ANALYSIS OF CATABOLIC GENE IN MARINE BACTERIA FOR POLYCYCLIC AROMATIC HYDROCARBON DEGRADATION

Dissertation submitted in partial fulfillment of the requirement for the degree of

MASTER OF SCIENCE IN LIFE SCIENCE

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DECLARATION

I Kirtimayee Sahoo, M.Sc. Life Science, Department of Life Science, N.I.T., Rourkela hereby declare that my research work incorporated in the dissertation titled “Analysis of catabolic gene in Marine bacteria for Polycyclic Aromatic Hydrocarbon degradation” is an authentic research work carried at Department of Life science, National Institute Technology, Rourkela under the direct guidance and supervision of Dr. Surajit Das, Asst. Professor, Department of Life science, NIT, Rourkela. The project work is original and no part of this work has been submitted for any other degree or diploma. All the given information is true to best of my knowledge.

Kirtimayee Sahoo

Date:

Place:

ACKNOWLEDGEMENT

This project is by far the most significant accomplishment in my life and it would not have been impossible without people who supported me and believed in my caliber.

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ABSTRACT

The aim of the present study was to analyze the biodegradation of Polycyclic Aromatic Hydrocarbons (PAHs) compound by the bacterial isolates and localization of the PAH-ring hydroxylating dioxygenase genes. (PAHs) are widely distributed in nature and are known to be toxic and carcinogenic. Some micro-organisms are capable of transforming and degrading PAHs. These potential abilities may be useful in removal of PAHs from the environment. Bacterial isolates capable of degrading Napthalene and Phenanthrene were isolated from Rushikuliya estuary (Odisha) water sample by selective enrichment. The isolates were screened for the ability to degrade Napthalene and Phenanthrene when provided as the sole source of carbon. Five isolates, designated as KR-2, KR-5, KR-7, KR-10 and KR-15 were selected after initial screening. Isolates were characterized by gram staining, citrate utilization, sugar fermentation, swimming and swarmming motility and antibiotic sensitivity test. In 7 days KR-2, KR-5, KR-7, KR-10 and KR-15, found to degrade 50.88%, 50.96%, 50.81%, 50.4% and 50.77% of phenanthrene and 70.01%, 66.01%, 78.18%, 74.1% and 71% of napthelene respectively. Genomic DNA was extracted and ring hydroxylating dioxygenase gene was amplified by primer- Forward primer - (GAG ATG CAT ACC ACG TKG GTT GGA) and Reverse primer - (AGC TGT TGT TCG GGA AGA YWG TGC MGT T). Of the five selected PAH degrading bacteria, KR-2 showed amplification of dioxygenase gene.

Key Words: Biodegradation, Phenanthrene, Napthalene, marine bacteria and Dioxygenase gene

1. INTRODUCTION

Bio degradation or biotic degradation is that the chemical dissolution of materials by bacteria or different biological aspects. Biodegradation is outlined as the use of biologically Catalyzed reduction in complexity of chemical compounds (Alexander, 1994). The term is usually employed in relation to ecology, waste management, bio medicine, and therefore the natural atmosphere(bio remediation) and is currently commonly related to environmentally friendly merchandise that are capable of decomposing back to natural components, it's primarily based 2 processes: growth and co-metabolism. Within the case of growth, organic pollutants are used as sole supply of carbon and energy. This method leads to an entire degradation (mineralization) of organic pollutants. Co-metabolism is outlined because the metabolism of organic compounds within the presence of a growth substrate that is employed because the primary carbon and energy supply. The predominant degraders of organo- pollutants within the oxic zone of contaminated areas are chemo-organotrophic species ready to use an enormous variety of natural and xenobiotic compounds as carbon sources and electron doners for the generation of energy. Though several bacteria are ready to metabolize organic pollutants, one bacterium doesn't possess the enzymatic capability to degrade all or maybe most of the organic compounds during a in a much polluted soil. Mixed microbial communities have the foremost powerful bio-degradative potential as a result of the genetic data of more than 1 organism is important to degrade the complicated mixture of organic compounds present in contaminated sites. The genetic potential and some environmental factors like temperature, pH and obtainable nitrogen and phosphorous sources, therefore, appear to work out the speed and and also the extent of degradation. Pseudomonades, aerobic gram-negative rods that never show fermentative activities, appear to possess the very best degradative ability e.g. P.putida and P.fluorescens. Additional necessary degraders of organic pollutants are often found inside the genera Comamonas Burkholderia, and Xanthomonas. The immense potential of the pseudomonas doesn't solely depend upon the catabolic enzymes, however additionally on their capability of metabolic regulation (Houghton and Shanley, 1994). A second necessary cluster of degrading bacteria are the gram-positive rhodococci and coryneform bacteria. Several species, currently classified as Rhodococcus spp. had originally been denoted as Mycobacterium group, and Corynebacterium group. Rhodococci are aerobic actinomycetes having considerable

morphological variability. A group of bacteria able to degrade PAHs, significantly low-molecular weight compounds (e.g. naphthalene and phenanthrene) are discovered. Such bacteria belong to the group Agmenellum, Aeromonas, Alcaligenes Mycobacterium, Rhodococcus,Sphingomonas, Stenotrophomonas, Vibrio, Paenibacillus, and others (Juhsz et al. 2000; Daane et al. 2002; Samanta et al. 2002; Van Hamme et al. 2003). However, few bacteria are known to degrade higher-molecular weight PAHs, like fluoranthene, pyrene, and benzo-pyrene. These consists of members of the group Bacillus, Pseudomons, Mycobacterium, and Stenotrophomonas (Kanaly and Harayama 2000).

Polycyclic aromatic hydrocarbons (PAHs) represent a bunch of priority environmental pollutants, that are ubiquitous contaminants in soils and sediments and are of environmental concern due to their toxic, mutagenic and/or carcinogenic effects (Mastrangelo et al., 1996; Marston et al., 2001; Xue and Warshawsky, 2005). Polycyclic aromatic hydrocarbons (PAH) represent an oversized and numerous category of organic compounds consisting of 3 or a lot of fused aromatic rings in numerous structural configurations. Increase in aromatic rings, structural angularity, and hydro-phobicity build PAH recalcitrant to degradation. In recent years, the biodegradation of PAHs has received considerable attention and a range of micro-organisms are reported to play necessary roles within the method (Pothuluri and Cerniglia, 1994; Shuttleworth and Cerniglia, 1995; Kanaly and Harayama, 2000; Habe and Omori, 2003; Tortella et al., 2005). Bioremediation technologies have increasingly been proposed to decontaminate PAH-contaminated sites (Harayama, 1997; Samanta et al., 2002; Parrish et al., 2004; Vinas et al., 2005). Enzymatic key reactions of aerobic biodegradation are oxidations catalyzed by oxygenases and peroxidases. Oxygenases which is a oxidoreductases that use O2 to include oxygen into the substrates. Degradative organisms like oxygen at its metabolic sites, which initially attack the substrate and at the top of the respiratory chain. This enzymatic system possesses increaseing significance for the co metabolic degradation of persistent organ pollutants. Some enzymes related to the degradation or detoxification of variety of documented organic compounds is: Polychlorodibenzodioxins - lignin peroxidases, the genes concerned in degrading petroleum enzyme production could also be located on chromosomal or plasmid DNA (Broderick, 1999). Biodegradation of hydrocarbons, each aliphatic and aromatic compound, might occur beneath anaerobic or aerobic conditions (Hamme et al., 2003). Alkane hydroxylases act as alkane-degrading enzymes that are distributed among various species of bacteria, yeast,

fungi, and algae (Beilen and Funhoff, 2007). The catechol dioxygenase category of bacterial iron-containing enzymes is an example of an enzyme category concerned within the degradation of aerobic aromatic hydrocarbons. Enzymes like catechol dioxygenases that are concerned in aromatic ring cleavage are liable for the wide range of microorganisms capable of degrading aromatic compounds (Broderick, 1999). Anaerobic degradation is additionally essential to the bioremediation method as a result of in many cases the environmental conditions will embrace limitations of the oxygen availability, like in mangroves, aquifers, and sludge digesters (Santos et al., 2011). Biocatalysis is producing new ways toward improving the event of merchandise and processes to cut back industrial prices and therefore the generation of toxic sub-products and, consequently, the impact on the atmosphere. Each enzymatic bioremediation and new clean energy production are contributing to minimizing fossil fuel damages (Alcalde et al., 2006). Enzymatic remediation are often less complicated than operating with whole organisms. The utilization of isolated enzymes doesn't generate toxic byproducts (Setti et al., 1997) and whole cell competitiveness isn't necessary (Alcalde et al., 2006). As an example of bioremediation by enzymes, PAH detoxifications are often achieved by the utilization of lactases (Alcalde et al., 2006) (enzymes capable of catalysing the oxidative process in phenols, polyphenols, and anilines, incorporated to the 4-electron reduction of molecular oxygen to water) (Smith et al., 1994). an excellent advantage of the enzymatic bioremediation of xenobiotics that are either hydrophobic or poorly soluble in aqueous solutions, like PAHs, is that enzymatic oxidation will occur within the presence of organic solvents (Alcalde et al., 2006). a drawback is that the relevant enzymes are often unstable, inhibited, or denatured in organic solvents. Aromatic hydrocarbons, e.g., benzene, toluene,and xylenes , and naphthalene belong to the massive volume petrochemicals, massively used as fuels and industrial fluids. Phenols and products of phenols such as chlorophenols are produced into the atmosphere as merchandise and waste materials from business. Aromatic compounds are shaped in massive amounts by these type of organisms, e.g., as aromatic amino acids, quinones. Thus, it's not shocking that a lot of microorganisms have evolved catabolic pathways to degrade aromatic type of compounds. In general, xenobiotics compounds i.e man-made aromatic compounds are often degraded by microorganisms, when the respective molecules are almost like natural compounds.

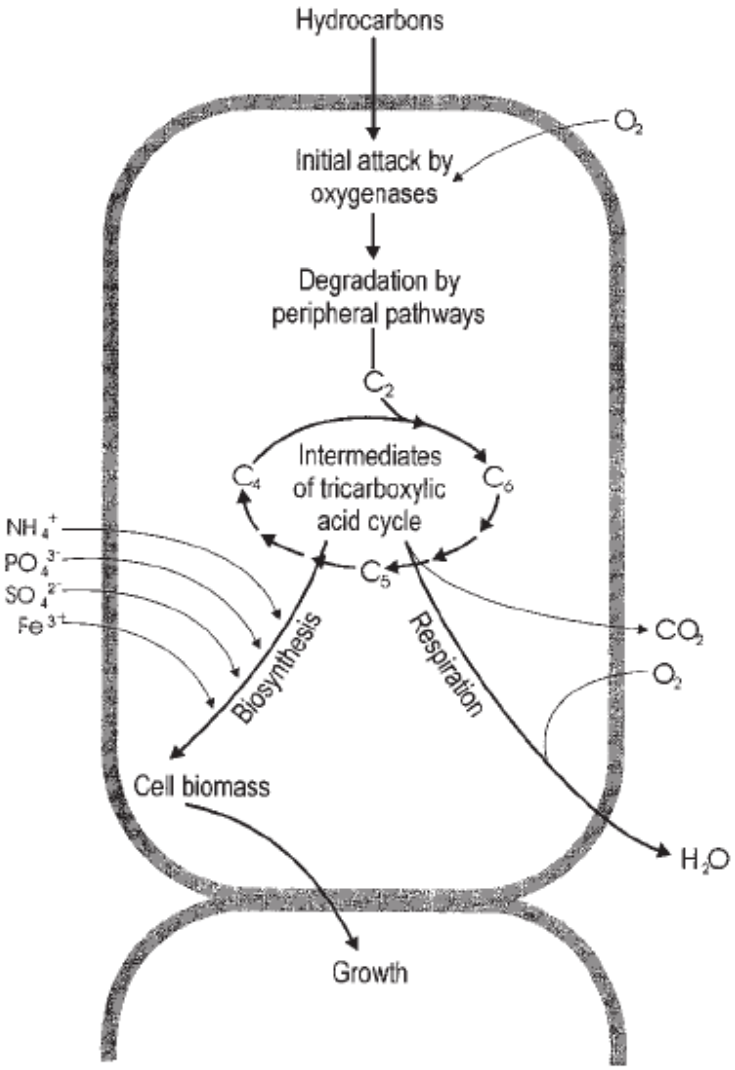

Fig.1. Main principle of aerobic degradation of hydrocarbons: growth associated processes.

