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Biodegradation of *meta*-fluorophenol by an acclimated activated sludge

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Abstract

An acclimated activated sludge was examined for its ability to degrade *meta*-fluorophenol as sole carbon source in aerobic batch cultures. The mechanism study revealed that the initial step in the aerobic biodegradation of *meta*-fluorophenol was their transformation to fluorocatechol. Following transformation of the fluorophenol to fluorocatechol, ring cleavage by catechol 1,2-dioxygenases proceeded *via* an *ortho*-cleavage pathway, then defluorination occurred.
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Keywords: Aerobic biodegradation; *ortho*-Cleavage pathway; Defluorination; Fluorophenol

1. Introduction

Halogenated compounds are important environmental pollutants of soil, water and air. Research investigating the environmental fate of halogenated compounds has largely focused on brominated and chlorinated organics [1]. Fluorinated organics have received less attention because they are perceived to be more inert biologically and therefore less likely to have an impact on human health or the environment. But the perception of inertness and its environmental significance are debatable: inert molecules tend to persist and accumulate, and they are more difficult to remediate. Moreover, organofluorine molecules actually do exhibit significant biological effects, as inhibitors of enzymes, cell–cell communication, membrane transport, and processes for energy generation [2].

In addition, the production and the use of fluorinated substances have been increased enormously in the recent years [3,4]. These compounds are used as propellants, surfactants, agrochemicals, adhesives, refrigerants, fire retardants and medicines. A large number of fluorinated compounds are intermediates or end products in the synthesis of agrochemicals. Because of the apparent stability, the bioactivity and the potential for accumulation in the environment of fluorinated organics, it is important to understand their environment fate and their biodegradation mechanism. The usage of fluorinated compounds, such as fluorophenols, in agricultural or industrial processes, has led to their accumulation in the environment. Therefore, the interest has been focused on the microbial degradation of fluorinated aromatics, especially on the metabolism of mono-fluorophenols by the acclimated activated sludge.

Several studies have described the oxidative degradation of mono-fluorinated aliphatics and aromatics by pure bacteria. Monofluoroacetate is the most investigated fluoroaliphatic compound since it is produced and stored by certain plants [5,6]. The bacterial metabolism of *p*-fluorophenylacetic acid, fluorobenzoic acid has been reported in detail [7,8]. A partial defluorination was observed. However, there is no report on the aerobic biodegradation of fluorinated phenols by acclimated activated sludge. Thus, it is the purpose of this paper to study the degradation of *meta*-fluorophenol by acclimated activated sludge and its mechanism.

2. Materials and methods

2.1. Chemicals

meta-Fluorophenol, used in the degradation studies were obtained from Xieshi Chemical Company (Shanghai, China), the purity of these chemicals was 99.9%. 3-fluorocatechol was purchased from ACROS Organics (New Jersery, USA). HPLC grade acetonitrile was obtained from Merk Company (Darm-

Abbreviations: AS, activated sludge; DO, dissolved oxygen; MLSS, mixed liquor suspended solids

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stadt, Germany). Ethyl acetate was got from Shanghai Chemical Company (Shanghai, China).

2.2. Microorganism and growth condition

The fluorophenol utilizing culture was obtained through acclimated activated sludge (AS) to a synthetic wastewater with fluorophenol as the sole carbon source for about 6 months. The seed activated sludge was got from Quyang wastewater treatment plants in Shanghai (China). The sequencing batch reactor (SBR) was used to acclimate sludge for *meta*-fluorophenol. In brief, the reactor had an active volume of 5 L, and were mixed and aerated by stirrers and aerators, with a hydraulic retention time (HRT) of 24 h. In the first stages of acclimation (2 months), the domestic sewage was taken as the assistant carbon source. Along with the improvement of the biodegradability of sludge, the concentration of fluorophenol in influent was increased. When the biodegradability of sludge became stable, acclimation turned to the second stage. In the second stage (4 months), the concentration of fluorophenol was 100 mg/L. The DO and temperature was kept at 2–3 mg/L and 25 °C, respectively, in all of experiment.

