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Phenanthrene degradation by *Pseudoxanthomonas* sp. DMVP2 isolated from hydrocarbon contaminated sediment of Amlakhadi canal, Gujarat, India

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ABSTRACT

Amlakhadi canal, flowing through Ankleshwar (Gujarat, India) has been impinged with various xenobiotic compounds, released in industrial discharges, over last many decades. Twenty five bacterial strains capable of phenanthrene degradation were isolated from sediments of Amlakhadi canal. The best strain amongst them was identified as *Pseudoxanthomonas* sp. DMVP2 based on 16S rRNA gene sequence analysis, and selected for further studies. Experiments were carried out for optimization of abiotic parameters for efficient phenanthrene degradation. Strain DMVP2 was able to degrade 300 ppm of phenanthrene completely in minimal medium containing peptone (0.1%, w/v) as nitrogen source with initial pH 8.0 at 37 °C under shaking condition (150 rpm) within 120 h. Strain DMVP2 was able to consume 1600 mg/l of phenanthrene even at high initial concentration (4000 mg/l) of phenanthrene. Identification of phthalic acid as major metabolite on GC–MS analysis and detection of protocatechuate dioxygenase activity revealed that phenanthrene was metabolized by phthalic acid-protocatechuate acid pathway. Strain DMVP2 was also able to utilize other xenobiotic compounds as sole carbon source and degrade phenanthrene in presence of other petroleum hydrocarbons. Consequently, *Pseudoxanthomonas* sp. DMVP2 has potential applications in bioremediation strategies.

1. Introduction

Pollution by polycyclic aromatic hydrocarbons (PAHs) in soil has recently become a matter of great concern due to their toxic, mutagenic and carcinogenic nature [1]. PAHs are persistent in environment due to the high thermodynamic stability of benzene moiety and hydrophobic nature. Most of them tend to sorb into the soil particulates, rendering them unavailable for biological uptake. The US Environmental Protection Agency have identified 16 PAH compounds as priority pollutants on the basis of their abundance and toxicity and phenanthrene is amongst them. Phenanthrene, a three-ring angular PAH, is known to be a human skin photosensitizer, an inducer of sister chromatid exchanger and a potent inhibitor of gap-junctional intercellular communication [2]. Therefore, it is necessary to establish effective methods for removal of PAH, to protect the environment. Bioremediation is considered as the most significant and influential for degradation or detoxification of xenobiotic compounds. Many reports have described phenanthrene degradation by various bacteria such as *Alcaligenes*, *Acinetobacter*, *Arthrobacter*, *Bacillus*, *Burkholderia*, *Brevibacterium*, *Acidovorax*, *Micrococcus*, *Moraxella*, *Mycobacterium*, *Pseudomonas*, *Rhodococcus*, *Sphingomonas*, *Stenotrophomonas*, *Paenibacillus* and others [2–8].

The catabolism of phenanthrene by bacteria has been studied. In general, phenanthrene is metabolized to 1-hydroxy 2,naphthoic acid (1N2HN) by initial dioxygenation. 1N2HN was further degraded through two different pathways. In one of the pathway, 1N2HN is oxidized to 1,2-dihydroxynaphthalene and is later converted to salicylic acid. In another pathway, 1N2HN undergoes ring-cleavage leading to formation of o-phthalic acid, which is further converted to protocatechuic acid [7,9].

Although *Pseudoxanthomonas* species have been found to be distributed in a wide variety of contaminated environment, there is no comprehensive biochemical report on the degradation of PAHs.

In this study, we have isolated phenanthrene degrading *Pseudoxanthomonas* sp. DMVP2 from hydrocarbon contaminated sediment and optimized various environmental parameters to achieve higher phenanthrene degradation. Phenanthrene degradation pathway was studied using gas chromatography and mass

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spectrometry. We have also examined the effect of presence of other petroleum hydrocarbons on phenanthrene degradation.

2. Materials and methods

2.1. Culture medium and chemicals

The minimal medium (MM) [KH_2PO_4 1.0 g l^{-1}, K_2HPO_4 1.0 g l^{-1}, NaCl 0.5 g l^{-1}, NH_4NO_3 1.0 g l^{-1}, $MgSO_4{\cdot}7H_2O$ 0.2 g l^{-1}, $CaCl_2$ 20 mg/l and $FeCl_3{\cdot}6H_2O$ 5 mg/l] supplemented with phenanthrene was used for enrichment of phenanthrene degrading bacteria. Phenanthrene, naphthalene and phthalic acid were purchased from Himedia (Mumbai, India). Pyrene and protocatechuic acid were obtained from Sigma–Aldrich (Steinheim, Germany). All other chemicals used were of analytical grade.

2.2. Enrichment, isolation and screening of phenanthrene degrading strains

Sediments samples were collected from long term polluted Amlakhadi canal, Ankleshwar, Gujarat, India. Sediment sample was inoculated in minimal medium amended with 50 ppm of phenanthrene and incubated at 37 °C under shaking condition (150 rpm) (Certomate, B. Braun Biotech International, Goettingen, Germany). After one week part of this enriched sample was used as inoculum in fresh MM amended with 70 ppm of phenanthrene and incubated at 37 °C under shaking condition (150 rpm). Fifteen transfers were carried out in fresh MM supplemented with phenanthrene and each time phenanthrene concentration was increased (increment of 20 ppm). The appropriate dilutions of enriched sample were spreaded on minimal agar and incubated at different temperature. Before use of minimal agar, 0.025% of phenanthrene was dissolved in acetone, spreaded on the surface of the medium as the sole carbon source. Based on morphological characteristic, 25 bacterial strains were isolated and subsequently maintained on minimal agar supplemented with phenanthrene (25 ppm). All isolated bacterial strains were examined for phenanthrene degradation.

