Chapter 7 Elucidation of metabolic pathways during degradation of anthracene and fluoranthene by *Sphingobium yanoikuyae* strain ANT3D

The biochemical processes involved in the environmental degradation of LMW and HMW PAHs are of great interest due to their environmental persistence and toxicity of these compounds. Microbial biodegradation is one of the most important natural processes which can influence the fate of pollutants in both terrestrial and aquatic environments. Both bacteria and fungi play important roles in the biotransformation of PAHs, but there are more reports on bacterial degradation as compared to fungi. Widely studied bacterial genera reported for PAHs degradation include *Rhodococcus, Arthrobacter, Burkholderia, Cyclocasticus, Mycobacterium, Rhodococcus, Sphingomonas* and *Pseudomonas*. Microbial degradation represents the major route for the ecological recovery of sites contaminated with PAHs (Lopez *et al.*, 2005).

*Sphingomonads* have wide versatility with respect to degradation of LMW and HMW PAHs. The initial reactions in the degradation of anthracene and fluoranthene are catalyzed by multicomponent dioxygenases that incorporate both atoms of molecular oxygen into the PAH nucleus to produce cis dihydrodiols (Seo *et al.*, 2012). *Sphingomonas yanoikuyae* B1 initially oxidizes anthracene at the 1,2 position to form cis-1,2-dihydroxy-1,2-dihydroanthracene, which is subsequently converted to 1,2-
dihydroxyanthracene, which is further metabolized to 2-hydroxy-3-naphthoic acid, salicyclate, and catechol by enzymes of the naphthalene pathway. The other alternate route for enzymatic attack on anthracene is at C-9 and C-10 position to form 9, 10 dihydroxy anthracene (Moody et al., 2001).

Based on literature survey, till date three alternative routes have been proposed for the bacterial degradation of fluoranthene on the basis of metabolite identification. The first is initiated by dioxygenation at C-7 and C-8 followed by meta cleavage and pyruvate release, leading to the formation of acenaphthenone (Weissenfels et al., 1991; Kelley et al., 1993), which may be further oxidized to naphthalene-1,8-dicarboxylic acid (Story et al., 2001). A second route involves dioxygenation at C-1 and C-2, meta cleavage, and the formation of 9-fluorenone-1-carboxylic acid, which is decarboxylated to 9-fluorenone (Kelley et al., 1993; Šepič et al., 1998). Mycobacterium sp. strain PYR-1, capable of growing in pyrene and fluoranthene if supplemented with small amounts of complex organic nutrients, possesses the C-7 and C-8 and the C-1 and C-2 dioxygenation routes (Kelley et al., 1993). The third route has recently been proposed on the basis of metabolites formed from fluoranthene by Mycobacterium sp. strain KR20 (Rehmann et al., 2001). These authors have proposed an initial dioxygenase attack at C-2 and C-3 followed by ortho cleavage to form a product identified as Z-9-carboxymethylenefluorene-1-carboxylic acid. A subsequent loss of 2C units would produce 9-fluorenone-1-carboxylic acid, which is further degraded to yield benzene-1,2,3-tricarboxylic acid (Lopez et al., 2006).
In the present chapter, anthracene a 3 ring PAH has been considered prototypic PAHs which serve as signature compound to detect PAH contamination, since its chemical structure is found in carcinogenic PAHs, such as benzo[a]anthracene. It has also been used as model PAHs to determine biodegradation potential. Degradation of fluoranthene a 4 ring HMW PAH has also been studied using S. yanoikuyae strain ANT3D.

**Materials and Methods**

**Chemicals**

Bushnell and Hass (BH) medium was purchased from Hi-media laboratory (India). Anthracene and fluoranthene were purchased from sigma Aldrich (USA) with high analytical standard (99.999%). Solvents used for GC-MS were HPLC grade. Glasswares were purchased from Thermo Scientific (India).

**Preparation of bacterial inoculum**

*S. yanoikuyae* strain ANT3D was inoculated in BH medium containing 0.1 % yeast extract and amended individually with anthracene and phenanthrene. The flasks were incubated at 30 °C and 150 rpm until the culture reached the late exponential phase. After incubation, the cells were centrifuged at 10,000 rpm for 10 min, washed twice with sodium-phosphate buffer and re-suspended in the same buffer to obtain cell suspension with optical density 1.00 at 600nm. The suspension was then used as an inoculum.
**Experimental set up**

1 mL of above inoculum was added to 50 mL BH medium in two flasks each amended with 100 ppm anthracene and fluoranthene. The flasks were incubated at 30 °C on rotary shaker (New Brunswick, USA) at 150 rpm. Metabolite detection and pH of the medium were measured at every 24h interval upto 6 days.