In general, benzene and connected compounds are characterised by a better thermodynamic stability than aliphatics are solely few reports on bacteria capable of attacking benzene are revealed. The primary step of benzene oxidation may be a hydroxylation catalyzed by a dioxygenase the merchandise, a diol, is then converted to catechol by a dehydrogenase enzyme. These type of initial reactions, dehydrogenation and hydroxylation, also are common to pathways of degradation of alternative aromatic hydrocarbons. The introduction of a substituent

cluster onto the benzene ring renders various mechanisms potential to attack aspect chains or to oxidize the aromatic ring. The flexibilityand flexibility of bacteria is predicated on the existence of catabolic plasmids. Catabolic plasmids are found to encode enzymes degrading naturally occurring aromatics like camphor balls, naphthalene, and salicylate. Most of the catabolic plasmids are self-transmissible and have a broad host vary. The bulk of Gram -ve soil bacteria collected from polluted areas possess degradative plasmids, mainly the therefore referred to as TOL plasmids. These pseudomonades are ready to grow on toluene compund, m- and p-xylene, and m-ethyltoluene. the most reaction concerned within the oxidation of toluene and connected arenas is that the methyl cluster hydroxylation. The methyl cluster of toluene is oxidized stepwise to the corresponding alcohol, aldehyde, and carboxylic cluster. Benzoate shaped or its alkylated derivatives are then oxidized by toluate dioxygenase and decarboxylated into catechol. The oxygenolytic type of cleavage of the aromatic ring happens via o- or m-cleavage.the importance of the range of degradative pathways and of the few key intermediates continues to be underneath discussion. Each pathway could also be gift in one bacterial group. “An alternative mechanism for the dissimilation of any compound becomes out there (ortho- versus metacleavage of ring structures, for example) management of every outcome should be imposed” (Houghton and Shanley, 1994). The metabolism of a good spectrum of aromatic compounds by one species needs the metabolic isolation of intermediates into distinct pathways.This type of metabolic compartmentation appears to be realized by metabolic regularization. The main enzymes of the degradation pathway of aromatic substrates are induced and synthesized in appreciable amounts solely when the substrate or structurally connected compounds are located. Enzyme inductance depends on the concentration of the inducting molecules. The substrate particular concentrations represent the edge of utilization and growth and are within the magnitude of μ M.A recent report on the regulation of TOL catabolic pathways has been revealed. Co-metabolism, the transformation of a substance while not nutritional profit within the presence of a growth substrate, may be a common phenomenon of microbial activities. it's the idea of bio-transformations (bioconversions) utilized in biotechnology to convert a substance to a chemically changed type. Microorganisms growing on a specific substrate gratuitously oxidize a second substrate (co-substrate). The co-substrate isn't assimilated, however the merchandise could also be out there as substrate for alternative organisms of a mixed culture. The conditions of co-metabolic transformations are the enzymes of the growing cells and therefore the synthesis

of cofactors necessary for enzymatic reactions, e.g., reducing equivalents, NADH for oxygenases. The example demonstrated, has been utilized in field for the elimination of trichloroethylene (Thomas et al., 1989). Methanotrophic bacteria utilized in this experiment will utilize methane and alternative C1 compounds as sole sources of carbon. They are responsible for the oxidation of methane to CO2 through methanol, formaldehyde. The absorption needs special pathways, and formaldehyde is that the intermediate assimilated. The primary step of methane oxidation is catalyzed by methane monooxygenase that attacks the inert CH4. It's an unspecific enzyme that additionally oxidizes numerous alternative compounds, e.g., alkanes, aromatic compounds, and trichloroethylene (TCE). TCE is oxidized to an epoxide excreted from the cell. The unstable oxidation product breaks right down to compounds, which can be employed by different micro-organisms. Methanotrophic bacteria are the group which comes under aerobic indigenous bacteria, in soil and aquifers, however methane has got to be added as growth substrate and inducer for the event of methanotrophic biomass. The summation of methane as substrate limits the applying for bioremediation. Cometabolism of chloroaromatics may be a widespread activity of bacteria in mixtures of commercial pollutants. Cometabolic transformation of 2-chlorophenol provides rise to dead finish metabolites, e.g., 3-chlorocatechol. This reaction product could also be auto-oxidized or polymerized in soil to humic-like structures. Irreversible binding of dead finish metabolites could fulfill the performance of detoxification. The buildup of dead finish merchandise inside microbial communities beneath choice pressure is that the basis for the evolution of latest catabolic traits.

2. REVIEW

Biodegradation or biotic degradation or biotic decomposition is that the chemical dissolution of materials by bacteria or alternative biological suggests that. The term is commonly employed in relation to ecology, waste management, biomedicine, and therefore the natural atmosphere (bioremediation) and is currently commonly related to environmentally friendly merchandise that is capable of decomposing back to natural components. The elimination of a large varies of pollutants and wastes from the atmosphere are an absolute demand to market a sustainable development of our society with low environmental impact. Biological processes play a serious role within the removal of contaminants and that they take benefit of the catabolic versatility of microorganisms to degrade or convert such compounds. Biosurfactant is an extracellular surfactant secreted by microorganisms, which reinforces the biodegradation method. Some microorganisms have a naturally occurrence,catabolic diversity to degrade, remodel or accumulate an enormous vary of compounds together with hydrocarbons (e.g. oil), polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), pharmaceutical substances, radionuclides and metals. Most microorganisms that assist the biodegradation would like light-weight, water and oxygen. Temperature is additionally a vital consideration determining the speed of biodegradation; as a result of microorganisms tend to breed faster in hotter conditions. In 1973 it had been proved for initial time that polyester degrades when disposed in bioactive material like soil. As a result, polyesters are waterproof and might be melted and formed into sheets, bottles, and alternative product, guaranteeing plastics currently obtainable as a biodegradable product. Following, Polyhydroxylalkanoates (PHAs) were created directly from renewable resources by microbes. Currently biodegradable technology could be a highly developed market with applications in product packaging, production and medication. Biodegradable technology thinks about with the producing science of biodegradable materials. Many microorganisms could also be concerned within the reactions of biogeochemical cycles, and in some cases they're the sole biological agents capable of regenerating kinds of components required for alternative organisms. The essential characteristics of aerobic microorganisms degrading organic pollutants are (1) Metabolic processes for optimizing the contact between the microbial cells and therefore the organic

pollutants. The chemicals should be accessible to the organisms having biodegrading activities. For instance, hydrocarbons are water-insoluble and their degradation needs the assembly of biosurfactants. (2) The starting intracellular attack of organic pollutants is an oxidative method, the activation and incorporation of oxygen is that the enzymatic key reaction catalyzed by oxygenases and peroxidases enzymes. (3) Peripheral degradation pathways of this change organic pollutants step by step into intermediates of the central intermediate metabolism, e.g., the Krebs cycle. (4) Biogenesis of cell biological mass from the central starting metabolites, e.g., acetyl- CoA, succinate. Sugars needed for numerous biosyntheses and growth should be synthesized by gluconeogenesis.

Degradation of PAHs

Polycyclic aromatic hydrocarbons (PAHs) are necessary pollutants found in air, soil and sediments. These compounds enter the surroundings in some ways. PAHs and their derivatives are widespread merchandise of incomplete combustion of organic materials arising from natural combustion like forest fires and volcanic eruptions, except for the foremost half by human activities. In recent decades the foremost supply of PAH pollution are industrial production, transportation, refuse burning, gasification and plastic waste incineration. The fate of polycyclic aromatic hydrocarbons in nature is of nice environmental concern because of their toxic nature, mutagenic effect, and carcinogenicity. For instance, phenanthrene is understood to be a person's skin photosensitizer and delicate allerge (Fawell and Hunt, 1988). It's additionally been found to be an inducer of sister chromatid exchanges and a potent inhibitor of gap junction intercellular communications (Weis et al., 1998). PAHs will sorb to organic-rich soils and sediments, accumulate in fish and alternative aquatic organisms, and should be transferred to humans through seafood consumption (Meador, et al., 1995). The biodegradation of PAHs are often thought-about on one hand to be a part of the traditional processes of the carbon cycle, and on the opposite because the removal of artificial pollutants from the surroundings. The utilization of microorganisms for bioremediation of PAH-contaminated environments appears to be a beautiful technology for restoration of polluted sites. Polycyclic aromatic hydrocarbons (PAHs) are a bunch of compounds containing carbon and hydrogen, composed of 2 or additional fused aromatic rings in linear,

angular, and cluster arranging pattern. 4 PAHs are taken into thought like Pyrene, phenanthrene, Napthalene and Anthracene.

Pyrene

It is a polycyclic aromatic hydrocarbon (PAH) consisting of 4 fused benzene rings, leading to a flat aromatic system. The chemical formula is denoted as C16H10. This colorless solid is that the smallest peri-fused PAH (one where the rings are fused through over one face). It's fashioned throughout the unfinished combustion of organic compounds. it absolutely was initial isolated from coal tar. As a peri-fused PAH, it's far more resonance-stabilized. Therefore, it's created during a wide selection of combustion conditions. Though it's not as problematic as benzo-pyrene however it's toxic to the kidneys and therefore the liver.

Phenanthrene

It is a polycyclic aromatic hydrocarbon composed of 3 benzene rings which are fused. Phenanthrene act as a composite of phenyl and anthracene. In its pure kind, it's found in cigarette smoke and as an irritant, photosensitizing skin to light-weight. It seems as a white powder having blue fluorescence. It's nearly insoluble in water however is soluble in most organic solvents like toluene, carbon tetrachloride, ether, chloroform and benzene.

Napthalene

It is an organic compound with the formula C10H8 that is that the simplest polycyclic aromatic hydrocarbon i.e PAH, and could be a white crystalline solid with a characteristic smell. It is an aromatic hydrocarbon; naphthalene's structure consists of a fused combine of benzene rings. It's best referred to as the most ingredient of ancient mothballs. A naphthalene molecule is often viewed because the fusion of a combine of benzene rings. It's classified as a benozenoid polycyclic aromatic hydrocarbon (PAH).

Anthracene

It is a solid polycyclic aromatic hydrocarbon consisting of 3 fused benzene rings. It's a element of coal-tar. It's utilized in the assembly of the red dye alizarin and substitute dyes. It is Colorless however exhibits a blue (400-500 nm peak) fluorescence underneath ultraviolet lightweight. Several PAHs contain a "bay-region" and a "K-region". The bay- and K-region epoxides, which might be fashioned metabolically, are highly reactive each chemically and biologically. Phenanthrene is that the simplest aromatic hydrocarbon that contains these regions. Consistent with the Schmidt-Pullman electronic theory, K-region epoxides ought to be additional carcinogenic than the parent hydrocarbon. Low-molecular weight (LMW) PAHs 2 or 3 rings) are comparatively volatile, soluble and additional degradable than are the upper molecular weight compounds. High molecular weight (HMW) PAHs (four or a lot of rings) sorbs strongly to soils and sediments and are additional proof against microbial degradation.

