

Proposal of possible pathway of fluorene biodegradation by *Citrobacter* sp. FL5

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Abstract: The biodegradation ability of *Citrobacter* sp. FL5 on fluorene was investigated in the present study. The bacterial isolate was identified based on biochemical test, physiological and 16S rDNA sequence analysis. Fluorene biodegradation was studied in the liquid media at the initial concentration of 50 mg L⁻¹ fluorene at different pH values (6, 7, 8 and 9) and temperatures (25, 30, 37 and 45°C). *Citrobacter* sp. FL5 showed maximum performance of fluorene degradation ability at pH 7 and 30°C where it degrades 98% fluorene in liquid media. Furthermore, the isolate degrades 97.5% and 96% of 100 and 150 mg L⁻¹ fluorene respectively at 168 hrs. The possible metabolic pathway for fluorene biodegradation by *Citrobacter* sp. FL5 was deduced by identification of metabolites through Gas Chromatography Mass Spectrometry (GC-MS) technique. 5 types of metabolites that were detected and identified were dibenzofuran, 9-fluorenylmethanol, methyl benzilate, piperonylic acid and catechol. This study was the first to report and describe the ability of *Citrobacter* sp. in detail for degradation of higher fluorene concentrations at various pH and temperatures ranges.

Keywords: *Citrobacter* sp., fluorene, dibenzofuran, degradation, metabolites, Gas Chromatography Mass Spectrometry (GC-MS)

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1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a wide group of xenobiotic pollutants, consisting of benzene rings fused into different arrangements, and its hydrophobic physical property makes it hard to degrade in the environment^[1,2]. Fluorene, a tricyclic PAHs with two fused benzene rings to a cyclopentane ring, is a xenobiotic compound of environmental concern associated with petroleum and oil spills, waste incineration, and industrial effluents^[3]. Although it has industrial applications in thermo and light sensitizers, luminescence chemistry, spectrophotometric analysis, and molecular chemistry, fluorene and its derivatives are potential toxic and carcinogenic com-

pounds that adversely affect living organisms primarily wildlife and human health^[4]. Moreover, PAHs with three ring fluorene are found abundantly in the ecosystem, and it is of prime importance to discover new bacterial strains for its successful degradation.

Although PAHs can be effectively treated by conventional physicochemical techniques, such treatments are very complex and expensive, in addition to associated high cost and low efficiency. In recent years, biodegradation has been regarded as one of the most promising technique for PAHs remediation. Microorganisms are able to utilize fluorene as a carbon and nutrient source, which leads to a high degree of elimination, although the high quantity of biomass produced may be considered a disadvantage.

Several reports are available on the biodegradation of fluorene by bacteria such as *Bacillus*, *Sphingomonas*, *Mycobacterium*, *Pseudomonas*, *Brevibacterium*, *Staphylococcus*, *Arthrobacter* and *Rhodococcus*^[5–11]. Microbial degradation of fluorene has been investigated for many years; however, there is still considerable interest in the metabolic capacity of fluorene degrading bacteria in various ecosystems. Nevertheless, there is a possibility for various types of bacterial isolates with a capability to show high efficacy in the degradation of fluorene than previously reported.

Rapid industrial growth in the past two decades have lead to soil and water pollution by PAHs, including fluorene in many cities in China. In the present study, *Citrobacter* sp. was utilized to degrade fluorene in liquid media. The intermediate metabolites in fluorene biodegradation were identified by GC-MS analysis.

2. Materials and Methods

2.1 Sampling Enrichment Culturing and Isolation of Indigenous Bacteria

The bacterium in this study was isolated from soil samples at an abandoned site in Shanghai, China. The samples were collected into a 100 mL sterilized bottle containing 50 mL minimal medium supplemented with 50 mg L⁻¹ fluorene and incubated at 30°C in a shaker at 130 rpm. After one week of incubation, the supernatant was discarded after centrifugation and re-suspended in similar fresh media. This procedure was repeated twice. Finally, the pellet in the bottle was harvested by centrifuging at 8000 rpm for 10 min and was used to isolate the bacteria.