The biomass mixture in bioreactor was centrifuged at 5000 rpm/min for 10 min, the activated sludge was then washed twice with 50 mL 0.01 M sodium phosphate buffer (pH 7.0), removing any additional growth substance contained in the mixed culture, and used to inoculate fluorophenol (3 g/L MLSS). Fluorophenol biodegradation experiments were performed in 500 mL conical bottle containing 250 mL of minimal medium and certain *meta*-fluorophenol as sole carbon source. The pH of the medium was adjusted to pH 7.0. The flasks were incubated in an orbital shaker at 150 rpm/min at 25 °C. Uninoculated control flasks were incubated in parallel. All values were corrected to account for evaporation loss. The tests were conducted in duplicate. Results of all analysis represent the mean values of replicate trial degradations.

The pH of the solution was adjusted to 7.5, 7.0 and 6.5 and 6.0 with buffer when the experiment about pH effect on the biodegradation was carried out. And the ratio of different metabolites was got from the peak area of chromatograph.

2.3. Enzyme analysis

Cells grown on the fluorophenol were harvested by centrifugation (5000 rpm/min, 10 min), and washed twice with 0.33 M Tris–HCl buffer (pH 7.6). The cells were broken by sonication and centrifuged at 20,000 rpm/min at 0–4 °C for 15 min. The cell extract was kept on ice and assayed for catechol dioxygenase activity.

Catechol 1,2-dioxygenase activity (*ortho*-cleavage activity) was measured by following the formation of 2-fluoromuconic acid, the *ortho*-cleavage product of 3-fluorocatechol. The following reagents were added to a quartz cuvette: 2 mL 50 mM Tris–HCl buffer (pH 8.0), 0.7 mL distilled water, 0.1 mL 100 mM 2-mercaptoethanol and 0.1 mL cell extract. The cuvette was mixed by inversion and 0.1 mL 3-fluorocatechol (1 mM) was then added and mixed again. 2-Fluoromuconic acid formation was followed by an increase in the absorbance at 260 nm over a period of 5 min.

Catechol 2,3-dioxygenase activity (*meta*-cleavage activity) was measured by following the formation of 2-hydroxymuconic semialdehyde, the *meta*-cleavage product of 3-fluorocatechol. The following reagents were added to a plastic cuvette: 2 mL 50 mM Tris–HCl buffer (pH 7.5), 0.6 mL distilled water, and 0.2 mL cell extract. After mixing 0.2 mL catechol (100 mM) was added and mixed again. 2-Hydroxymuconic semialdehyde production was followed by an increase in the absorbance at 375 nm over a period of 5 min.

2.4. Analytic methods

A pH electrode was used to measure the pH value (model: pHS-3C). Fluoride anion release was followed with a fluoride anion sensitive electrode (model: pF-1-01). Fluoride anion concentrations were calculated with reference to a standard curve constructed with NaF standards. All optical density measurements were carried out using spectrophotometer (model: 752N). Dry weight measurements were determined by filtering a specific volume of suspended culture through preweighed 0.45 μm pore size filters, drying the cells at 105 °C for 2 h and reweighing them. This method is based on the procedure described in Standard Methods for the Examination of Water and Wastewater.

2.5. Qualitative analysis of fluorophenol and fluorocatechol

Qualitative analysis of fluorophenol was made by using a 4-aminoantipyene colorimetric method based on the procedure described in Standard Methods for the Examination of Water and Wastewater [9]. Samples were centrifuged at 5000 rpm/min for 10 min and the resulting supernatants were diluted to bring the concentration into the range 0–5 mg/L. The samples were treated by placing 10 mL in a test tube and adding 0.25 mL 0.5N NH_4OH. The pH was then adjusted to 7.9 ± 0.1 with approximately 200 μL potassium phosphate buffer (pH 6.8). Hundred microliters of 2% (w/v) 4-aminoantipyene solution was added and the tubes were allowed to stand for 15 min at room temperature. The absorbance was read at 500 nm.

