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BIODEGRADATION OF HIGH MOLECULAR WEIGHT POLYCYCLIC AROMATIC HYDROCARBON CHRYSENE BY SOIL BACTERIAL ISOLATES

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ABSTRACT

PAHs (polycyclic aromatic hydrocarbons) are common and dangerous organic pollutants made primarily of carbon and hydrogen. Chrysene is also a potential human carcinogen and a high molecular weight PAH. The current research focuses on chrysene biodegradation, and effective chrysene degrading microbial strains were identified from soil samples taken from the hydrocarbon-contaminated surrounding soil of the Mathura petroleum refinery in Uttar Pradesh, India. The isolated isolates were morphologically examined, and basic biochemical tests were performed. 16sRNA analysis was performed for molecular analysis, and it was discovered that 3AS belongs to *Enterobacter* sp. and 5AS belongs to *Ochrobactrum* sp. The biodegradation efficiency of strain 3AS was 60%, while strain 5AS could only digest 55% of chrysene in MSM (Minimal Salt Media). The principal metabolite of chrysene biodegradation discovered in the cultures of strain 3AS using the GC-MS (Gas chromatography-mass spectrophotometry) technique, as well as significant metabolites such as phenol, 2, 5-bis (1,1- dimethyl ethyl), 1-naphthoic acid, 8-methoxy, and 3-nitrophthalic acid. In 5AS cultures, phthalic acid and di-isobutyl ester was detected. The catechol-1, 2-dioxygenase gene was not found, but the presence of catechol-2, 3-dioxygenase suggests that chrysene can be degraded via the meta cleavage pathway.

KEY WORDS: Polycyclic aromatic hydrocarbons, Biodegradation, Chrysene, GC-MS, catechol

INTRODUCTION

Polycyclic aromatic hydrocarbons are organic compounds composed of two or more fused benzene rings organized in a linear, angular, or cluster arrangement (Cerniglia, 1992). PAHs (polycyclic aromatic hydrocarbons) are classified as either low molecular weight (LMW) or high molecular weight (HMW) based on their complexity (Cerniglia and Heitkamp, 1989). A PAHs compound's solubility in water decreases as its molecular weight rises, whereas its melting and boiling points rise as vapor pressure rises (Clar, 1964 and Patnaik, 1999). The United States Environmental Protection Agency has designated 16 PAHs, including chrysene, as priority pollutants that are carcinogenic, genotoxic, and mutagenic to humans and other living things (Menzie *et al.,* 1992). They are released into the environment as organic carbon from a variety of sources, including incomplete combustion of fossil fuels (gasoline, kerosene, coal, and diesel fuel), wood, automobile engine exhausts, petrochemical industrial effluents, tobacco and cigarette smoke, oil storage wastes-accidental spills from oil tankers, refineries, and municipal solid waste (Lundstedt *et al.,* 2003).

PAHs can be removed from the environment by a variety of physical and chemical processes such as volatilization, adsorption, chemical degradation, and photolysis. Microbial degradation, on the other

hand, is regarded to be the most important process for removing PAH pollution from contaminated areas (Yuan *et al.,* 2002). A variety of bacterial species that are known to breakdown PAHs have been identified from PAH-contaminated soil or sediments. Several of these are PAH-degrading bacteria that belong to the *Pseudomonas aeruginosa*, *Pseudomonas fluoresens*, *Mycobacterium* spp., *Haemophilus*spp., *Rhodococcus* spp., and *Paenibacillus* spp. families (Haritash and Kaushik, 2009). However, there are few reports of bacteria that use chrysene as their only source of carbon, and those that can digest it include *Alcaligenes faecalis* (John *et al.,* 2012), *Bacillus* sp., and *Pseudomonas* sp. (Dhote *et al.,* 2010). Dioxygenases are the most important enzymes involved in PAH degradation. These enzymes are made up of a multicomponent enzyme system that catalyses the early breakdown of PAHs by incorporating two molecular oxygen atoms (Haryama, 1992). Catechol is usually formed as an intermediate in bacterial biodegradation polycyclic aromatic hydrocarbon pathways, which is often cleaved by ortho- or meta cleavage dioxygenases (Meyer *et al.,* 1999; Mesarch *et al.,* 2000; Cerniglia 1984, Smith, 1990 and Bamforth and Singleton, 2005). So far, no comprehensive chrysene biodegradation mechanism has been discovered. The pathway's three metabolites have been identified: hydroxyl phenanthroic acid, 1-hydroxy-2-naphthoic acid, and salicylic acid (Anand *et al.,* 2011). The goal of this study was to isolate plausible chrysene degraders from petroleum-contaminated areas and determine key metabolites generated during bacterial catabolism of chrysene.

