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ORIGINAL PAPER

Metabolism of acenaphthylene via 1,2-dihydroxynaphthalene and catechol by *Stenotrophomonas* sp. RMSK

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Abstract *Stenotrophomonas* sp. RMSK capable of degrading acenaphthylene as a sole source of carbon and energy was isolated from coal sample. Metabolites produced were analyzed and characterized by TLC, HPLC and mass spectrometry. Identification of naphthalene-1,8-dicarboxylic acid, 1-naphthoic acid, 1,2-dihydroxynaphthalene, salicylate and detection of key enzymes namely 1,2-dihydroxynaphthalene dioxygenase, salicylaldehyde dehydrogenase and catechol-1,2-dioxygenase in the cell free extract suggest that acenaphthylene metabolized via 1,2-dihydroxynaphthalene, salicylate and catechol. The terminal metabolite, catechol was then metabolized by catechol-1,2-dioxygenase to *cis,cis*-muconic acid, ultimately forming TCA cycle intermediates. Based on these studies, the proposed metabolic pathway in strain RMSK is, acenaphthylene → naphthalene-1,8-dicarboxylic acid → 1-naphthoic acid → 1,2-dihydroxynaphthalene → salicylic acid → catechol → *cis,cis*-muconic acid.

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) are compounds of environmental and health concern. Some of the PAHs and their biotransformation products have been shown to be toxic, mutagenic and carcinogenic in nature (Cerniglia 1992). Acenaphthylene belongs to the class of PAHs and is a component of crude oil, coal tar, tobacco smoke (Schocken and Gibson 1984) and a product of combustion which may be produced and released to the environment during natural fires. Emissions from petroleum refining and coal tar distillation are the major contributors of acenaphthylene to the environment. Acenaphthylene may be released to the environment via manufacturing effluents and the disposal of manufacturing waste byproducts. Because of the widespread use of materials containing acenaphthylene, releases to the environment also occurs through municipal waste water treatment facilities and municipal waste incinerators.

Microorganisms play an important role in the degradation of aromatic hydrocarbons both in the terrestrial and aquatic ecosystems. Few reports available are on the initial oxidation of acenaphthylene by microorganisms. Chapman (1979) reported that the

naphthalene grown *Pseudomonas* sp. oxidized the acenaphthylene to *cis*-1,2- acenaphthenediol. Schocken and Gibson (1984) reported that *Sphingomonas yanoikuyae* B8/36 cometabolized acenaphthylene with succinate by dioxygenating acenaphthylene to form *cis*-1,2-acenaphthene dihydrodiol and 1,2-dihydroxyacenaphthylene and finally to form acenaphthenequinone which cannot be further oxidized. Siriwat et al. (2006) reported the pathway of acenaphthylene degradation in *Rhizobium* sp. Strain CU-A1 through Naphthalene-1,8-dicarboxylic acid.

In this study we describe the metabolism of acenaphthylene by a *Stenotrophomonas* sp. RMSK. Mass spectral, chromatographic and enzymatic evidence are presented for the identification of metabolites and a pathway for the oxidation of acenaphthylene.

Materials and methods

Chemicals

Acenaphthylene, 1-naphthoic acid, NADH and NAD^+ were purchased from Sigma–Aldrich (Steinheim, Germany). 1,2-dihydroxynaphthalene, salicylate and catechol were procured from Lancaster chemicals, England. All other chemicals used in this study were of analytical grade.

Media and culture conditions

The phosphate buffered mineral salts (PMS) medium was used for the cultivation of the *Stenotrophomonas* sp. RMSK (Nayak et al. 2009). The medium was sterilized for 15 min by autoclaving at 121°C at 15 lb pressure. Liquid cultures (100 ml) were incubated in flasks (250 ml) at 37°C on a rotary shaker at 180 rpm (B. Braun, Ceretomat-SII, Germany) for 6 days. The growth of the culture was monitored by measuring the protein concentration.

Isolation and characterization of a bacterial strain

The bacterial strain capable of degrading acenaphthylene was isolated from left over coal fields of Raichur Thermal Power Station, India by enrichment technique. About 1 g of soil sample was added into 100 ml of PMS medium supplemented with 70 mg of acenaphthylene and cultivated at 37°C for 6 days on a rotary shaker at 180 rpm. The culture was then transferred to fresh PMS medium containing acenaphthylene and incubated under similar conditions. The purity of the culture was confirmed by plating on LB agar. The bacterial strain was identified based on its morphological, physiological tests and 16 s rDNA sequencing. This culture has been deposited in "National Collection of Industrial Microorganisms (NCIM)," Pune, India with accession number NCIM 5310.

