KINETICS AND METABOLISM OF ANTHRACENE BY *ALCALIGENES FAECALIS* MVMB1

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Abstract

The biodegradation of anthracene, a perilous model Polycyclic aromatic hydrocarbon (PAH) was examined by employing aerobic bacterial strain *Alcaligenes faecalis*, isolated from contaminated soil. The degradation and growth of the organism on anthracene was experimented at different inoculum concentrations and pH. The optimum conditions were observed to be at 2% (v/v) inoculum concentration and 6.5 pH. The effect on initial substrate concentration proved anthracene as an inhibitory type substrate as the maximum degradation was at 40 mg l^{-1}. Within 40 h and at 30 ° C, the strain metabolized 92.34% of anthracene under optimized conditions. With the simulated and experimental data's, kinetic constants were calculated for Monod, Haldane and competitive inhibition models. Metabolites were identified using High performance liquid chromatography and Gas chromatography mass spectrum for elucidating the biodegradation pathways. Three degradation pathways were proposed, one of which has been demonstrated as a new branch in anthracene degradation pathway because of 9 hydroxy fluorene a novel metabolite. Altogether these results signify the effectual PAH degradation by the promising isolated strain *Alcaligenes faecalis* MVMB1.

Key words: Anthracene, *Alcaligenes faecalis,* Biodegradation, Polycyclic aromatic hydrocarbons, Kinetic models

I. INTRODUCTION

Anthracene is an anthropogenically formed polycyclic aromatic hydrocarbon (PAH) due to incomplete combustion of organic substances from petrochemical industries as well as from oil refinery activities (1). Animal PAH's exposure occurs by breathing, ingestion and dermal exposition. On exposure to humans, PAHs' metabolized to produces epoxide compounds with mutagenic and carcinogenic properties (2). Thus substantial treatments are to be carried out to eradicate them in the environment. But the complex molecular structure and low solubility in water of PAH, limits the application of conventional treatments.

Biodegradation has become the most reliable mechanism by which thousands of xenobiotic pollutants, including crude oil, are eliminated from the environment. Environmental bacteria are considered to be the most important organisms capable of natural biodegradation. Many organisms like *Burkholderia* sp. VUN10013 (3), *Pseudomonas* sp. (4) *Rhodococcus* sp. (5), *Mycobacterium* sp. (6) etc have been isolated which degraded anthracene. Only few reports have demonstrated a substantial degree of anthracene or petroleum contaminant oxidation under aerobic conditions by *Alcaligenes faecalis* (7-9). The biodegradation of PAHs by microbes has been generally known to be inhibited by PAH itself. Thus to describe the biodegradation of a substrate, it is necessary to evaluate the relationship between the specific growth rate and the concentration of PAH for which kinetic models can be used.

Biotransformation pathways for anthracene have been elucidated for various microorganisms suggesting dioxygenation and dehydration by which 1,2-dihydroxyanthracene is formed. But till date, no degradation pathway for anthracene by *Alcaligenes faecalis* has been proposed. Therefore, on the basis of the metabolites identified in this paper, a new insight to the biotransformation of *Alcaligenes faecalis* will be provided.

With these perspective, the present study is intended (i) to optimize inoculum concentration and pH for the degradation of anthracene by *Alcaligenes faecalis* MVMB1, (ii) to describe the biodegradation of anthracene by *Alcaligenes faecalis* MVMB1 in an optimized medium, (iii) to determine the appropriate kinetic model for describing the biodegradation of anthracene and to estimate the corresponding kinetic parameters and (iv) to conclude about the pathway of degradation.