MATERIALS AND METHODS

Chemicals

Sigma-Aldrich Chemie supplied the chrysene (Steinheim, Germany). All other reagents were purchased from Himedia Laboratories Pvt. Ltd and were of analytical grade (India).

Sample collection

Petrochemical soil sludge samples were gathered from the effluent released from the Mathura petroleum refinery's main refinery campus in order to isolate chrysene degrading bacteria for the current study.

Isolation of chrysene degrading bacteria

The technique of enrichment culture was utilized to isolate efficient chrysene degrading bacteria. The mineral salt media (MSM) was made according to Verma's methodology, with significant changes (Verma *et al.,* 2006). It's made up of the following ingredients (in grammes per liter): Potassium dihydrogen orthophosphate, (KH_2PO_4) 8.50g, Dipotassium hydrogen orthophosphate, (K_2HPO_4) 21.75g, Disodium hydrogen orthophosphate dehydrate, ($Na_2HPO4.2H_2O$) 33.40g, Ammonium chloride, (NH4Cl) 0.50g, Calcium chloride, anhydrous, (CaCl2) 27.50g, Calcium chloridedehydrates, ($CaCl_2.2H_2O$)36.40g, Magnesiumsulfate heptahydrate, ($MgSO_4.7H_2O$) 22.50 g and Iron(III) chloridehexahydrate, ($FeCl_3.6H_2O$) 0.25g. B (0.026 percent), Cu (0.05 percent), Mn (0.05 percent), Mo (0.006 percent), and Zn (0.07 percent) are the trace elements in the solution (2ml/l), which has a pH of 7.2. Chrysene was employed at a concentration of 0.2 mL in MSM (mineral salt media) agar plates, and an acetone solution containing 0.1 percent w/v of chrysene was created (0.1 g chrysene in 100 ml acetone). The chrysene solution was sprayed onto pre-dried MSM-agar plates, which were then incubated for 14 days at room temperature at 25 °C. The control plates, which were not coated with the test PAH, were also manufactured. Colonies that were prominent and clear on chrysene coated plates were chosen. These colonies were then duplicated on fresh MSM-agar plates enriched with chrysene and cultured for another 14 days.

Biochemical and Molecular Characterization of the strain

Morphological and physiological basis were used to identify the bacterial strains at first. The bacteria were identified and phylogenetically characterized using 16s rRNA analysis. To amplify the 16s rRNA gene, the following primers were used.

Forward Primer- 5'-ACTCCTACGGGAGGCA GC3'

Reverse Primer-5'-CCGTCAATTCATTTG AGTTT-3' andinitial denaturation at 94 °C for 1 minute, annealing at 52 °C for 1 minute, and extension at 72 °C for 1 minute were followed by a 10-minute final extension at 72 °C.

Biodegradation Studies

In order to investigate bacterial isolates' chrysene biodegradation potential in MSM, overnight LB (Luria- Bertani Broth) grown cultures of the isolated strain were inoculated in chrysene supplemented 20

ml MSM broth (pH 7.2) with 50 ppm and 100 ppm of chrysene as a substrate in 100 ml flasks, and then culture flasks were incubated at 37 °C and 120 rpm in anincubator shaker for seven days. A control without bacterial inoculum was maintained in the same incubator. To track the growth of the cultures, OD (optical density) values were recorded at regular intervals. The metabolites formed during chrysene biodegradation were studied using gas chromatography and mass spectrophotometry.

The analysis of the volatile constitu ents wa srunona Shimadzu QP-2010 GC-MS system equipped with AB- Innowa x 7031428 WCOT (wall coated opentubular column) column (60m × 0.25 mm × 0.25 µm) directly coupled to the MS. The carrier gas was helium with a flowrate of 1.21mL/ min. Oven temperature was programmed as 100 ºC for 1 min and subsequently held isothermal for 2 min.injectorport: 260 ºC, detector: 280 ºC, split ratio 1:50, volume injected: 1 µl of the sample. The recording was performed at 70 eV, scantime 1.5s; mass range 40-750amu. Chemstationsoft ware was adopted to handle mass spectra and chromatograph.