Fluorocatechol was qualified by the method of Arnow [10]. Samples were centrifuged at 5000 rpm/min for 10 min to remove sludge. One milliliter of sample was placed in the test-tube. To each test-tube 1 mL 0.5N HCl was added. Tubes were mixed well and to this 1 mL nitrite–molybdate reagent was added resulting in a yellow colour. Nitrite–molybdate reagent was prepared by dissolving sodium nitrite and sodium molybdate in water to a concentration of 0.1 g/mL. After mixing, 1 mL of 1N NaOH was added resulting in a red colour. To this 1 mL distilled water was added. Following mixing, the absorbance was read at 510 nm.

2.6. Quantitative analysis of fluorophenol and fluorocatechol

Analysis of fluorophenols and fluorocatechols was made using a Hewlett-Packard 1050 high performance liquid chromatograph with a reverse phase column (4.6 mm × 250 mm,

packed with KR100-5C18). The sample was filtered through a 0.45 μm filter before analysis by HPLC. The mobile phase consisted of distilled-deionized water and acetonitrile was used in a step gradient with a flow rate of 1 mL/min. Acetonitrile in the mobile phase began at 15% (v:v), increased to 30% in 5 min, then to 70% in 20 min, and 90% acetonitrile and 10% water was reached at 30 min and was sustained until 35 min past injection. The injection volume is 10 μL. Detection of fluorophenols and fluorocatechols was at 220 nm using a diode array detector. The peaks were identified on the basis of added reference compounds, and the detection data were compared with calibration plots of peak areas of standard. 4-Fluorocatechol was not available, concentration is given using 3-fluorocatechol standard curve.

2.7. Identification of metabolites

Identification of fluorocatechols was carried out using GC–MS. After centrifugation to remove cells, the resulting supernatant (30 mL) was acidified to pH 2.0 with 2 M HCl, and extracted with ethyl acetate in three successive extractions. The extract obtained was dried with anhydrous Na_2SO_4 and concentrated in volume to 1 mL with gently blowing nitrogen over the surface. Then the sample was assayed immediately or stored at 4 °C for 2–3 days. If it was necessary, the concentrated metabolites were derived by bis(trimethylsilyl) tri-fluoroacetamide or trifluoroacetic anhydride. Derivatization is used to improve either the volatility or the thermal stability of compounds to make them more accessible to gas chromatography.

Separation and identification of the extracts were carried out by Hewlett 5890 Packard SERIES II fitted with a HP 5 capillary column (30 m length, 0.25 mm diameter, 0.25 μm film thickness). One microliter extract was injected. The oven temperature was programmed to hold at 90 °C for 3 min, then increase to 280 °C at 10 °C/min and hold constant at 280 °C for 5 min when the extract was not derived, while to ramp at 15 °C/min to 150 °C from an initial temperature of 70 °C (hold 2 min), increase to 200 °C at 2 °C/min, then increase to 280 °C at 15 °C/min, finally keep at 280 °C for 5 min when the extract was derived. Helium was used as the carrier gas at the constant flow of 1 mL/min. Effluents from GC column were transferred to a 70 eV electron impact source held at 200 °C. The inject temperature was kept at 250 °C. Identification was obtained by probability-based matching with mass spectra in NIST library as well as by matching with the mass spectra and retention time of the standard reference compounds used.

3. Results

3.1. Biodegradation of meta-fluorophenol

The transformation of the *meta*-fluorophenols by the acclimated activated sludge was shown in Fig. 1. The biodegradation of *meta*-fluorophenol was accompanied by concurrent release of fluoride anion. Accompanying with metabolism of 100 mg/L *meta*-fluorophenol, approximately 100% fluoride anion was released.