Aerobic and anaerobic degradation

Microorganisms will either degrade or manufacture hydrocarbons (Ehrlich and Dekker, 1995), looking on the presence of sure metabolic pathways, specific to every operate within the environmental conditions. Various microorganisms, like bacteria, cyanobacteria, green algae, and fungi, are capable of degrading completely different parts of petroleum underneath different environmental conditions (e.g., aerobic and anaerobic conditions at varied salinities and pH). The extracellular enzymes created by white rot basidiomycetes are believed to underlie the flexibility of those fungi to degrade PAHs and alternative organo-pollutants (Bumpus, 1989; Field et al., 1993; Bezalel et al., 1996; Novotny et al., 2004). The peroxidases showing the catalytic action generates additional polar and water-soluble metabolites, like quinones, that are a lot of vulnerable to additional degradation by indigenous bacteria present in soils and sediments (Meulenberg et al., 1997). The enzymatic equipment provides these capabilities to microorganisms. Petroleum degradation happens gradually by sequential metabolism of its compounds. The presence of a high enzymatic capability permits microbial communities to degrade advanced hydrocarbons (Alexander, 1994). The genes concerned in degrading petroleum enzyme production could also be located on chromosome(Broderick,

1999). Bio-degradation of hydrocarbons, each aliphatic and aromatic compound, might occur beneath anaerobic or aerobic conditions or showing the degradation (Hamme et al., 2003). Beneath aerobic conditions, oxygenase enzymes introduce oxygen atoms into hydrocarbons (mono-oxygenases introduce one oxygen atom to a substrate whereas dioxygenases introduce two oxygen atom). The degradation which is anaerobic in nature is catalysed by anaerobic bacteria, like sulphate-reducing bacteria, using totally different terminal electron acceptors (Hamme et al., 2003). Degradation of hydrocarbons aerobically are often faster, because of the metabolic advantage of getting the supply of O2 as an electron acceptor (Cao et al., 2009). Aerobic catabolism of hydrocarbons is often faster, because of the metabolic advantage of getting the supply of O2 as an electron acceptor (Cao et al., 2009). The ultimate product of the oxidation of saturated aliphatic hydrocarbons is acetyl-CoA that is catabolised within the citric acid cycle, alongside the assembly of electrons within the electron transport chain. This chain is repeated, additional degrading the hydrocarbons that are normally oxidised to CO2 (Madigan et al., 2010). Aromatic hydrocarbons, like benzene, toluene and naphthalene, also can be degraded in aerobic conditions. The degradation of those compounds sometimes is an initial step within the formation of catechol or a structurally connected compound. Once fashioned, catechol are often degraded, leading to compounds that may be introduced into the citric acid cycle. Additionally these compounds are often fully degraded to CO2 (Madigan et al., 2010, Cao et al., 2009). Alkane hydroxylases are alkane-degrading enzymes that are distributed among many various species of bacteria, yeast, fungi, and algae (Beilen and Funhoff, 2007). Furthermore, van Beilen and Funhoff proposed 3 classes of alkane-degrading enzyme systems: C1–C4 (oxidation of methane to butane which is oxidised by methane-monooxygenase-like enzymes), C5– C16 (oxidation of pentane to hexadecane, which is oxidised by integral membrane nonheme iron or cytochrome P450 enzymes), and C17+ (longer alkanes, oxidised by basically unknown enzyme arrangement). They they reported about the compositions, co-factors and presence of the most teams of alkane hydroxylases (soluble methane monooxygenase, particularly methane monooxygenase , AlkB-related alkane hydroxylases enzyme, eukaryotic P450, Bacterial P450 oxygenase enzyme organization and dioxygenase (CYP153, class I). Microorganisms that are ready to degrade alkanes will contain multiple alkane hydroxylases and may therefore consume totally different substrate ranges (Beilen and Funhoff, 2007). One among the foremost studied alkane

degradation pathways is that described for Pseudomonas putida Gpo1, encoded by the OCT plasmid (Beilen et al., 1994, Beilen et al., 2001). During this case, the conversion of an alkane into an alcohol is initial mediated by a membrane monooxygenase and rubredoxin reductase (Hamme et al., 2003). Van Hamme and colleagues (Hamme et al., 2003) presented a model for alkane metabolism in gram-negative bacteria and described the locations and functions of the ALK gene merchandise. The catechol dioxygenase category of bacterial iron-containing enzymes is an example of an enzyme class concerned within the degradation of aerobic aromatic hydrocarbons. These enzymes are ready to catalyse the addition of molecular oxygen atoms to one, 2-dihydroxybenzene (catechol) and its derive products compounds, with succeeding cleavage of the aromatic ring (Madigan et al., 2010, Cao et al., 2009, Hamme et al., 2003). Despite the actual fact that petroleum degradation underneath aerobic conditions happens faster than underneath anaerobic conditions, it's necessary to notice that anaerobic degradation is additionally essential to the bio-remediation method as a result of in many cases the environmental conditions will embrace limitations of the oxygen availability, like in mangroves, aquifers, and sludge digesters (Santos et al., 2011). In metabolism which is anaerobic, aromatic compounds are cahnged into benzoyl-CoA, that is target of the benzoyl-CoA reductase (BCR) action (Hosoda et al., 2005). Looking on the environmental conditions, totally different terminal electron acceptors are often used, like nitrate, sulphate, and Fe (III); typically, the degradation pathways converge to benzoyl-CoA (Cao et al., 2009).

PAH pollution strategies

Petroleum may be a heterogeneous mixture of hydrocarbons, as well as aliphatic (n-alkanes), alicyclic, and aromatic hydrocarbons (i.e., polycyclic aromatic hydrocarbons), that varies in compositional and physical properties in keeping with the reservoir’s origin (Hamme et al., 2003). These hydrocarbons are organic compounds containing carbon and hydrogen, that are highly insoluble in water. Microorganisms will either degrade or manufacture hydrocarbons (Ehrlich and Dekker, 1995), counting on the presence of bound metabolic pathways, specific to every perform within the environmental conditions. Recently, anthropogenic practices like industrial actions, petroleum derivatives (such as gasoline and kerosene spills), and partial combustion of fossil fuels have caused an accumulation of petroleum hydrocarbons within the

atmosphere (Santos et al., 2011). In fact, petroleum and derivatives have a significant ecological impact on contaminated marine and terrestrial ecosystems (Santos et al., 2011). Several necessary processes influence the destination of hydrocarbons within the atmosphere. Among these are sorption, volatilisation, abiotic transformation (chemical or photochemical), and biotransformation (Korda et al., 1997). Sorption and volatilisation don't destroy contaminants, but, instead, they solely accumulate or transport them to a different location. Abiotic chemical transformations involving organic contaminants aresometimes slow, whereas photochemical reactions are insignificant in most environments (Korda et al., 1997, Crapez et al., 2002, Santos et al., 2011). The chief benefit of the contaminant-degrading process is the complete mineralisation of compounds, as well as biomass formation (Atlas, 1991, Cunha and Leite, 2000, Watanabe and Hamamura, 2003).

Enzymes

The characterization of genes involved in bacterial organic pollutant degradation has promoted the development and application of molecular techniques to study the microbial ecology of contaminated environments (Widada et al., 2002a; Widada et al., 2002b). These proficiencies admit the use of polymerase chain reaction (PCR) to amplify specific target catabolic sequences. The degradative gene probes XYlE, XYlA, M (toluene and Xylene), ndoB (napthalene) and alkB (C6-C12n-paraffin) have been used to detect the presence of catabolic genes in the bacterial populations of petroleum hydrocarbon contaminated soils, and the biodegradation potential of such bacteria has been assessed on the basis of these studies. Nucleic acid based methods have been used to determine the potential for successful bioremediation before deploying a field system and to monitor the performance of in situ bioremediation.

Biodegradation of pah

Microbial biodegradation of polynuclear aromatic hydrocarbons (PAHs), especially simple ones such as naphthalene and phenanthrene, has been extensive considered over last few decades (Cerniglia and Heitkamp. 1989, Harayama, S. 1997).

Napthalene

Naphthalene biodegradation is the best studied of the PAHs because it is the simplest and most soluble PAH, and naphthalene-degrading microorganisms are relatively easy to isolate. Bacterial strains that are able to degrade aromatic hydrocarbons have been repeatedly isolated, mainly from soil. These are usually gram-negative bacteria; most of them belong to the genus Pseudomonas. The biodegradative pathways have also been reported in bacteria from the genera Mycobacterium, Corynebacterium, Aeromonas, Rhodococcus, and Bacillus (Cerniglia, 1984, Smith, 1994). The biochemical sequence and enzymatic reactions leading to the degradation of naphthalene were first presented by Evans (Evans, 1964). Initially bacteria oxidize naphthalene by incorporating both atoms of molecular oxygen into the aromatic molecule to form cis-1,2-dihydroxy-1,2- dihydronaphthalene. Naphthalene dioxygenases from Pseudomonas sp. NCIB 9816 and Pseudomonas putida ATCC 17 484 act as multicomponent enzyme systems which are responsible for naphthalene cis-dihydrodiol formation (Cerniglia, 1984, Smith, 1994). These consist of three protein components: a flavoprotein (reductaseNAP), a two-iron, two sulphur ferredoxin NAP, and terminal oxygenase ISPNAP enzyme. Naphthalene dioxygenase is very unstable. Terminal dioxygenase has a molecular weight of 158 kD and is composed of two subunits which were established to be 55 and 20 kD. A naphthalene oxygenase enzyme were isolated from cells of Corynebacterium renale, which was also utilize naphthalene as a prime source of carbon and energy (Cerniglia, 1984). The enzyme has a molecular weight of about 99 kD and formed cis-1,2- dihydroxy-1,2-dihydronaphthalene as a predominant metabolite. Metabolism of naphthalene by a strain of Mycobacterium sp. requires both monooxygenation and dioxygenation with the formation of both cis- and trans-1,2-dihydrodiols. The reaction is catalized by cytochrome P-450 monooxygenase that forms naphthalene 1,2-oxide which is further converted to the trans-diol by an epoxide hydrolase enzyme. Many investigations have indicated that genes that encode for naphthalene oxidation in pseudomonads are found on plasmids. There are three known plasmids that determine the degradation of naphthalene: NAH7, NPL1 and pND13, but the plasmid which has been studied most intensively is NAH7. This plasmid specifies a complete

degradative pathway for the degradation of naphthalene, with formation of salicylate as a main intermediate. Plasmid NAH7 is 83-kB, and consists of two clusters, which form nah1 and nah2 operons (nahA-F = nah1 and nahGM = nah2 = sal). The nah1 operon includes nahA-F genes coding for the conversion of naphthalene to salicylate and the nah2 (sal) operon includes nahG-M coding for the metabolism of salicylate to pyruvate and acetaldehyde. The sal operon (56 MD in size) specifies a complete salicylate degradation including a functional meta pathway. In contrast Pseudomonas stutzeri strain AN10 is a naphthalene-degrading strain whose dissimilatory genes are chromosomally-encoded. Genetic organization of its naphthalene-catabolic pathway is similar to that found in other well-characterized naphthalene-degradation pathways such as the archetypal plasmid NAH7 from P. putida G7 (Harayama et al., 1987). The degradation pathway of napthalene in P. stutzeri AN10 is consist of many genes which is encoded by 19 genes distributed within two operons: upper and lower pathways and shows high degrees of similarity of homologous catabolic genes. Such events accelerate the evolution of modern degradative pathways, supplying new genetic matters beneath the environment and resulting in an enhanced natural bioremediation potential.

Anthracene and Phenanthrene

These tricyclic aromatic hydrocarbons are widely distributed throughout the environment. They have been used as model substrates in studies on the environmental degradation of polycyclic aromatic compounds, because each structures are contain carcinogenic PAHs such as benzo[a]pyrene and benz[a]anthracene. Pure cultures and mixed cultures of bacteria isolated from fresh-water and marine environments have the ability to metabolize anthracene and phenanthrene as the sole source of carbon. Anthracene can be completely mineralized by Pseudomonas, Sphingomonas, Nocardia, Beijerinckia, Rhodococcus and Mycobacterium with the initial oxygenated intermediate being a dihydriol (Evans et al., 1965). Apart from Mycobacterium, these species oxidize anthracene in the 1,2 positions to form cis-1,2-dihydroxy- 1,2-dihydroanthracene and then convert it to 1,2- dihydroxyanthracene via NAD+-dependent dihydrodiol dehydrogenase. Various Mycobacterium species metabolize phenanthrene at different sites of the particle, presumptively through dioxygenase and monooxygenase attack on the aromatic nucleus. Studies have been conducted on many strains

from Pseudomonas, Arthrobacter, Aeromonas, Sphingomonas, Brevibacterium, Mycobacterium and Nocardia (Cerniglia , 1984, Samanta, et al., 1999, Pinyakong et al., 2000), described and characterized metabolites in the degradation of phenanthrene by the newly isolated phenanthrene utilizing strain Sphingomanas sp. strain P2. Results obtained by PCR suggest that PAH degradation genes are arranged in a polycistronic operon in the chromosome or a plasmid. Phenanthrene degradation genes were found in plasmids in different strains of Comamonas testosteroni, Beijerinckia sp. And Alcaligenes facealis AFK2 showed that Comamonas testosteroni strain contains the genes which is capable enough for the degradation of this compound on the chromosome.

Pyrene

Mycobacteria have been repeatedly isolated as bacteria that are able to degrade pyrene and benzopyrene. Although these bacteria are known for their comparatively slow growth, their growth on PAHs is faster than other bacteria; for example, the growth range of Mycobacterium species BB1 on pyrene was twice faster than Rhodococcus sp. UW1 (Heitkamp et al., 1988). Mycobacterium sp. mineralized pyrene when grown in mineral salt medium supplemented with organic nutrients. In a recent study it has been shown that Sphingomonas yanoikuyae strain R1 can transform pyrene to cis-4,5- dihydro-4,5-dihydroxypyrene and pyrene-4,5-dione. Mycobacterium sp. strain RJGII-135 has been found to mineralize not only pyrene but also a benzo[a]pyrene (Harayama, 1997).