The bacterial strains were isolated via serial dilution technique on nutrient agar plates and colonies were transferred to high selection pressure minimal media containing 50 mg L⁻¹ fluorene. Minimal media used consisted of the following components per liter: 3 g KH₂PO₄, 8 g NaH₂PO₄, 1 g NH₄Cl, 0.5 g NaCl, 0.2 g MgSO₄·7H₂O, and 2.5 mL trace element solution. The trace element solution was composed of 23 g MnCl₂·2H₂O, 30 mg MnCl₄·H₂O, 20 mg NiCl₂·6H₂O, 50 mg ZnCl₂, 36 mg CoCl₂·6H₂O, 10 mg CuCl₂·2H₂O, 30 mg Na₂MoO₄·2H₂O, and 30 mg H₃BO₃ in 1 L distilled water. Media pH was adjusted to 6.5. During every experiment, fluorene was dissolved in dimethylsulfoxide (DMSO). Minimal media was added after DMSO evaporated. A potential bacterial isolate, designated as FL5 was selected for fluorene degradation studies. The strain was characterized physiologi-

cally and biochemically according to the procedures in Bergey's manual^[12].

2.2 Molecular Characterization

Genomic DNA was isolated from overnight bacterial culture in Luria broth using genomic DNA extraction kit (Sangon Biotech Co. Ltd., Shanghai, China). DNA was dissolved in TE (Tris-EDTA) buffer at 4°C overnight. To identify the bacterial isolates, 16S rRNA gene was amplified via polymerase chain reaction (PCR) using genomic DNA as template. The primers used for the amplification and sequencing of the 16S rRNA gene are 5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-AAGGAGGTGATCCAGCCGCA-3' corresponding to the forward and reverse primers of 16S rDNA, respectively. The PCR program used was according to Achal and Pan^[13]. 16S rRNA amplicon was gel eluted and ligated into pGEM-T vector as per manufacturer's instruction (Promega, USA). The sequences were generated by chain termination method using an Applied Biosystem automated sequencer. The 16S rRNA gene sequence was compared with Ribosomal Database Project-II and with those from GenBank using the BLASTN program. The 16S rRNA gene sequences for FL5 determined in this study were deposited into NCBI's GenBank under the accession number KT890283.

2.3 Biodegradation Studies of Fluorene

Biodegradation studies were performed by inoculating bacterial strain FL5 (10⁷ cfu/mL) in a flask containing 50 ml of minimal media supplemented with 50 mg L⁻¹ of fluorene. The flasks were incubated at 30°C at 130 rpm for one week (168 hrs). Bacterial growth was determined by measuring the absorbance at 600 nm, and fluorene degradation was quantified by GC-MS at regular time intervals.

The effect of pH on the degradation of fluorene was studied by growing FL5 at an initial pH of 6.0, 7.0, 8.0, or 9.0 in minimal media supplemented with 50 mg L⁻¹ of fluorene as the sole carbon source at 30°C. The effect of temperature on fluorene degradation was also determined by incubating the samples at 25°C, 30°C, 37°C or 45°C in a shaker in minimal media supplemented with 50 mg L⁻¹ of fluorene, at an optimal pH of 7.0.

The effects of fluorene concentration on the growth of strain FL5 and on its fluorene degrading ability was also examined. Isolate FL5 was inoculated in a flask containing 50 ml minimal media supplemented with different concentrations (50, 100, and 150 mg L⁻¹) of

fluorene. The flasks were incubated at 30°C in a shaker at 130 rpm for 168 hrs. Bacterial growth was measured using spectrophotometric method at 600 nm and fluorene degradation efficacy in the culture was measured using GC-MS. Degrading efficiency (D) was calculated using the following equation:

$$D (\%) = (C_i - C_f / C_i) \times 100,$$

C_i — initial concentration of fluorene

C_f — final concentration of fluorene

2.4 Analytical Method

A gas chromatography mass spectrometer (Agilent 5975C GCMS) with an HP-5MS (30 m × 0.25 mm I.D. × 0.25 μm) and 5% phenyl methyl silox (Agilent 19091S-433: HP-5MS) fused-silica capillary column, was used for the separation, identification, and quantification of fluorene and its metabolite products. The column temperature program was set at 55°C held for 2 min, 20°C/min to 280°C and 10°C/min to 310°C held for 5 min, with a total run time for 24 mins.

The injection volume was set to 1 μL in running mode of pulse splitless. The MS was operated in electron impact (EI) ionization mode with an electron energy of 70 eV, and the scan to determine the appropriate masses for the selected ion monitoring ranged from 50 to 350 amu (atomic mass unit). The mass spectra of the samples were identified by comparison with standard compounds in the NIST Mass Spectral Search Program (Version 2.0) for the NIST/EPA/NIH Mass Spectral Library.

