constructed. The nucleotide sequence of a Rieske non-heme iron oxygenase was determined; the putative fluorobenzene dioxygenase genes were cloned and expression was tested under several different conditions.

This chapter compiles the main conclusions of the study presented in this thesis and presents some suggestions for further research.

8.1. GENERAL CONCLUSIONS

8.1.1. Biodegradation experiments

The effect of three metals, iron, copper and silver, on fluorobenzene (FB) biodegradation by *L. portucalensis* F11 was assessed. At the concentration tested (1 mM), Fe^{2+} revealed a positive effect on bacterial growth and no effect on the degradation of 1 and 2 mM of FB. This result is not surprising, since iron is an important co-factor for several enzymes of cells metabolism. An inhibitory effect on FB biodegradation was observed with the addition of Cu^{2+} and Ag^+ (1 mM). Cu^{2+} completely inhibits cells growth and reduces FB biodegradation by 50% in growing cells (1 and 2 mM of FB) and by 20% in resting cells (2 mM of FB), with liberation of 80 and 45% of the stoichiometric expected value, respectively. Ag^+ was the most potent FB degradation inhibitor, with reduction to one third of FB consumption and no fluoride released in growing cells experiments. In resting cells, the degradation of FB was reduced by 25% and only 20% of expected fluoride was released. In the presence of these two metals, the accumulation of catechol and 4-fluorocatechol as dead-end metabolites was observed. This result suggests inhibition of catechol 1,2-dioxygenase, a key enzyme on FB metabolism, by Cu^{2+} and Ag^+. The results of this study stress the importance to evaluate the impact of co-contamination when designing biodegradation processes.

The capability of strain F11 to degrade other xenobiotic compounds of environmental concern was evaluated. The degradation of chlorobenzene (CB) was achieved in co-metabolism with FB. Cultures of F11 were able to degrade CB when induced by pre-growth on FB or when FB was simultaneously present in the culture medium. Cells previously grown on FB degraded 0.5 mM of CB with liberation of 50% of the stoichiometric chloride and 85% of 1 mM of CB with liberation of 15%. CB degradation rates of 7.95 ± 0.39 µmol l^{-1} day^{-1} and 16.9 ± 0.81 µmol l^{-1} day^{-1}, respectively, were observed. Total degradation of 0.5 mM of each substrate was observed when both were simultaneously present on the culture medium. Concomitant degradation was observed, at degradation rates of 105 ± 6.07 µmol l^{-1} day^{-1} for CB and 126 ± 16.2 µmol l^{-1} day^{-1} for FB, and stoichiometric halogen liberation. The growth yield was lower when both substrates were supplied simultaneously compared to the values obtained when they were individually added, which suggest a phenomenon of

synergistic inhibition. The study of biodegradation of CB, the chlorinated analogue of FB, stressed the importance of the nature of the halogen, especially on the induction of biodegradation.

The biodegradation of difluorobenzenes (DFBs) was evaluated when the compounds were supplied as sole carbon source and in the presence of FB. *L. portucalensis* F11 was able to degrade 1,3-DFB (0.5 mM) as sole carbon source, with stoichiometric liberation of fluoride. The presence of FB in the culture medium increase the velocity of 1,3-DFB degradation and allowed the degradation of 50% of 1,4-DFB, with stoichiometric fluoride release. This result indicates that FB is able to degrade 1,4-DFB in cometabolism with FB. The simultaneous supply of FB and 1,2-DFB resulted in 30% consumption of each substrate but no fluoride was released, indicating that the presence of 1,2-DFB inhibited FB degradation. The effect of cell density and substrate concentration were also investigated. An higher initial cell density showed to be positive for the degradation of 1,4-DFB (0.5 mM) in cometabolism with FB (0.5 mM). Both compounds were completely degraded, with stoichiometric fluoride release. Complete biodegradation was achieved with 1 mM of FB and 0.5 or 1 mM of 1,3-DFB and 1 mM of FB and 0.5 mM of 1,4-DFB. The different biodegradation patterns obtained with each of the DFBs, revealed that the position of the halogen substituents on the aromatic ring have a great influence on its biodegradation.