2.3. Identification by 16S rRNA gene sequencing

Genomic DNA was extracted from strain DMVP2 as described by Ausubel et al. [10]. The 16S rRNA gene was amplified in a 30 μl PCR reaction consisting of 1× buffer (10 mM Tris pH 9.0, 50 mM KCl, 1.5 mM MgCl2, 0.1% Triton X-100), 0.33 mM each of dNTPs, 0.66 pmol each of custom synthesized universal primer 8F 5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′), and 1.5 U of Taq DNA polymerase. Amplification program was performed with initial denaturation step at 94 °C for 5 min; followed by 30 cycles of 1 min denaturation step at 94 °C, 1 min annealing step at 55 °C, and 1.2 min elongation step at 72 °C and a final extension step at 72 °C for 20 min using Biorad icycler version 4006 (Biorad, CA, USA). The amplified 16S rRNA gene products were purified using Wizard PCR-Clean-up kit (Promega, Madison, USA) The purified 1.5 kb PCR product was sequenced by automated DNA Analyser 3730 using ABI PRISM® BigDye™ Terminator Cycle Sequencing 3.1 (Applied Biosystems, Foster City, CA). Nearly full-length bacterial 16S rRNA gene sequence was analyzed using BLAST (n) program at NCBI server [11] to identify and downloaded the nearest neighbour sequences from the NCBI database. All the sequences were aligned using Clustal W version 1.6 program. The phylogenetic tree was constructed using aligned sequences by the neighbour joining (NJ) algorithm using Jukes–Cantor evolutionary distances and Kimura 2 parameter with more than 1000 replicates in MEGA (Molecular Evolutionary Genetic Analysis) version 4.0 software [12].

2.4. Preparation of bacterial inoculum

The bacterial strain DMVP2 was inoculated in MM containing 300 ppm of phenanthrene and incubated at 37 °C under shaking condition (150 rpm) till the growth reached late exponential phase. Cells were centrifuged at 5000 × *g* for 10 min, washed twice with 0.85% normal saline and finally resuspended in the same buffer to obtain a cell suspension with an absorbance (A_{600}) of 1.0 and this was then used as the inoculum.

2.5. Effect of abiotic factors on phenanthrene degradation

All experiments were carried out in 250 ml Erlenmeyer flasks containing 100 ml of MM amended with appropriate amount of phenanthrene. The residual phenanthrene was analyzed using gas chromatography (GC) as described in Section 2.6.

2.5.1. Effect of inoculum quantity

Minimal medium supplemented with 300 ppm of phenanthrene were inoculated with different inoculum quantity such as 1, 2, 4 and 6% (v/v), of strain DMVP2 and incubated at 37 °C under shaking condition (150 rpm). A control, without inoculation of strain DMVP2 was kept under similar conditions to determine abiotic loss of phenanthrene. Samples were collected at regular intervals of time (24 h) up to 120 h and analyzed for residual phenanthrene.

2.5.2. Effect of nutrients

Minimal medium supplemented with 300 ppm of phenanthrene along with different nutrients such as peptone, yeast extract, sodium succinate and ammonium nitrate (0.1%, w/v) were inoculated with strain DMVP2 (4%, v/v) and incubated at 37 °C under shaking condition (150 rpm). A control, without any nutrient, was kept under similar conditions. Samples were collected at regular intervals of time (24 h) up to 120 h and analyzed for residual phenanthrene.

2.5.3. Effect of temperature

Minimal medium supplemented with 300 ppm of phenanthrene along with peptone (0.1%, w/v) were inoculated with strain DMVP2 (4%, v/v) and incubated at different temperatures such as 30 °C, 37 °C and 40 °C under shaking condition (150 rpm). Controls, without inoculation of strain DMVP2, were kept under similar conditions. Samples were collected at regular intervals of time (24 h) up to 120 h and analyzed for residual phenanthrene.

2.5.4. Effect of static as well as different shaking condition

Minimal medium supplemented with 300 ppm of phenanthrene along with peptone (0.1%, w/v) were inoculated with strain DMVP2 (4%, v/v) and incubated at 37 °C under static as well as under different shaking condition such as 50, 100 and 150 rpm. Controls, without inoculation of strain DMVP2, were kept under similar conditions. Samples were collected at regular intervals of time (24 h) up to 120 h and analyzed for residual phenanthrene.

2.5.5. Effect of pH

Minimal medium supplemented with 300 ppm of phenanthrene and peptone (0.1%, w/v) was adjusted to different initial pH such as 7.0, 8.0, 9.0 and 10.0 using either HCl or NaOH. The flasks were inoculated with strain DMVP2 (4%, v/v) and incubated at 37 °C under shaking condition (150 rpm). Controls, without inoculation of strain DMVP2, were kept under similar condition with inoculated flasks. Samples were collected at regular intervals of time (24 h) up to 120 h and analyzed for residual phenanthrene.

2.5.6. Effect of initial concentration of phenanthrene on degradation

Minimal medium supplemented with peptone (0.1%, w/v) along with different concentrations of phenanthrene such as 300, 500, 750, 1000, 1500, 2000, 4000 and 5000 ppm of phenanthrene were inoculated with strain DMVP2 (4%, v/v) and incubated at 37 °C under shaking condition of 150 rpm. Controls, without inoculation of strain DMVP2, were kept under similar conditions. Samples were collected after 120 h to determine residual phenanthrene.

2.5.7. Effect of surfactants

Minimal medium supplemented with 300 ppm of phenanthrene along with 0.02% (w/v)/(v/v) of different surfactants such as sodium dodecyl sulfate (SDS), cetyl trimethyl ammonium bromide (CTAB), Tween 80 and Triton X-100 were inoculated with strain DMVP2 (4%, v/v) and incubated at 37 °C under shaking condition of 150 rpm. A control, without any surfactant, was kept under similar conditions. Samples were collected after 96 h to determine residual phenanthrene.