II. MATERIALS AND METHODS

A. Microorganism isolation and acclimatization

Soil sample was collected from petroleum contaminated site near Ennore, Chennai. PAH degrading bacteria were isolated from the soil sample by enrichment culture technique on MSM by using PAH compounds as sole carbon and energy source. The MSM composed per liter (pH 7): KH_2PO_4 - 1.0 g, Na_2HPO_4. $2H_2O$ - 1.25 g, $MgSO_4$. $7H_2O$ - 0.5 g, $CaCl_2$. $2H_2O$ – 0.05 g, $FeSO_4$. $7H_2O$ – 0.005 g, $(NH_4)_2SO_4$ - 1.0 g sterilized by autoclaving at 121 ° C for 20 min. PAH were dissolved in 250 ml Erlenmeyer flasks containing 5 ml acetone, evaporated and MSM (100 ml, pH 7.0) were added to the culture flask to a final concentration of 120 mg l^{-1} PAH, sterilized by autoclaving at 121 C for 20 min. 1 gm soil sample was inoculated into MSM PAH and incubated in the dark for 7 days at 30 C with agitation of 150 rpm. The turbidity growth was visualized and at the end of 7 th day, 5 ml of the enrichment cultures were transferred to a fresh MSM PAH and incubated under the same Strain MVMB1 was identified through morphological, biochemical and 16S rRNA gene sequence analysis. Nucleotide sequence similarity searches were conducted by Genebank BLAST (N). Phylogenetic tree was constructed using mega software 4.1 version.

B. Carbon-source utilization

In addition to anthracene, the purified strain was also tested for growth on one of the following compounds at 0.01%: naphthalene, phenanthrene, fluorene, fluoranthene and pyrene which were added as sole carbon sources to liquid MSM. Sterilized MSM medium containing appropriate amounts of PAH were inoculated with the tested strains and incubated in an orbital shaker as described above. Growth test was followed by measuring the increase of OD600 nm of the culture.

C. Batch Experiments

The minimal salts medium composition for further experiments was as follows (g per liter) KH_2PO_4 - 2 g, Na_2HPO_4. $2H_2O$ - 1.5 g, $MgSO_4$. $7H_2O$ - 0.2 g, $CaCl_2$. $2H_2O$ – 0.3 g, $FeSO_4$. $7H_2O$ – 0.02 g, $(NH_4)_2SO_4$ – 1.0 g and anthracene – 0.1 g. To optimize the biodegradation of anthracene, batch experiments were carried out at 37 ° C & pH 7 and substrate concentration of 0.1 g l^{-1} unless otherwise stated. After each optimization, the optimized conditions were adapted for further experiments. The range of inoculum concentration studied was from 1 % to 5%. For pH studies, the desired pH in the range of 5.5 to 7.5 was maintained by adding HCl or NaOH at the beginning of the experiment and not controlled afterwards. The kinetic study was conducted from the results obtained by optimization experiments at different initial substrate concentration ranging from 10 - 100 mg l^{-1}. Suitable substrate models found in the literature and adopted in this study has been described below:

Monod model (10), the earliest model on microbial growth kinetics assumes exponential growth rate with a constant specific growth rate until some substrate becomes growth limiting.

$$\mu_g = \frac{\mu_{max}[S]}{K_S + [S]} \quad [1]$$

where μ_g is the specific growth rate (h^{-1}), μ_{max} the maximum specific growth rate (h^{-1}), [S] the substrate concentration (mg l^{-1}) at time t, K_s is the half saturation constant (mg l^{-1}). However, this equation becomes unsatisfactory for explaining inhibitory growth of microorganism at higher substrate concentrations. This is corrected by incorporating (K_i) inhibition constant which can be used to describe the growth linked kinetics. Haldane model (11), originally proposed in 1968 is most widely used for substrate inhibition kinetics.

$$\mu_g = \frac{\mu_{max}[S]}{K_s + [S] + ([S^2]/K_i)} \quad [2]$$

The competitive growth inhibition model represents rate expression due to inhibition of toxic compounds other than the substrate or product, in analogy to enzyme inhibition (11):

$$\mu_g = \frac{\mu_{max}[S]}{\{K_s\left(1 + \frac{I}{K_i}\right)\} + [S]} \quad [3]$$

where I is the toxic compound concentration (mg l^{-1})

D. Analytical methods

Bacterial concentrations were determined using a UV–visible spectrophotometer (Hitachi U-2000, Japan) at 600 nm by measuring the absorbance of the cell solutions. Biomass concentration was estimated from

correlation between OD and dry cell weight. Anthracene was analyzed and quantified by hexane exaction, evaporation, acetonitrile resuspension (12) and high-performance liquid chromatography (HPLC) (Shimaduzu, 10 ATVP, Japan) estimation (13). Degrading efficiency was calculated as follows Degrading Percentage (%) = Ci - Ct / Ci × 100% where C stands for the concentration of Anthracene.