3. Results and Discussion

3.1 Isolation and Identification of Fluorene Degrading Strain

Bacterial isolate FL5 was selected based on its ability to grow abundantly on fluorene amended medium. The isolate was Gram-negative, rod shaped as depicted in SEM (Figure 1), with opaque creamy appearance in agar plate, non-motile, catalase positive and oxidase negative. FL5 is unable to hydrolyze phenylalanine, gelatin or esculin; however, it utilizes citrate, arabinose, fructose, glucose, maltose, mannitol and xylose. Acid production was noted with glucose source. It showed growth between a pH range of 6–9 and a temperature range of 25–50°C. Furthermore, the bacterial isolate reduced nitrate to nitrite. The limited diversity of bacterial community in a sample with fluorene was unsurprising due to its extreme toxic environment. Only organisms capable of utilizing fluorene

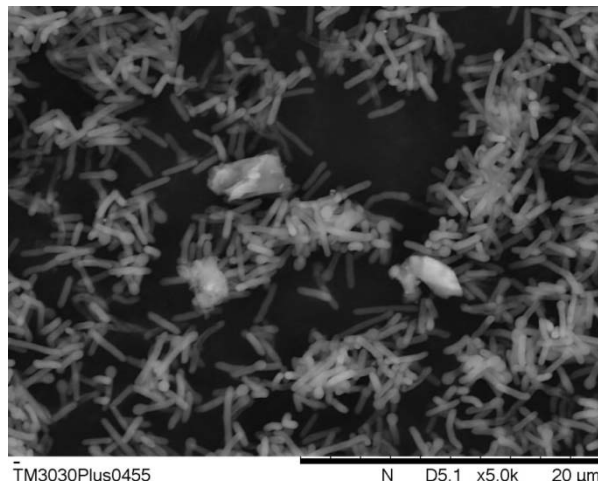


Figure 1. SEM showing rod-shaped bacterial isolate FL5.

can survive. Many phenotypic, biochemical, and physiological properties of this isolate resembled the *Citrobacter* species, which was further confirmed via 16S rRNA gene analysis.

Nucleotide BLAST and RDP-II analysis showed that isolate FL5 belonged to the phylum Gammaproteobacteria and Enterobacteriaceae family. Phylogenetic analysis revealed it to be of the *Citrobacter* species (Figure 2). Seven sequences of *Citrobacter* were included in the dataset. *Plesiomonas shigelloides* was included as an outgroup taxon for rooting purposes. Isolate FL5 was identified as *Citrobacter* sp. when compared with the other sequences present in the NCBI database, and was deposited under the accession number KT890283. This was the first report on the extensive characterization of fluorene degrading *Citrobacter* sp.

3.2 Biodegradation Studies of Fluorene

The ability of *Citrobacter* sp. FL5 to degrade fluorene in cultures was investigated. The isolate was able to grow and utilize fluorene as a sole carbon and energy source. Bacterial growth analysis was done up to 168 hours, and was observed to be growing significantly in media containing 50 mg L⁻¹ of fluorene. The growth of FL5 increased with respect to time (Figure 3). Fluorene was rapidly degraded in the medium, concomitant with bacterial growth. The data showed that *Citrobacter* sp. FL5 was able to remove high amounts of fluorene within 168 hrs.

It has often been observed that pH and temperature play an important role in biodegradation processes^[14,15]. The effect of pH on fluorene biodegradation was depicted in Figure 3 in which the removal of

