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Degradation of Anthracene by a *Pseudomonas* strain, NGK1

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***Pseudomonas* sp. NGK1, isolated by naphthalene enrichment culture technique, is capable of degrading anthracene as a sole source of carbon and energy. The organism degraded anthracene through the intermediate formation of 1,2-dihydroxyanthracene, 2-hydroxy-3-naphthoic acid, salicylate, and catechol. The intermediates were isolated and characterized by TLC, spectrophotometry, and HPLC analysis. The cell free extract of anthracene-grown cells showed activities of anthracene dioxygenase, 2-hydroxy-3-naphthylaldehyde dehydrogenase, 2-hydroxy-3-naphthoate hydroxylase, salicylate hydroxylase and catechol 2,3-dioxygenase. The formed catechol as a metabolite is degraded through *meta*-cleavage with the formation of α-hydroxymuconic semialdehyde.**

Key words: *Pseudomonas*, anthracene, degradation, metabolism

Polycyclic aromatic hydrocarbons in nature are encountered as environmental pollutants, owing to their toxic, mutagenic or carcinogenic properties. Anthracene, phenanthrene and their derivatives are emitted to the environment during coal gasification and the liquefication process (9, 11, 16, 24, 26). These chemicals are also used in the manufacture of dye precursors, fertilizers and pesticide residues (8). The structure of anthracene and phenanthrene resembles those found in acute carcinogenic PAHs, benzo(a) pyrene and benzo(a) anthracene (14). There are several reports on the degradation of naphthalene when compared to anthracene and phenatherene degradation (2, 3, 5, 10, 13, 19, 20). Studies on the degradation of anthracene by *Pseudomonas* sp. were initiated by Evans *et al.* (7) on the basis of sequential induction. They reported that the naphthalene-grown cells of *Pseudomonas* sp. converted 1,2-dihydroxyanthracene into *cis*-4(2-hydroxynaphtha 3yl)-2-oxobut 3-enoic acid and salicylate was shown to accumulate in the culture filtrates. The initial studies of Cola *et al* (4) on the degradation of anthracene by *Flavobacterium* resulted in the accumulation of anthracene dihydrodiol. Subsequent steps of anthracene catabolism were examined by Jarina *et al.* (15) with a mutant strain of *Pseudomonas putida* strain 119 and *Beijerinckia* sp. strain B-836 and conferred this diol to be 1,2-dihydroxy 1,2-dihydroanthracene. Sanseverno *et al.* (25) and Menn *et al.* (22) isolated three strains of *Pseudomonas fluorescens* and reported 2-hydroxy -3-naphthoic acid as the dead end product of anthracene degradation. In this paper, we present our results on the degradation of anthracene by a *Pseudomonas* sp. NGK1 isolated by naphthalene enrichment culture.

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Materials and Methods

Chemicals

Anthracene (ANT) was purchased from Merck, India. 2-hydroxy-3-naphthoic acid (2H3NA), NADH, NAD, and NADPH were procured from Sigma Co. (USA). 1-Hydoxy-2-naphthoic acid (1H2NA), 3-hydroxy-2-naphthoic acid (3H2NA), 1,2-dihydroxnaphthalene (1,2DHN), salicylic acid (SAL), catechol (CAT), and gentisic acid were purchased from Aldrich (USA). All other chemicals used in this study were of analytical grade.

Microorganism and growth medium

Pseudomonas sp. NGK1 (NCIM 5120) was isolated in our laboratory from biological waste water treatment effluent (19, 21). This culture was maintained on slants of naphthalene mineral salts medium and also cultivated in Luria-Bertani (LB) agar medium. The organism was grown in the mineral salts medium supplemented with anthracene (1.4 mM in

dimethyl formamide)as a sole source of carbon and energy on a rotary shaker at 150 rpm at 30 ± 2°C. Growth of the bacterium was determined by monitoring the optical density of the culture medium at 660 nm. The viable cell numbers in the culture medium was estimated by the spread plate method. Colonies which appeared on the plates were counted by a Lapiz colony counter. The utilization of anthracene by this bacterium was determined by HPLC and UV spectrophotometric methods.