Metabolite isolation and identification was carried out with the samples from the batch culture cultivated up to late log phase (25 h). 100 ml of supernatant was acidified for pH 2 which was then extracted twice with 30 ml of ethyl acetate each time. This was evaporated, condensed and derivatised with N methyl –N-(trimethylsilyl) trifluoroacetamide (14) for Gas Chromatography Mass Spectrum analysis (GC-MS) (Thermo-Finngan Trace DSQ) whereas it was fully evaporated and re-dissolved in acetonitrile for HPLC.

E. Data analysis

Regression analysis was performed with the data analysis tool pack of Microsoft Excel®. The model equations were solved using the non-linear regression method using MATLAB® 7.0.

III. RESULTS AND DISCUSSIONS

A. Identification and acclimatization of microorganism

The Strain MVMB1 isolated from petroleum contaminated soil was facultative anaerobic, Gram negative, motile, rods singly or in short chains. Colonies were yellow, smooth with punctuate and diffuse edges. The shape of growing cells was cocobacilli. Temperature range for growth was 30 – 37 ° C; no growth occurred at 45 ° C. They were negative for Indole, methyl red and Voges proskauer and positive for Citrate utilization, oxidase, catalase and decarboxylase (lysine decarboxylase and Ornithine decarboxylase). Acids were produced from D-glucose, lactose, D-mannose and Sorbitol. Nitrate was not reduced. The bacterial 16S r DNA was aligned against representatives of the genus Alcaligenes from the GenBank Databases. 1403 bp sequence has been submitted to GenBank under accession number HM103385.

The PCR-amplified product was sequenced and a phylogenetic tree was drawn on the basis of the sequences. The evolutionary history was inferred using the Neighbor-Joining method (15). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (16). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 1403 positions in the final dataset. Phylogenetic analyses were conducted in MEGA 4 (17).

The phylogenetic tree constructed for comparing the out group clearly indicated that the isolate MVMB1 belongs to genus *Alcaligenes* and, hence, it was designated *Alcaligenes faecalis*. The bases of the 16S rRNA gene of strain MVMB1 showed 97% identity with *Alcaligenes faecalis subsp. faecalis strain persicum*. (phylum: *Proteobacteria*; class: *Beta Proteobacteria*; order: *Burkholderiales*; family: *Alcaligenaceae*) as shown in the phylogenetic tree in Fig. 1.

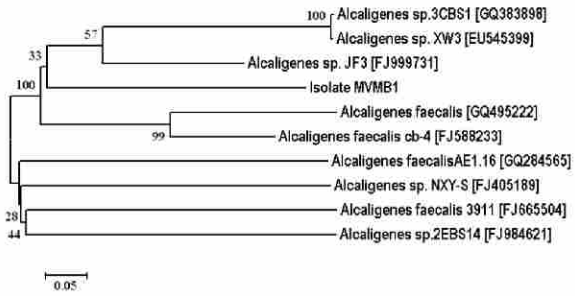

Fig. 1. Phylogenetic tree of the strain MVMB1. Bootstrap consensus tree was drawn by multiple sequence alignment with Neighbour-Joining method using software Mega, version 4.

Table 1. Utilization of Substrates by *Alcaligenes faecalis* MVMB1

Substrates	MVMB1
Naphthalene	++
Phenanthrene	+++

Substrates	MVMB1
Fluorene	+
Fluoranthene	–
Pyrene	–

This strain can utilize Phenanthrene, Naphthalene and flourene as sole carbon source with optical density > 0.2 (++), > 0.3 (+++) & > 0.1 (+), measured after 48 h of growth respectively (Table 1).