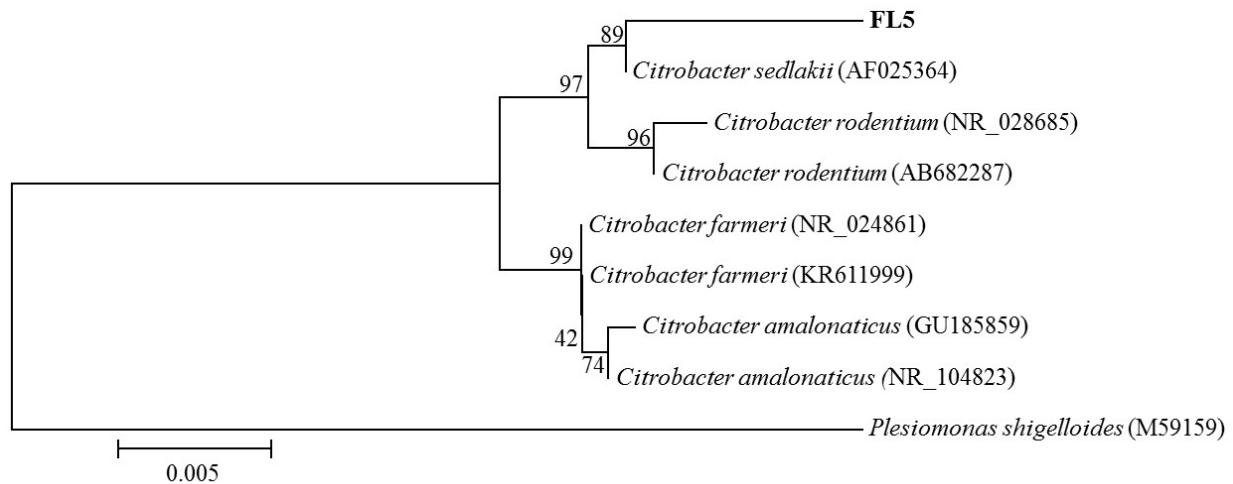


Figure 2. Phylogenetic tree of FL5 generated from Neighbor-Joining (NJ) analysis of 16S rDNA sequences rooted with *Plesiomonas shigelloides*. Numbers at the branch points indicate bootstrap values (%) based on NJ analysis of 1000 replicate datasets.

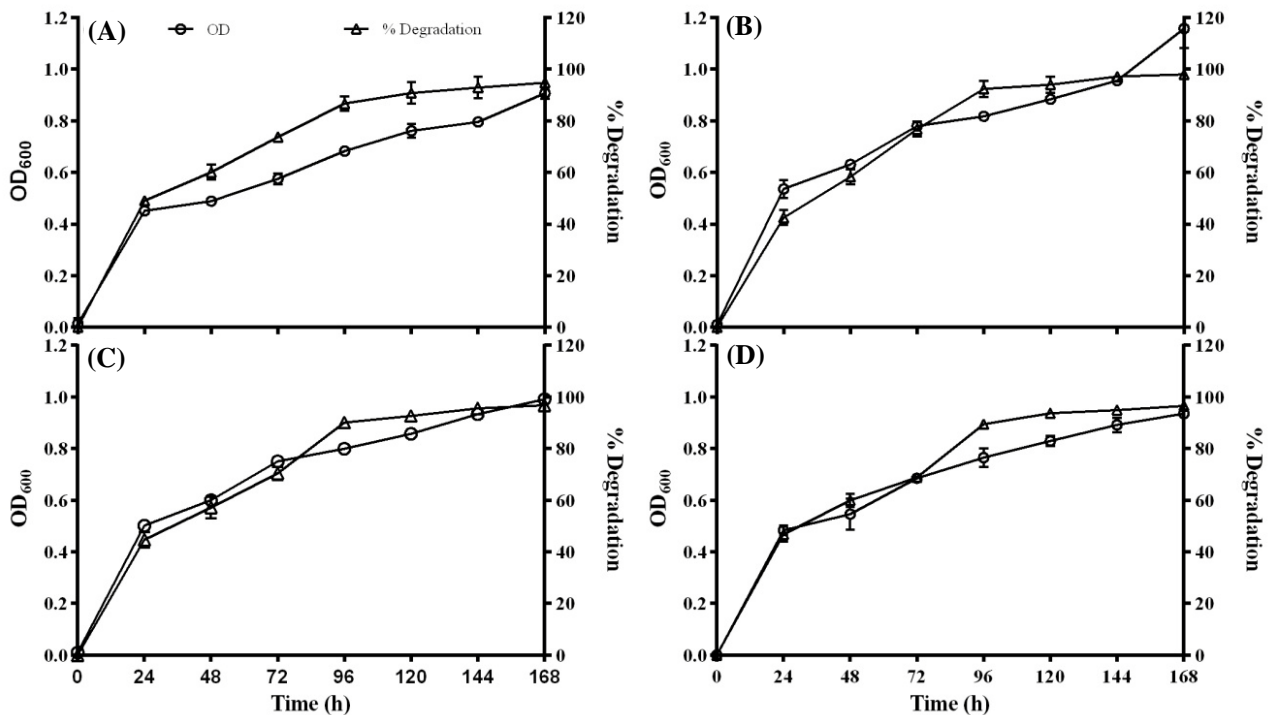


Figure 3. Growth and fluorene degradation by *Citrobacter* sp. FL5 at (A) pH 6, (B) pH 7, (C) pH 8, and (D) pH 9 at 50 mg L⁻¹ initial concentration of fluorene. Error bars represent the standard deviation. (OD: Optical Density – absorbance wavelength)