Isolation and identification of metabolites

The bacterium was cultured in the mineral salts medium with anthracene for 30~32 h. The obtained medium was extracted with ethyl acetate for isolation of metabolites. The extraction procedure was carried out as shown in the flow chart (Fig. 1). The separated solvent fractions were dried over anhydrous sodium sulfate and ethyl acetate was evaporated under vacuum. The obtained residue was dissolved in methanol and analysed for metabolic products by thin layer chromatography (TLC) and HPLC with an ultraviolet detector (wavelength 276 nm), shimpack CLC-C8(M) ODS column (4.6 × 15 cm) was employed with methanol at a flow rate of 1 ml per min. The metabolites were isolated by preparative thin layer chromatography with different solvent systems. The metabolites were visualized with a UV lamp (λmax 254 nm) and the compounds were eluted with methanol and determined by UV analysis by comparison to standard solutions.

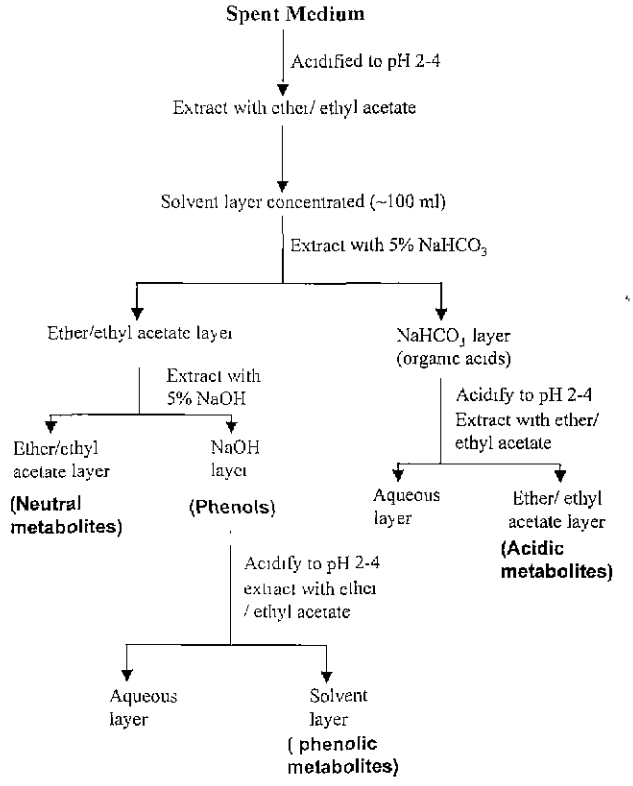

Fig. 1. Flow chart for extraction of metabolites from the spent medium.

Oxygen uptake study

The oxygen consumption by intact cells was performed in the conventional Warburg constant volume apparatus at 30°C. Cells were harvested in the early logarithmic growth phase by centrifugation (12,000 × g for 20 min) and washed twice with Mphosphate buffer(pH 7). The incubation mixture (3.2 ml) contained 0.05 M phosphate buffer (pH 7), cells (2 mg dry weight) and substrates (10 µl). The central well contained 0.2 ml of freshly prepared 20% KOH solution. Oxygen consumption was measured after tipping the substrate from a side arm. All values were corrected for endo- genous oxygen consumption.

Preparation of cell free extract

Cell-free extract was prepared from the bacterium grown in the mineral salts medium containing respective substrates. Fresh cells from bacterium of the early logarithmic growth phase were harvested and washed with buffer. Then the cells were resuspended in the same buffer containing 0.01% Triton X-100. The cells were disrupted by ultrasonication (Vibra Cell Ultrasonicator model 375, USA) at a nominal power (70 w) for a period of 6.5 during which the disrupted cells and oscillator probe were cooled in ice. Unbroken cells and cell debris were removed by centrifugation (44,000 × g for 45 min at 4°C). The supernatant was used as an enzyme source for all the enzyme assays.