This identified species was acclimatized and numerous generations had been successfully cultured for the organism to attain the capacity to consume anthracene. Several external factors like inoculums concentration, pH, substrate concentration, physical properties of contaminants and the oxygen content can limit the rate of biodegradation of organic compounds. Thus it becomes necessary to optimize each of these factors for the selected organism for achieving maximum compound degradation. The optimization of the substrate concentration in any biodegradation is particularly important because inhibition may occur at higher concentrations.

B. *Effect of inoculum concentration*

Experiments were conducted at different inoculums concentration, an important factor to be considered for the maximum degradation. Fig. 2 represents the results on the effect of various inoculum concentrations on growth and degradation by *Alcaligenes faecalis*. The highest inoculum concentration did not stimulate the biodegradation when compared to the minor inoculums concentration used. 2% v/v inoculum proved to be optimum with a cell density of 12 mg l^{-1}. The rate of degradation was 1.147 mg l^{-1} hr^{-1} for 2% initial inoculum concentration representing 45.89% degradation in 35 minutes. The failure of the small inoculum to degrade anthracene could be a result of the failure of the bacterium to survive. Each inoculum concentration seems to have limited ability to perform anthracene biodegradation which is lost after a certain period of time.

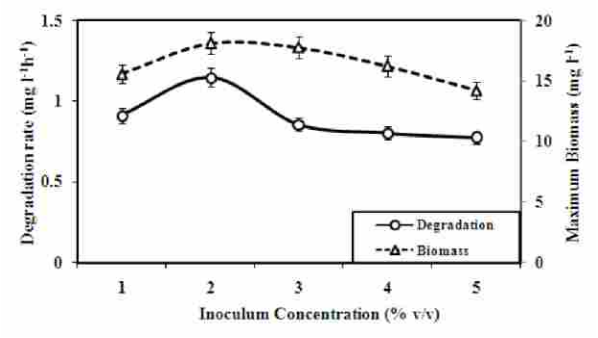

Fig. 2. Effect of inoculum concentration on % degradation of anthracene (circles) and maximum growth (triangles) (Conditions: pH 7.0; anthracene 0.1 g l^{-1}).

C. *Effect of pH on anthracene degradation*

The cells structural organization, permeability, availability of macronutrients and the enzyme activity are highly pH reliant. The % degradation of the aromatics can be enhanced in the microbial environment either at acidic pH (18, 19) or alkaline pH (20) or neutral pH (21) depending on the ability of the strain. In the present study, high biomass of 20.8 mg l^{-1} and a degradation rate of 1.221 mg l^{-1} hr^{-1} were obtained at an initial pH of 6.5 after 35 h of incubation in the liquid culture. (Fig. 3) Nearly 1.2 to 1.5 folds increase for pH 6.5 to 7.5 and decrease for 6.5 to 5.5 representing optimum degradation of 48.85% for pH 6.5. In a study of Kim et al. (22) a fourfold increase in the degradation rates of phenanthrene and pyrene has been observed at pH 6.5 compared with those at pH 7.5. Thus good metabolic activity of the organism suggesting proton extrusion and proton removal (23) for *Alcaligenes faecalis* has occurred at pH 6.5.

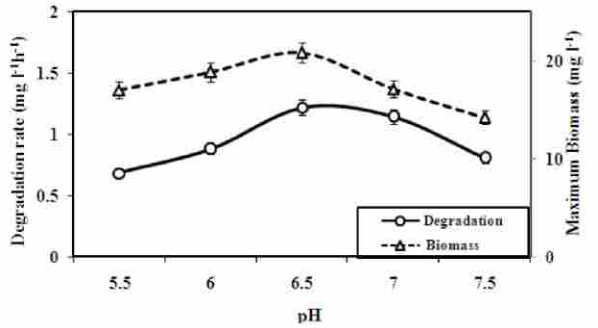

Fig. 3. Effect of pH on % degradation of anthracene (circles) and maximum growth (triangles) (Conditions: inoculum concentration 2%; anthracene – 0.1 g l^{-1})