All the results represent the average of three independent experiments. Significant difference was calculated using mean and standard deviation. Error bars represent the standard deviation from the mean in graph of all experiment.

2.6. Phenanthrene extraction, analysis and quantification

The entire content (100 ml) of flask was taken for determination of residual phenanthrene in all the experiments. For phenanthrene extraction, 20 ml dichloromethane (DCM) was added in flask and incubate at 37 °C under shaking condition (150 rpm) for 1 h. The content was then centrifuged at 5000 × *g* for 10 min and the extract was dried using anhydrous sodium sulfate to remove aqueous phase. Residual phenanthrene in extract was analyzed using gas chromatography (Clarus 500, Perkin Elmer, USA) equipped with flame ionization detector (FID) and a Stabliwax column (30 m length × 0.32 mm inner diameter, crossbond-PEG) (Restek, USA). Nitrogen gas was used as the carrier gas at a flow rate of 3 ml/min. The injector temperature and the detector temperature were kept at 270 °C.

2.7. Analysis of metabolites during phenanthrene degradation by strain DMVP2

Strain DMVP2 was grown in MM amended with phenanthrene (300 ppm) and peptone (as nitrogen source). Flasks were incubated under optimized conditions. At regular interval of time (0 h, 72 h and 144 h), metabolites were extracted from medium as described by Samanta et al. [13].

Metabolites were analyzed using GC (Autosystem XL, Perkin Elmer, USA), equipped with PE-5MS capillary column (30 m long; 0.25 mm internal diameter) and coupled to a MS (Turbo mass, Perkin Elmer, USA). GC temperature programme was 80 °C (1 min), 80–280 °C (10 °C/min) and 280 °C (30 min). The injection volume was 1 μl and helium was used as carrier gas at the flow rate of 1 ml/min. The mass spectrometer was operated at electron ionization energy of 70 eV. Injector and detector temperature were kept at 250 °C.

In addition to this, activity of protocatechuate dioxygenase and catechol dioxygenase were determined as described by Chatterjee and Dutta [14].

2.8. Growth of Pseudoxanthomonas sp. DMVP2 on various other xenobiotic compounds

MM containing 0.1% [(w/v)/(v/v)] of different xenobiotic compounds [namely, ketone (acetone), alkanes (*n*-hexane), monoaromatic hydrocarbons (benzene, toluene and xylene), PAH (naphthalene, fluoranthene and pyrene), other petroleum hydrocarbons (carbazole, phenol, petroleum oil and diesel fuel) and intermediates of phenanthrene degradation (phthalic acid, protocatechuate, catechol and salicylic acid)] were inoculated with strain DMVP2 and incubated under optimized conditions. The samples were withdrawn at regular intervals of time (24 h) up to 72 h to monitor growth of strain DMVP2 by measuring absorbance at A_{660} nm using spectrophotometer (Spectronic 20D+, Milton Roy, USA).

2.9. Effect of presence of other petroleum hydrocarbons on phenanthrene degradation

In order to examine the effect of other petroleum hydrocarbons on phenanthrene degradation, strain DMVP2 was inoculated in MM amended with 300 ppm phenanthrene along with other petroleum hydrocarbons [0.1% (w/v)/(v/v)] such as benzene, toluene, xylene, petroleum oil, diesel fuel, carbazole, naphthalene, pyrene and fluoranthene. All flasks were incubated under optimized conditions. Control flask, without other petroleum hydrocarbons, was kept under similar conditions. Abiotic controls, without inoculation of strain DMVP2, were kept under similar conditions. Samples were collected after 96 h and analyzed for growth of strain DMVP2 as well as for residual phenanthrene.

3. Results and discussion

Ankleshwar industrial estate (Gujarat, India) consists of nearly 3000 manufacturing units. Majority of the industries manufacture chemicals, dyes, paints, fertilizers, pharmaceuticals, pesticides and other xenobiotic compounds. The treated and untreated effluents from various industries of Ankleshwar industrial estate are finally released in Amlakhadi canal. Amlakhadi canal further ends up in Narmada estuary that enters the Arabian Sea, Gujarat. Kathuri [15] found that this canal highly polluted with different toxic metals such as chromium, cadmium, mercury, zinc and others. Beside this, canal is also contaminated with various organic compounds such as chlorinated benzene, phenolics, petroleum hydrocarbons, polychlorinated biphenyl and others [15]. Many of these pollutants (PAHs) adsorb on to particulate matter due to their hydrophobic nature and settle down as sediment. Therefore, sediment of this canal has become sink of recalcitrant compounds and pose a major threat to the ecosystem. Contamination with xenobiotic compounds exerts selective pressure on microorganisms which results in microbial community shifts towards bacterial species with enhanced PAH degrading capability. Hence, Amlakhadi canal sediment was selected for isolation of PAH degrading organisms. Pathak et al. [16] had isolated *Pseudomonas* sp. HOB1 from the same Amlakhadi canal, showing ability to tolerate 60,000 ppm of naphthalene. In present study, 25 phenanthrene degrading bacterial strains were isolated, on the basis of their distinct morphological characteristic, from enriched sample. The best phenanthrene degrading strain was selected and identified as *Pseudoxanthomonas* sp. DMVP2 (accession number JF440626) based on 16S rRNA gene sequence analysis. The phylogenetic cluster of strain DMVP2 along with other closely related bacterial strains is depicted in Fig. 1. *Pseudoxanthomonas* sp. belongs to the family *Xanthomonadaceae* in the order *Xanthomonadales* and class *Gammaproteobacteria*. *Pseudoxanthomonas* sp. DMVP2 was found to be aerobic, gram-negative, non-endospore forming rod shaped bacterium and grown in smooth colonies with entire margin and yellow pigmentation on nutrient agar plate. Moreover the strain has the ability to utilize various xenobiotic compounds as a sole carbon source. The genus *Pseudoxanthomonas* was first isolated by Finkmann et al. [17] from contaminated sediment. There are few reports on the isolation of

