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ORIGINAL PAPER

Degradation of difluorobenzenes by the wild strain *Labrys portucalensis*

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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.

Keywords Difluorobenzenes · Biodegradation · Cometabolism · Inhibition · *Labrys portucalensis*

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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.

Materials and methods

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 25°C. Growth was monitored by measuring the optical density at 600 nm (OD600).

Biodegradation experiments

After growth, cells of *L. portucalensis* strain F11 were harvested by centrifugation (10,000 rpm for 15 min at 4°C), 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 OD600 of 0.05, in a working volume of 200 mL. The cultures were incubated at 25°C 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 (OD600 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.

Analytical methods

Concentrations of FB and DFBs were analysed by gas chromatography as previously described (Carvalho et al. 2005). Biodegradation of these compounds was determined by measuring fluoride release, using a fluoride selective electrode, as previously described (Carvalho et al. 2002).

Chemicals

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

Results

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 Fig. 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 OD600, was observed during 1,3-DFB degradation (Fig. 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 (Fig. 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 (Fig. 1b, c). 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 (Fig. 1d). In the control flasks without inoculum addition, both removal of DFBs and fluoride release were not observed (data not shown).

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 (Fig. 2a). It is important to notice that when FB was present in the medium,

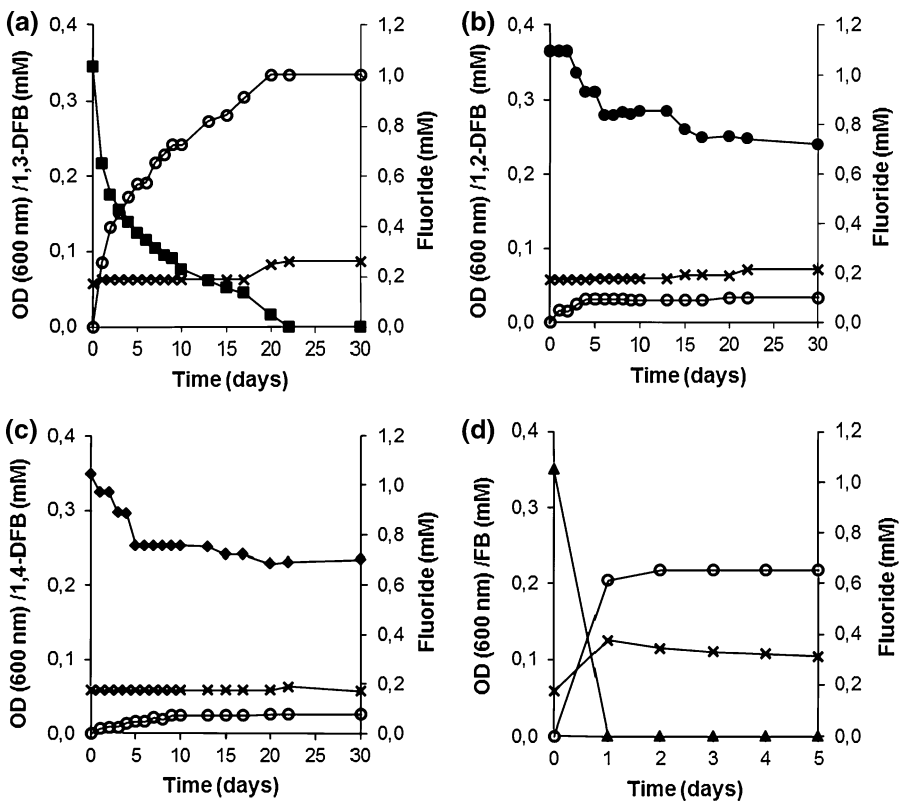

Fig. 1 Typical growth of *L. portucalensis* strain F11, pregrown on FB, on 0.5 mM of **a** 1,3-DFB, **b** 1,2-DFB, **c** 1,4-DFB or **d** FB, in batch culture. The experiment was conducted in triplicate. Optical density (x), 1,3-DFB (*square*), 1,2-DFB (*filled circle*), 1,4-DFB (*diamond*) and FB (*upward triangle*) concentrations in the culture medium and fluoride release (*open circle*) are indicated