D. Effect of initial substrate concentration

The experimental study on different substrate concentrations resulted in slow and constant growth with degradation rate less than the optimum rate of 2.3085 mg l^{-1} h^{-1} observed at 40 mg l^{-1} (Fig 4). The cells grew well without the adaptation phase upto 40 mg l^{-1} initial concentration achieving 92.34% degradation within 40 h (Fig. 5). Somtrakoon et al. (3) experienced efficient utilization of 90% (150 mg l^{-1}) anthracene in 10 days using *Burkholderia sp*, anthracene-grown VUN10013. Using isolated *Pseudomonas* strains nearly 72% degradation for 250 ppm anthracene in 48 days had been achieved by Santos et al. (4).

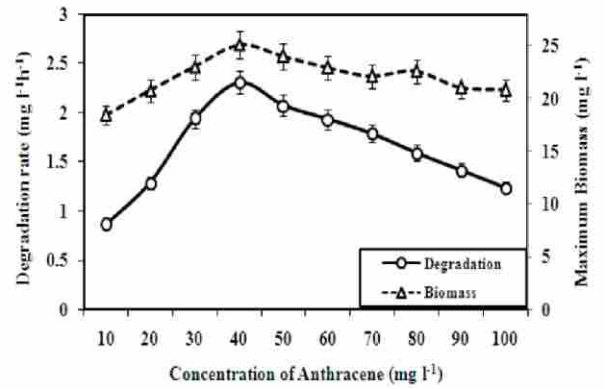

Fig. 4. Effect of substrate concentration on % degradation of anthracene (circles) and maximum growth (triangles) (Conditions: inoculum concentration 2%; pH 6.5)

E. Cell growth kinetics

Growth models allow quantitative description of the substrate utilization or growth associated product formation. The correlation between the specific growth rate (%) of a microorganism and the substrate concentration is an important tool in the biodegradation process. These are helpful in the understanding the behavior of biological systems in treating hazardous compounds accumulating in the environment (11). Various single substrate growth kinetic models are available in the literature. The model parameters were evaluated using MATLAB 6.5, based on Windows XP. This software utilizes the curve fitting tool box for minimizing the sum of square of the residuals. Except for the monod model, all substrate concentration values were fitted to the experimental data, but the data up to 40 mg l^{-1} were alone considered for the monod model. The possible reasons for the loss of activity towards anthracene include high concentration inhibition and formation of toxic by product inhibition. Fig. 6 gives the results of the three kinetic models and the results of experimental run.

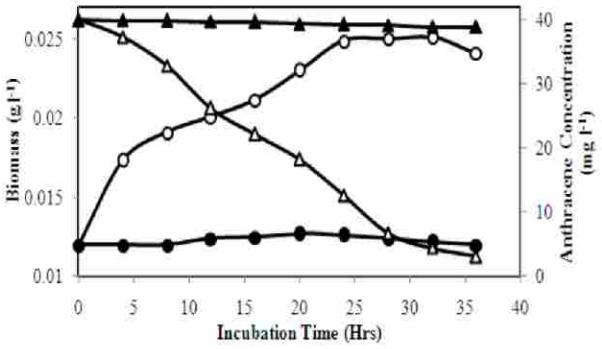

Fig. 5. Growth (circles) of MVMB1 in anthracene at 40 mg l^{-1} and anthracene biodegradation (triangles) by MVMB1. Control was performed by inoculating with cells without carbon source. (Solid symbol is about the control and hollow symbol is about the test degradation). The culture was kept at 37 ° C with 150 rpm shaking.

The fitted equations for the models were as follows:

$$\mu_g = 0.03156[S]/13.3 + [S]$$

$$\mu_g = 0.07174\ [S]/42.66 + [S] + ([S^2]/37.02)$$

$$\mu_g = 0.02162\ [S]/0.5831\ [1 + (1/0.209)] + [S]$$

The specific growth rate was found to be in the range of 0.0184 to .0251 h^{-1} which may be due to the optimized parameters used in the present study. Roy et al. (24), reported a specific growth rate of 0.0188 h^{-1} and K_S of 139 mg l^{-1} for anthracene degradation by a mixed culture of *Acinetobacter* sp. and *Alcaligenes* sp. Tian et al. (25) reported that *Pseudomonas mendocina* as an organism is capable of degrading phenanthrene as the sole source of carbon. The degradation was 95% for 100 ppm of within two days with specific growth rate of 0.033 h^{-1}.