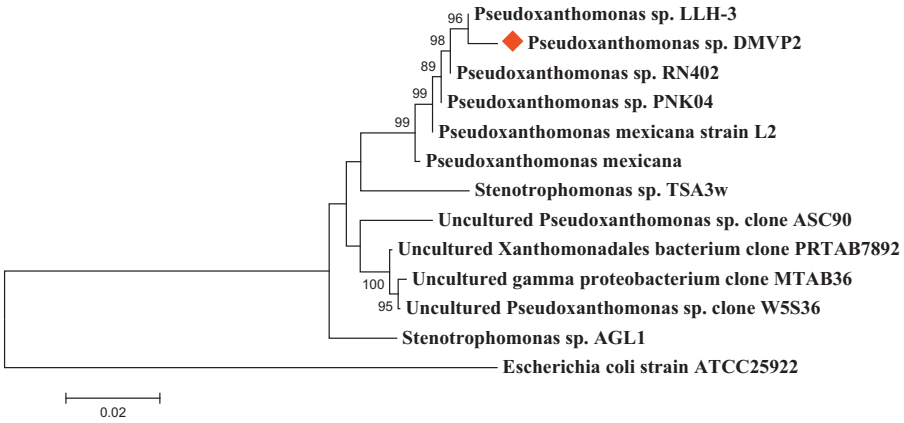

Fig. 1. Phylogenetic tree derived from 16S rRNA gene sequence of *Pseudoxanthomonas* sp. DMVP2 and sequences of closest phylogenetic neighbours obtained by NCBI BLAST(n) analysis. The NJ-tree was constructed using neighbour joining algorithm with Kimura 2 parameter distances in MEGA 4.0 software. *Escherichia coli* strain ATCC25922 has been taken as an out-group. Numbers at nodes indicate percent bootstrap values above 50 supported by more than 1000 replicates. The bar indicates the Jukes–Cantor evolutionary distance.

Pseudoxanthomonas genera from hydrocarbon contaminated sediment such as *P. kalamensis* JA40 and *P. spadix* [18,19]. Klankeo et al. [20] have reported that *Pseudoxanthomonas mexicana* carries nidA gene, situated on megaplasmid, responsible for degradation of pyrene. To the best of our knowledge, this is the first report describing the optimization of various abiotic factors for maximum phenanthrene degradation and analysis of metabolic pathway for phenanthrene degradation by *Pseudoxanthomonas* sp. DMVP2.

3.1. Effect of abiotic factors on phenanthrene degradation

Fig. 2(a) shows the effect of inoculum quantity of strain DMVP2 on phenanthrene degradation. When inoculum of strain DMVP2 increased up to 4% (v/v), lag period for growth of strain DMVP2 decreased and subsequently resulted in higher phenanthrene degradation (88%). As the inoculum of strain DMVP2 was increased further, it resulted in decreased degradation. Similar phenomenon was reported by Abdelhay et al. [21].

Various carbon and nitrogen sources such as glucose, tryptophan, glycerol, sodium acetate, yeast extract, peptone, sodium succinate and ammonium nitrate were studied for phenanthrene degradation, amongst them peptone, yeast extract, sodium succinate and ammonium nitrate were found to enhance phenanthrene degradation (data not shown). To validate, time course study was carried out to determine the effect of selected nutrients (peptone, yeast extract, sodium succinate and ammonium nitrate) on phenanthrene degradation (Fig. 2(b)). As observed from Fig. 2(b), 95% phenanthrene was degraded in presence of peptone within 120 h. Strain DMVP2 was able to degrade 92, 91 and 90% phenanthrene in presence of yeast extract, sodium succinate and ammonium nitrate, respectively within 120 h. Consequently, peptone was selected as the nitrogen source for further studies. Zong et al. [22] had also reported that supplementation of nutrients in the medium enhanced PAH degradation.

Fig. 2(c) shows phenanthrene degradation at different temperatures (30, 37 and 40 °C), with maximum degradation (95%) obtained at 37 °C within 120 h. Strain DMVP2 was able to degrade 90% phenanthrene within 120 h at 40 °C, whereas it was unable to grow at 50 °C. At 30 °C, strain DMVP2 was able to degrade 84% of phenanthrene within 120 h. This was attributed to the fact that at lower temperature, enzyme catalyzed reaction slowed down and also the growth of organism was slower and subsequently phenanthrene degradation decreased. This indicated that isolated strain DMVP2 is mesophilic in nature.

Fig. 2(d) shows phenanthrene degradation profile of strain DMVP2 under static as well as under shaking conditions (50, 100 and 150 rpm). Under all shaking condition, strain DMVP2 was able to degrade more than 80% of phenanthrene within 120 h, while under static condition only 49% of phenanthrene was degraded within 120 h at 37 °C. The maximum degradation (97%) was obtained under shaking condition of 150 rpm. The dissolution of phenanthrene and availability of oxygen increased under shaking condition and thus resulted in increased rate of degradation.

pH is an important environmental factor affecting microbial metabolism. Consequently, its effect on phenanthrene degradation was studied as illustrated in Fig. 2(e). Generally, >90% of phenanthrene was degraded in alkaline condition within 120 h. When the initial pH of medium was 8.0, higher phenanthrene degradation 99.6% was observed. This can be taken as an advantage in using this isolate for bioremediation of phenanthrene in coastal environment. Hambrick et al. [23] have also shown that soil bacteria which degrade PAH prefer alkaline condition rather than acid condition. Zhao et al. [4] also found that 37 °C temperature and pH 8.0 were favorable conditions for phenanthrene degradation by *Pseudomonas stutzeri* ZP2.