3. OBJECTIVES

- **Collection of marine water samples.**
- **Enrichment of Polycyclic aromatic hydrocarbon adapted Consortia.**
- **Isolation and screening for Phenanthrene and napthalene degrading Bacteria.**
- **To study the degradation of phenanthrene and napthalene by isolates.**
- **Amplification of dioxygenase gene locus.**
- **Characterization of the isolates.**

4. MATERIAL AND METHODS

1. Isolation

Water sample was collected from Rushikulya, Odisha in sterilized falcon tubes, kept in ice and transferred to the laboratory immediately. 1 ml of sample was suspended into Minimal media broth (MMB) (Dipottasium phosphate-7g/L, Monopottasium phosphate-2g/L, Sodium citrate-0.50g/L, Magnesium sulphate-0.10g/L, Ammonium sulphate-1g/L) (Sambrook et al.,1989) supplemented with 100mg/L of Phenanthrene and Naphthalene each as the sole carbon and energy source for growth and then it was kept in the incubator shaker at 37ºC /160 rpm for 15 days. After 15 days of incubation, the innoculum was diluted and spread on Sea Water Nutrient Agar media plates. The isolated colonies were selected for further screening.

2. Screening

The Minimal Media Agar plates were prepared and each plate was incorporated with polycyclic aromatic hydrocarbon i.e. Phenanthrene and Naphthalene. The stock solution of PAHs (5mg/ml) was prepared in Hexane and 0.5 ml of it was spread over Minimal Media Agar plates and the plates were kept for few minutes forming layer of PAHs over agar surface. After that, the isolated colonies were streaked over the mat of corresponding PAH. The same colonies were also inoculated in Minimal media broth with PAH (Phenanthrene and Napthalene) 100mg/L and kept in the incubator shaker for 7 days, in order to monitor the growth which is further kept for degradation studies.

3. Carbon source utilization

- Isolates were taken and inoculated in Luria bertani broth which was incubated for 24 hours at 370C.

- Then the cell mass was centrifuged at 4 ºC/7000 rpm for 10 minutes.
- After that, supernatant was discarded and pellet was resuspended in 1 ml of MMB (Haimou et al., 2004).
- 100µl of suspended cell mass was transferred to MMB tubes with 100 mg/L of Phenanthrene and naphthalene as a sole carbon source.
- 1 % of glucose without any PAH (phenanthrene and Napthalene) in MMB was kept as control for growth.
- After that in order to monitor the growth, the OD595 at 595 nm were taken at regular time interval i.e 0 day, 1 day ,3 day, 5 day and 7 day.

4. Phenanthrene and Napthalene Standard curve

- 5 mg/ml of concentration, PAH (Phenanthrene and Napthalene) solution was prepared in Hexane.
- From the prepared stock solution 0.5µg, 1µg, 10 µg, 50 µg and 100 µg per ml of corresponding PAH solution in hexane was prepared in duplicate form for standard curve.
- Standard curve was prepared by taking the scanned absorbance of PAH solution in between 200- 400nm range using UV-Vis Spectrophotometer, and from this standard curve we can get the λmax for Phenanthrene and Napthalene.

5. Degradation study

- The isolates were inoculated into test tubes containing 5 ml of minimal media broth which is supplemented with PAH (Phenanthrene and Napthalene) at concentration of 100mg/L and kept for 24 hours at 370C in order to check the degradation.
- PAH was delivered to media from stock solution (5mg/ml) in hexane. It was kept for few hours to completely remove hexane from the media. Thereafter BMM-PAH tubes were subsequently incubated at 370C/180 rpm for 7 days.
- After 7 days, cell was harvested by centrifugation and it was again transferred to BMM-PAH tubes (Phenanthrene and Napthalene) at concentration of 200mg/L.

Thereafter these tubes were incubated at 37ºC/180 rpm for another 7 days (Kiyohara et al, 1982).

After 7 days, BMM-PAH tubes were centrifuged for 10 minitu at 6000 rpm. The supernatant was disposed and the pellet was washed with saline water for 2 times.

- Basal minima media which were autoclaved was put into the above pellet without any carbon source.
- Then the OD was taken at 595 nm and adjusted to 0.1.
- 500µl was taken from 0.1 OD adjusted culture and transferred to BMM media with 100mg/L of PAH.
- These were incubated at 37ºC/ 160 rpm for 7 days.
- After 7 days of incubation above media were used for extraction and quantification of Phenanthrene and Napthalene.

6. Quantification

- After 7 days of shaking, residual PAH was extracted from culture (5ml).
- For extraction equal volume dichloromethane was added to the degradation set up.
- The tubes were then vortex for 10 min and kept for another few minute to separate aqueous and organic phases.
- Upper aqueous layer was extracted and dried over sodium sulfate.
- There after organic phase was pipette out and kept for drying over night.
- The residual was re-suspended in equal volume of n-hexane.
- The extracted phenanthrene and naphthalene was 10 times diluted in n-hexane.
- The residual phenanthrene and naphthalene concentration was determined from standard curve of respective PAH.
- Absorbance of Phenanthrene and naphthalene was taken at 252 and 221nm respectively.

7. Characterization of isolates

The morphological properties of the cell were examined under compound microscope through which we can get the information about Gram staining, Motility and citrate utilization test were done to characterize the isolates.

7.1. Physical characterization

Gram staining:

Gram staining is an important method of differentiating bacterial species into two large groups such as Gram-positive and Gram-negative, based on the chemical, chiefly the presence of high levels of peptidoglycan in its cell wall, and physical properties. The Gram staining is almost always the first step in the identification of a bacterial organism, it is mainly performed to know whether the culture is pure or not. While Gram staining is a valuable diagnostic tool in both clinical and research backgrounds, not all bacteria can be classified by this type of process, thus producing Gram-variable and Gram-indeterminate groups as well. A Gram positive reaction results in a purple/blue color while a Gram negative reaction results in a pink/red color.

Staining mechanism:

Gram-positive bacteria have a thick mesh-like cell wall made of peptidoglycan (50- 90% of cell wall), which are absorb purple colour stain by crystal violet, whereas those are Gram-negative bacteria have a thinner layer (10% of cell wall), which are stained pink by the counter-stain,safranin. There are four basic steps of the Gram staining:

Applying a primary stain (crystal violet) to a heat-fixed smear of a bacterial culture for 2 min.

The addition of a trapping agent (Gram's iodine)

Rapid decolorization with alcohol and

Counterstaining with safranin.

Gram reaction:

A clean grease free slide was taken and a smear of the bacterial culture was made on it with a sterile loop. The smear was air-dried and then heat fixed. Then it was subjected to the following staining reagents:

ii) Colony morphology: Shape, size, colour, elevation and margin of colony and appearance are observed in overnight plate culture on Nutrient agar media and noted down.

iii) Cell morphology: The gram stained cells were viewed under light microscope under 100x oil immersion to determine the morphological characteristics of the cells.

iv)Motility test: It is used to check the ability of bacteria to migrate away from the stabbing line which is the line of inoculation. The bacteria was inoculated by a needle into motility media, i.e, mannitol agar by stabbing the culture in a straight line and was observed after 24-48 hours incubation. If the test organism migrates away from the line of inoculation, the bacteria are motile.

7.2. Biochemical test

7.2.1. Sugar fermentation tests

The sugar test was done by using Bacillus Hi-media biochemical kit (A, B1 and C) which contain different sugars. Kit A consists of Dextrose, Lactose, Maltose, Fructose, Raffinose, Xylose, Galactose, Galactose, Trehalose, Melibiose, Sucrose, L-Arabinose, and Mannose. Kit B consists of Inulin, Sodium gluconate, Dulcitol, Glycerol, Inositol, Salicin, Sorbitol, Mannitol, Adonitol, Arabitol, Erythritol, α- methyl-D- glucoside. Kit C consists of Rhamnose, Melezitose, α-methyl-D-mannoside, Cellobiose, Xylitol, Onpg, Esculin hydrolysis, D-Arabinose, Citrate utilization, Malonate utilization, Sorbose.

7.2.2. Antibiotic resistance

All the strains were tested for antimicrobial resistance by the method of Bauer et al. (1966) with antibiotic impregnated discs (Hi-Media).The following antibiotic discs with concentration of the drug as stated in the parenthesis were used, Gentamicin 10µg, Amphicilin 30µg, Azithromycin 30µg, Chloramphenicol 50µg, Erythromycin 15 µg,

Vancomycin 30 µg, Norfloxacin 10µg, Streptomycin 10µg, Methicillin 5µg, Tetracyclin 30µg, Kanamycin 30µg, AC 10µg. After that the strains were characterized as nonresistant, intermediate or resistive based on the diameter of the inhibition zones around the disc.

7.2.3. Swimming and swarming

Swimming and swarming motility is a rapid and a coordinated translocation of a bacterial population across solid or semi- soft solid surfaces. This type of characteristic motility is one of the example of an emerging concept in microbiology. Swarming type motility was first described by and has been mostly studied in genus Serratia (Alberti and Harshey, 1990), Salmonella (Harshey, 1994), Aeromonas (Kirov et al., 2002), Bacillus (Kearns and losick, 2004), Yersinia (Young et al.,1999), Pseudomonas, Proteus (Caiazza et al.,2005), Vibrio (Rather, 2005) and Escherichia (Harshey, 1994). This multicellular behavior has been mostly observed in controlled laboratory conditions and relies on two critical elements: 1) the nutrient composition and 2) viscosity of culture medium (i.e. % agar).One particular feature of this type of motility is the formation of dendritic fractal-like patterns formed by migrating swarms moving away from an initial location.
For swimming motility test 0.2% of nutrient agar was prepared and for swarming motility test 0.5% of nutrient agar was prepared, after that both were poured into plates. The poured plates were in semi-solid condition and 10 µl of culture was inoculated at the centre of the plates, then the plates were kept in the incubator for 24 hrs at 37ºC.

8. Amplification of PAHs (Phenanthrene and Napthalene) Dioxygenase locus

Polymerase chain reaction allows the production of more than 10 million copies of a target DNA sequence from only small amount molecules. The sensibility of this technique means that the sample should not be contaminated with any other DNA or previously amplified products (amplicons) that may reside in the laboratory environment.

8.1. Preparation of lysates

- 1.5 ml of micro centrifuge tubes was taken.
- 1 ml of culture was brought and cell mass was harvested by centrifuging it at 7500rpm for 10 min.
- Supernatant were discarded.
- 200µl of autoclaved milli Q water was added to each tube.
- It was vortex properly.
- Centrifuged at 7500 rpm for 10 min.
- Supernatant were discarded.
- Again 200µl of autoclaved milli Q water was added.
- Again it was vortex properly.
- The top of the each tube was pierced with the help of needle.
- These tubes were kept on boiling water for 10 min.
- After proper boiling these were immediately kept on ice for 5 min.
- Then it was again centrifuged at 10,000 rpm for 5 min.
- Fresh aliquots were taken.
- Supernatants were transferred to the fresh aliquots.
- These were stored at 4ºC.

8.2. Preparation of reaction mixture

The preparation of a master mix or reaction mixture comprising buffer, dNTPs, primers, water and Taq DNA polymerase enzymes, MgCl2 in a single eppendrof is one of the most crucial step in PCR. This can then be suspended into individual aliquote tubes. Template DNA solutions are then added to this. This method minimizes the possibility of pipetting errors and saves time by reducing the number of reagent transfers in each tube.

8.3. Reagents

1. Template DNA:
2. Primers :

The primers (Aurelie et al., 2008) for the amplification of the DNA were as follows

Forward primer - (GAG ATG CAT ACC ACG TKG GTT GGA)

Reverse primer - (AGC TGT TGT TCG GGA AGA YWG TGC MGT T)

3.10X buffer assay

4. dNTP

5. Taq DNA polymerase:

This was obtained in a stored buffer containing 50% glycerol.

8.4. Methodology

- Genomic DNA was amplified in sterile PCR tubes with reaction volume of 25µl containing the following components-
- 10X assay buffer-2.5µl, MgCl2 – 1.5µl, dNTP – 0.5µl, Forward primer – 0.5µl , Reverse primer- 0.5µl, Taq DNA polymerase- 1µl, Template - 4µl, autoclaved MQ water was added to make up the volume to 25µl.Table.1.
- The tubes were then placed in the thermal cycler and amplification was carried out. Then the PCR products were then stored at -20ºC for further use.

21 µl of the master mix & 4 µl of Template were taken in 0.5 ml PCR tubes and then it was mixed properly.

- Spinning was done briefly to pellet liquid to the bottom and the following thermal cycle program was used to amplify the DNA
- PCR products may store in -20º for further use or may used for Gel run to observe the bands.