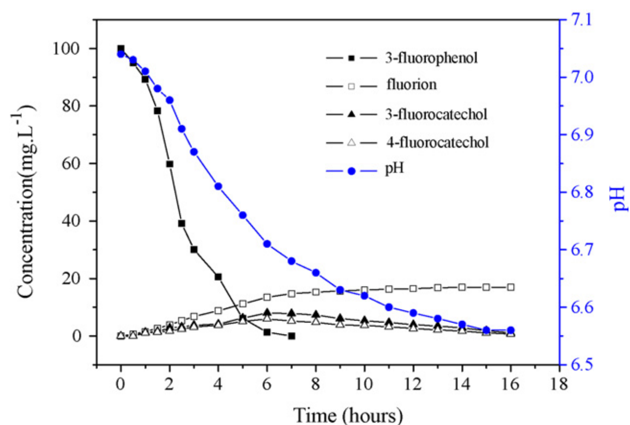

Fig. 1. Biodegradation of *meta*-fluorophenol by the acclimated activated sludge: (■) *meta*-fluorophenol; (□) fluoride anion; (▲) 3-fluorocatechol; (△) 4-fluorocatechol; (●) pH.

From Fig. 1 it can be seen that the degradation of fluorophenol resulted in a drop of pH. This drop was due to not only the release of fluoride, but also the production of organic acids which were described as being intermediates of the degradation of halophenols [11]. The initial fluoride anion release went with a corresponding drop in pH which reached a plateau as the fluoride anion release also completed. The pattern of fluoride release could mirror the pH drop, which suggested that the pH drop resulted mainly from the fluoride anion release.

3.2. Conversion of meta-fluorophenol by acclimated activated sludge as determined by GC–MS and HPLC

Extracts of the biodegraded sample were analyzed using GC–MS and HPLC operations. The *meta*-fluorophenol metabolites were identified on the basis of molecular, fragment ion in mass spectra and the chromatographic retention time (Rt) of authentic compounds. Results obtained were presented in Figs. 2 and 3. The retention time of the peaks indicated which metabolites were present, while the peak area reflected the amount of the various metabolites. The peak at 6.33 and 8.80 min in Fig. 2 could be identified as 3-fluorocatechol and 4-fluorocatechol, respectively. This suggested that *meta*-fluorophenol could be hydroxylated to give catechol-type intermediates by the phenol hydroxylase. From the HPLC in Fig. 3 the same results could be derived.

Data of fluoride anion release and the metabolites identified during the degradation of the fluorophenols by the acclimated activated sludge were shown in Fig. 1. Fluorocatechol measured by HPLC was formed gradually when fluorophenol metabolism continued. The increase of fluoride anion indicated that the biodefluorination had occurred. When the initial concentration of *meta*-fluorophenol was 100 mg/L, the amount of accumulated 3-fluorocatechol reached the maximal at 6 h and began to drop, accompanied by a further increase in fluoride anion as expected.

From the GC/MS total ion chromatogram presented in Fig. 2 it could be derived that fluorinated biodegradation metabolites were mainly fluorocatechols. In addition to the fluorocatechol,

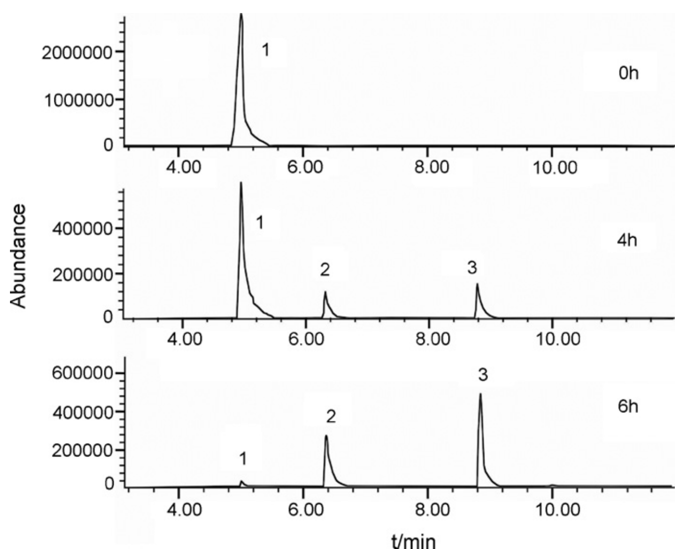

Fig. 2. GC–MS total ion chromatogram of metabolites of 100 mg/L *meta*-fluorophenol biodegraded by the acclimated activated sludge at different time: (peak 1) *meta*-fluorophenol (Rt 4.97 min); (peak 2) 3-fluorocatechol (Rt 6.33 min); (peak 3) 4-fluorocatechol (Rt 8.80 min).

