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RESEARCH ARTICLE

ISOLATION AND CHARACTERIZATION OF PHENANTHRENE DEGRADATING BACTERIA FROM DAIRY WASTE SAMPLES

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Abstract

Phenanthrene is the commonly used hazardous chemical in various industries and the detoxification is a big problem till today. In this study, phenanthrene degrading bacteria were isolated from dairy waste samples, characterized by molecular techniques. A total of 10 samples were collected respectively from different spots of dairy industry. Minimum salt agar medium with phenanthrene composition was used to isolate the pure culture of resistant bacterium. Morphological, biochemical tests were done to identify the phenanthrene degrading bacteria. The colonies of the isolates were circular to irregular in dairy samples. Phenanthrene tolerant bacteria were isolated after the dairy waste soil samples was serially diluted and transferred onto M9 minimal agar medium amended with 10mg/l of phenanthrene. A concentration of 10 mg/l was chosen based on the prevailing concentration. Four Phenanthrene tolerant bacteria from dairy waste (HW2, HW1, HW3, HW5) were isolated. From the biochemical and 16srRNA PCR amplification, the bacterium identified was Micrococcus leuteus. The resistance to Phenanthrene by the isolate was tested with various concentrations of Phenanthrene from 0 to 10mM. The Micrococcus luteus showed a MTC value of 28Cfu/ml at 8mM and Bacillus cereus showed least.

Introduction:-

One of the most severe issues facing our world today is the contamination of polycyclic aromatic hydrocarbons (PAHs). This PAH pollution is becoming a global environmental issue. PAHs are found in the environment by oil spills, leakage and inefficient management of industrial waste, shipping, wood processing and fossil fuel combustion [1]. It also has a detrimental impact on the climate and human health. In Malaysia, soil polluted with PAHs has been the product of agricultural and industrial activities over the last 50 years [2-5]. Many manufacturing plants discharge their effluents and dispose of their untreated chemicals into the atmosphere. In reality, considering the growing number of environmental problems and the deterioration in environmental quality, it has a negative effect on the environment and on humankind. It is very important to look after and restore a healthy clean environment [6].

Bioremediation of soil polluted with PAHs has therefore gained growing international attention. Biodegradation is one way of using microorganisms to extract PAHs from polluted sites[7-9]. It refers to the use of biodegradation reactions to convert toxic compounds to non-toxic compounds. In addition, bioremediation is considered to be a promising environmentally sustainable treatment for remediation of hydrocarbon compounds. There are many cases of phenanthrene-contaminated soil in India especially in the Telangana state where oil processing plants are located.

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In certain cases, phenanthrene has been infected by soil for more than eight years [10]. Phenanthrene was classified as one of the 16 PAHs mentioned as a priority pollutant in the US EPA. Some studies have shown that phenanthrene can be degraded by microorganisms such as bacteria, fungi, yeast and microalgae [11]. However, bacteria have a significant role to play in this degradation. Several researchers have documented the degradation of individual PAHs, in particular phenanthrene, by single and mixed bacterial cultures[12-16]. Bacteria can degrade this organic pollutant by using their own metabolic activity to convert pollutants into different compounds [17]. In other words, the metabolic processes of the bacteria have the potential to use chemical contaminants as an energy source and to transform them to a less toxic form than the initial contaminants. The goal of this study is therefore to isolate potential bacteria from soil contaminated with PAHs to remediate phenanthrene at different concentrations.

Methodology:-

Collection of Samples:

Soil samples from dairy waste disposal sites were collected from Vijaya Dairy, Uppal, Hyderabad City. A total of 10 samples were obtained from different spots separated by 5-10 m from each other to cover a high concentration of soil metal. The soil and sediment cores were sampled from these sites. The soil samples from the first designated contaminated site were obtained from outside the factory in sterilized containers or falcon tubes. These tubes were sealed and brought in autoclaved bags to avoid contamination from external sources and examined by the Department of Microbiology, University of Osmania. Samples were held at or below 4°C. The soil samples from the second polluted site (metal disposal site) were collected in sterilized bags and held at or below 4°C.

Isolation of Bacteria:

The soil samples were analyzed in the laboratory within 24 hours of collection. Serial dilutions (up to 10-6) of the samples (one g for fresh soil and one ml for water) were made using sterile distilled water. The diluted 0.1 ml sample was inoculated on nutrient agar and incubated at 37°C for 24 hours. Pure colonies would be streaked to selective media. This method was replicated in a span of two weeks to keep them in a pure condition. Crops were processed at cooling temperature.

Isolation of pure culture Phenanthrene resistant bacterium:

Minimum salt agar medium with phenanthrene composition was used to isolate the pure culture of resistant bacteria. 100 ml of the minimum salt agar medium was prepared and sterilized in the autoclave at a pressure of 15 lbs and a temperature of 121o C, 0.5 ml of the final medium (minimal salt medium) was transferred to the solidified Minimal Salt Agar medium by the streaking plate method[18].