Table.1. Showing the total cycle for the PCR

		TEMPERATURE	TIME
	Initial denaturation	95ºC	5 min
30 CYCLES	Denaturation	95ºC	30 sec
	Annealing	57ºC	30 sec
	Extension	72ºC	30 sec
	Final extension	72ºC	7 min

Precaution for pcr:

- PCR reagents prepared in large amounts should be distributed in 1.5 ml microfuge tube and stored at -20°C.
- Water used for PCR reagents, DNA and primers should be sterilized, and then distributed in 1.5 ml microfuge tubes and stored at -20°C.
- When primers are made, the stock solution is usually highly concentrated. From this extremely concentrated stock solution, a proper diluted stock solution are made for personal use and stored separately. This system allows massive lab wide contamination problem.
- Different sets of pipette should be designated for different procedures. One set of pipette should be designated for preparing PCR reactions. One pipette should be designated to be used only in loading samples in agarose gels.

5. RESULTS

1. Isolation and screening

The water sample was transferred to Basal Minimal media broth (Sambrook et al.,1989) supplemented with 100 mg/L of Phenanthrene and Naphthalene each as the sole carbon and energy source for growth and then it was kept in the incubator shaker at 37ºC /160 rpm for 15 days. After 15 days of incubation, it was serially diluted and 100µl was spread on Sea Water Nutrient Agar media plates. After 24hrs of incubation 15 isolated colonies with different colony morphology were obtained. The isolates were further screened for their potential to degrade Phenanthrene and Napthalene. Preliminary screenings were simply done by continuously monitoring the growth of isolates on PAH-BMM Agar plate's and PAHs-Basal minimal broth for 7 days. After 7 days of monitoring, among 15 isolates (Table .1) continuous growth was observed in 5 isolates (KR-2, KR-5, KR-7, KR-10, KR-15) and the OD was found to be more than 0.5 at 595 nm.

Table 2. Growth of isolates on Sea Water Nutrient Agar plates.

Isolates	Growth on Sea Water Nutrient Agar Plates
KR-1	+++
KR-2	+
KR-3	±
KR-4	+++
KR-5	–
KR-6	++
KR-7	–
KR-8	±
KR-9	+++
KR-10	++
KR-11	+

KR-12	_
KR-13	_
KR-14	+++
KR-15	++

+++: Excelent, ++: Good: +: Satisfactory, -: No growth

2. Screening of isolates

Table 3. Growth of isolates on BMM-PAH agar plates.

Isolates	Phananthrene	Napthalene
KR-2	+	+
KR-5	+	+
KR-7	+	+
KR-10	+	+
KR-15	+	+

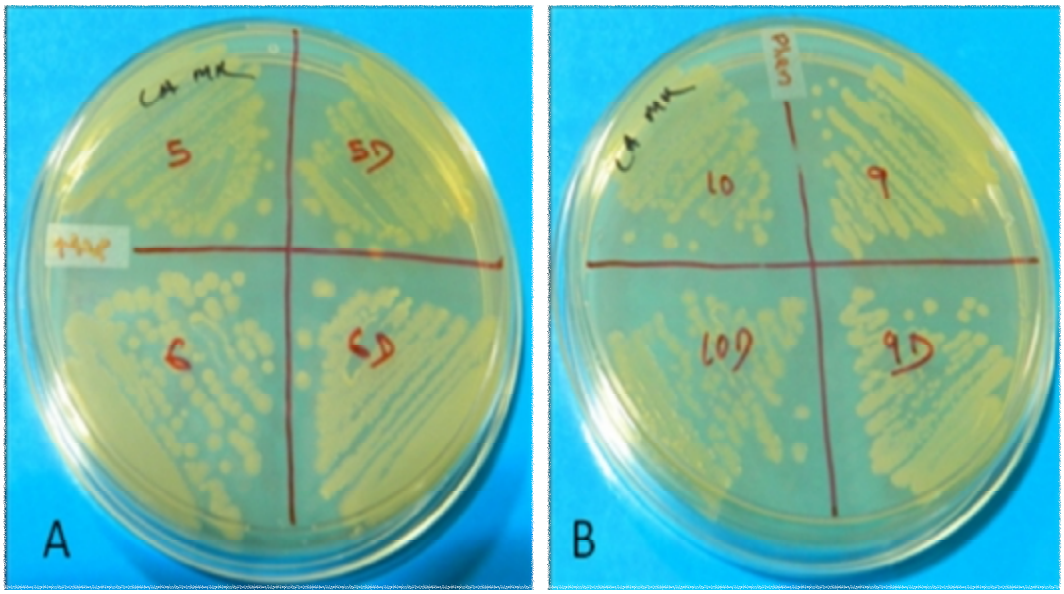

Fig: 2 Isolated colonies on Basal minimal media A- Napthalene and B- Phenanthrene

3. Carbon source utilization

Isolates were taken and inoculated in Luria bertani broth which was incubated for 24 hours at 37^0C. After incubation, the cell mass was collected by centrifugation at 4^0C/7000 rpm for 10 mintues. Then supernatant was discarded and pellet was re-suspended in 1ml of BMM. 100 µl of suspended cell was transferred to 5ml BMM tubes with 100 mg/L of phenanthrene and Napthalene separately as a sole carbon source 1% glucose in BMM without any PAHs was kept as control for growth. After that, these tubes were kept at 37^0C/160 rpm for 7 days. In order to monitor the growth, OD_{595}(fig3.1 and 3.2) were taken at regular time interval i.e. 0 day, 1 day, 3 day, 5 day and 7 day by ELISA plate reader (Victor plus). The growth pattern of isolates in terms of OD595 in different carbon source is illustrate in table 4 and 5

Table.4 OD_{595} for Phenanthrene

Isolates	0 Day	1 Day	3 Day	5 Day	7 Day
KR-2	0.032	0.12	0.324	0.567	1.001
KR-5	0.025	0.23	0.354	0.532	1.243
KR-7	0.038	0.33	0.386	0.683	1.332
KR-10	0.045	0.24	0.367	0.645	1.489
KR-15	0.034	0.43	0.57	0.668	1.662

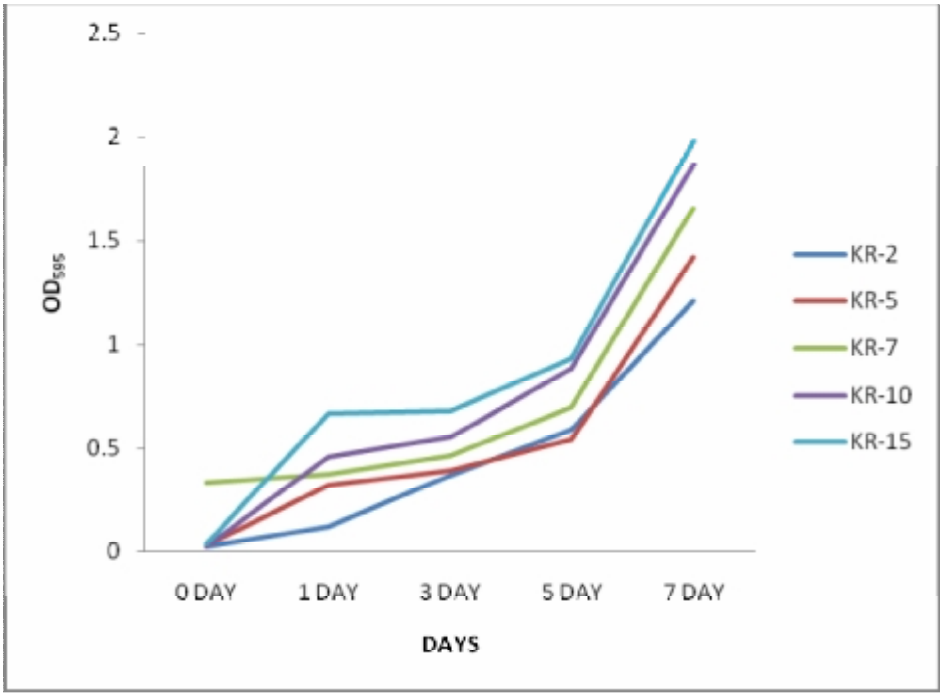

Fig. 3 OD on each day interval for phenanthrene

Table.5 OD_{595} for Napthalene

ISOLATES	0 DAY	1 DAY	3 DAY	5 DAY	7 DAY
KR-2	0.024	0.12	0.37	0.587	1.21
KR-5	0.031	0.32	0.39	0.545	1.42
KR-7	0.33	0.37	0.46	0.698	1.66
KR-10	0.027	0.46	0.56	0.889	1.87
KR-15	0.039	0.67	0.68	0.937	1.98

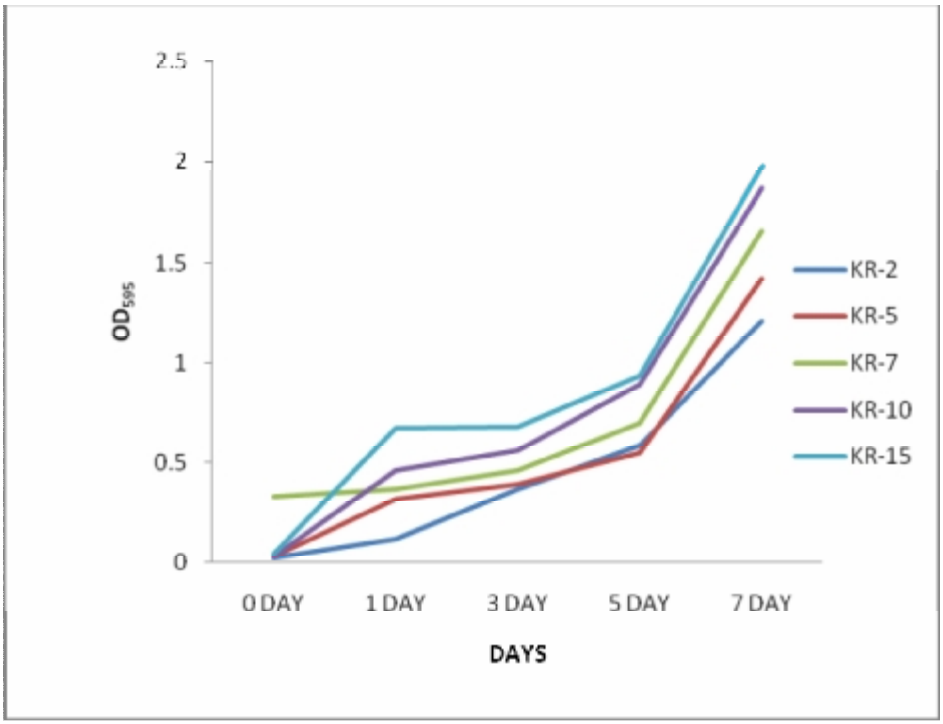

Fig. 4 OD on each day on interval Napthalene

6.

4. Penanthrene and Napthalene Standard curve

5 mg/ml of concentration, PAH (Phenanthrene and Napthalene) stock solution was prepared in Hexane. From the above prepared stock solution 5 ml of Phenanthrene and Napthalene working solution in hexane with concentration of 0.5µg/ml, 1µg/ml, 10µg/ml, 5oµg/ml and 100 µg/ was prepared in triplicated for standard curve. It was scan to get the λ_{max} value and absorbance in between 200- 400nm range using UV-Vis Spectrophotometer. For Phenanthrene and Napthalene λ_{max} was found to be 292nm and 221nm respectively and absorbance at this λ was considered to prepare the standard curve. λ_{max} and standard curve of Phenanthrene and Napthalene is shown in fig.3 and 4. Absorbance value for different concentration is illustrated in table 4.