**Extraction of metabolites during degradation (Pinyakong et al., 2000)**

The bacterial cell culture was centrifuged at 10,000 rpm for 10 min, followed by filtration through Whatman filter paper No. 1 to remove crystalline hydrocarbons. Supernatant was transferred to separating funnel, pH adjusted 2-3 with 12 N HCl, followed by triplicate extraction with equal volume of ethyl acetate. The ethyl acetate phase was further extracted with 10 mM NaOH for better phase separation. The ethyl acetate phase was pooled and dried over anhydrous Na₂SO₄. Solvent phase was reduced to 1-2 mL using rotary vacuum evaporator (Büchi, Switzerland). The residue was dissolved in methanol or acetonitrile for further purification and analysis.

**Trimethylsilyl- derivatization of metabolite (Zeinali et al., 2008)**

Derivatization was routinely carried out to transform the chemical structure which increased both volatility and thermal stability of the compounds to make them more readable by GC. The frequently used derivatization methods are silylation and methylation, which are used to convert compounds containing active hydrogen groups, thus changing polar-reactive compounds into nonpolar inert ones. In the
present study, silylation was carried out using trimethylsilyl (TMS) acetamide. Derivatives of the metabolite were prepared at 60 °C for 30 min using derivatization reagent, bovine serum albumin (BSA) + 10% TMCS [N,O-bis(trimethylsilyl)acetamide + 10% trimethylchlorosilane].

Gas chromatography-Mass spectrometry (GC-MS) analysis

A modified method of Hadibarata et al., (2013) was used, in which analysis was performed on GC-MS Shimadzu QP-2010 equipped with Rtx-5 MS fused silica capillary column (30m, 0.25mm ID, 0.025 µm thickness). The column temperature for GC was set at 60 °C hold for 3 min, the temperature was then raised up to 280°C at rate of 10°C/min and hold for 10 min. Helium (99.999%) was used as carrier as gas at flow rate of 1 mL/min using electrical control pressure. The GC injector was held isothermally at 280 °C with split mode of injection. The interface temperature was maintained isothermally at 280 °C. The MS was operated in electron impact (EI) mode with electron energy 90 eV, 1-s scan interval, mass range M/z of 50-500 amu (atom to mass unit). The mass spectra from individual total ion peaks were identified by comparing with external standard and internal library database of NIST and Wiley.

Unless otherwise mentioned the experiments were conducted in triplicates.
Results and Discussion

Detection of metabolite of anthracene degradation

*S. yanoikuyae* strain ANT3D extensively metabolized anthracene as determined in chapter 3. The metabolites were detected at every 24h interval upto six days. Based on the metabolites detected, pathway for anthracene degradation had been elucidated as shown in Fig 7.1. Metabolites detected during the degradation of anthracene by GC-MS with their Mass pattern, retention time, molecular formula and their possible structures analyse are as represented in Table 7.1.

Based on literature survey, there major routes for the degradation of anthracene by bacteria have been proposed (1) 3-hydroxy-2-naphthoic acid, 2,3-dihydroxynaphthalene and further via a pathway similar to the naphthalene degradation pathway to form salicylate (Cerniglia, 1984) (2) 3-hydroxy-2-naphthoic acid and then via o-phthalic acid (van Herwijnen et al., 2003) (3) 9,10 anthraquinone via phallic anhydride or pthalic acid similar to the fungal pathway (Swaathy et al., 2014).

The identification and characterization of major initial and ring fission products indicated multiple route of enzymatic attack. In the present study, two alternative pathways have been proposed for degradation of anthracene by *S. yanoikuyae* strain ANT3D in which oxidation of anthracene occurs at C-9 and C-10 position to form 9,10 anthraquinone is further oxidized to
Figure 7.1 Proposed metabolic pathway for anthracene degradation by *S. yanoikuyae* strain ANT3D.
pthalic anhydride followed by benzene 1,2 dicarboxylic acid (pthalic acid). Pthalic acid is further converted to benzoic acid, which enters the TCA cycle.