Enzyme assays

Anthracene 1,2-dioxygenase (ADO) was assayed by the oxidation of NADH at 340 nm. The reaction mixture contained 2 mM NADH, anthracene (1 µM dissolved in 0.1 ml methonal) in 0.05 M phosphate buffer (pH 6.5) containing Triton X-100 and suitable amount of cell-free extract in a 1 cm cuvette. The reaction was initiated by the addition of anthracene. The activity of 1,2-dihydroxynaphthalene dioxygenase (12DHNO) was measured as described by Kuhm *et al.* (17). The enzyme activity was measured by monitoring the change in absorbance at 331 nm. Salicylaldehyde dehydrogenase activity (27) was determined by measuring the rate of increase in the absorbance at 340 nm due to the formation of NADH. Salicylate hydroxylase (SALH) activity (28) was measured at 340 nm due to the oxidation of NADH. 1-Hydroxy-2-naphthoate hy-

droxlase (1H2NH) assay system was performed by the modified method (28). The activity was measured spectrophotometrically by monitoring the decrease in absorbance at 340 nm. 2-Hydroxy-3-naphthoate hydroxylase (2H3NH) activity was carried out as described for 1-hydroxy-2-naphthoate hydroxylase. 1-Hydroxy 2-naphthoate dioxygenase assay system in a total volume of 1 ml contained 0.026 mM 1-hydroxy-2-naphthoate (pH 7) and 0.05 M phosphate buffer (pH 7) at 30°C. The reaction was started by adding the cell free extract and the rate of increase in absorbance at 340 nm was measured spectrophotometrically. Catechol 1,2-dioxygenase (C12O) activity was performed by the method of Hageman (12). The enzyme activity was measured spectrophotometrically by monitoring the increase in absorbance at 260 nm due to the formation of *cis, cis*-muconic acid in the reaction mixture. Catechol 2,3-dioxygenase assay (C23O) (23) system consisted of in a total volume of 3 ml containing 10 mM catechol and suitable amounts of cell free extract in a 1 cm covette. The reaction was initiated by adding catechol and an increase in absorbance at 375 nm due to the formation of α-hydroxy muconic semialdehyde. The protein concentration in the cell free extract was determined by the method of Lowry *et al.* (18). The specific activity of the enzyme is determined as μM of substrate converted or product formed per min per mg protein. The mode of ring cleavage of catechol was determined by using a cell free extract (6).

Preparation of anthracene metabolites by using cell free enzyme

The initial metabolites of bacterial degradation of anthracene was isolated enzymatically. The 100 ml reaction mixture containing 5 mM phosphate buffer (pH 6.0~6.2), 30 mM NADH and 30 mM anthracene was incubated with enzyme (10 mg protein) at 30℃ with gentle shaking on an incubated shaker for one h. At the end of the incubation period, the pH of the medium was adjusted to 7 with 5% sodium bicarbonate solution. The reaction mixture was then extracted with ethyl acetate. The neutral extract was concentrated and dried over anhydrous sodium sulphate and traces of sulphate were removed by barium chloride. The solvent was evaporated by vacuum. The residue was dissolved in dry chloroform and subjected to TLC. The major compound obtained was purified by preparative TLC using benzene:ethanol (9:1). The lower spot gave positive colour with Gibb's reagent. The spots were scrapped off and eluted with dry chloroform. The solvent was evaporated and the compound was recrystallized with dry hexane.

Results and Discussion

The growth behaviour of *Pseudomonas* sp. strain NGK1 which was isolated by naphthalene enrichment was tested for its ability to utilize anthracene. The results on the growth of the bacterium on anthracene and utilization of anthracene at different incubation periods are given in Fig. 2. The results indicated that there is an increase in cell population with an increase in incubation period. The initial cell population (6×10^5 cfu ml^{-1}) reached (9×10^9 cfu ml^{-1}) after 4.5 days of incubation. The maximum growth of the bacterium was observed at about 5 days of incubation. The bacterium showed the complete utilization of anthracene (2.8 mM) at about 6 days of incubation.