50 mg L⁻¹ of fluorene with different initial pH value has been shown. This suggested that the pH value of minimal media could affect fluorene degradation to a certain extent. The results indicated that *Citrobacter* sp. FL5 degrades 94.8% fluorene with the initial pH value of 6, relatively lower compared to the other pH due to slower bacterial growth. At the initial pH value of 7, *Citrobacter* sp. FL5 removed 98% of fluorene. When the initial pH value was 8, the isolate removed

fluorene by 96.8%. The bacterial isolate showed better growth profile at pH 7 followed by at pH 8 and 9. Fluorene degradation, D (%) was 96.6% at pH 9. pH is one of the crucial abiotic factors for the survival and activity of microorganisms^[16]. In this study, neutral to alkaline conditions (pH 7.0–9.0) were more favorable for the degradation of fluorene than in acidic conditions (pH 6.0), and optimal degradation was achieved at pH 7.0.

Citrobacter sp. FL5 was able to degrade significant amounts of fluorene at a wide range of temperatures (25–45°C). The optimal temperature for fluorene degradation was 30°C where 98% degradation was achieved (Figure 4). At 37°C, *Citrobacter* sp. FL5 degraded 90% fluorene, and 67% at 45°C. Optimum temperature enhanced microbial activity. The optimum conditions for maximum fluorene biodegradation were pH 7.0 at 30°C. The result was consistent with other reports on PAHs biodegradation efficiency. *Bacillus fusiformis* isolated from wastewater sludge of an oil refinery showed optimal naphthalene degradation at pH 7 and 30°C^[17]. Furthermore, among the four temperatures studied, 118 microbial isolates (78.6%) had an optimal temperature of 30°C for PAHs degradation, as previously reported^[18]. Temperature and pH of the medium affected PAHs degradation by increasing the solubility of the compound^[19].

Citrobacter sp. FL5 effectively degraded high concentrations of fluorene although at higher concentrations it may induce poor bacterial growth. *Citrobacter* sp. FL5 degraded 96% fluorene at a concentration of 150 mg L⁻¹, while 97.5% of fluorene was degraded at a concentration of 100 mg L⁻¹ by the end of 168 hrs. The rate of fluorene biodegradation by *Citrobacter* sp. FL5 was higher than previously reported including *Bacillus megaterium*^[11]. Fluorene-degrading *Pseudomonas putida* ATCC17514 degraded 100 mg L⁻¹ fluorene; however, it was completed in more than 100 hrs^[20]. *Pseudomonas* sp. JM2 was reported to degrade 40% fluorene in 96 hrs at an initial concentration of 50 mg L⁻¹^[21]. The isolate in the present study had an uncommonly high tolerance and a rapid degradation rate at a broad pH and temperature range among many documented fluorene-degrading bacteria. These properties made it potentially useful for the degradation of

high concentrations of fluorene in various environments.

3.3 Metabolites During Fluorene Biodegradation

In order to determine the intermediate metabolites and pathway during fluorene degradation by *Citrobacter* sp. FL5, GC-MS analysis was carried out. Unlike direct common metabolites of fluorene biodegradation, such as 9-fluorenol and 9-fluorenone reported in many studies^[5,6,10], *Citrobacter* sp. FL5 produced five different major metabolites, identified as dibenzofuran, 9-fluorenylmethanol, 2,2-diphenylacetic acid, piperonylic acid and catechol (Figure 5). The metabolites showed five main peaks with retention times of 13.035, 21.612, 22.219, 22.794, and 23.384 min (Table 1). As a result, a pathway for fluorene biodegradation by *Citrobacter* sp. FL5 was proposed (Figure 6). Dibenzofuran is susceptible to angular dioxygenase attack^[22] and its production suggested initial oxidation of fluorene to form its heteroatomic analog which oxidizes to 9-fluorenylmethanol. Upon further ring cleavage and probable involvement of dehydrogenase, catechol was formed as a simple metabolite of fluorene degradation. Further studies are required in order to purify the compound and identify if other metabolites are involved in between the process.