The 16S rDNA was amplified from genomic DNA of *Stenotrophomonas* sp. RMSK as described previously (Kim et al. 2004). In brief, 16S rDNA was amplified by PCR (94°C for 5 min, 30 cycles consisting of 94°C for 30 s, 55°C for 30 s, 72°C for 90 s followed by a terminal incubation at 72°C for 10 min) with universal 16S rDNA F and R primers from isolated strains. PCR amplified product was purified and nucleotide sequence was determined at SolGent Co. Ltd (Taejeon, Korea) with an automated sequencing apparatus (ABI PRISM 377, PE Biosystems Inc.) using 16S rDNA F/R primers. The sequences thus generated were deposited in NCBI nucleotide sequence database under the accession FJ603459. The 16S rDNA sequence was compared to sequences in public databases with the BLAST search program on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/) to find closely related bacterial 16S rDNA gene sequences. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura et al. 2007). Phylogenetic tree was constructed by Neighbor-Joining method and Maximum Composite Likelihood model with bootstrap values at 500 replicates.

Acenaphthylene degradation experiments

Acenaphthylene degradation experiments were carried out by growing culture with acenaphthylene and monitoring the disappearance of the acenaphthylene by quantitative UV analysis. Simultaneously incubated culture flasks without carbon source as well as uninoculated flasks containing the acenaphthylene served as controls. Experiments were conducted in triplicate. Stock solution of acenaphthylene was prepared in minimum amount of acetone. Appropriate amount of the filter sterilized stock solution of

acenaphthylene was introduced in the flask containing 100 ml of sterilized PMS medium to obtain 70 mg acenaphthylene per flask and the acetone was removed by evaporation. Acenaphthylene grown cells from late exponential growth phase (OD at 660 nm = 0.6) were used as inoculums (2% v/v). The culture was incubated on a rotatory shaker (180 rpm, 37°C). Growth at the expense of acenaphthylene was verified by demonstrating an increase in bacterial protein with a concomitant decrease in the concentration of acenaphthylene. Acenaphthylene utilization and accumulation of metabolites were demonstrated by changes in the UV-Visible spectra of supernatants and by the examination of metabolites accumulated in the spent medium by HPLC.

Growth of *Stenotrophomonas* sp. RMSK on various aromatic hydrocarbons

The bacterium was tested for the utilization of various aromatic hydrocarbons, viz 2-hydroxynaphthoic acid, 1-naphthoic acid, phthalic acid, salicylic acid, homophthalic acid and catechol. In all the cases bacterium was grown on PMS medium (pH 7), with appropriate carbon source added to the shake flask (1 g/l). The culture was incubated on a rotatory shaker (180 rpm, 37°C). Growth at the expense of respective aromatic compounds was verified by demonstrating an increase in bacterial protein.

Isolation and chemical analysis of acenaphthylene metabolites

Bacterium was grown in 100 ml of PMS medium containing 120 mg of salicylic acid at 37°C with shaking at 180 rpm for 76 h. The cells were removed by centrifugation and cell pellet was washed with phosphate buffer (50 mM, pH 7.0) for three times. The cells were then transferred to PMS medium containing 1 g/l acenaphthylene. The culture was incubated at 37°C and 180 rpm for 24 h. The culture medium was centrifuged at 5,000*g* for 10 min to remove the cells and then the spent medium was acidified with 6 M HCl to pH 2 and extracted twice with equal volume of ethyl acetate. The ethyl acetate extract was dried over anhydrous sodium sulphate and concentrated under vacuum. The residue was dissolved in 1 ml of methanol and used for analysis.

The metabolites were analyzed by thin layer chromatography (TLC) on silica gel 60 plates using the solvent system cyclohexane: chloroform: acetone (3:1:2 v/v/v). Acenaphthylene metabolites were further analyzed by HPLC (Shimadzu, Shimadzu Corporation, Japan) equipped with LC 10ATVP pump and a 250 × 4.6 mm C18 Inertsil ODS-3 column (particle size, 5 μ; Phenomenex, USA.) at a flow rate of 1 ml/min. Injection was via a Rheodyne injector equipped with a 20 µl sample loop. UV absorption was measured at 254 nm. The compounds were eluted by using a linear gradient of 30–80 % methanol-water over 30 min.