no other intermediate metabolites were observed. These metabolites resulted from *ortho*-hydroxylation of the fluorophenols by a phenol hydroxylase. Consumption of fluorophenol resulted in the transient accumulation of fluorocatechol metabolites and considerable fluoride anion release. This suggested that the ring-fission of fluorocatechol by the catechol dioxygenase was inhibited to a certain extent. From the fact that fluorocatechol was the only intermediate accumulating it was concluded that the key step in the enzyme-catalyzed transformation of fluorophenol by the acclimated activated sludge was related to the enzyme catalyzing the conversion of the fluorocatechol.

Since *meta*-fluorophenol had two unsubstituted *ortho*-positions, the phenol hydroxylase had two possible positions to attack. So 3-fluorocatechol and 4-fluorocatechol could be detected during biodegradation of *meta*-fluorophenol. The results suggested that the regioselective hydroxylation depended on the pH value. At pH value 7.5, *meta*-fluorophenol was preferentially hydroxylated at C2 *ortho*-position, resulting in no 4-fluorocatechol detected (C6 hydroxylated intermediate). When pH value is 7.0, C2/C6 hydroxylation ratio was 7.9. Along with decreasing of pH value, the C6 *ortho*-hydroxylation increased relative to C2 hydroxylation, yielding the C2/C6 hydroxylation ratio of 1.7 at pH value 6.0.

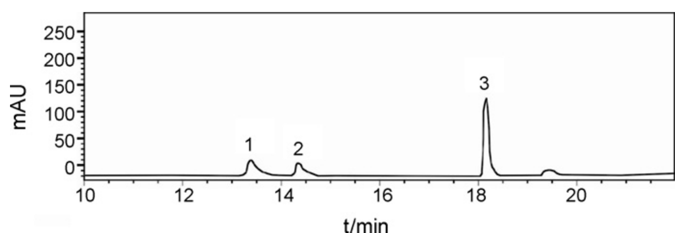

Fig. 3. Liquid chromatogram of metabolites of 100 mg/L *meta*-fluorophenol biodegraded by the acclimated activated sludge at different time: (peak 1) 3-fluorocatechol (Rt 13.370 min); (peak 2) 4-fluorocatechol (Rt 14.327 min); (peak 3) *meta*-fluorophenol (Rt 18.133 min).

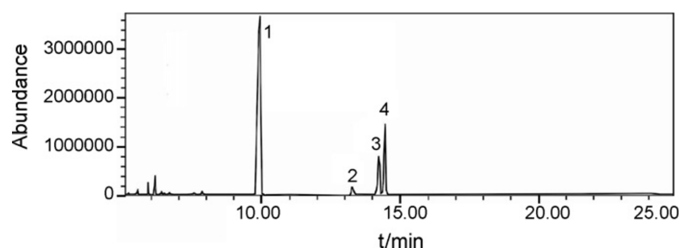

Fig. 4. GC–MS total ion chromatogram of metabolites of 128 mg/L 3-fluorocatechol biodegraded by the acclimated activated sludge at 8 h (derived by bis(trimethylsilyl) tri-fluoroacetamide): (peak 1) silanized 3-fluorocatechol; (peak 2) silanized dienelactone; (peaks 3 and 4) silanized 2-fluoromuconate isomers.