Amplification of catechol dioxygenasegene

For the amplification of the CD-1, 2O (catechol-1,2 dioxygenese) and CD-2,3O (catechol-2,3-dioxygenase) genes, the following primer pairs were developed.

Thirty cycles of amplification were performed at 94 °C for 1 minute, 56 °C for 1 minute, 72 °C for 1 minute, and 72 °C for 10 minutes.

RESULTS AND DISCUSSION

Under laboratory condition, 3AS and 5AS were the only strains that successfully used chrysene as their sole source of carbon and energy. John *et al.,* 2012, described a similar procedure for isolating PAH-degrading bacteria from the aviation fuel spill site.

Isolation, identification and characterization of chrysene degrading bacteria

When viewed under a microscope, the isolated strains are gram-negative rods, with strain 3AS showing positive findings for catalase, Voge-Proskauer, indole test, methyl red, and citrate test but a negative oxidase test. Only the catalase and oxidase tests showed a positive result for strain 5AS. 16S rRNA analysis was used to further characterize the strains' phylogenetic relationships, and a 600bp DNA fragment was amplified by PCR. Sanger's sequencing technique was used to sequence the PCR product. The 16S rRNA gene sequence of isolated strain 3AS was deposited in the NCBI database with the accession number KT362156.1, indicating that it belongs to *Enterobacter* sp. It was found to be identical to *Enterobacter sp.* strain BacI70 in every way (KX641554). The strain 5AS had a similar documentation pattern, and its 16S rRNA gene sequence was similarly uploaded in the NCBI database under the gene bank accession number KR584658.

Biodegradation studies and chrysene Metabolite identification by GC-MS technique

The development, enrichment, and characterization of the bacterial consortium ASDC (*Rhodococcus* sp., *Bacillus* sp., and *Burkholderia* sp.,) were reported and the consortium used chrysene as its sole source of carbon and energy, with a maximum breakdown rate of 1.5 mg/l/day and a maximum growth rate of 0.125/h, all under optimal circumstances of pH 7.0, 37 °C, and 150 rpm aeration on gyrating shaking (Vaidya *et al.,* 2018). It was observed; over the course of seven days, GC-MS research revealed that 3AS could destroy 60% of chrysene given at a concentration of 100 ppm. In the cultures of the strain 3AS, a variety of metabolites with varying retention times, molecular weights (MW), molecular formulas, and concentrations were discovered.

In the cultures of isolated strains, metabolites such as phenol, 2,5-bis (1,1-dimethylethyl)-, 1-naphthoic acid, 8-methoxy, 3-nitrophthalic acid, and phthalic acid, diisobutyl ester were found, as well as other non-relevant metabolites in terms of the chrysene biodegradation pathway. However, in the parent state, none of the metabolites were detected? *Pseudoxanthomonas* sp. PNK-04, a chrysene biodegrading strain, was isolated from the coal sample, and three novel metabolites, hydroxyphenanthric acid, 1-hydroxy-2-naphthoic acid, and salicylic acid, were identified in its cultures by TLC (Thin Layer Chromatography), HPLC (High Performance Liquid Chromatography), and MS (Mass-Spectrophotometry) (Nayak *et al.,* 2011). The synthesis of catechol as an intermediate compound in phenol degradation occurs, and the ultimate products are TCA cycle intermediates (Nair *et al.,* 2008). *Rhodococcus* sp. 602 was found to be capable of metabolizing both naphthalene and naphthyl-1-dodecanoate as the sole carbon source, according to Silva *et al.,* 2010. Under nitrogen-limiting