4. Conclusion

This work demonstrated the exceptional ability of *Citrobacter* sp. FL5 in degrading fluorene. The strain was able to significantly degrade fluorene even at higher concentrations in a short time period. In conclusion, due to broad pH ranges, strong degradation ability, and adaptability to temperature variations, *Citrobacter* sp. FL5 is a promising candidate bacterium for the bioremediation of fluorene from contaminated environments. Based on our knowledge and review, this was the first study on the biodegradation of fluorene by *Citrobacter* sp.

Conflict of Interest and Funding

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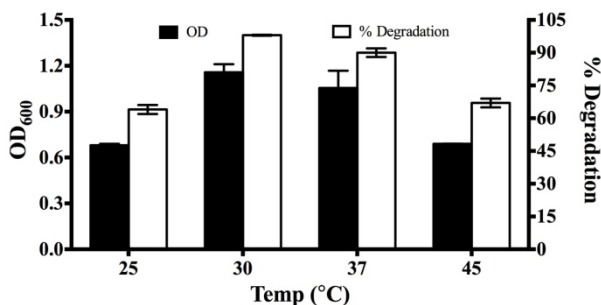


Figure 4. Effect of temperature on the growth and degradation efficiency by *Citrobacter* sp. FL5 at 50 mg L⁻¹ initial concentration of fluorene. Error bars represent the standard deviation.