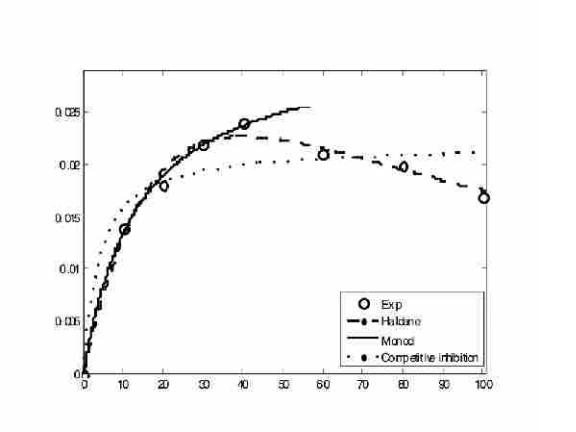

Fig. 6. Models fitted to the results of experimental data at optimized conditions.

F. Identification of metabolite

Six metabolites were found from the extract, analyzed using HPLC and also MSTFA derivatised extract using GC MS. The mass spectrums of the metabolites (Fig. 7) were compared with those of reference and authentic standards. Four metabolites had their base ion at m/z 73 and the other specific ion at m/z 147 which are characteristics of MSTFA derivatisation (14). Metabolite II, eluted at 13.055 min, contained a molecular ion peak at m/z 416 and fragment ions at m/z values of 399 (M+ –15, CH3 loss), 383, 341, 327, 281, 221, 207, 191, 147 and 73. This metabolite was identified as 3-(2-carboxyvinyl) naphthalene-2-carboxylic acid compared with the authentic standard, which is known as a product of ortho-cleavage of 1, 2 dihydroxyanthracene.

The mass spectrum of metabolite III with 15.059 min retention time, contained a molecular ion peak at m/z 489 and fragment ions at m/z values of 416 (M + –73) $[(CH_3)_3Si]^+$ loss), 401,385,355, 341 $[2(CH_3)_3Si]^+$ loss), 327, 281, 267, 221, 207, 191, 147 and 73. This metabolite was identified as 4, 5 dihydroxyphthalate compared with the authentic standards.

One of the spectrum, metabolite IV, had its molecular ion at m/z 356 which was consistent with a di-TMS ether of an anthracene dihydrodiol (26) detected at 16.783 min in the GC-MS analysis. The major fragment ions of this metabolite were 341 (M+ – 15,) CH3 loss), 281,267 (M+ –89,) OSi (CH3)3 loss), 221, 191,147 and 73. The position of hydroxyl substitution on the anthracene ring could not be determined because of the limited material and lack of authentic standard.