LC–ESI–MS of acenaphthylene metabolites were recorded on a MICROMASS QUATTROII triple quadrupole mass spectrometer (Water's, UK) having JASCO PU-980 HPLC pump connected to it. The column was PARTISIL-10 ODS-3, 250 × 4.6 mm 5 m, wave length 254 nm. Solvent system was methanol/water gradient, 0.8 ml/min.

Preparation of cell-free extract and enzyme assay

The cell free extract was prepared from the *Stenotrophomonas* sp. RMSK grown in PMS medium containing acenaphthylene according to the method of Veeranagouda et al. (2006). Different enzyme assays were performed by using the crude enzyme. The reaction mixture of 1,2-dihydroxy naphthalene dioxygenase (Kuhm et al. 1991) contained 1 ml acetic acid–NaOH buffer (50 µmol, pH 5.5) and enzyme (0.1 ml). The reaction was initiated by addition of 1,2-dihydroxy naphthalene (0.4 µmol) in tetrahydrofuran (10 µl). Increase in absorbance was measured at 331 nm due to the formation of 2-hydroxy chromene-2-carboxlic acid (ε = 26,000). Salicylaldehyde dehydrogenase was determined from the rate of increase of the absorbance at 340 nm due to the formation of NADH (ε = 38,400). The reaction mixture contained 2.75 ml of 20 mM tetrasodium pyrophosphate HCl (pH 8.5), 0.1 ml salicylaldehyde (3 mM aqueous solution of freshly redistilled aldehyde), and 0.1 ml NAD (150 mM) (Shamsuzzaman and Barnsley 1974). Catechol-1,2-dioxygenase (Hegeman 1966) activity was measured spectrophoto metrically by increase in absorbance at 260 nm due to formation of *cis, cis* muconic acid (ε = 16,900). Catechol-2,3-dioxygenase was measured by determining the rate of accumulation of 2-hydroxymuconic semialdehyde

($\varepsilon = 36{,}000$) at 375 nm (Feist and Hegeman 1969). Reaction mixtures contained 100 μ moles of Tris-hydrochloride buffer (pH 7.6) and 0.2 µmol of catechol. The reaction was initiated by the addition of 0.1 ml of enzyme. Gentisate-1,2-dioxygenase (Crawford et al. 1975) was measured spectrophotometrically by increase in absorbance at 334 nm due to formation of maleylpyruvate. The assay mixture contained 0.15 µmol of gentisic acid in 3 ml of 0.1 M Na–K phosphate buffer (pH 7.4) and the reacton started by addition of enzyme. A value of ε equal to 10,800 was used to calculate the enzyme activity. Protein concentration of enzyme solution was determined by using bovine serum albumin as standard (Lowry et al. 1951). Specific activity of crude extract was expressed as µmol of substrate degraded/product formed per minute per mg of protein under assay conditions.

Results

Isolation and characterization of strain RMSK

The bacterial strain was isolated by selective enrichment culture technique with acenaphthylene as a sole source of carbon and energy. The strain is gram-negative, motile, small straight to curved rods. The cells occurred singly or in pairs. Spore formation was not observed. Colonies are smooth, circular, yellowish and convex. The culture has the ability to reduce nitrite and nitrate. Further, the strain showed positive tests for oxidase, catalase and gelatin hydrolysis. Tests for starch, urea, Tween-80 hydrolysis and indole production are negative. This bacterium is resistant to tetracycline, amikacin, gentamicin, ciprofloxacin, but sensitive to amoxicillin, penicillin and cefalotin.

The almost compete sequence (1,429 bases) of the strain was determined. Which amounts to more than 90% of the 16r DNA. The isolated strain RMSK clustered within *Stenotrophomonas acidaminiphila* and showed 96% identity (with 99% sequence coverage) with type strain of *Stenotrophomonas acidaminiphila* NK2.Ha-5. Thus confirming its identity as *Stenotrophomonas* sp. and renamed as *Stenotrophomonas* sp. RMSK. The phylogenetic relationship of the strain has been shown in Fig. 1.

Growth of bacterium on acenaphthylene

Stenotrophomonas sp. RMSK was able to grow on acenaphthylene as the sole source of carbon and energy. The typical pattern of growth reported in Fig. 2 showed an increase in protein concentration in relation to utilization of carbon source. The growth is stabilized after 5 days and entered into the stationary phase. At the same time the concentration of acenaphthylene decreased in the medium. It is observed that more than 82% of the acenaphthylene is degraded within 6 days of incubation.