The phenanthrene consumption at various initial concentrations of phenanthrene is shown in Fig. 2(f). Increase in phenanthrene consumption (1600 mg/l) was observed till initial concentration of phenanthrene was increased to 4000 mg/l. When initial phenanthrene concentration was increased further (5000 mg/l), strain DMVP2 was able to grow. However, the phenanthrene consumption decreased significantly. The reason for decreased consumption of phenanthrene at high concentration is attributed to toxicity and stress of phenanthrene on strain DMVP2. Phenanthrene degradation by isolated *Pseudoxanthomonas* sp. DMVP2 was higher than *P. mexicana*, which degraded 100 ppm phenanthrene in 8 days [20]. *Pseudoxanthomonas* sp. DMVP2 was also able to degrade phenanthrene more efficiently than earlier reported by Zhao et al. [4] and Kim et al. [5].

The effect of surfactants on phenanthrene degradation by strain DMVP2 is depicted in Fig. 3. Strain DMVP2 was able to degrade 92, 86, 22 and 6% phenanthrene (300 ppm) in the presence of Tween 80, Triton X100, SDS and CTAB, respectively within 72 h. Whereas, strain DMVP2 was able to degrade 86% phenanthrene within 72 h without any surfactants. Tween 80, a good dispersing agent, can improve the dispersion and solubility of phenanthrene. Bautista et al. [24] had also reported that Tween-80 was the best amongst non-ionic surfactants in improving the degradation of PAHs. However, addition of non-ionic surfactants did not show any significant

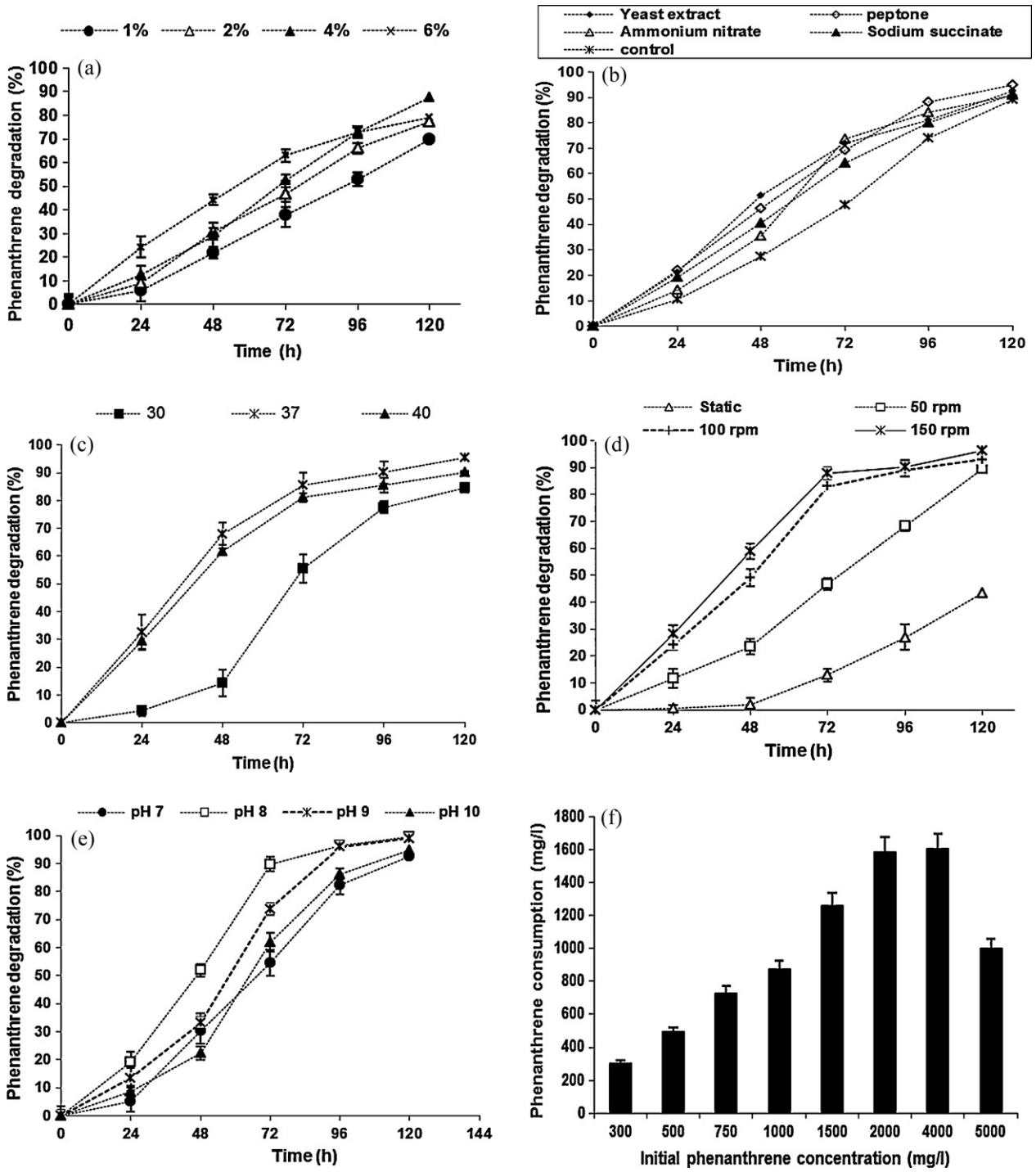

Fig. 2. Effect of various abiotic factors on phenanthrene (300 ppm) degradation by strain DMVP2. (a) Effect of inoculum quantity, (b) effect of various nutrients, (c) effect of temperature, (d) effect of static and shaking condition, (e) effect of initial pH of MM medium and (f) effect of initial phenanthrene concentration.

increase in phenanthrene degradation in comparison to control (without surfactants). Possibly, strain DMVP2 might be producing bio-surfactants, which increased pseudo solubilization of phenanthrene in the medium. Nayak et al. [25] had reported rhmanolipid production by *P. mexicana* in presence of PAH which stimulated the degradation of various xenobiotic compounds.