The initial step in the aerobic degradation of phenolic compounds was their transformation to catechols by the enzyme phenol hydroxylase following which ring cleavage occurred *via* either the *ortho*- or *meta*-cleavage pathway [12,13]. In order to study the ring cleavage pathway of the fluorocatechol by the acclimated activated sludge in detail, the following experiment was carried out. The metabolite extract of 128 mg/L 3-fluorocatechol (intermediate of *meta*-fluorophenol) by the same acclimated activated sludge was divided into three parts. One was analyzed directly by GC–MS, the other two was derived with either bis(trimethylsilyl) tri-fluoroacetamide or trifluoroacetic anhydride before the sample was analyzed by GC–MS. The further biodegradation metabolites of 3-fluorocatechol were only found in the sample derived by bis(trimethylsilyl) tri-fluoroacetamide. This result indicated that the metabolites of 3-fluorocatechol should not contain –OH because bis(trimethylsilyl) tri-fluoroacetamide could derive not only –OH but also –COOH while trifluoroacetic anhydride could derive only the –OH. Namely, 3-fluorocatechol was further biodegraded not through *meta*-cleavage pathway but *ortho*-cleavage pathway because containing-hydroxyl metabolites of halogenated phenol were formed when it was biodegraded through *meta*-cleavage pathway [14,15]. For example, the *meta*-cleavage of 4-chlorophenol resulted in the production of a chlorinated aliphatic compounds, 5-chloro-2-hydroxymuconic semialdehyde. The peaks 3 and 4 in Fig. 4 were identified as silanized 2-fluoromuconate isomers which resulted from the *ortho*-cleavage of the 3-fluorocatechol by a catechol 1, 2-dioxygenase. All results suggested that *meta*-fluorophenol was transformed mainly *via ortho*-cleavage pathway.

Experiments carried out on the degradation of fluorophenol by the mixed culture indicated that ring cleavage had been mainly *via* the *ortho*-cleavage pathway. In order to confirm this, the key enzymes involved in catalyzing ring fission (catechol 1,2-dioxygenase, catechol 2,3-dioxygenase) were assayed [16]. No catechol 2,3-dioxygenase activity was detected, indicating the absence of *meta*-cleavage capability in the mixed culture. Enzyme assays confirmed that degradation was mainly *via* the *ortho*-cleavage pathway.

The peak 2 in Fig. 4 was identified as silanized dienelactone formed through defluorination of 2-fluoromuconate. According to literature data on the conversion of 2-chloro-, 3-chlorophenol by (chloro) muconate cycloisomerases [17,18], possible path-