Table.6 Standard Curve of Phenanthrene and Napthalene at different Concentration

CONCENTRATION	PHENANTHRENE	NAPTHALENE
0.5	0.0002	0.003
1	0.009	0.0008
10	0.0508	0.0602
50	0.318	0.326
100	0.565	0.566

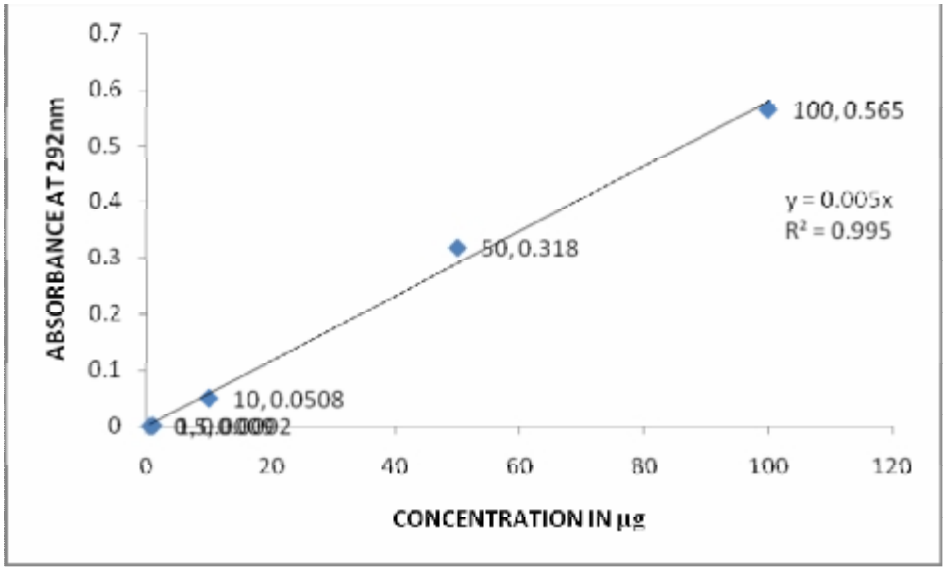

Fig. 5 Standard Curve of Phenanthrene at different concentration

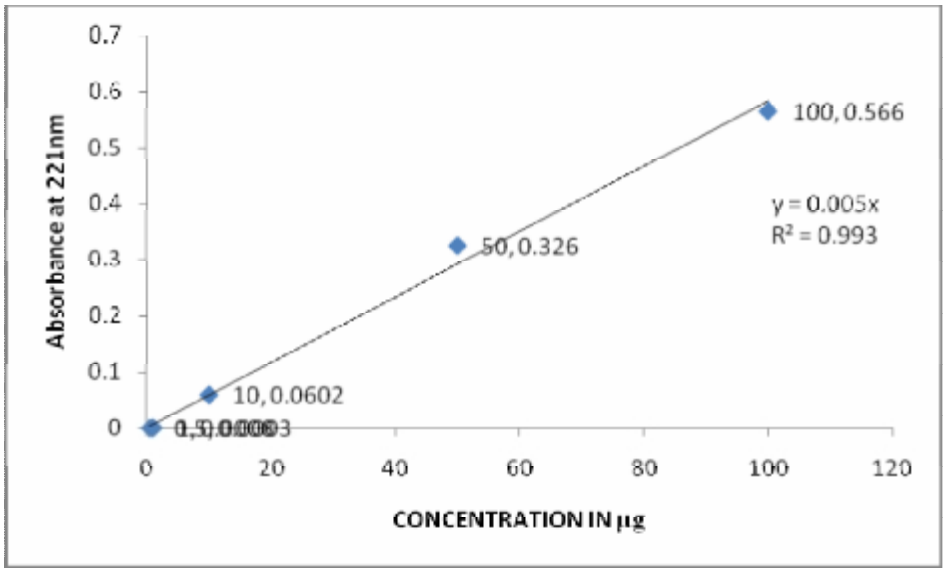

Fig. 6 Standard Curve of Napthalene at different concentration

5. Degradation study

The isolates were inoculated into test tubes containing 5 ml of minimal media broth which is supplemented with PAH (Phenanthrene and Napthalene) at concentration of 100mg/L and kept for 24 hours at 37^0C in order to check the degradation. PAH was delivered to media and was kept for few hours to completely remove hexane from the media. There after BMM-PAH tubes were subsequently incubated at 37^0C/180 rpm for 7 days. After 7 days, cell was harvested and transferred to BMM-PAH tubes (Phenanthrene and Napthalene) at concentration of 200mg/L. Again these tubes were incubated at 37ºC/180 rpm for another 7 days (Kiyohara et al, 1982). BMM-PAH tubes were centrifuged for 10 min at 6000 rpm after 7 days of incubation. The supernatant was discarded and the pellet was washed with saline water for 2 times. Autoclaved basal minima media was added to the above pellet without any carbon source and the OD was taken at 595nm. 500µl was taken from 0.1 OD adjusted culture and transferred to BMM media with 100mg/L of PAH. These were incubated at 37ºC/ 180 rpm for 7 days. After 7 days of

incubation above media were used for extraction and quantification of Phenanthrene and Napthalene.

6. Quantification and extraction:

Before quantification and extraction, the prepared stock at different concentration 0.5µg, 10µg, 50µg and 100µg was scanned within 200-400 nm in UV-Visible Spectrophotometer. Then Phenanthrene show its peak at 292nm (fig. 5) and Napthalene show its peks at 221nm (fig. 6). Then quantification and extraction was done using Dichloro-methane, then it wsa reconstituted with hexane. After extraction each sample KR-2, KR-5, KR-7, KR-10 and KR-15, OD was taken at 292nm for Phenanthrene and 221nm for Napthalene. The results of degradation studies of Phenanthren were illustrated in table 5 and in naphthalene in table 6.

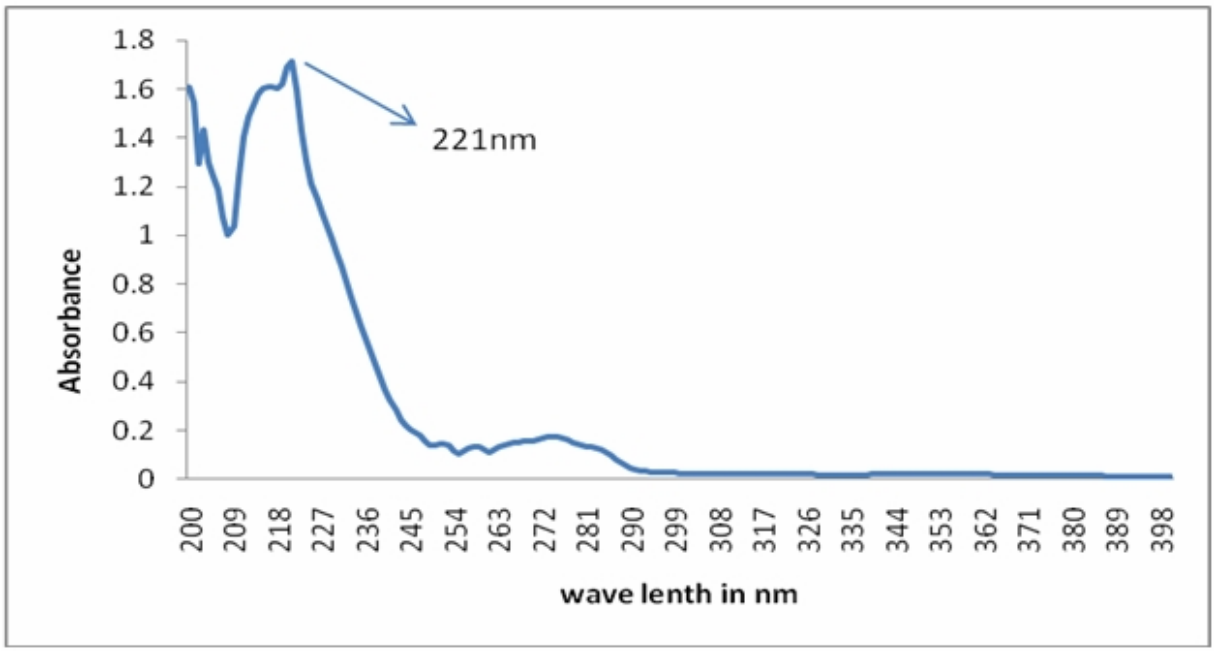

Fig.7 λ_{max} for naphthalene

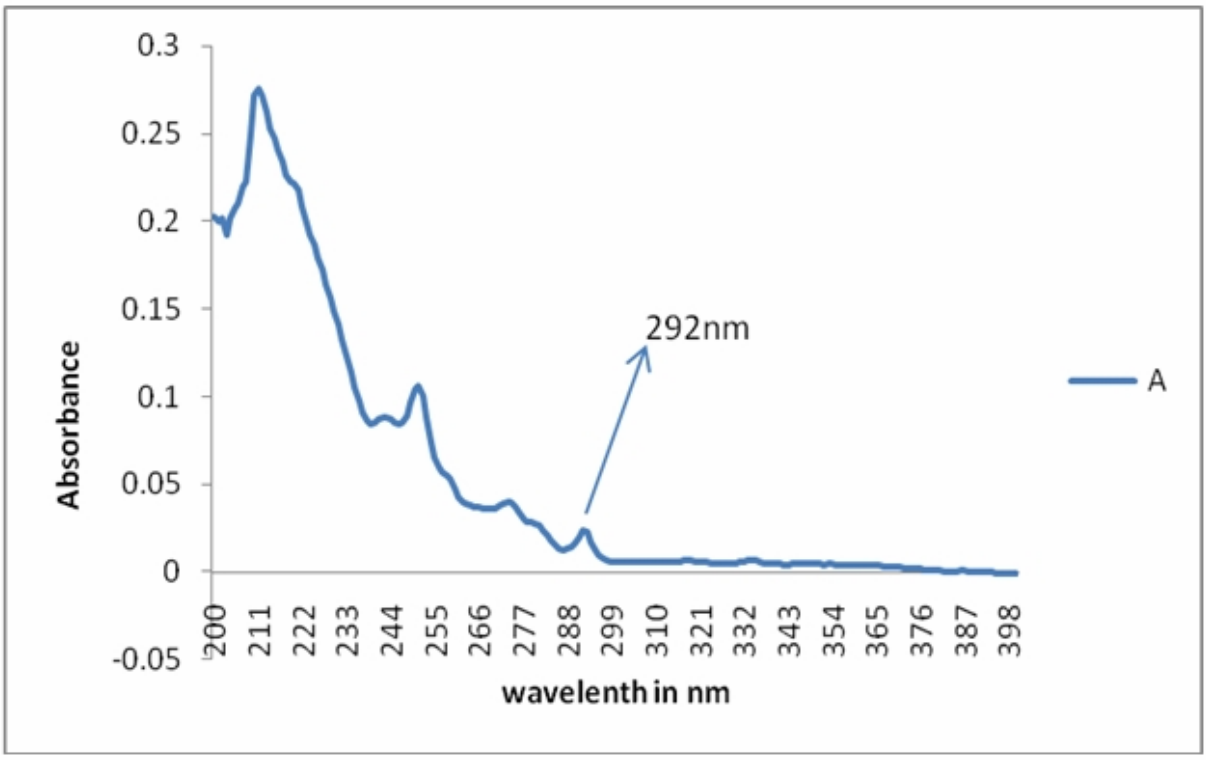

Fig.8 λ_{max} for phenanthrene

Table.7 Percent degradation of phenanthrene in basal minimal media:

Isolates	Initial concentration (I) mg/l	Concentration (c) after 7 days mg/l	% OF Degradation(I-C)/I*100
CONTROL	100	0.956	0.956
KR-2	100	50.88	50.88
KR-5	100	50.96	50.96
KR-7	100	50.81	50.81
KR-10	100	50.4	50.4
KR-15	100	50.77	50.77

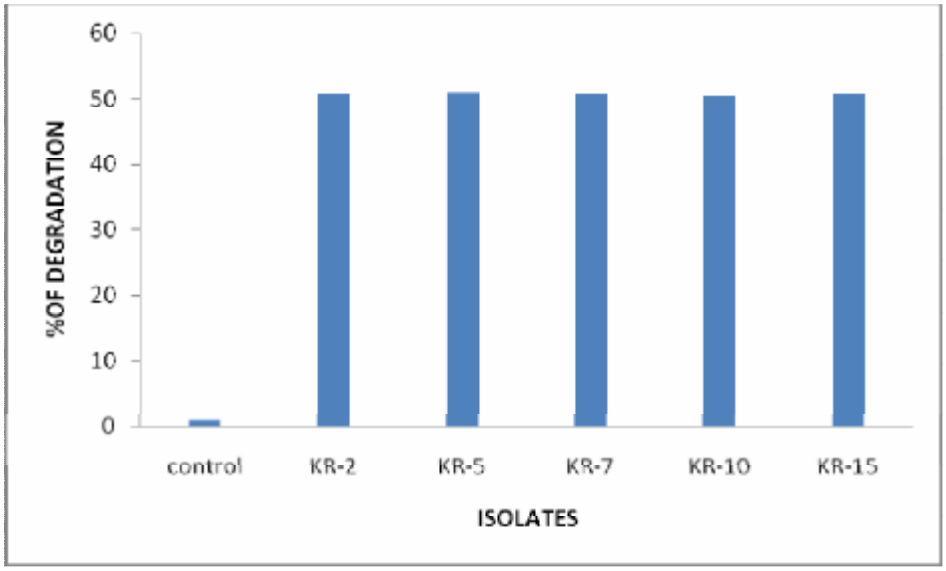

Fig.9 % of degradation of phenanthrene

Table. 8 Percent degradation of napthalene in basal minimal media:

Isolates	Initial concentration (I) Mg/l	Concentration (c) after 7 days mg/l	% of degradation
CONTROL	100	0.876	0.876
KR-2	100	29.9878	70.012
KR-5	100	33.9878	66.012
KR-7	100	21.817	78.182
KR-10	100	25.89	74.109
KR-15	100	29	71

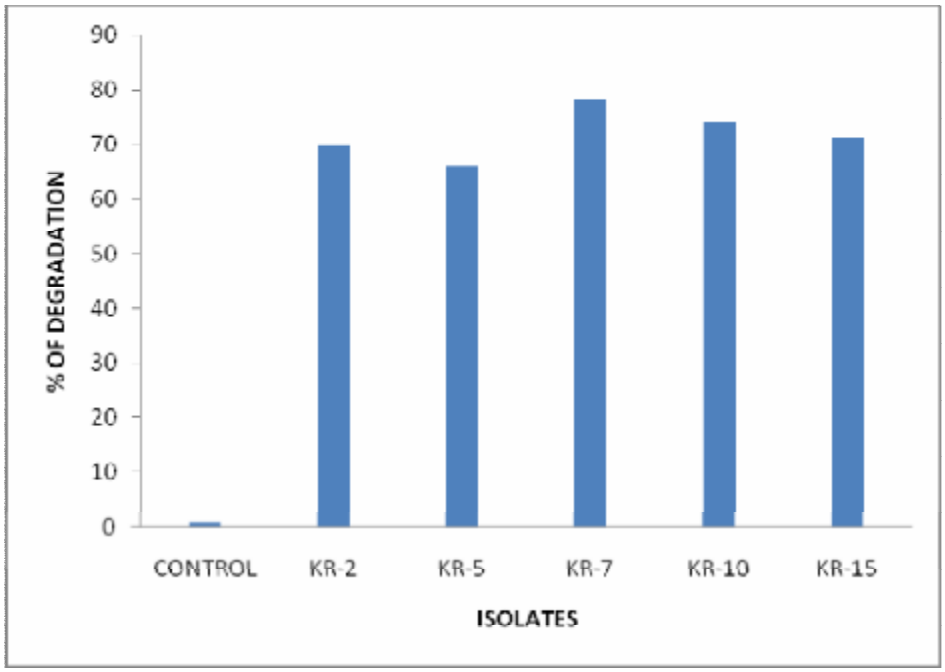

Fig. 10 % of degradation of naphthalene

7. Gel picture of polymerase chain reaction

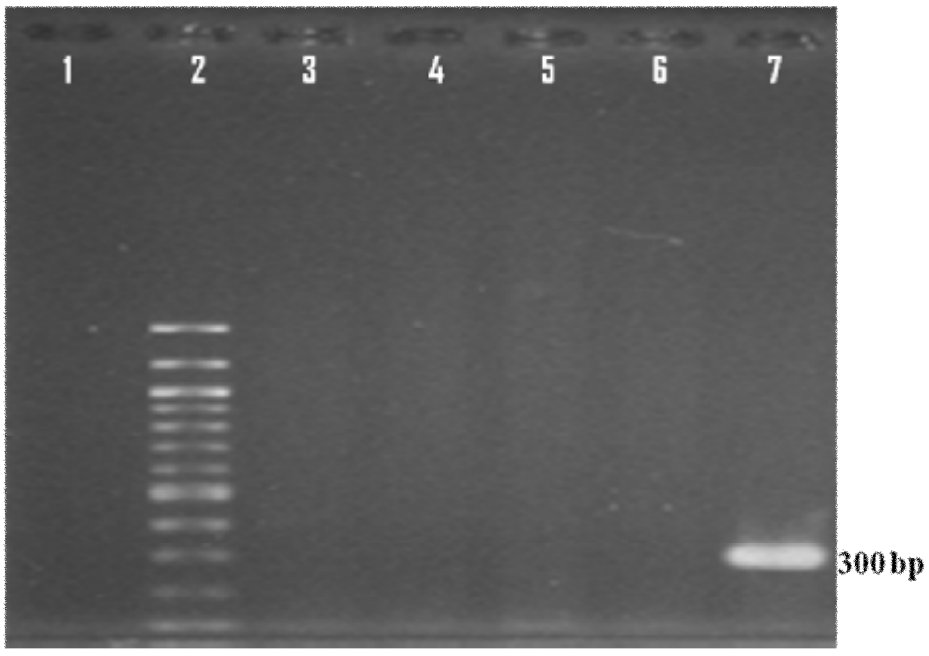

Fig. 11 Gel Photograph showing amplification of *pah dioxygenase* gene. Lane 1: -ve Control, Lane 2: 100bp ladder, Lane 3-7: KR-5, KR-7, KR-10,KR-15 and KR-2

8. Characterization of isolates:

From the five isolates (KR-2, KR-5, KR-7, KR-10, KR-15) KR-5was found to be Gram positive, and the rest were Gram-negative, Motile and show positive Citrate Utilization test. The results of characterization of these isolates were illustratd in Table 7.

Table.9 Characterization of isolates

Characteristics	KR-2	KR-5	KR-7	KR-10	KR-15
Gram stain	-	+	-	-	-
Colour of colonies	Cream	White	White	Cream	White
Motility	+	Highly +	+	+	+
Citrate utilization	+	-	+	-	-

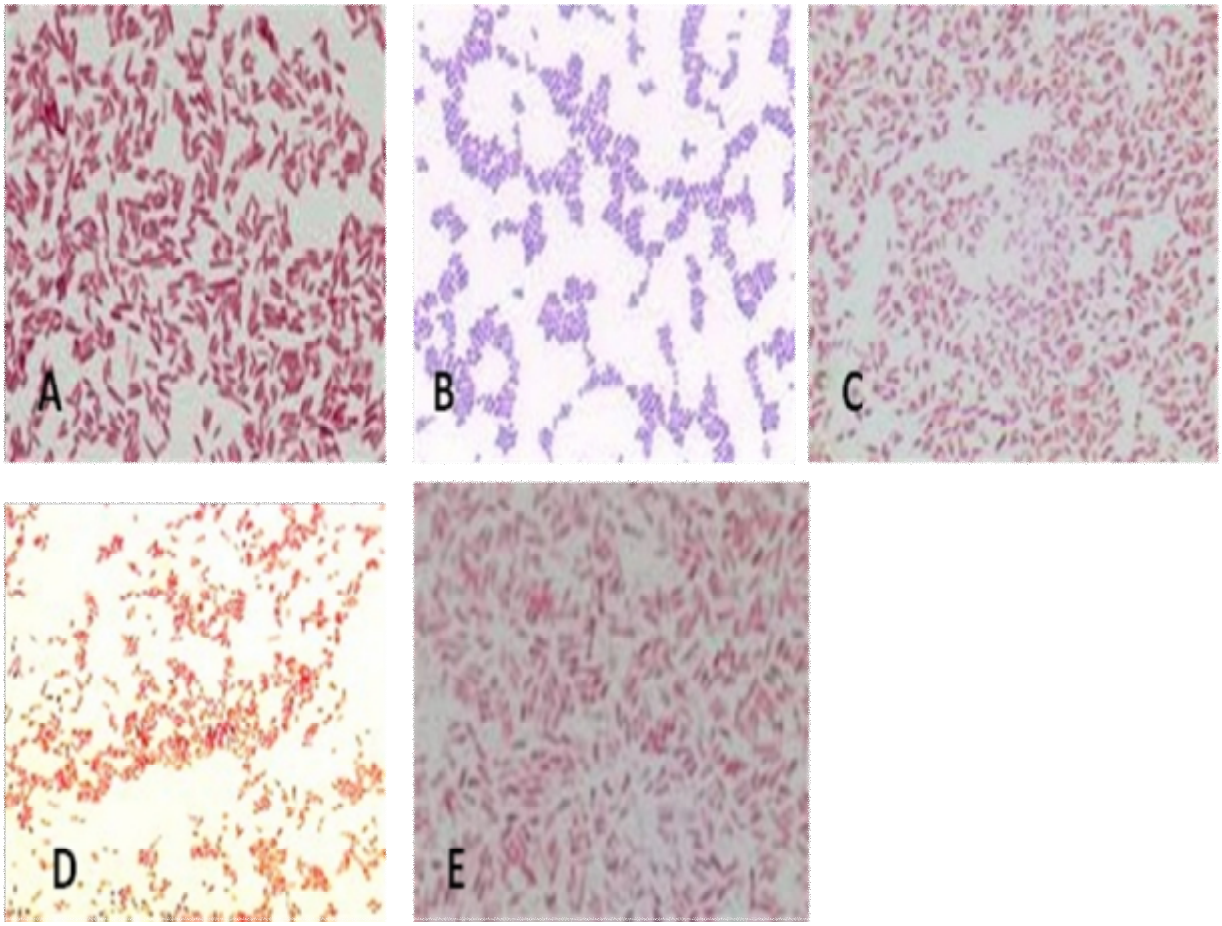

Fig. 12 Gram staining of 5 isolates: A- KR-2, B-KR-5, C-KR-7, D-KR-10 and E-KR-15

Table.10 Swimming and swarming motility test

Motility Test	KR-2	KR-5	KR-7	KR-10	KR-15
Swimming (0.2%)	+	+	+	+	+
Swarming (0.5%)	+	+	+	+	+

9. Sugar utilization test

Sugar fermentation test was performed to detect bacteria's ability to ferment sugar and produce gas and/or acid as a end product. The result of the carbohydrate/sugar utilization test of the five isolates is given in the Table 9. Isolates show varied pattern of utilization of 34 different sugars.

Table.11 Utilization of sugar by different isolates

Sugars	KR-2	KR-5	KR-7	KR-10	KR-15
Lactose	-	+	-	-	-
Xylose	-	+	-	-	-
Maltose	-	+	-	-	-
Fructose	-	+	-	-	-
Dextrose	-	+	-	-	-
Galactose	-	+	-	-	-
Raffinose	-	+	-	-	-
Trehalose	-	+	-	-	-
Melibiose	-	+	-	-	-
Sucrose	-	+	-	-	-
L- Arabinose	-	+	-	-	-
Mannose	-	+	-	-	-
Inulin	-	+	-	-	-
Glycerol	-	+	-	-	-
Sodium gluconate	-	-	-	-	-
Salicin	-	+	-	-	-
Dulcitol	-	+	+	-	-
Inositol	-	+	-	-	-
Sorbitol	-	+	-	-	-
Mannitol	-	+	-	-	-

Adonitol	-	+	-	-	-
Arabitol	-	+	-	-	-
Erythritol	-	+	-	-	+
α-Methyl-D-glucoside	-	+	-	-	-
Rhamnose	-	+	-	+	-
Cellobiose	-	+	-	-	-
Melezitose	-	+	+	-	-
α-Methyl-D-Mannoside	-	+	-	-	-
Xylitol	-	-	-	-	-
ONPG	-	+	-	-	-
Esculin hydrolysis	+	-	+	+	+
D-Arabinose	-	+	-	-	-
Malonate utilization	+	-	+	+	+
Sorbose	-	-	-	-	-

+:Positive, -: Negative

10. **Antibiotic sensitivity test**

All the five isolates were tested for antimicrobial resistance by the method of Bauer et al., 1966 with antibiotic impregnated discs (Hi-Media). After that, the isolates were characterized as sensitive or resistant based on the diameter of the inhibition zones around the disc. Zone inhibition was measured in millimeter.

Table. 12 Zone of inhibition (in mm) and resistant/sensitivity towards antibiotics

Isolates	Gen	Amp	Azi	Chl	Ery	Van	Nor	Str	Met	Tet	Kan	Ac	Inference
KR-2	**30**	**9**	**29**	**24**	**24**	**X**	**30**	**19**	**X**	**19**	**24**	**14**	**Van^R, Met^R**
KR-5	**30**	**X**	**24**	**12**	**28**	**24**	**32**	**19**	**X**	**19**	**20**	**X**	**Amp^R, Met^R, Ac^R**
KR-7	**22**	**X**	**25**	**21**	**16**	**X**	**28**	**20**	**X**	**17**	**22**	**X**	**Amp^R, Met^R, Van^R**
KR-10	**23**	**X**	**25**	**24**	**13**	**X**	**24**	**X**	**X**	**19**	**20**	**X**	**Amp^R, Met^R, Van^R, Ac^R**
KR-15	**21**	**X**	**23**	**15**	**15**	**X**	**20**	**19**	**X**	**14**	**18**	**X**	**Amp^R, Met^R, Van^R, Ac^R**

Gen 10- Gentamicin, Amp 30- Amphicillin , Azi 30- Azithromycin, Chl 50- Chloramphenicol, Ery 15- Erythromycin, Van 30- Vancomycin, Nor 10- Norfloxacin, Str 10- Streptomycin, Met 5- Methicillin, Tet 30- Tetracyclin, Kan 30- Kanamycin, Ac 10- Ac.

