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# Biodegradation of fluoroanilines by the wild strain *Labrys portucalensis*

Catarina L. Amorim [a,b], Maria F. Carvalho [a], Carlos M.M. Afonso [b], Paula M.L. Castro [a,*]

[a] CBQF – Centro de Biotecnologia e Química Fina - Laboratório Associado, Escola Superior de Biotecnologia, Universidade Católica Portuguesa/Porto, Rua Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal
[b] CEQUIMED-UP, Laboratório de Química Orgânica e Farmacêutica, Departamento Ciências Químicas, Faculdade de Farmácia da Universidade do Porto, Rua Jorge Viterbo Ferreira n° 228, 4050-313 Porto, Portugal

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ABSTRACT

Aniline and halogenated anilines are known as widespread environmental toxic pollutants released into soil and water. In contrast to aniline, which is rapidly metabolized via catechol, halosubstituted anilines are more resistant to microbial attack. A fluorobenzene-degrading bacterium, *Labrys portucalensis* strain F11, was tested under different culture conditions for the degradation potential towards 2-, 3- and 4-fluoroaniline (2-, 3- and 4-FA). Strain F11 was able to use FAs as a source of carbon and nitrogen however, supplementation with a nitrogen source improved substrate consumption and its dehalogenation extent. When F11 cells were previously grown on fluorobenzene (FB), higher biodegradation rates were achieved for all isomers. Complete 2-FA biodegradation with stoichiometric fluoride release was achieved when FB-induced cells were used. On the other hand, the degradation of 3- and 4-FA was characterized by incomplete defluorination of the target compounds suggesting accumulation of fluorinated intermediates. F11 cultures simultaneously supplied with FB and the fluorinated anilines showed a concomitant degradation of both substrates, suggesting co-metabolic biodegradation. To our knowledge, this is the first time that biodegradation of 2- and 3-FA as a sole carbon and nitrogen source and co-metabolic degradation of FA isomers in the presence of a structural analogous compound is reported.


## 1. Introduction

Environmental contamination by toxic xenobiotic compounds has become a serious worldwide problem. Aromatic amine compounds, many of them with halogenated substituents, such as fluoroanilines, constitute a major class of environmental pollutants that have been released into soil and water due to their extensive use in industries and agriculture (Travkin et al., 2003). These compounds are widely used in the industrial production of dyes, pharmaceutical products, perfumes, pesticides, herbicides and plant growth regulators (Okazaky et al., 2003). They can cause various toxic effects on humans and some may exhibit acute carcinogenic and mutagenic effects (Cao et al., 2009).

Microbial degradation of aromatic pollutants plays an important role in environmental cleanup avoiding their accumulation in the environment Biodegradation of haloaromatic compounds has been extensively studied, however these studies have mainly focused on the microbial degradation of chloroaromatics (Loidl et al., 1990; Boon et al., 2001; Radianingtyas et al., 2003; Vangnai and Petchkroh, 2007) whereas very little is known about the bacterial utilization of fluoroaromatics. In contrast to other haloaromatic compounds, fluoroaromatics are generally considered to be more stable to microbial attack (Key et al., 1997). The incorporation of fluorine into a molecule supplies it a thermal and oxidative stability, which generally characterizes these compounds (Natarajan et al., 2005; Dolbier Jr., 2005). The removal of the halogen substituent, which is usually the major responsible for the toxic and xenobiotic character of haloaromatic compounds, constitutes a key reaction during the biodegradation of this class of compounds. Thus, dehalogenation usually reduces both recalcitrance to biodegradation and the risk of forming toxic intermediates during subsequent metabolic steps (Janssen et al., 2001).

Only a few studies concerning the biodegradation of fluoroanilines (FA) can be found in the literature. Zeyer et al. (1985) describe for the first time a microbial strain able to use several substituted anilines, including 4-FA. More recently, the aerobic degradation of 3,4-difluoro- and 3,4-dichloroanilines by a *Pseudomonas* strain was reported (Travkin et al., 2003). In both cases, the degrading strains were able to use the target compounds as a sole source of carbon, nitrogen and energy and the presence of a co-substrate was found

* Corresponding author. Tel.: +351 225580059; fax: +351 225090351.
*E-mail addresses:* camorim@porto.ucp.pt (C.L. Amorim), mfcarvalho@porto.ucp.pt (M.F. Carvalho), cafonso@ff.up.pt (C.M.M. Afonso), plcastro@porto.ucp.pt (P.M.L. Castro).

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to accelerate the rate of degradation. 3,4-difluoro- and 3,4-dichloroanilines were also reported to be biodegraded under nitrate reducing conditions by a *Rhodococcus* sp. (Travkin et al., 2002). Recently, a strain of *Acinetobacter baylyi* GFJ2, originally isolated from an enrichment with 4-chloroaniline (4-CA) (Hongsawata and Vangnai, 2011) was reported to be able to convert FA isomers using a minimal medium supplemented with yeast extract, ammonium sulfate and succinate (MMSAY), however, total biodegradation of the isomers was not achieved.