The bacterium was grown in mineral salts medium containing tween-80 and amended with anthracene. The ethyl acetate (phenolic) extract of the replacement culture on TLC corresponded well with those of 2-hydroxy-3-naphthoic acid, salicylic acid, catechol and α-hydroxymuconic semialdehyde. The neutral extract showed accumulation of 1,2-dihydroxy-1,2-dihydroanthracene with λmax at 238, 287, and 297 nm, which corresponded well with those of reported values. The TLC Rf values and UV absorbance values are given in Table 1 and the HPLC elution profile is shown in Fig. 3. Furthermore, the cell free extract of anthracene grown cells were incubated in 5 mM phosphate buffer (pH 6.2) containing anthracene and NADH (1 mM each), and the analysis of neutral extract of this incubation mixture by benzene:ethanol (9:1) showed the pres-

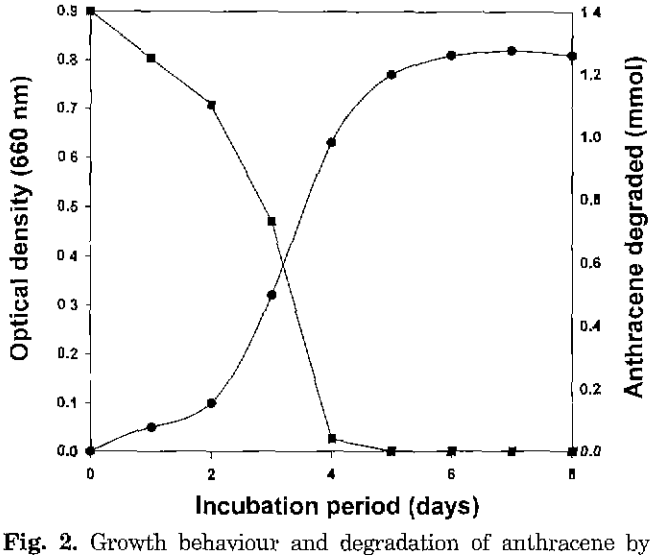

Fig. 2. Growth behaviour and degradation of anthracene by *Pseudomonas* sp. strain NGK1 in the mineral-salts medium containing tween-80 (5-cmc) supplemented with anthracene (in DMF). (●), growth and (■), degradation of anthracene.

Table 1. The R_f and λ_{max} values of anthracene metabolites and authentic compounds

Compound	A		B		C		λ_{max} (nm) in methanol	
	a	b	a	b	a	b	a	b
ANT	0.96	–	0.90	–	0.81	–	254	–
2H3NA	0.74	0.74	0.98	0.97	0.48	0.50		
2H3NA	0.60	0.06	0.90	0.88	0.37	0.39	247, 282	247, 282
SAL	0.62	0.61	0.74	0.75	0.50	0.48	234,296	234, 296
CAL	0.96	0.95	0.69	0.66	0.29	0.31	220,275	220, 275

a-authentic; b-metabolite.
Solvents systems: A-Butanol, aqueous ammonia: water (20:2:1)
B-Benzene: acetic acid, water (125:73:2)
C-Benzene: dioxane: acetic acid (74:2:2)

ence of unutilized anthracene (R_f=0.8) and two other metabolites. Metabolite 'a' (R_f=0.08) showed a positive diol test and metabolite 'b' (R_f=0.18) showed a positive dihydroxy test (a deep blue colour with $FeCl_3$-$K_3Fe(CN)_6$ reagent). These metabolites 'a' and 'b' were purified on preparative TLC. Metabolite 'a' showed the λ_{max} at 238, 287, and 297 nm, identical to 1,2-dihydroxy-1,2- dihydroanthracene (Fig. 4) and metabolite 'b' showed a melting point of 165°C which is identical to that of 1,2-dihydroxyanthracene. The dilute cell free extracts of anthracene grown cells in 5mM phosphate buffer (pH 6.2) containing 100 μM NAD^+ converted metabolite 'a' to a product whose properties are similar to metabolite 'b'. The dilute cell free extracts of anthracene grown cells converted metabolite 'b' to a colourless product, which was extracted by the acidic fraction. This metabolite showed strong absorbance at 247, 290, and 305 nm in acidic medium and in alkaline medium the absorption was changed to 320 and 448 nm (Fig. 5) and orange yellow colour