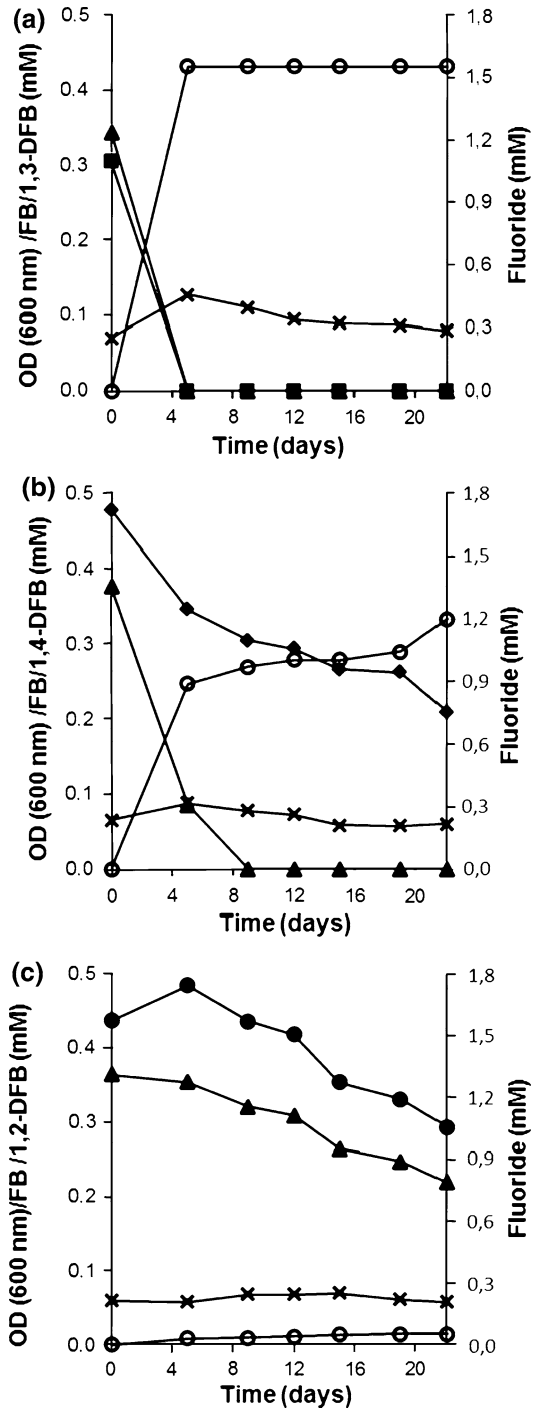

Fig. 2 Typical growth of *L. portucalensis* strain F11, pregrown on FB, on 0.5 mM of FB and 0.5 mM of **a** 1,3-DFB, **b** 1,4-DFB or **c** 1,2-DFB, in batch culture. The experiment was conducted in triplicate. Optical density (x), FB (*upward triangle*), 1,3-DFB (*square*), 1,4-DFB (*diamond*) and 1,2-DFB (*filled circle*) concentrations in the culture medium and fluoride release (*open circle*) are indicated

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 (Fig. 2b). The biomass increase in these cultures was lower than that observed with 1,3-DFB (Fig. 2a, b). 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 (Fig. 2c).

Degradation of DFBs was also investigated with a higher initial cell density (OD600 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 Fig. 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 (Fig. 3b). Nevertheless, this fluoride release was higher than the one obtained in the low density cultures (Fig. 2c). No differences were observed in the degradation of 1,3-DFB in the cultures with a higher initial cell density (Figs. 2a, Fig. 3c). Control flasks without inoculum addition showed no consumption of DFBs and FB and no fluoride release (data not shown).