Growth of *Stenotrophomonas* sp. RMSK on various aromatic hydrocarbons

The bacterium *Stenotrophomonas* sp. RMSK utilized acenaphthylene as the sole source of carbon and energy. It also utilizes 2-hydroxynaphthoic acid, 1-

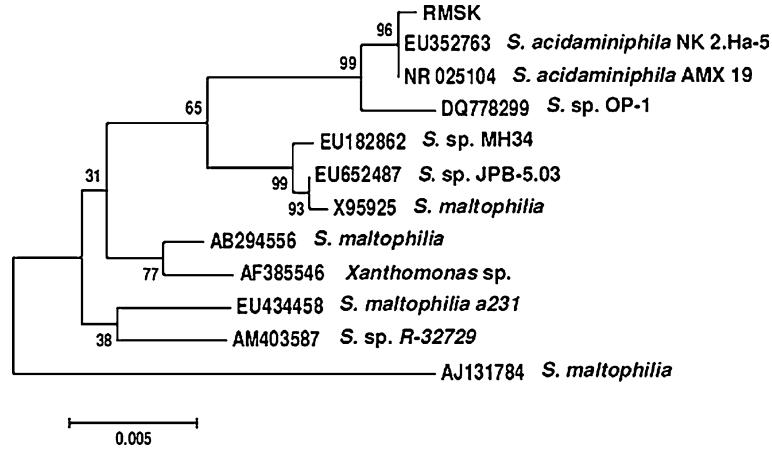

Fig. 1 Phylogenetic tree showing the position of isolate RMSK with reference strains of Xanthomonadaceae. The strain RMSK showed 96% homology with type strain *Stenotrophomonas acidaminiphila* NK2.Ha-5 (EU352763)

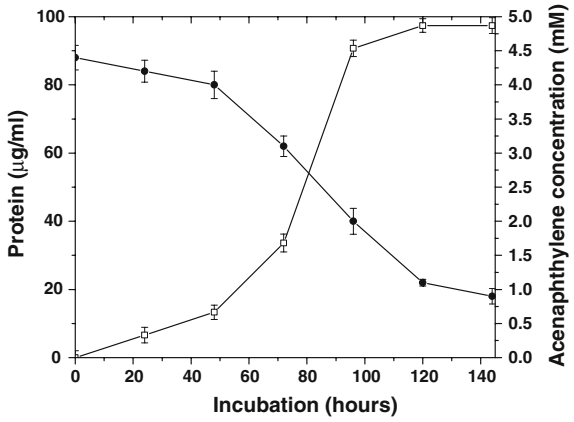

Fig. 2 Growth profile of *Stenotrophomonas* sp. RMSK on acenaphthylene (□) and disappearance of acenaphthylene (•)

naphthoic acid, phthalic acid, salicylic acid, homophthalic acid and catechol as source of carbon and energy. However, it fails to grow on 1-methylnaphthalene and 2-methylnaphthalene.

Identification of acenaphthylene metabolites

TLC analysis of the ethyl acetate extract of the acenaphthylene spent medium showed the presence of polar metabolites. Based on the comparison of the R_f values of the metabolites with the standard compounds, 1,2-dihydroxynaphthalene (R_f 0.871), 1-naphthoic acid (R_f 0.610) and salicylic acid (R_f 0.085) were identified as the probable intermediates.

Ethyl acetate extract of the resting cells incubation with acenaphthylene were analyzed by HPLC and LC–ESI–MS. Retention times from HPLC analysis (Fig. 3) and LC–ESI–MS characteristics of the metabolites are shown in Table 1. The LC–ESI–MS of the first metabolite gave a molecular ion at m/z 199 (M^+–OH) and subsequent at 198, 172(M^+–CO_2), 168, 155 (M^+–OH), 144 and 128(M^+–CO_2). The pattern of fragmentation is the characteristic of naphthalene-1, 8-dicarboxylic acid (Siriwat et al. 2006). The mass spectrum of the second metabolite showed a molecular ion peak M^+ 161 and subsequent