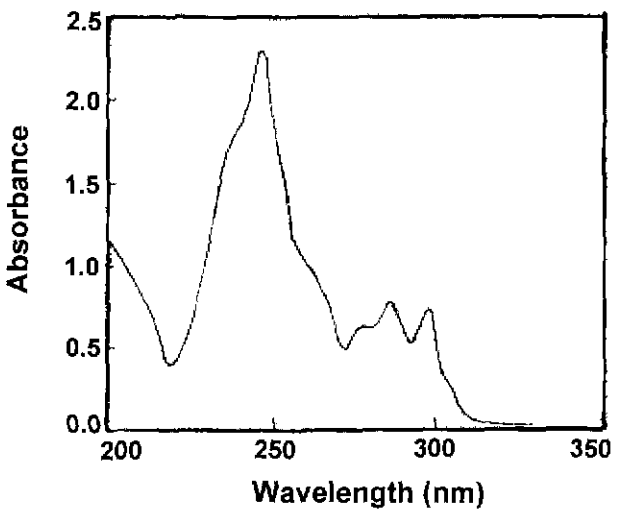

Fig. 4. UV spectrum of *cis*-1,2-dihydroxy-1,2-dihydroanthracene formed from anthracene degradation by *Pseudomonas* sp. NGK1.

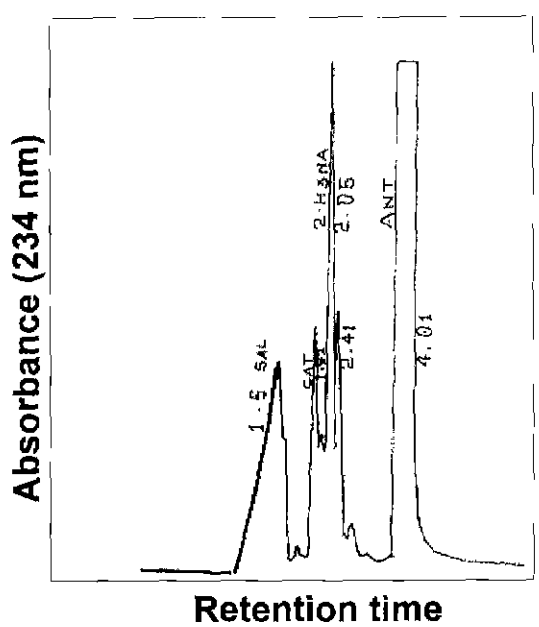

Fig. 3. HPLC elution profile of anthracene metabolites formed by *Pseudomonas* sp. NGK1.

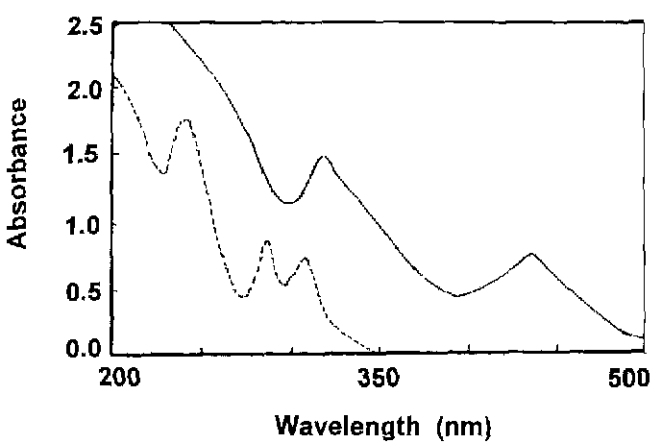

Fig. 5. UV-VIS spectrum of *cis*-(2-hydroxynaphth 3-yl) 2-oxobut-3-enoic acid formed from anthracene degradation by *Pseudomonas* sp. NGK1. (--) in 0.1 N HCl and (—) in 0.1 N NaOH.