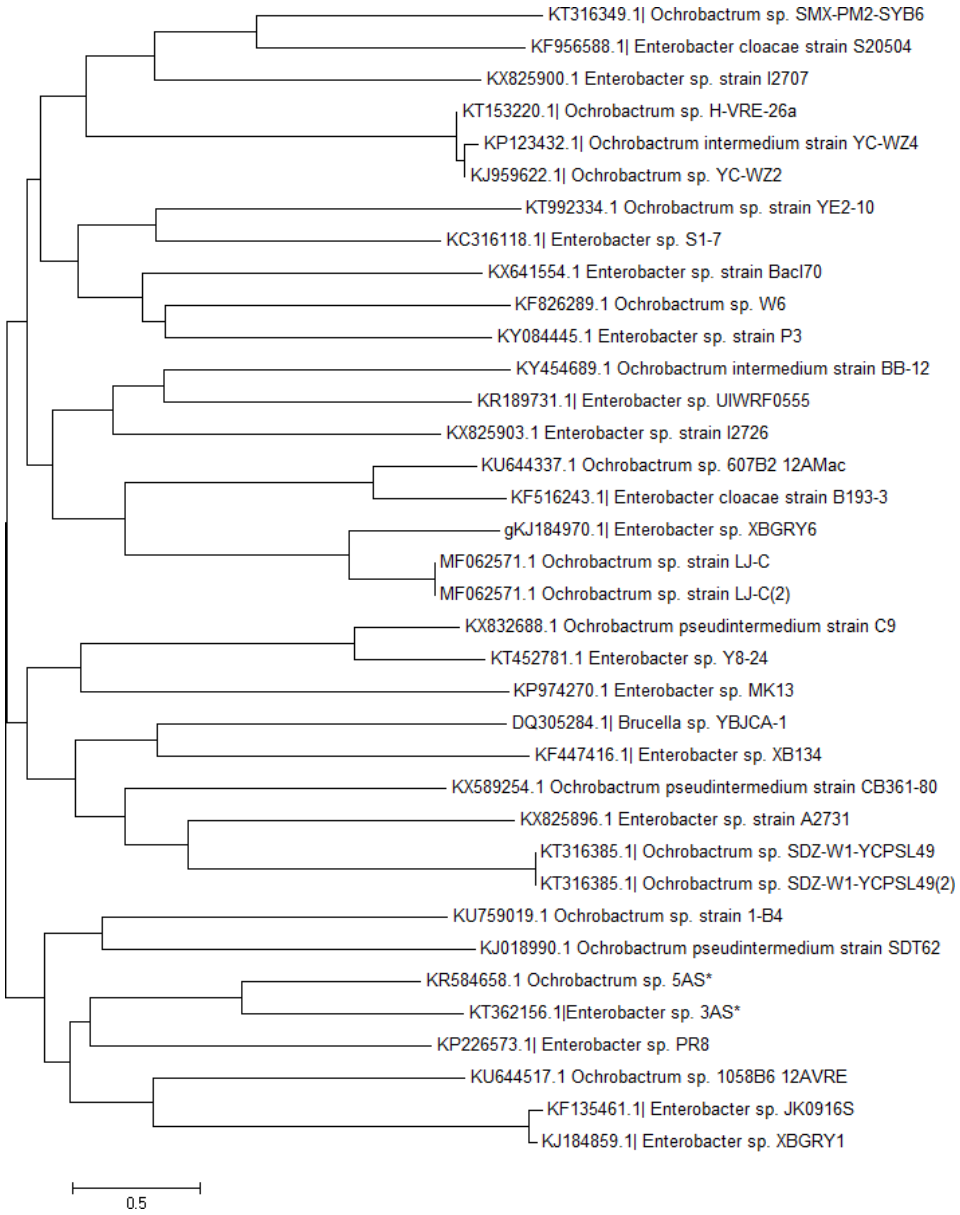

Fig. 1. The isolated strains 3AS and 5AS were placed in phylogenetic positions among related bacteria using MEGA 6.0 (Neighbor-Joining technique) software, and the isolated strains were marked with an asterisk symbol.

circumstances, this strain was able to accumulate triglyceride from naphthyl compounds. These triglycerides may be subjected to -oxidation, resulting in the formation of tri carboxylic cycle intermediates (TCA) (Silva *et al.*, 2010).

Amplification of catabolic genes of chrysene biodegradation

Microbes can digest resistant poly aromatic hydrocarbons, resulting in the formation of catechol as an intermediate byproduct during catabolic breakdown. Catechol biodegradation begins with oxidative ring cleavage in one of two ways: intraradiol (or ortho) cleavage to produce muconic acid, or extradiol (or meta) cleavage to produce extradiol. Finally, pyruvate + acetaldehyde and sucinnate + acetyl coA are formed from catechol (Zeyaullah *et al.*, 2009). All of the aforementioned products are TCA intermediates (tri carboxylic acid cycle). As a result, the hazardous persistent pollutant is completely mineralized into a non-toxic form, and it is removed from the environment. The isolated bacteria' catechol catabolic genes were amplified using PCR. In two of the isolated isolates,

the catechol 1,2- dioxygenase gene was not found. In both strains, the Catechol -2,3-dioxygenase (CD-2,3-O) gene was found.