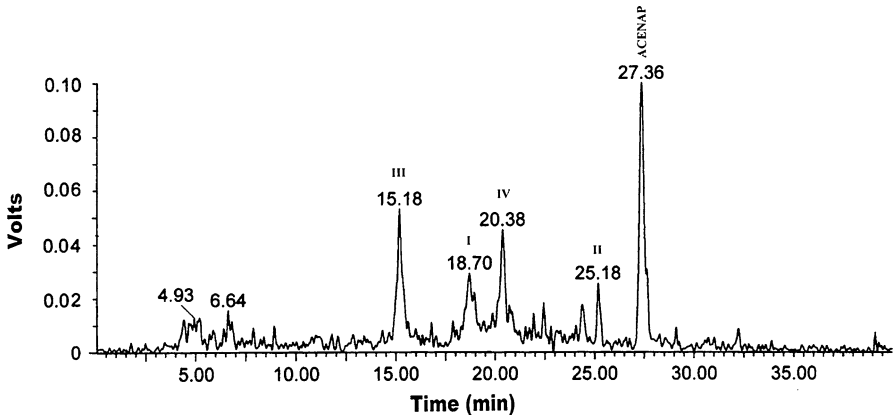

Fig. 3 HPLC elution profile of acenaphthylene and its metabolites in the culture supernatant during resting cell transformation of acenaphthylene for 48 h by strain RMSK. *I* Naphthalene-1, 8-dicarboxylic acid, *II* 1,2-dihydroxy naphthalene, *III* 1-naphthoic acid, *IV* salicylic acid, *ACNAP* acenaphthylene

Table 1 LC–ESI–MS properties of the acenaphthylene metabolites accumulated by *Stenotrophomonas* sp. RMSK

Metabolite[b]	R_t (min)	*m/z* of major ion peaks (%)[a]	Suggested structure
I	18.70	199(12), 198(100), 172(55), 155(67), 128(24)	Naphthalene-1,8-dicarboxylic acid
II	25.18	161(M^+ 100), 159(58), 143(66), 142(39), 128(32), 115(19), 103(38), 98(24)	1,2-Dihydroxy naphthalene
III	15.18	172(M^+ 68), 168(12), 161(8), 155(59), 154(100), 151(72), 144(25), 127(32), 115(25), 101(34), 98(22), 89(12)	1-Naphthoic acid
IV	20.38	138(M^+ 35), 121(23), 120(100), 93(55), 80(23), 77(14), 63(42)	Salicylic acid

[a] The ion abundance percentages are shown in parentheses

[b] Identification was based on the match of mass spectra (fragmentation and peak intensity) and HPLC retention times with data for authentic samples other than naphthalene-1,8-dicarboxylic acid, which is identified based on their mass spectral data and comparison with reported mass spectra for the same compound

Table 2 Specific activities of enzymes of acenaphthylene metabolism in cell free extracts of *Stenotrophomonas* sp. RMSK grown on acenaphthylene and 1-naphthoic acid

Enzyme	Specific activity[a]	
	Acenaphthylene	1-Naphthoic acid
1,2-Dihydroxy naphthalene dioxygenase	0.17 ± 0.002	0.14 ± 0.004
Salicylaldehyde dehydrogenase	0.23 ± 0.003	0.20 ± 0.009
Catechol-1,2-dioxygenase	0.41 ± 0.004	0.38 ± 0.007
Catechol-2,3-dioxygenase	ND	ND
Gentisate-1,2-dioxygenase	ND	ND

ND not detected

[a] Specific activity is expressed as μmol of substrate oxidized/min/mg of protein

fragmentation ion at 159,143 (M^+–OH), 142 (M^+–H_2O), 103, 128 and 98. The fragmentation pattern of this metabolite matched well with that of standard 1,2-dihydroxynaphthaene. The fragmentation of the third metabolite gave the molecular ion peak M^+ at 172 followed by 168 (M^+–CO_2), 155(M^+–OH),154,151,101 is identical with that of 1-naphthoic acid. The fourth metabolite was found to be salicylic acid which gave molecular ion peak at 138 and 121 (M^+–OH), 120, 93, 80 and 63.

Enzymatic studies

The cell free extracts prepared from acenaphthylene and 1-naphthoic acid grown cells showed moderate activity of 1,2-Dihydroxynaphthalene dioxygenase, and high activity of salicylaldehyde dehydrogenase and catechol-1,2,-dioxygenase (Table 2).

Discussion

The acenaphthylene degrading bacterium was identified as *Stenotrophomonas* based on its morphological, physiological and biochemical characteristics (Garity et al. 2004). The 16S rDNA sequence showed 96% identity with type strain of *Stenotrophomonas acidaminiphila*, further confirming its affiliation to genus *Stenotrophomonas* and it is renamed as *Stenotrophomonas* sp. RMSK. The strain is deposited in the culture collection centre with an accession number NCIM 5310.