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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 4b). F11 cultures fed with the highest

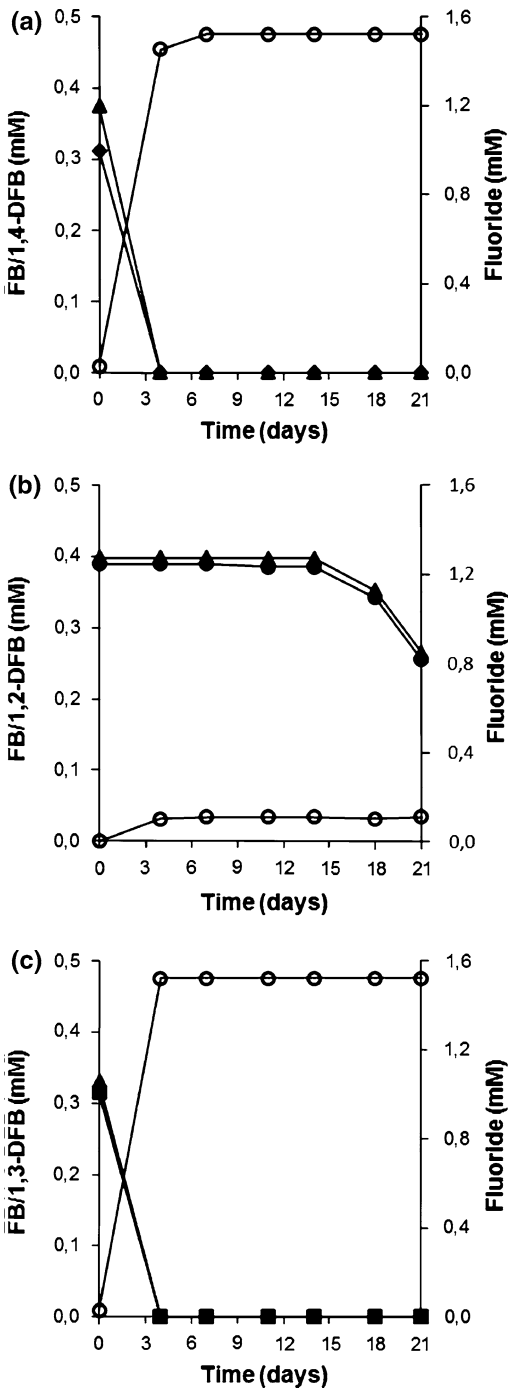

Fig. 3 Degradation of DFBs by high density cells of *L. portucalensis* strain F11. An FB-pregrown culture was used to start batch cultures with an initial OD (at 600 nm) of ca. 0.2, with 0.5 mM of FB and 0.5 mM of **a** 1,4-DFB, **b** 1,2-DFB or **c** 1,3-DFB. The experiment was conducted in triplicate. FB (*upward triangle*), 1,4-DFB (*diamond*), 1,2-DFB (*filled circle*) and 1,3-DFB (*square*) concentrations in the culture medium and fluoride release (*open circle*) are indicated

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 (Fig. 4d, f).

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. 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 *Rhocococcus 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