The aim of this study was to investigate the degradation of 2-, 3- and 4-FA by a previously isolated fluorobenzene (FB) degrading bacterium, *Labrys portucalensis* strain F11, under different culture conditions, providing useful information for further application of strain F11 in the bioremediation of FAs.

## 2. Material and methods

### 2.1. Microorganism and culture conditions

Strain F11, a FB-degrading bacterium identified as *Labrys portucalensis*, was previously isolated from a sediment sample collected from an industrially contaminated site in Northern Portugal (Carvalho et al., 2008). Strain F11 has been shown to grow at temperatures of between 16 and 37 °C and has an optimum temperature range of 28–32 °C while the pH range found to support growth was between 4.0 and 8.0, with optimum pH values of between 6.0 and 8.0 (Carvalho et al., 2008). In this study, growth conditions used in other studies on the metabolism of haloaromatic compounds by strain F11 (Carvalho et al., 2006; Moreira et al., 2012a; 2012b) were followed. As such, the microorganism was aerobically grown in sterile mineral salts medium (MM) (Moreira et al., 2012b) supplemented with 1 mM of FB as the sole carbon and energy source. Cultures were incubated on an orbital shaker at 25 °C.

### 2.2. Fluoroanilines degradation assays

#### 2.2.1. Biodegradation of FA as sole carbon and nitrogen source

The ability of strain F11 to degrade 2-, 3- and 4-FA as sole carbon and nitrogen source was tested in batch cultures. For these experiments, ammonium sulfate was excluded from the MM. Non-induced cells obtained by growing strain F11 on nutrient agar (NA) plates were used to inoculate MM in order to attain an optical density at 600 nm ($OD_{600\ nm}$) of 0.05, corresponding to a dry cell weight of 0.022 $g_{cells}$/L. The cultures were fed with each FA, supplemented individually at a final concentration of 0.5 mM.

#### 2.2.2. Biodegradation with induced and non-induced cells

Biodegradation of the FAs by F11 cells previously induced for haloaromatics degradation was assessed. Strain F11 pre-grown on 1 mM of FB was used as induced cells. The cells were harvested by centrifugation (10,000 rpm for 15 min at 4 °C), washed with MM and resuspended in the same medium. F11 cells obtained from NA plates were used as non-induced cells. The initial $OD_{600\ nm}$ was 0.05 and each culture was fed with 0.5 mM of 2-, 3- or 4-FA, supplemented individually as the sole carbon source.

#### 2.2.3. Biodegradation in co-metabolism with FB by induced cells

The degradation of FAs by strain F11 was investigated in the presence of FB as an additional carbon source. In these experiments, F11 cultures were simultaneously fed with 0.5 mM of FB and 0.5 mM of one of the target FA. Induced cells of *Labrys portucalensis* were used to inoculate each flask, to obtain an $OD_{600\ nm}$ of 0.05.

In all biodegradation experiments, the flasks were incubated on a rotary shaker at 25 °C. Samples were periodically taken for fluoride release, fluoroaromatic consumption and $OD_{600\ nm}$ analysis. The experiments were run in triplicate. Non-inoculated flasks containing MM and 2-, 3- or 4-FA, supplemented individually, were incubated under similar conditions as an abiotic control.

### 2.3. Analytical methods

#### 2.3.1. Fluoride analysis

Concentration of free fluoride in the culture supernatants were measured with an ion-selective combination electrode (model Orion 96-09, Thermo Electron Corporation, Beverly, MA) after removing the biomass by centrifuging at 8000 rpm for 10 min. A calibration curve was prepared by using freshly prepared standard solutions of sodium fluoride (0.01–5 mM) in MM. The ionic strength of the standards and samples was adjusted with a buffer solution, named 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 $C_6H_5Na_3O_7.2H_2O$ 0.002 M.

#### 2.3.2. FA analysis

Concentrations of 2-, 3- and 4-FA in the culture supernatants were determined by high performance liquid chromatography (HPLC), carried out using a System Gold 126 (Beckman Coulter, Fullerton, USA) chromatograph equipped with a LiChrospher 100 RP-18 (5 μm), HPLC cartridge, Merck. The injection volume was set to 20 μL. The flow rate was set to 0.6 ml/min and the compounds were detected at 230 nm with a UV detector. The eluent consisted of water/methanol 50/50 (v/v). Each analysis was performed isocratically over a 12 min period.

#### 2.3.3. FB analysis

FB was analysed by gas chromatography using a CP-Wax 52 CB capillary column under a temperature programmation starting at 50 °C for 2 min, increasing to 150 °C at a rate of 25 °C/min and reaching the final temperature of 250 °C at a rate of 50 °C/min. Injector and detector temperatures were 250 °C. 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.

#### 2.3.4. Optical density measurement

The OD of the cultures was measured at 600 nm using a spectrophotometer (Helios gamma, Unicam Instruments, UK).

### 2.4. Reagents

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

### 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. Results and discussion

### 3.1. Biodegradation of FAs as sole carbon and nitrogen source

The FB degrading bacterium, *Labrys portucalensis* strain F11, was previously shown to be capable to degrade different haloaromatic compounds, mainly fluorinated ones (Carvalho et al., 2005; Moreira et al., 2012a,b). The capacity of strain F11 to use FAs as a carbon and