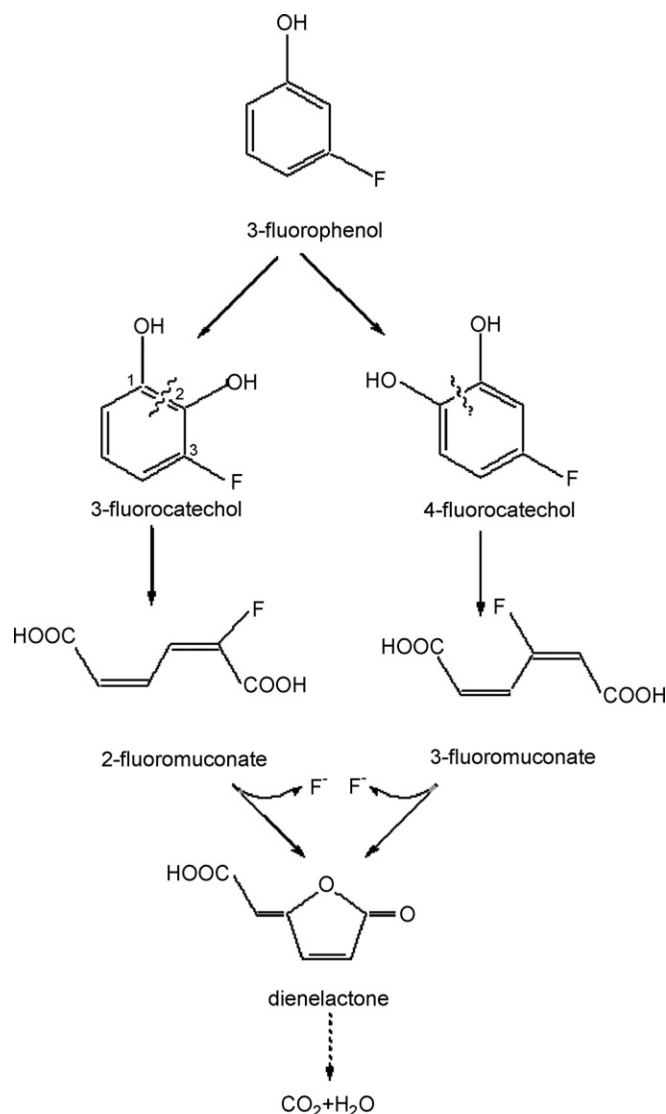

Fig. 5. The proposed degradation pathway of *meta*-fluorophenol by the acclimated activated sludge.

way for the defluorination of fluoromuconate by muconate cycloisomerase can be proposed. Fig. 5 schematically presents these possible pathways. Cyclisation of fluoromuconate to fluoromuconolactone cannot lead to dehalogenation. Fluoromuconolactone is not stable, then defluorined to result in formation of dienelactone. This reaction would be analogous to the dechlorination of 3-chloromuconate proceeding by its initial conversion to 4-chloromuconolactone catalyzed by chloromuconate cycloisomerase, followed by chloride elimination resulting in the dienelactone [19].

4. Discussion

4.1. Regioselectivity hydroxylation of fluorophenol by the acclimated activated sludge

The results suggested that the regioselective hydroxylation of *meta*-fluorophenol depended on the pH value. Molecular orbital calculations in combination with frontier orbital theory could provide insight into the reactivity of the C2 and C6 position in *meta*-fluorophenol. The electron density of C2 and C6 of *meta*-fluorophenol was calculated through the *hyperchem* software. The result indicated that C2/C6 electron density of *meta*-fluorophenol was 0.1364, while that of fluorophenolate anion was 0.9565. Thus, with pH value increased, C2/C6 hydroxylation ratio increased.

4.2. Aromatic ring was cleaved via ortho-cleavage pathway

Cleavage of the aromatic ring may occur using either the *ortho*- or the *meta*-cleavage pathway. Aromatic compounds found naturally in the environment, such as phenol and benzene, were typically broken down *via* the *meta*-pathway. While methyl-substituted aromatic compounds were also successfully degraded *via* the *meta*-cleavage pathway, chlorinated aromatic compounds were generally broken down *via* the *ortho*-cleavage pathway. The *ortho*-cleavage pathway of mono-fluorophenol biodegraded by this acclimated activated sludge was proposed on the basis of the following results: (1) mono-fluorophenol was transformed completely with approximately 100% fluoride anion release. *meta*-Cleavage by catechol 2,3-dioxygenase was known to generally result in incomplete metabolism due to the production of dead-end or suicide-metabolites [20]. (2) Fluoromuconate was identified in the biodegradation of fluorocatechol by the same acclimated activated sludge, which was the typical metabolite of the halogenated phenol *via* an *ortho*-cleavage pathway [21]. (3) The fact that there was no containing-hydroxyl metabolites during the biodegradation of fluorocatechol proven that the fluorophenol was biodegraded not *via* a *meta*-cleavage pathway but an *ortho*-cleavage pathway because containing-hydroxyl metabolites were formed during the ring cleavage of fluorophenol *via* the *meta*-cleavage pathway. No apparent biodegradation by the *meta*-cleavage pathway was found in the test, so it was concluded that the fluorophenol was transformed by the acclimated activated sludge mainly *via* the *ortho*-cleavage pathway.

For complete degradation of halogenated phenols to occur, two steps were necessary, cleavage of the aromatic ring and the removal of the halogen. This study revealed that the initial step in the aerobic biodegradation of mono-fluorophenol by this acclimated sludge was their transformation to fluorocatechol. Fluorocatechols were central metabolites in the aerobic degradation of fluorophenol. Following transformation of the fluorophenol to fluorocatechol, *ortho*-ring cleavage by 1,2-dioxygenases proceeded. Defluorination occurred after the ring was cleaved.

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