Table 2. Oxygen uptake of various compounds by cell suspension of *pseudomonas* sp. NGK1 grown on various substrates

Growth substrate	Oxygen uptake * (nmol min^{-1} mg^{-1} dry cells)						
	ANT	2H3NA	3H2NA	1,2-DHN	SAL	CAT	GLC
ANT	020	035	036	046	022	015	068
2H3NA	010	092	098	062	072	040	080
3H2NA	008	082	092	052	060	038	068
NAP	018	054	050	360	19	070	095
SAL	028	065	060	300	168	090	092
SAL'	030	036	030	080	034	020	080
GLC'	003	004	004	014	416	045	095
GLC	002	005	006	012	006	015	130

SAL', salicylate-grown cells induced with anthracene
GLC', glucose-grown cells induced with catechol
*, The values are corrected for endogenous consumption.

was developed gradually. The properties of this compound are identical to that of the ring fission product of 1,2-dihydroxyanthracene, the *cis*-4-(2-hydroxynaphtha 3-yl)2-oxo, but 3-enoic acid as reported previously. In addition, the cell free extract of anthracene grown cells in phosphate buffer (5 mM, pH 5.5) converted anthracene to a product, which showed a positive aldehyde test with o-dianisidine in acetic acid and yellow fluorescence under UV light. This aldehyde positive compound was purified by preparative TLC using benzene : ether : ethanol : acetic acid (120:60:1:18). The purified metabolite (R_f=0.6) showed a melting point of 100°C which is identical to that of 2-hydroxy-3-naphthaldehyde. The dilute cell free extracts of anthracene grown cells converted this aldehyde to 2-hydroxy-3-naphthoic acid.

The results on the oxygen consumption of *Pseudomonas* sp. grown on various substrates (Table 2) indicated that cells grown on anthracene oxidized anthracene, 2-hydroxy-3-naphthoic acid, 3-hydroxy-2-naphthoic acid, 1,2-dihydroxynaphthalene, salicylate and catechol and cells grown on naphthalene and salicylate oxidized anthracene, 2-hydroxy-3-naphthoic acid, 1,2-dihydroxynaphthalene, salicylate and catechol without lag. However cells grown on 2-hydroxy-3-naphthoic acid and 3-hydroxy-2-naphthoic acid oxidized 2-hydroxy-3-naphthoic acid, 3-hydroxy-2-naphthoic acid and salicylate readily and anthracene was oxidized at a lower rate. The glucose (GLC) grown cells did not show oxidation of the above intermediates. However, 1.2-dihydroxynaphthalene and catechol were oxidized at lower rates, but oxidized glucose rapidly.

The various enzymatic assays were performed by using the cell free extracts of the bacterium grown on anthracene, 2-hydroxy-3-naphthoic acid, salicylic acid, and glucose (Table 3). Cell free extracts obtained from cells grown on anthracene showed activities of all the enzymes tested except for catechol 1,2-dioxygenase and less activity of 1,2-dihydroxynaphthalene dioxygenase. Cells grown on 2-hydroxy-3-naphthoic acid or 3-hydroxy-2-naphthoic acid showed high activities of 2-hydroxy-3-naphthoate hydroxylase, 3-hydroxy-2-naphthoate hydroxylase, salicylate hydroxylase and catechol 2,3-dioxygenase and low activity of anthracene 1,2-dioxygenase and very low activity of catechol 1,2-dioxygenase. However, extracts of cells grown on salicylate and salicylate induced glucose grown cells, showed moderate to high activities of all the above enzymes except catechol 1,2-dioxygenase. The cell free extracts of cells grown on glucose did not show activities of the above enzymes. The cell free extracts of anthracene-grown cells in tris buffer (pH 7.8) converted catechol to a yellow coloured product, which showed strong absorbance at 375 nm, which is an indicative of the formation of the *meta*-cleavage product of catechol, the α-hydroxymuconic semialdehyde.