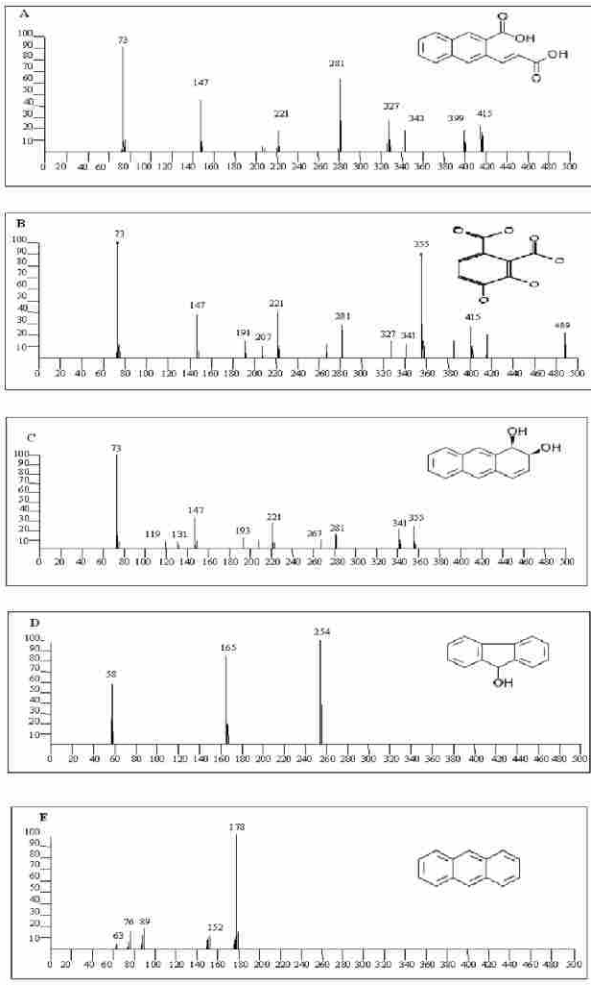

Fig. 7. Mass spectrum of the identified metabolites as trimethyl silyl derivatised products with the conformed structure of the metabolites (A - 3-(2-carboxyvinyl) naphthalene-2-carboxylic acid, B - 4, 5 dihydroxyphthalate, C - Anthracene dihydrodiol, D - 9-hydroxyfluorene and E - anthracene)

The fifth metabolite in the extract was found to be the remaining anthracene similar to NIST, Korade and Fulekar (27) as well as the authentic standard. The sixth metabolite obtained at 22.0 min was identified as MSTFA 9-hydroxyfluorene which had a base peak at m/z 254. The other fragment ions were present at m/z 165 and 58. The spectrum was compared with that obtained by the authentic standard and by the some author's work (14, 28).

G. Interpretation of degradation pathway

Based on rigorous chemical structure identification from mass spectrum (Fig. 8), three pathways are evident in the degradation of anthracene by *Alcaligenes faecalis*. Metabolites IV, anthracene cis 1,2 dihydrodiol have been described as an intermediate of anthracene degradation by *Rhodococcus* sp. (5) and *Mycobacterium vanbaalenii* PYR-1 (6). Dihydrodiols can be the intermediate compound, because most bacterial anthracene cleavage reactions occur at the 1,2-position coursed by dioxygenation and dehydration reactions (39-31). The enzymatic attack in the C-1 and C-2 positions of the anthracene moiety was similar to the pathways previously reported in phenanthrene degradation by *Micrococcoccus* sp (32). A ring fission product, 3-(2-carboxyvinyl)naphthalene-2-carboxylic acid identified by Moody et al. (6) was also identified in the present investigation. *ortho*-Ring cleavage of 1,2-dihydroxyanthracene can lead to the formation of 3-(2-carboxyvinyl)naphthalene-2-carboxylic acid. The detection of these *ortho*-cleavage ring fission products is analogous to evidence in a recent report on naphthalene degradation in *Geobacillus* sp *G27*(33). This work confirms and extends the catabolic pathways previously proposed for anthracene degradation by *Alcaligenes faecalis*. This predicted pathway forms one of the catabolic pathway for anthracene by *Alcaligenes faecalis*. With the detection of dihydroxy phthalate in our study, the next pathway that is similar to the phthalate pathway in other bacterial strains (*Mycobacterium vanbaalenii* PYR-1, *Sphingomonas paucimobilis* EPA, *Sphingomonas* sp.126 LB, *Rhodococcus* sp 505, *Pseudomonas rhodesiae* KK1 sp.) was considered as the second pathway.

The observation of 9 hydroxy flourene never before detected in anthracene metabolism by bacteria, suggests that there is also a novel pathway through this compound which forms the third pathway in *Alcaligenes faecalis*. This proceeds through catalyzes of 9,10-hydroxylation of anthracene to produce anthracene-9,10-dihydrodiol, which can be converted to 9,10 dihydroxyanthracene, which spontaneously tautomerizes to 9,10-anthraquinone. (*Mycobacterium vanbaalenii* PYR-1) The benzil benzilic acid rearrangement can convert diketone to hydroxyl carboxylic acid in the presence of hydroxide ions (34). This 9 hydroxy- fluorene-9-carboxylic acid with the help of decarboxylase would help in the formation of 9 hydroxy flourene which can proceed through the dihydroxy pthlate.