On the basis of the characterization of the isolated metabolites by HPLC, LC–ESI–MS and demonstration of activity of the enzymes of the pathway and growth of the organism on various metabolic intermediates we propose a pathway of acenaphthylene degradation in the *Stenotrophomonas* sp. RMSK.

Most of the reports on bacterial degradation of acenaphthylene are confined to formation of naphthalene-1,8-dicarboxylic acid as an accumulated end product (Komatsu et al. 1993; Schocken and Gibson 1996). Siriwat et al. (2006) reported the complete pathway of oxidation of acenaphthylene by *Rhizobium* sp. Strain CU-A1, wherein acenaphthyquinone and naphthalene-1,8-dicarboxylic acid were identified as initial metabolites of acenaphthylene oxidation. The initial oxidation of acenaphthylene in strain RMSK occurs in the same manner (Table 1, metabolite I and metabolite III). Identification of metabolite 1,2-dihydroxynaphthalene with molecular mass of 161, growth of bacterium on 1-naphthoic acid and the presence of 1,2-dihydroxynaphthalene dioxygenase activity in cell free extracts indicate that 1-naphthoic acid after decarboxylation and successive hydroxylation is converted to 1,2-dihydroxynaphthalene by a hydroxylase. This contrast with the oxidation of 1-naphthoic acid in *Pseudomonas maltophilia* Csv89 (Phale et al. 1995), wherein 1-naphthoic acid was twice hydroxylated to form 1,2-dihydroxy-8-carboxy naphthalene. 1,2-dihydroxy naphthalene was converted to salicylaldehyde. The formed salicylaldehyde is oxidized by an NAD^+ requiring enzyme salicylaldehyde dehydrogenase to salicylic acid (metabolite IV). Activity of this enzyme is noticed in the cell-free extracts of cells grown on acenaphthylene and 1-naphthoic acid. The salicylate after decarboxylation and successive hydroxylation is converted to a terminal aromatic metabolite, catechol. The catechol is further converted to *cis-cis*-muconic acid via ortho-cleavage pathway by catechol-1,2-dioxygenase. A tentative pathway for the degradation of acenaphthylene by *Stenotrophomonas* sp. RMSK is shown in Fig. 4. However, an insite in to the enzymatic activities involved in the upper pathway is necessary to provide better knowledge of the bacterial metabolism of acenaphthylene. The ability to degrade acenaphthylene and other aromatic compounds makes this strain ideal candidate for application in remediation at the contaminated sites.

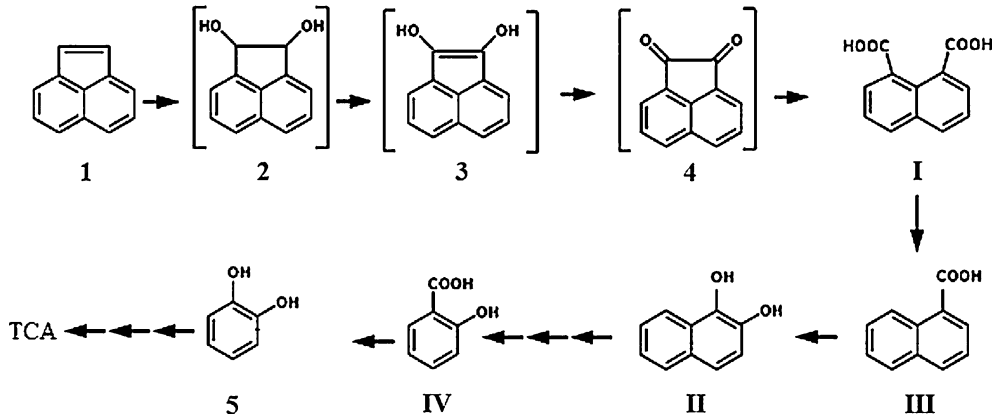

Fig. 4 Proposed pathway for the degradation of acenaphthylene by strain RMSK. The transient intermediates, which were not characterized, are shown in bracket. *1* acenaphthylene, *2* acenaphthene-1,2-diol, *3* 1,2-dihydroxy acenaphthylene, *4* acenaphthenequinone, *5* catechol, *I* naphthalene-1,8-dicarboxylic acid, *II* 1,2-dihydroxynaphthalene, *III* 1-naphthoic acid, *IV* salicylic acid

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