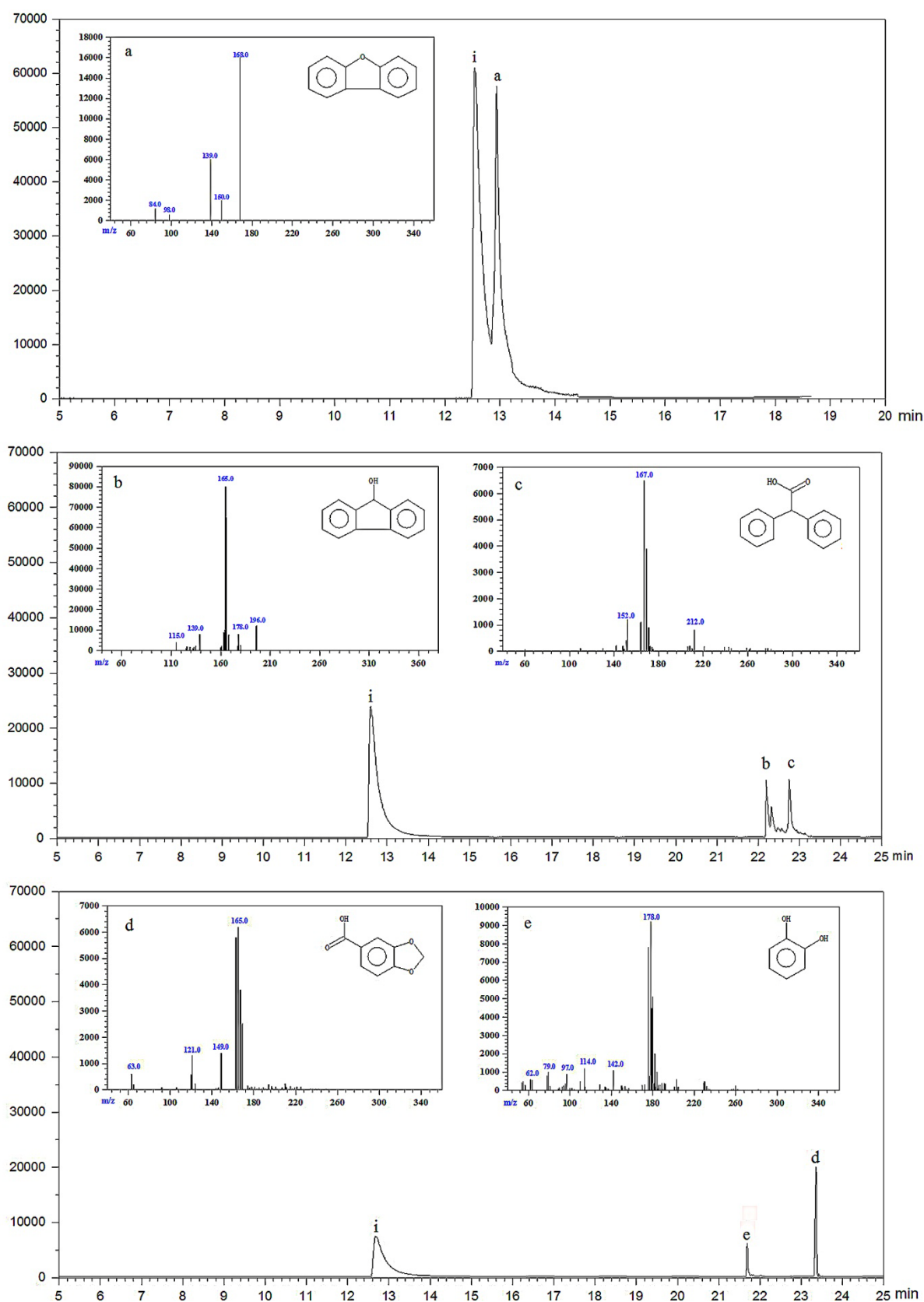
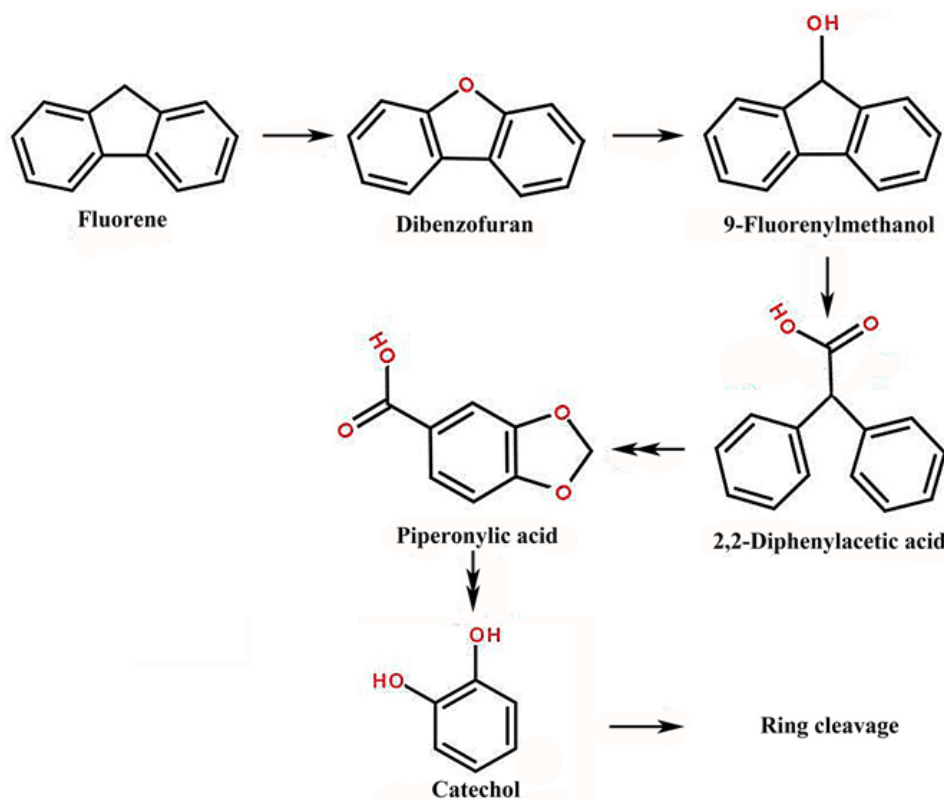


Figure 5. GC chromatogram of metabolites (i) fluorene, (a) dibenzofuran, (b) 9-fluorenylmethanol, (c) 2,2-diphenylacetic acid, (d) piperonylic acid, and (e) benzenediol produced during fluorene degradation by *Citrobacter* sp. FL5.

Table 1. GC retention time and electron impact mass spectral properties of metabolites formed during fluorene degradation by *Citrobacter* sp. FL5

Metabolite	Retention time (min)	m/z of fragment (% relative abundance)	Identification
a	13.035	84(10), 98(5), 139(42), 160(15), 163(88)	Dibenzofuran
b	22.219	115(5), 139(9), 165(88), 178(11), 196(13)	9-Fluorenylmethanol
c	22.794	152(17), 167(92), 212(14)	2,2-Diphenylacetic acid
d	23.384	63(8), 121(20), 149(21), 165(90)	Piperonylic acid
e	21.612	62(6), 79(9), 97(8), 114(11), 142(10), 178(93)	Benzenediol

**Figure 6.** Proposed possible pathway of fluorene degradation by *Citrobacter* sp. FL5.

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