In the present proposed pathway, the initial reaction of anthracene degradation is similar to reports by Dean Ross et al., (2001); Moody et al., (2001); Ahmed et al., (2012); Swaathy et al., (2014), in which reaction was catalyzed by multicomponent dioxygenase that introduced molecule of oxygen to aromatic ring of anthracene at C-9,10 positions to form 9,10 anthracenedihydrodiols. However, the formation of quinone in present study is in accordance to the Ahmed et al., (2012). According to Cerniglia, (1992); Ye et al., (2011); Swaathy et al., (2014) the formation of anthraquinone is the most common abundant product of degradation of anthracene. The pathway as shown in Fig 7.1 corroborates to the pathway for anthracene degradation by fungus Aspergillus fumigatus as reported by Ye et al., (2011), in which formation of anthraquinone was mediated by formation of anthrone. Metabolite pthalic acid and its anhydride have frequently reported in both bacteria and fungi (Ye et al., 2011; Ahmed et al., 2012). Fig 7.2 shows the change in colour of the medium from colorless to purple indicating the formation of metabolites during degradation of anthracene. During degradation of anthracene, it has been observed that the pH of the medium changes from neutral to alkaline (7.09-9.34) (Fig 7.3), an observation supported by Swaathy et al., (2014) who stated that ring opening and subsequent cleavage of PAHs ring takes place above neutral pH. This might be due to the formation of hydroxyl quinone compound. Moreover
alkaline pH selectively increases the permeability of the molecule thereby increasing solubility.

Figure 7.2 Color changes in the medium during degradation of anthracene by *S. yanoikuyae* strain ANT3D. A, control B, color change during degradation on (i) 2nd (ii) 4th (iii) 5th (iv) 6th day.

Figure 7.3 Change in pH of the medium during degradation of anthracene.
Alternate pathway for anthracene degradation

*S. yanoikuyae* strain ANT3D oxidized anthracene to anthracene *cis*- 1,2-dihydroxydiol a reaction similar to that reported for *Pseudomonas* and *Mycobacterium* spp. (Jerina *et al.*, 1976; Moody *et al.*, 2001). Information from this and other studies on the degradation of PAHs by microorganisms suggests that both monooxygenases and dioxygenases catalyze the initial attack on the aromatic ring. Based on the metabolites detected, alternate pathway for anthracene degradation had been elucidated as shown in Fig 7.4.

The formation and characterization of initial oxidation and ring fission products show that *S. yanoikuyae* strain ANT3D degraded anthracene by an alternate route initiating with dioxygenation of anthracene at C-1 and C-2 position to yield anthracene *cis* 1,2- dihydrodiol. The resulting anthracene dihydrodiol is further dehydrogenated to 1,2 dihydroxyanthracene. However these two intermediates have not been detected in ethyl acetate extract of *S. yanoikuyae* strain ANT3D. Similar kind of intermediate has also been reported by the Cerniglia, (1984); Moody *et al.*, (2001). The next step is the cleavage of aromatic ring in *ortho* position to produce 3-[(2-carboxyvinyl) naphthalene-2-carboxylic acid, transformed to 2,3 dicarboxy napthpathalene. However, these two metabolites have also not been detected in culture extract of *S. yanoikuyae* strain ANT3D. 2-hydroxynaphthalene has been transformed from the 2-hydroxy 3 naphthanoic acid. Similar transformation has been reported by van Herwijnen *et al.*, (2003) in culture extract of *Mycobacterium* sp.
strain LB501T. The transformation of 3-hydroxy 2-naphthanoic acid to 2-hydroxynaphthalene, is different from Zeinali et al., (2008) who reported 2,3 dihydroxynaphthalene by *Nocardia otitidiscaviarum*. Detection of 2-hydroxynaphthalene is probably the first report of degradation product of anthracene by *S. yanoikuyae* strain ANT3D. 2-hydroxy naphthalene is subsequently converted to benzoic acid, an observation in accordance to Hadibarata et al., (2012) by *Polyporus* sp. S133. The metabolism of 2-hydroxynaphthalene may be similar to metabolism of naphthalene that forms catechol (Zeinali et al., 2008) or through a protocatechuate-degrading pathway (van Herwijnen et al., 2003) thereby entering TCA cycle. Hence based on the metabolites detected, we herein report complete mineralization of anthracene.