3.2. Analysis of metabolites during phenanthrene degradation

To investigate phenanthrene degradation pathway followed by strain DMVP2, the intermediates were analyzed using GC–MS at regular time intervals. The GC–MS analysis of the zero day extract gave one major peak at retention time (R_t) 16.09 min which was identified as phenanthrene based on the published mass spectra from National Institute of Standard and Technology (NIST) (Fig. 4(a)). After 72 h incubation, peak of phenanthrene decreased and many other new peaks were observed by GC–MS analysis suggesting the onset of phenanthrene degradation and formation metabolites were formed (Fig. 4(b)). Amongst all peaks, seven peaks were identified (Table 1). Major peaks found were of phthalate anhydrade (R_t 10.69 min) and its ester derivatives such as DI-N-octyl phthalate (R_t 23.55 min) which are known

Table 1
GC–MS data for the metabolites of phenanthrene obtained from the extracts of the strain DMVP2.

Metabolites	Retention time (min)	Molecular ion and fragmentation pattern	Suggested metabolites
1	10.67	149(M^+), 104, 76, 50, 74	Phthalate anhydrade
2	11.34	198(M^+), 85,71, 57, 43	1-Tetradecane
3	14.09	213(M^+),99, 85, 71, 57, 43	1-Pentadecane
4	18.34	312(M^+), 129,73, 57, 43	Eicosanoic acid
5	22.69	302(M^+), 287,105, 77	Phenol 2,4-bis(1-phenyl ethyl)
6	23.07	168(M^+), 149, 104, 71, 57	Phthalic acid, diisoctyl ester
7	23.55	280(M^+), 167, 149, 113, 104	DI-N-octyl phthalate

intermediates of phenanthrene degradation. Besides that, many long chains alkenes such as 1-pentadecane (R_t 14.095 min), 1-teradecane (R_t 11.345 min) and ecosanoic acid (R_t 18.349 min) were found during phenanthrene degradation. This corroborates with the fact, reported by Nayak et al. [25], that in presence of phenanthrene, *P. mexicana* produced rhmanolipid which contain long chain alkenes. Thus strain DMVP2 might also be producing some surfactant in presence of phenanthrene, leading to formation of long chain alkanes. In addition to this, peak (R_t 22.69) was identified as phenol 2,4-bis(1-phenyl-ethyl) according its mass fragmentation 302 (M^+ $C_{22}H_{22}O$), 287 (M^+–OH), 105 (M^+–C_6H_5–CH–CH_3) and 77 (M+–C_6H_5–CH–CH_3). The reason for presence of this metabolite is not known. In present study, we could not find any metabolites of upper metabolic pathway of phenanthrene degradation. Therefore, further study is required to characterize upper metabolic pathway of phenanthrene degradation. All these peaks completely disappeared by 144 h (GC–MS analysis) indicating that strain DMVP2 completely degraded phenanthrene (300 ppm).

Further investigation of phenanthrene degradation pathway was carried out by determining protocatechuate dioxygenase activity in cell free extracts of strain DMVP2 grown with phenanthrene (Fig. 5). Protocatechuate was added as substrate to the crude cell extract and UV–vis spectrum was recorded at intervals of 4 min (from 0 to 24 min). Characteristic decrease in maxima absorption (250 nm and 290 nm) indicated the formation of β-carboxy-cis, cis-muconate, ortho-cleavage product of protocatechuic acid [14]. Thus it is assumed that protocatechuate is degraded by ortho-cleavage dioxygenase of strain DMVP2. However, no catechol dioxygenase activity was detected in cell free extracts of strain DMVP2 grown with phenanthrene (data not shown). These results indicated that *Pseudoxanthomonas* sp. DMVP2 might be degrading phenanthrene by phthalic acid and protocatechuic acid pathway.

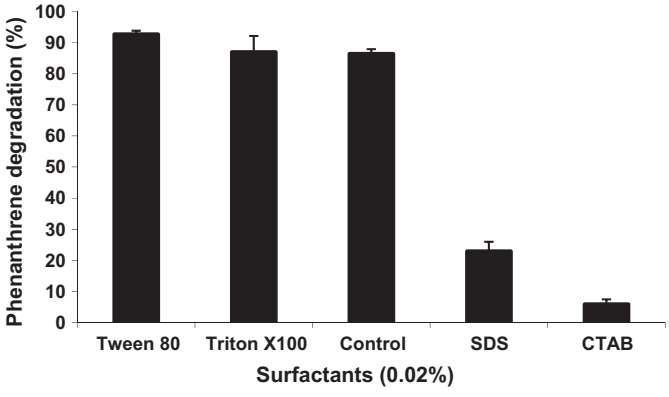

Fig. 3. Effect of surfactants on phenanthrene degradation by strain DMVP2 at 37 °C under shaking condition (150 rpm).