The following are the pathways proposed for degradation by Alcaligenes faecalis sp MVMB1 (Fig. 10) (i) Anthracene → *cis*-1,2-Dihydroanthracene-1,2 diol → Anthracene-1,2-diol → 3-[(Z)-2-Carboxyvinyl]-2-naphthoate (ii) Anthracene → *cis*-1,2-Dihydroanthracene-1,2-diol → Anthracene-1,2-diol → [4-(2-Hydroxynaph-3-yl)-2-oxobut-3-enoate] → 3-Hydroxy -2 naphthoate → Phthalate → dihydroxy phthalate (iii) Anthracene → [*cis*-9,10-Dihydroanthracene-9,10-diol] → 9,10-Anthraquinone → [Benzil benzilic acid rearrangement]9 hydroxy-fluorene-9-carboxylic acid → [Decarboxylase] 9 hydroxy flourene → dihydroxy phthalate. Thus three pathways were proposed in which the 9 hydroxy flourene pathway in a novel one. All the three pathways were evident to enter the central metabolism, as no above identified metabolites were seen in the culture extract taken after 50 h.

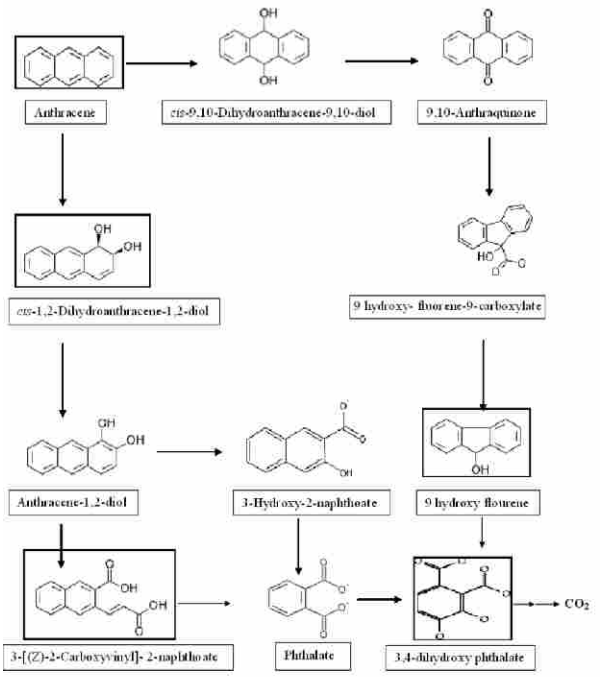

Fig. 8. The proposed catabolic pathway for anthracene degradation by *Alcaligenes faecalis*. The compounds in box were identified in this study.

IV. CONCLUSION

Batch degradation of anthracene using *Alcaligenes faecalis was* investigated and the factors affecting the degradation were optimized. The optimum conditions supporting the growth and degradation were found to be 2% inoculum concentration and 6.5 pH. The effect of substrate concentration showed the optimum growth to be 40 ppm. The kinetic models analyzed (Monod, Haldane and Competitive inhibition) were found to fit well. The pathway of degradation was proposed based on the metabolites mass spectrum analysis. *Alcaligenes faecalis* MVMB1 is unique in degrading anthracene by three pathways. The pathway of degradation was proposed based on the metabolites mass spectrum analysis which showed formation of 9 hydroxy flourene as a metabolite. A novel pathway involving 9 hydroxy flourene has also proved that *Alcaligenes faecalis* MVMB1 can degrade even high molecular weight compound Flourene. This paper has thrown insight on the *Alcaligenes faecalis* in biodegradation of PAH as a model microbe and its catabolic pathway. With these results it is suggested that *Alcaligenes faecalis* can be used in any consortium for handling various hydrocarbons efficiently.

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