**Metabolic pathway for fluoranthene**

GC-MS analyses of the acidic extract from *S. yanoikuyae* strain ANT indicated formation of three metabolites. Metabolites detected during the degradation of fluoranthene by GC-MS with their mass pattern, retention time, molecular formula and possible structure analyses are represented in Table 7.1.

Metabolite 1-acenaphthylene, 1,8 naphthalic anhydride and pthalic acid were detected during degradation of fluoranthene by *S. yanoikuyae* strain ANT3D.
Figure 7.4 Alternate metabolic pathway for anthracene degradation by *S. yanolkuyae* strain ANT3D. Structures in parentheses are proposed intermediates and have not been detected in culture extract.
Fig 7.5 shows the proposed degradation pathway for fluoranthene degradation by S. yanoikuyae strain ANT3D. Change in pH of the medium was from 7.09 to 9.25 (Fig 7.6) similar to that obtained for anthracene degradation suggesting that it could be due to the formation of quinone compound. Fig 7.7 shows change in color of the medium during degradation of fluoranthene.

Where I - Fluoranthene, II- 7,8 dihydro fluoranthene  III- acenapthanone, IV- 1,2,3 tricarboxylic acid, V- dicarboxylic acid VI- 1,8 naphthalic anhydride, VII- pthalic acid

**Figure 7.5 Proposed pathway for the degradation of fluoranthene by S. yanoikuyae strain ANT3D. Structures in Brackets are proposed intermediates and have not been detected in culture extract.**

In present study S. yanoikuyae strain ANT3D initially hydroxylated fluoranthene at C-7 and C-8 positions which is similar to the initial oxidation by Sphingomonas and Mycobacterium spp. proposed by Ho et al., (2000); Lòpez et al., (2005). The 7,8 dihydrofluoranthene is then converted to 1,2,3 tricarboxylic acid.
1,2,3 tricarboxylic acid is further transformed to naphthalene-1,8-dicarboxylic acid, which is in equilibrium with its anhydride. The detection of naphthalene-anhydride in cultures of *S. yanoikuyae* strain ANT3D indicates that, *S. yanoikuyae* strain ANT3D degraded fluoranthene using the 7,8-dioxygenation route. In this study, pthalic acid was detected which is product of decarboxylation of benzene-1,2,3-tricarboxylic acid whose further oxidation would link this route with to the central metabolism, thereby permitting the complete mineralization of fluoranthene. This route of degradation of fluoranthene was also noted by the López *et al.*, (2006).

![Figure 7.6 Change in pH of the medium during degradation of fluoranthene.](image)

Fluoranthene degradation route involving initial dioxygenation at C-7 and C-8 has been extensively studied for *Sphingomonas* and *Mycobacterium* spp. (Kelley *et al.*, 1993; Ho *et al.*, 2000; Rehmann *et al.*, 2001). *Mycobacterium* sp. was observed to initially form hydroxylate fluoranthene at C-2 and C-3 positions. (Rehmann *et al.*, 2001; López *et al.*, 2006). *Sphingomonas yanoikuyae* R1 was also reported to
transform fluoranthene into fluoranthene-2,3-dione via initial oxidation at C-2 and C-3 positions (Kazunga et al., 2001). Similar kind of reactions has also been proposed for the oxidation of acenapthanone formed during growth using fluoranthene by *Sphingomonas paucimobilis* strain EPA 505 (Story et al., 2001). The cascade of reactions was similarly noted by the López et al., (2005), who demonstrated different route for the initial oxidation of fluoranthene by *Mycobacterium* sp. strains CP1, CP2, CFt2 and CFt6 were isolated from creosote-contaminated soil due to their ability to grow in pyrene (CP1 and CP2) or fluoranthene (CFt2 and CFt6). The formation of benzene-1,2,3-tricarboxylic acid from naphthalene-1,8-dicarboxylic acid could be explained by an ortho pathway similar to that utilized by certain bacterial strains to produce phthalate from either 1-hydroxy-2-naphthoic or 2-hydroxy-3-naphthoic acids during degradation of phenanthrene (Kiyohara and Nagao 1978; Kästner 2000) or anthracene (van Herwijnen et al., 2003) respectively.

![Figure 7.7 Formation of metabolites during degradation by *S. yanoikuyae* strain ANT3D as indicated by color change of medium on 3rd (B) and 6th (C) day as compared to control (A).](image-url)