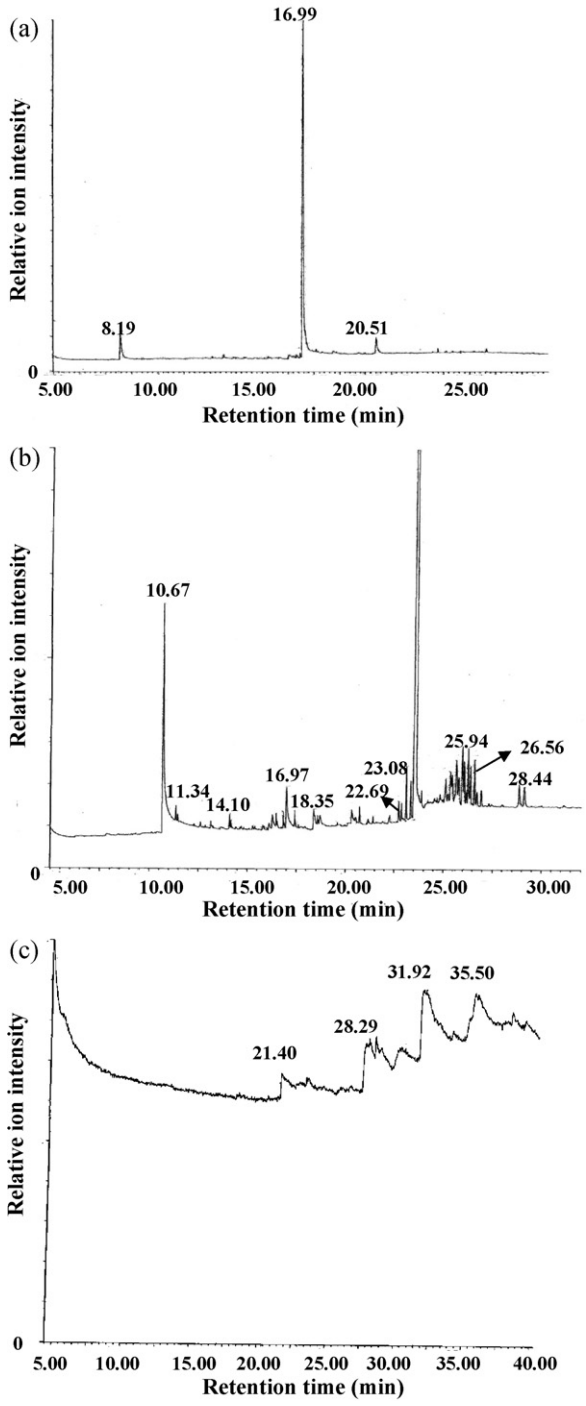

Fig. 4. GC–MS analysis of metabolites during phenanthrene degradation by strain DMVP2 at regular interval (a) 0 h extract, (b) 72 h extract and (c) 144 h extract.

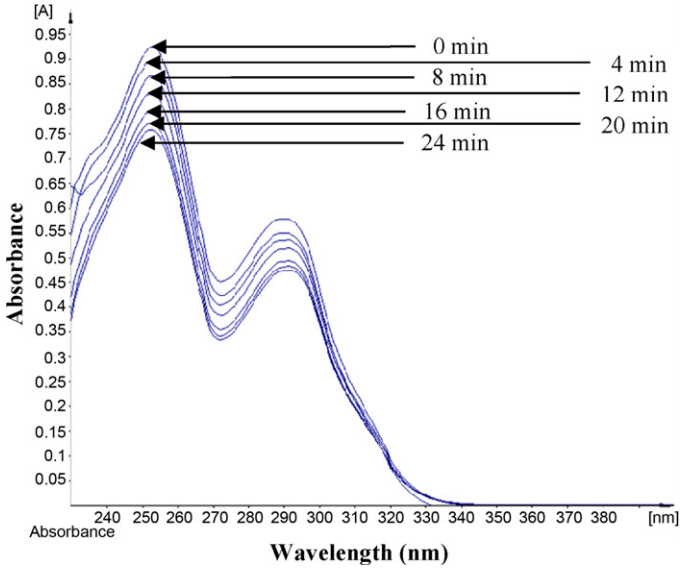

Fig. 5. Protocatechuate dioxygenase activity by cell free extract of strain DMVP2 grown on phenanthrene.

3.3. Growth of *Pseudoxanthomonas* sp. DMVP2 on other xenobiotic compounds

The efficiency of strain DMVP2 to utilize other xenobiotic compounds as sole source of carbon and energy is presented in Table 2. Besides being able to degrade phenanthrene, strain DMVP2 was also able to utilize other aromatic hydrocarbons like pyrene, fluoranthene, benzene, acetone, carbazole and aliphatic hydrocarbons such as petroleum oil, diesel fuel and hexane as sole carbon source. Comparing the results of our study with those available in the literature, it is evident that broad catabolic ability is common amongst all PAH-degrading strains due to structural similarity of many aromatic hydrocarbons and broad substrate specificity of dioxygenase enzyme. Moreover, successive cleavage of fused aromatic rings during biodegradation of multi-ring aromatic compounds eventually produces monoaromatic compounds as intermediates; hence one of the possibilities for the multi-ring degraders is that they may be competent monoaromatic-degraders [5]. Churchill et al. [26] also observed that *Mycobacterium* sp. strain CH1 could degrade pyrene and was also capable of using a wide range of branched alkanes and *n*-alkanes as sole carbon and energy sources.

Table 2
Growth profile of the *Pseudoxanthomonas* sp. DMVP2 on various xenobiotic compounds as sole carbon source.

Xenobiotic compounds	Growth profile
PAH	
Phenanthrene	+++
Naphthalene	–
Fluoranthene	+++
Pyrene	+++
Monoaromatic hydrocarbons	
Benzene	+
Toluene	–
Xylene	–
Asphaltenes	
Acetone	++
Phenol	–
Resins	
Carbazole	++
Other petroleum hydrocarbons	
n-Hexane	++
Petroleum oil	+++
Diesel fuel	+++
Metabolites	
Salicylic acid	+++
Phthalic acid	+++
Protocatechuic acid	++
Catechol	–

+++: very high growth, ++: high growth, –: no growth.

Strain DMVP2 was not able to utilize toluene, xylene and naphthalene as sole carbon source. The inability of strain DMVP2 to tolerate monoaromatic hydrocarbon (xylene and toluene) is generally believed to be due to disruption of biological membranes. Alteration of membrane structure can disrupt energy transduction and the activity of membrane-associated proteins [27,28]. There are reports suggesting that bacteria degrading phenanthrene by phthalate pathway cannot grow on naphthalene as sole source of carbon [8,29].