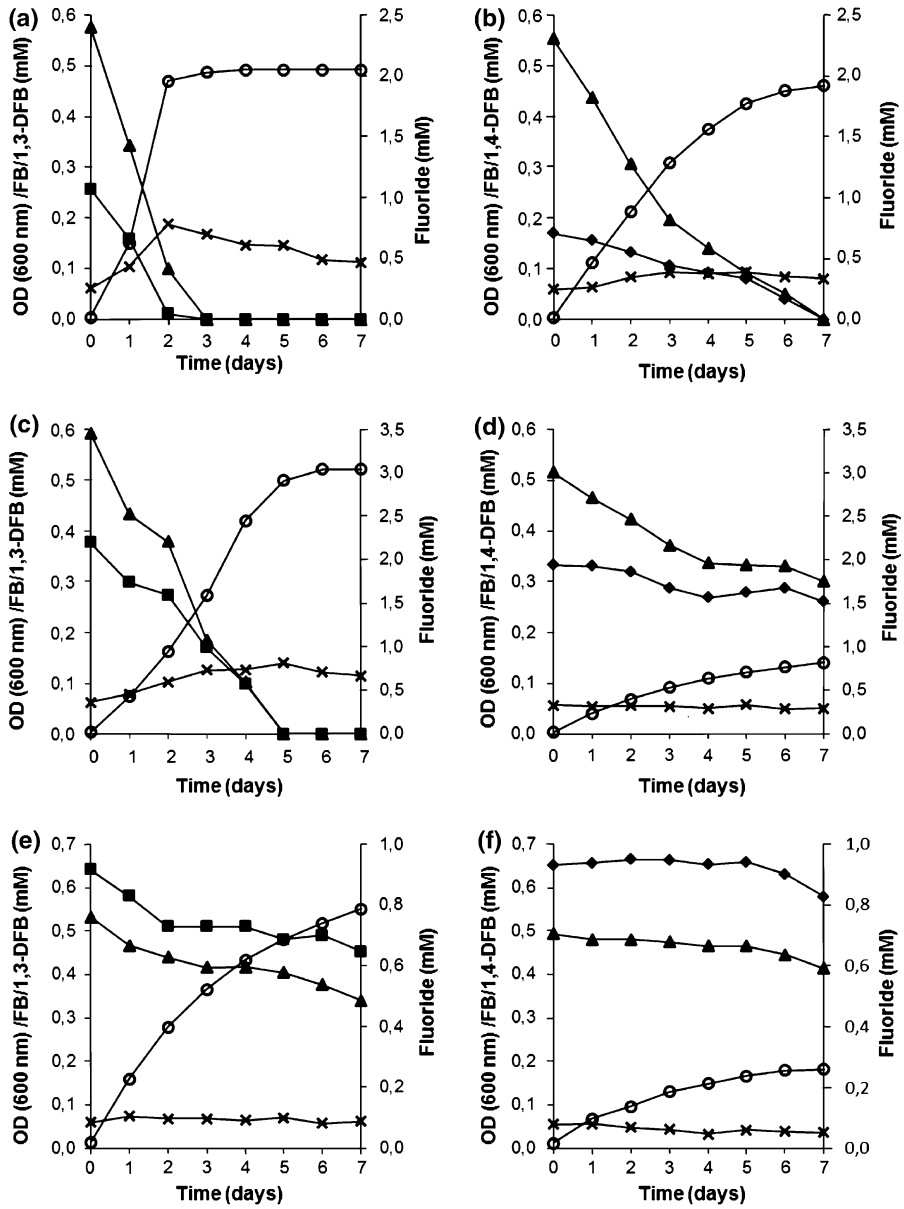

Fig. 4 Effect of concentration on the degradation of 1,3- and 1,4-DFB in the presence of FB. Cultures of *L. portucalensis* strain F11, pregrown on FB, were incubated in batch mode with 1 mM of FB and **a** 0.5 mM of 1,3-DFB, **b** 0.5 mM of 1,4 DFB, **c** 1 mM of 1,3-DFB, **d** 1 mM of 1,4-DFB, **e** 2 mM of 1,3-DFB or **f** 2 mM of 1,4-DFB. The experiment was conducted in triplicate. Optical density (x), FB (*upward triangle*), 1,3-DFB (*square*) and 1,4-DFB (*diamond*) and concentrations in the culture medium and fluoride release (*open circle*) are indicated

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 metabolised 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. 2006), 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. (2006), 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, 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 (fluoro)catechol dioxygenase. The fluorinated muconate is then expected to be converted, with concomitant defluorination, into maleylacetate, which is then channeled 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. 2006).

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.

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.

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