L. portucalensis F11 was also able to degrade fluoxetine (FLX), a chiral pharmaceutical compound, as sole carbon and energy source. Complete biodegradation of both enantiomers of 2 μM racemic FLX was observed with stoichiometric fluoride release. More than 80% degradation was observed for 4 and 9 μM of racemic FLX, with release of more than 50% of the stoichiometric fluoride. The addition of a supplementary carbon source revealed to have a positive effect on FX degradation. Different results were obtained for the tested concentrations (4, 9, 21, 45 and 89 μM), but the overall degradation was always superior to 80% with at least 35% of the stoichiometric fluoride released. At 4, 9 and 21 μM of racemic FLX, total degradation of both enantiomers were observed. The half-life of biodegradation varies between 5 and 8 days (for 4 and 89 μM) in co-metabolism and between 8 and 16 days (for 2 and 9 μM) in the experiments as single carbon source. Cumulative degradation experiments revealed that stain F11 could tolerate and degrade successive FLX additions. Preferential degradation of the *R*-enantiomer was observed in all

experiments. A single enantiomer of NFLX was identified as major intermediate during FLX degradation. These data demonstrate that enantioselective analysis is required for a full understanding of biological behavior of chiral compounds.

8.1.2. Genetic studies

A genomic library of *L. portucalensis* F11 was constructed with the aim to find the genes encoding the aromatic dioxygenase responsible for the attack of the aromatic ring, named fluorobenzene dioxygenase (Fbd). Aromatic ring-hydroxylating dioxygenases belong to a class of Rieske non-heme iron oxygenases that are responsible for the generation of *cis*-dihydroxylated metabolites in the first step of the bacterial degradation of many aromatic compounds. These enzymes are interesting for two major reasons, for their central role in the biodegradation processes of many xenobiotic compounds and for the production of valuable chiral building blocks. The library was constructed by using the vector pLAFR3 and partial *Sau*3A-digested genomic DNA fragments. The ligation mixture was packaged in lambda phages and introduced into *E. coli* VCS 257 by transfection. The screening for the aromatic dioxygenase genes was performed through a sequence-based Polymerase Chain Reaction (PCR) strategy and using a colorimetric method to detect dioxygenase activity. From the negative screening results it was assumed that the FB dioxygenase genes were not present in the constructed cosmid library. However, the genomic library can be a useful tool for future research projects, for the generation and confirmation of sequencing data, comparative genomics or to search for other genes of interest. This can be very interesting due to extraordinary biodegradation capabilities of this strain.

Due to the failure in the use of the genomic library to find genes encoding the aromatic dioxygenase another approach was taken. Fbd genes were cloned by cassette ligation-mediated PCR with the TaKaRa LA PCR in vitro cloning kit. This strategy allowed the nucleotide sequencing of a 4 kb DNA fragment of *L. portucalensis* F11, containing four open reading frames, namely the gene coding for 1,2-catechol dioxygenase (*catA*) and three genes encoding a ring hydroxylating dioxygenase (*fbdABC*) (alpha and beta subunit of the dioxygenase component and the oxidoreductase component). BlastP analysis revealed that FbdA, FbdB and FbdC are closely related (≥80% sequence identity) to benzoate and

halobenzoate dioxygenases. The phylogenetic tree constructed based with alignment with others previously characterized dioxygenases revealed that FbdA forms a distinct cluster with the large subunits of benzoate and halobenzoate dioxygenase enzymes. The conserved amino acid residues that are involved in cofactor binding were identified in the protein sequence. The putative fluorobenzene dioxygenase genes were cloned into different vectors and transformed into several expression strains, resulting in different recombinants, which were tested for expression of the dioxygenase by SDS-PAGE analysis and for substrate conversion under different conditions. The SDS-PAGE analysis revealed that most of the expressed protein was on the pellet fraction and not on the soluble form. This could be due to improper folding of the enzyme components. In the bioconversion experiments a decrease in the substrate concentration was consistently observed, indicating bioconversion, but it was not possible to detect/ identify the product formed. The obtained Blast and alignment results also also strongly suggest that the sequenced genes *fbdABC* are in fact encoding a ring-hydroxylating dioxygenase, corroborating the suggested bioconversion.

8.2. FUTURE WORK

During the development of this research and from the results obtained, some suggestions for further research are given in this section:

- Further explore the biotransformation capacities of strain F11, e.g,. for other fluorinated drugs,

- Investigate the possibility of using *L. portucalensis* F11 in biaugmentation strategies for the treatment of industrial and domestic wastewaters and on bioremediation of contaminated sites;

- Perform proteomic and metabolic studies to determine the metabolic pathway(s) used for the degradation of the tested xenobiotic compounds;

- Design a synthetic gene to optimize the expression of the alpha subunit of the dioxygenase by *E. coli* strains.

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