A 900bp product was amplified effectively, and the PCR amplicon was observed on a 1% agarose gel. The gene for catechol-2,3-dioxygenase was found on chromosomal DNA rather than plasmid. The PCR product was sequenced, yielding an 860-bp nucleotide sequence. The isolated strain 3AS's sequence was submitted to the NCBI gene database under the accession number MF943250, while the strain 5AS's sequence was submitted under the accession number MF737203. The catechol 2,3-dioxygenase gene's protein sequences were evaluated using the NCBI BLAST program's blastp software, and it belongs to the glyoxylase gene superfamily, which is a conserved domain. Cat E gene superfamily, which codes for catechol-2,3-dioxygenase, is also found in that domain (this enzyme plays important role in secondary metabolites biosynthesis, transport and catabolism of catechol via meta cleavage pathway). This subfamily of the VOC superfamily also contains *Bacillus subtilis* CatE and related proteins, as well as HpaD 3,4-dihydroxyphenylacetate 2,3-dioxygenase.

CONCLUSION

Air pollution is the biggest cause of death worldwide, with polycyclic aromatic hydrocarbons accounting for the majority of the contaminants. Researchers have successfully used microorganisms to treat polycyclic aromatic hydrocarbons contaminated soils, demonstrating that bioremediation has significant promise for remediating contaminated soils. The chrysene is a pollutant that is xenobiotic, recalcitrant, and poisonous. In a well-planned manner, the two efficient chrysene biodegrading microorganisms were identified. They breakdown chrysene very well in the lab, and because the method of chrysene degradation is still unknown, the metabolite profile

Table 1. Sequence of Primers to amplify the catechol dioxygenase gene from isolated strains 3AS and 5AS

Name of the Primer .		Primers	Annealing Temperature
Primers for CD-1,2-O gene		Forward Primer 5´-GAAGTGC/GGCATGGACGACG/AGC-3´ Reverse Primer 5´-CG/ATCCCACAGGTACTTGT-3´	52 ºC
Primers for CD-2,3-O Gene	For 3AS strain	Forward Primer 5´-ATGCGTGATTTTGGCCTGACGG -3´ Reverse Primer 5´-CCGGTTCTCGAGTCATACGGCCTCCGGTTTTTTT-3´	56 ºC
	For 5AS strain	Forward Primer 5´-ATGCGTGATTTTGGCCTGAC-3´ Reverse Primer 5´- CTATACGGCCTCCGGTTTTTT -3´	

Table 2. Metabolites observed in the GC-MS spectra of the isolated strain 3AS and 5ASy

Retention Time	Area%	Name of Compound
Compounds observed in the GC-MS spectra of the strain 3AS		
12.250	1.77	Phenol, 2,5-bis(1,1-dimethylethyl)- ($C_{14}H_{22}O$)
14.342	9.89	Benzothiazole-2-Thiol ($C_7H_5NS_2$)
14.742	1.54	2,2-Dimethyl-1,3-cyclohexanedione ($C_8H_{12}O_2$)
16.283	0.17	1-Naphthoic Acid, 8-Methoxy- ($C_{12}H_{10}O3$)
24.408	0.43	3-Nitrophthalic Acid ($C_8H_5NO_6$)
26.417	0.20	Cyclohexane, 1,1-dimethyl- (C_8H_{16})
Compounds observed in the GC-MS spectra of the strain 5AS		
6.365	3.02	Propanamide, N-(2-fluorophenyl)-3-(4-morpholyl)- ($C_{13}H_{17}FN_2O_2$)
14.660	1.06	Phthalic acid, diisobutyl ester ($C_{16}H_{22}O_4$)
20.960	**31.33**	1,2-Benzenedicarboxylic Acid, 3-Nitro ($C_8H_5NO_6$)
10.144	0.49	Germacrane B ($C_{15}H_{30}$)

of the chrysene biodegradation pathway was investigated. Complete knowledge of each pollutant's degrading cycle could be important in genetically engineering bacteria that can live in a range of environments. These isolated strains have the potential to breakdown environmentally unfriendly contaminants, and their ability to degrade other high molecular weight hazardous pollutants could be investigated further. The current study's major goal is to isolate robust and efficient microbial strains that may repair harmful contaminants in the air, water, or soil for human welfare, as a pollution-free environment is everyone's right.

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