R- Resistant, S- Sensitivity

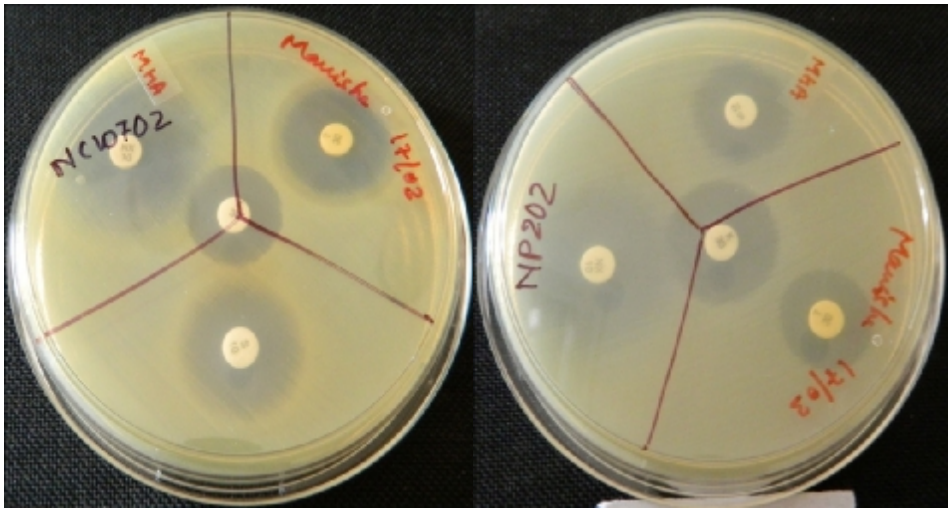

Fig. 13 picture showing Zone of inhibition (in mm) and resistant/sensitivity towards antibiotics of isolates

5. DISCUSSION

The present study focused on the isolation and screening of potential Phenanthrene and Napthalene degrading bacteria from the marine source. Marine water and sediments receives waste from all terrestrial, atmospheric and freshwater source and being polluted by variety of ornaic and inorganic polliutants. After selective enrichment of PAHs degrading bacteria, 5 isolates named as KR-2, KR-5, KR-7, KR-10, KR-15 were studied for their degradation capability . In 7 days of incubation KR-2, KR-5, KR-7, KR-10 and KR-15, found to degrade 50.88%, 50.96%, 50.81%, 50.4% and 50.77% of phenanthrene and 70.01%, 66.01%, 78.18%, 74.1% and 71% of napthelene respectively. Genomic DNA was extracted and ring hydroxylating dioxygenase gene was amplified by primer- Forward primer - (GAG ATG CAT ACC ACG TKG GTT GGA) and Reverse primer - (AGC TGT TGT TCG GGA AGA YWG TGC MGT T). The results obtained with the above test demonstrated that these particular isolates degrade more potentially Napthalene than Phenanthrene. A total of 5 PAH degrading bacteria isolated from water sample were analyzed phenotypically for their ability to degrade PAH and whether they contain dioxygenase locus or gene which is responsible for the degradation of PAH. The analysis was designed to screen PAH degrading isolates as potential sources for degradation of PAH i.e. Phenanthrene and Napthalene. All isolates were subjected to PAH spray plate method, degradation, quantification. Physical and biochemical characterization were done through Gram staining, citrate utilization test, sugar utilization test, swimming and swarming motility test, antibiotic sensitivity test. All the isolates show different morphological aspects in terms of Gram staining, color of colonies, motility and citrate utilization. Among all the isolates only KR-5 is Gram positive and rest were Gram negative. This particular isolates is highly motile. All the above isolates showing positive result in Swimming and Swarming motility test. In carbohydrate utilization test, the isolate KR-5 showing positive result towards all the sugars i.e. utilize all the sugars but all the other isolates showing negative result. All isolates were subjected to the carbon source utilization process; degradation and quantification which show different types of results i.e. all strains were differentially potentially to degrade polycyclic Aromatic hydrocarbons. Standard curve of the particular PAH i.e. Phenanthrene and Napthalene show maximum absorbance at 292

and 221nm respectively. Degradation and quantification study suggested strong evidence about the % of degradation of isolates in PAHs. Among all the isolates KR-5 is most potentially degrade the phenanthrene showing about 50.96 % of degradation and in case of Napthalene KR-7 is the most potential one showing about 78.18 %of degradation. The result of antibiotic sensitivity test shows, all isolates were resistant towards the antibiotic methicillin, and all isolates show different inhibition zone towards different antibiotics. The PAH compound taken for the studies were Phenanthrene and Napthalene. Metabolic pathway is important for degradation study. These two compound show different degradation pathways by forming different intermediates. Although biodegradation of phenanthrene has been widely reported in Gram-positive bacteria and Gram-negative bacteria, very little amount of knowledge is experienced about degradation hydrocarbon ability in the genus Staphylococcus (Mrozik & Labuzek, 2002; Survery et al., 2004). In addition, there are a few reports on the assimilation of phenanthrene via initial dioxygenation at the position of 1,2-phenanthrene. Jerina et al. (1976) found cis-1,2-phenanthrenedihydrodiol as a minor product of phenanthrene degradation in the mutant strains Beijerinckia B-836 and Pseudomonas putida. Moreover, dioxygenation occurs at the position of 1,2-phenanthrene by Sphingomonas group strain P2, Mycobacterium vanbaalenii and Sinorhizobium group and Bukohrdia sp. C4 has been reported recently (Balashova et al., 1999; Pinyakong et al., 2000; Keum et al., 2006). The accumulation of 1-hydroxy-2-naphthoic acid and 2-hydroxy-1-naphthoic acid as the major metabolites in phenanthrene metabolism in Pseudomonas and Burkholderia spp. Metabolism of phenanthrene by two distinct paths, through either phthalate or salicylate, are well documented (Iwabuchi and Harayama 1997; Saito et al. 2000; Samanta et al. 1999). Apart from dioxygenation of phenanthrene at the position of 3,4, there are information of phenanthrene degradation involving initial dioxygenation at the 9, 10-position of phenanthrene (Sutherland et al., 1990). The genes and the proteins for aromatic ringhydroxylating dioxygenases have yet been reported for Staphylococcus species, has not done the full genome analysis of various Staphylococcus species present in the database reveal the presence of ring-hydroxylating PAH dioxygenases (Mallick et al, 2007). The plasmid cured strain was unable to grow on either phenanthrene or 2-hydroxy-1-naphthoic acid or salicylic acid as sole carbon energy Sources, indicating that the particularly plasmid pPHN possibly harbours genes involved in phenanthrene assimilation (Mallick et al,

2007). Keum et al. (2006) reported 2- hydroxy-1-naphthoic acid as one of the metabolic intermediates along with 1-hydroxy-2-naphthoic acid in the degradation of phenanthrene by a Sinorhizobium sp Recently. 1-hydroxy-2-naphthoic acid and 2-hydroxy-1-naphthoic acid were reported to be identified as metabolic intermediates in the degradation of phenanthrene by Burkholderia sp. C3 (Seo et al., 2006a, b), which were detected to be converged to 1,2-dihydroxynaphthalene and further degradation occurs by ortho- and metacleavage dioxygenases. Metabolites accumulating during growth and resting cell incubations with phenanthrene indicate that isolates initiates its attack on phenanthrene by dioxygenation at the C-1 and C-2 positions to contribute cis-1,2-phenanthrenedihydrodiol. It is believed that the dehydrogenation of the metabolites supports the enzymic dioxygenation of phenanthrene at the C-1 and C-2 positions and subsequent meta-cleavage occurs in 1,2 position of dihydroxyphenanthrene which is the intermediate in Phenanthrene degrading pathway. 5,6-Benzocoumarin was identified earlier as one of the compounds produced from phenanthrene-1,2-dihydrodiol (Pinyakong et al., 2000). Among the possible metabolic intermediates in the degradation of phenanthrene which is a PAH, 2-hydroxy-1-naphthoic acid were found to be utilized. The cells grown on phenanthrene, 2-hydroxy-1-naphthoic acid failed to respire on 1-hydroxy-2-naphthoic acid, naphthalene-1,2-dicarboxylic acid or o-phthalic acid, pointing out that none of these compounds are involved in the phenanthrene degradation pathway. Although assimilation of phenanthrene via 2-hydroxy-1- naphthoic acid was reported by Pinyakong et al. (2000). Keum et al. (2006) reported 2- hydroxy-1-naphthoic acid as one of the metabolic intermediates along with 1-hydroxy-2-naphthoic acid in the degradation of phenanthrene by a Sinorhizobium sp. Balashova et al. (1999) described the accumulation of 1-hydroxy-2-naphthoic acid and 2-hydroxy-1- naphthoic acid as the major metabolites in phenanthrene metabolism in Pseudomonas and Burkholderia group of spp. Metabolic process of 2-hydroxy-1-naphthoic acid yielded salicylaldehyde, catechol and a trace amount of 2-naphthol in the exhausted culture. Although salicylaldehyde and catechol were also produced as intermediates in the metabolism of 1- hydroxy-2-naphthoic acid through the 1,2-dihydroxynaphthalene (Evans et al., 1965; Gibson & Subramanian, 1984). Napthalene biodegradation occurs through the formation of salicylate as intermediate. Its oxidation may follow either a gentisic acid (Grund et al., 1992) or catechol pathway in order to produce compounds to be integrated in the Kreb cycle. At the initial stage bacteria oxidize

naphthalene by incorporating both atoms of molecular oxygen into the aromatic molecule to form cis-1,2-dihydroxy-1,2- dihydronaphthalene. The second step in bacterial oxidation of naphthalene is the conversion of cis-1,2-dihydroxy-1,2-dihydro naphthalene to 1,2-dihydroxynaphthalene. Next step leads to the enzymatic breakdown of 1,2-dihydroxynaphthalene to cis-2-hydroxybenzalpyruvate, which is then converted via series of dioxygenases to salicylate and pyruvate. Salicylate is oxidized by salicalate hydroxylase to catechol, which can undergo either ortho or meta fission depending upon bacterial metabolism (Dagley and Gibson, 1965; Dagley, 1971)

6. SUMMARY AND CONCLUSION

The main of the above study is the Analysis of catabolic gene for PAH degradation in bacteria isolated from water sample. For the above study of degradation water sample was collected from Rushikulya, Odisha in sterilized falcon tubes. Sample was suspended into minimal media broth supplemented with 100mg/L of Phenanthrene and Napthalene each as the sole carbon and energy source for growth. 15 isolated colonies with different colony morphology were obtained. After 7 days of monitoring among 15 isolates continuous growth was observed in 5 isolates designated as KR-2, KR-5, KR-7, KR-10 and KR-15. Among the above 5 isolates which were grown in sea water nutrient agar only KR-5 is Gram positive and rest isolates were Gram negative. All isolates show different morphological aspects in terms of color of colonies, motility and citrate utilization test. All the isolates were motile but KR-5 was highly motile. Regarding swimming and swarming motility test, isolates were showing positive result. All the isolates show negative result towards sugar utilization test except KR-5. In antibiotic sensitivity test all the isolates were sensitive towards methicillin and show different inhibition zone for different antibiotics. Isolates were taken and inoculated in Luria bertani broth for carbon source utilization and the OD were taken at regular interval. The maximum absorbance for Phenanthrene and Napthalene are 292 and 221nm respectively. Degradation and quantification suggested about the % of degradation of isolates in PAHs. In 7 days KR-2, KR-5, KR-7, KR-10 and KR-15, found to degrade 50.88%, 50.96%, 50.81%, 50.4% and 50.77% of phenanthrene and 70.01%, 66.01%, 78.18%, 74.1% and 71% of napthelene respectively. Among all the isolates which are mentioned earlier KR-5 is most potential degrader for Phenanthrene and KR-7 is the most potential degrader for Napthalene. From this study of degradation it is concluded that the above mentioned isolates are more potentially degrade Napthalene than Phenanthrene. Among the 5 PAH degrading bacteria, KR-2 showing amplification with specific primers. The amplification in PCR indicates the presence of particular dioxygenase gene to degrade the PAH compound. Although the presence of dioxygenase gene confirm the degradation of PAH but due to the above result of degradation study, it denotes that not only dioxygenase gene but also various gene and other mechanism are involved for the degradation of PAHs.

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