It is evident from the above results on metabolite characterization, oxygen uptake studies, and enzymatic assays and from knowledge of anthracene degradation to conclude that anthracene was ini-

Table 3. Activities of various enzymes in cell-free extract of *Pseudomonas* sp. grown on different substrates

Growth substrate	Specific activity (μmol substrate converted or product formed min^{-1} mg^{-1} protein)							
	ADO	2H3ND	2H3NH	3H2NH	12DHNO	SALH	C23O	C12O
ANT	0.18	0.36	0.14	0.15	0.24	0.12	0.10	0.006
2H3NA	0.08	0.30	0.41	0.32	0.32	0.28	0.19	0.008
3H2NA	0.10	0.34	0.38	0.39	0.30	0.25	0.13	0.006
SAL	0.16	0.56	0.40	0.40	4.20	0.65	0.41	0.090
SAL'	0.24	0.34	0.20	0.18	0.44	0.22	0.15	0.006
GLC	0.02	0.015	0.003	0.003	0.02	0.002	ND	0.120

SAL. salicylate-grown cells induced with anthracene ; ND, not detected.

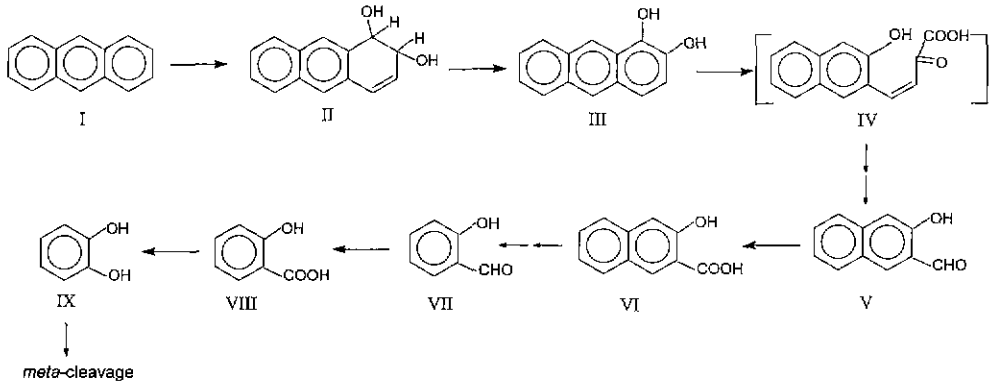

Fig. 6. Metabolic pathway for the degradation of anthracene to catechol. Chemical designations: I, Anthracene; II, *cis*-1,2-dihydroxy-1,2-dihydroanthracene; III, 1,2-dihydroxyanthracene; IV, *cis*-(2-hydroxynaphtha 3-yl) 2-oxobut-3-enoic acid; V, 2-hydroxy-3-naphthylaldehyde; VI, 2-hydroxy-3-naphthoic acid; VII, salicylaldehyde; VIII, salicylic acid; IX, catechol.

tially dihydroxylated by a dioxygenase to form 1,2-dihydroxyanthracene by a NAD^+ requiring dehydrogenase. The dihydroxy-anthracene after successive enzymatic hydrolysis is converted to 2-hydroxy-3-naphthaldehyde, which with the help of a dehydrogenase, was further converted to 2-hydroxy-3-naphthoic acid in the presence of NAD^+. This 2-hydroxy-3-naphthoate, after decarboxylation and successive hydroxylation, is converted to 2,3-dihydroxynaphthalene by a hydroxylase. The 2,3-dihydroxynaphthalene was convered to salicylaldehyde. The formed salicylaldehyde is oxidized to salicylate by a flavin linked dehydrogenase. The salicylate after decarboxylation and successive hydroxylation by salicylate hydroxylase is converted to a terminal aromatic metabolite, catechol. The catechol is further converted to α-hydroxymuconic semialdehyde via a *meta*-cleavage pathway by catechol 2,3-dioxygenase. A tentative pathway for the degradation of anthracene by *Pseudomonas* sp. strain NGK1 is shown by Fig. 6. Cola *et al.* (4), Gibson (8), and Jerina *et al.* (15) reported the accumulation of *cis*-1,2-dihydroxy-1,2-dihydroanthracene from the culture filtrates of *Flavobacterium*, *Pseudomonas putida*, and *Morexalla* sp. respectively. The cell free extracts of salicylate grown anthracene induced cells showed considerable activities of anthracene dioxygenase and 2-hydroxy-3-naphthoate hydroxylase. These results suggest that salicylate, but not 2-hydroxy-3-naphthoic acid, acts as an inducer of anthracene catabolic enzymes.

Acknowledgments

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