Strain DMVP2 was able to utilize intermediates of phenanthrene degradation pathway such as phthalic acid and protocatechuic acid as sole carbon source but not others such as catechol, 1-naphthol and salicylic acid. The growth of strain DMVP2 on phthalic acid and protocatechuic acid again showed that the strain DMVP2 degrades phenanthrene via phthalic acid–protocatechuic acid pathway.

3.4. Effect of presence of other petroleum hydrocarbons on phenanthrene degradation

Generally bioremediation studies are focused on degradation of single hydrocarbon and neglect the effect of other co-contaminants which are generally present at the hydrocarbon contaminated sites. In the open environment, PAHs exist as diverse multi-component mixtures at contaminated sites. Therefore, studies on biodegradation using single PAH compounds does not accurately reflects the true complexity of PAH degradation required in natural environments. In some cases, these interactions may be positive, resulting in increased degradation of one or more components, whereas in other cases negative effects have also been observed [30]. Keeping this in view, we determined the effect of presence of other petroleum hydrocarbons on phenanthrene degradation by strain DMVP2 (Fig. 6(a) and (b)).

When phenanthrene was present alone in the medium, 97% of the phenanthrene was degraded within 96 h. The addition of pyrene and fluoranthene to the medium along with phenanthrene did not affect the pattern of phenanthrene degradation, as 95% of phenanthrene was degraded within 96 h in presence of pyrene and fluoranthene. Moreover, growth of strain DMVP2 increased when fluoranthene and pyrene were present along with phenanthrene (Fig. 6(b)). Somtrakoon et al. [31] had also reported that phenanthrene degradation was not affected in the presence of high molecular weight PAHs. In contrast, Zong et al. [22] reported that *Sphingomonas* sp. Phe4 took longer time to degrade phenanthrene in presence of other PAHs. Growth of strain DMVP2 as well as phenanthrene degradation decreased in presence of naphthalene.

The growth of strain DMVP2 was not hindered when diesel fuel and petroleum oil were present along with phenanthrene, rather it supported the growth of bacteria and enhanced the rate of phenanthrene degradation. Strain DMVP2 was able to degrade phenanthrene (300 ppm) completely within 96 h. One of the possible reason, as suggested by Yap et al. [32], is that lipids present in the oil weakens the interfacial tension and change the property of interface between hydrophobic phase and aqueous phase. This promotes the solubilizing of phenanthrene in the medium and thus increased degradation was observed. Pizzul et al. [33] had also reported that addition of vegetable oil increased the biodegradation of phenanthrene by actinomycetes. Therefore, diesel fuel and petroleum oil could be used as biostimulant agent for bioremediation of phenanthrene contaminated environment.

Interestingly, the presence of carbazole in medium along with phenanthrene significantly increased the growth of strain DMVP2,

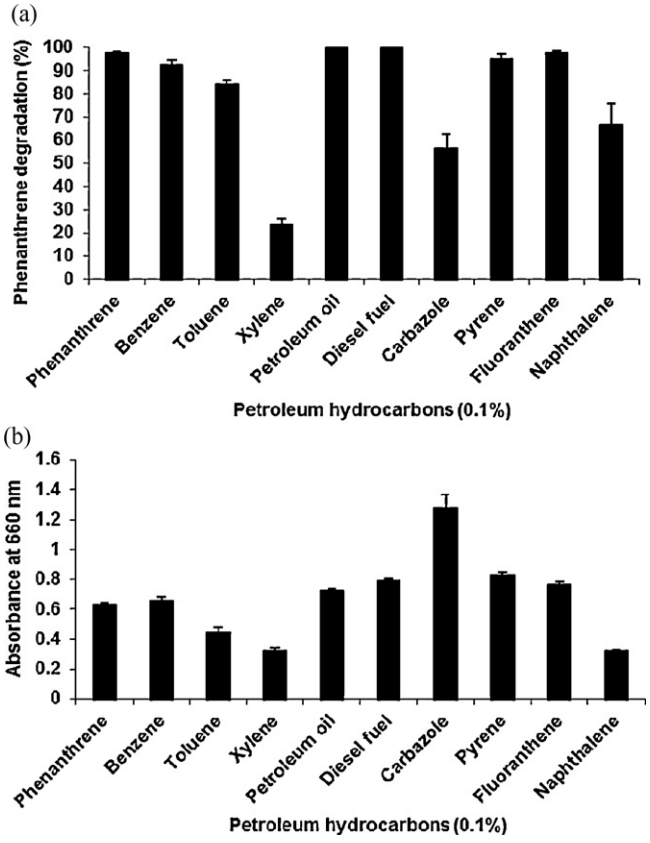

Fig. 6. Effect of petroleum hydrocarbons on (a) phenanthrene degradation and (b) growth of strain DMVP2 in presence of phenanthrene.

however phenanthrene degradation decreased to 56%. Decreased degradation in the presence of multiple substrates can be due to formation of toxic metabolites and competitive inhibition [34,35]. Strain DMVP2 could degrade 92, 83 and 23% of phenanthrene (300 ppm) in the presence of benzene, toluene and xylene, respectively. Even growth of strain DMVP2 increased in the presence of benzene whereas decreased in the presence of toluene and xylene. Therefore, presence of toluene and xylene showed inhibitory effect on phenanthrene degradation.

4. Conclusion

To the best of our knowledge this is the first report on optimization as well as analysis of metabolic profile of phenanthrene degradation by *Pseudoxanthomonas* genera. Strain DMVP2 was able to degrade phenanthrene (300 ppm) completely within 120 h under optimized conditions. This study provides valuable information on optimization of critical parameters to enhance phenanthrene degradation by *Pseudoxanthomonas* genera. Strain DMVP2 was able to utilize many xenobiotic compounds, other than phenanthrene, as sole carbon source. Moreover, *Pseudoxanthomonas* sp. DMVP2 can degrade phenanthrene in presence of other petroleum hydrocarbons and thus it can play an important role in biodegradation of phenanthrene in sites contaminated with mixture of pollutants.

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