Isolation of bacteria on nutrient agar containing concentration of Phenanthrene:

Phenanthrene-containing nutrient agar was used for the isolation of bacteria from various samples. Phenanthrene 10mg/liter stock solutions is prepared in deionized water. Work solutions with various concentrations have been prepared from each stock solution. Working solutions of various concentrations were added to the molten state nutrient agar, which was then poured into petri dishes and allowed to solidify.

Identification of Phenanthrene resistant bacterium and their characterization

Microscopic examination of Gram's stained preparations:

A glass slide was extensively washed with 70% ethanol to make it free of grease. A thin clotting of the isolated colony was collected and placed on a drop of normal saline. The fine thin coating was air dried and the heat was fixed to the flame without interruption. Gram's staining was achieved as follows: the stain was overlaid with crystal purple stain and allowed to operate for one minute, the excess of stain was drained and the stain was washed with distilled water. Gram's iodine solution was then applied over the frottis and allowed to work for one minute, the excess iodine was drained. For a few seconds, Destaining was performed with 95% ethyl alcohol. The counter stain saffron was then added and permitted to function. The slide was washed in distilled water and dried with adsorbent paper.Observed under a high-power microscope and an oil-emergence for the presence of bacteria. Gram-positive microorganisms appeared dark purple while gram-negative microorganisms appeared pink. Their morphology and arrangement were recorded

Biochemical tests:
Various biochemical studies have been carried out to classify various isolates, such as indole test, citrate utilization test, nitrite reduction test, catalase test, MR–VP, urease test, sugar fermentation test (such maltose, glucose, lactose and fructose).

Isolation of genomic DNA:
The DNA was extracted by the process of alkaline analysis. Bacterial colonies from a 24-hour culture were inoculated into 2 ml of Luria-Bertani (LB) broth incubated overnight at 37°C with vigorous shaking. Bacterial cells were harvested by centrifuging 1.5 ml of the culture in centrifugal tubes for 2 min at 13,000 rpm. The supernatant was discarded and the pellets were suspended in 100 µl of ice cold cell resuspension buffer consisting of tris-EDTA by mixing with pipette followed by incubation with 10 µglysozyme (50 mg/ml) at 37 C for 30 min. 5 µl proteinase K (20 mg/ml) and 30 µl 10 per cent SDS were added and mixed for lysing of bacterial cells for 1 h at 37 °C. 100µl of 5M sodium chloride (NaCl) was applied to the lysic solution, followed by incubation at 65°C for 2 min. This was accompanied by addition of 80µl (ctyltrimethylammonium bromide) CTAB/NaCl (sodium chloride) and further incubation at 65°C for 10 min. This mixture was treated with phenol/chloroform/isoamyl alcohol (25:24:1) followed by centrifugation at 12,000 rpm for 5 minutes. The move was repeated three times. The DNA was precipitated with 95% ethanol at room temperature by inverting the tubes 4 to 6 times. The DNA was allowed to stand at room temperature for 2 min and then to be centrifuged at 12,000 rpm for 5 min. The supernatant was gently discarded and the tubes were inverted on a paper towel to allow the fluid to drain away. The genomic DNA pellet was rinsed with one ml of 70% ethanol. The supernatant was discarded and the DNA pellet was allowed to dry in the air for 10 minutes and stared at-20°C for the night. Genomic DNA washed in 70% ethanol and dissolved in 10 min and stared at -20°C for overnight. Genomic DNA was washed in 70% ethanol and dissolved in 100µl TE buffer for further use [19].

1. Agarose gel electrophoresis of DNA
2. The agarose gel was prepared for analysis of the isolated DNA. One gram of agarose was dissolved in a 100 ml (1X) TAE buffer. The mixture was heated in a microwave for 2min while shaking at 30 sec intervals to release some gas. A comb with well-spaced teeth was mounted on agarose electrophoresis plate to make wells. The agarose solution was allowed to cool slightly and stained with 5µl of ethidium bromide before it was poured onto the plate. The stained agarose gel was allowed to polymerize in the plate for one hour. The comb was carefully removed and the plate was placed in the chamber of electrophoresis. The TAE buffer was applied before the gel was fully submerged in the buffer. Approximately 4µl of DNA was combined with 5µl (6X) of blue bromophenol and loaded into the wells with the aid of a pipette. The chamber was connected to the power supply and the gel was run for three hours at 92 volts.
3. Analysis of bacterial genomic DNA
4. The agarose gel stained with ethidium bromide has been visualized by a UV-trans illuminator.
5. 16S rDNA amplification by polymerase chain reaction (PCR) and study of PCR products
6. 16S rDNA was amplified with bacterial universal primers unique to eubacterial 16S rDNA genes, Forward primer: 5'-TCGGTTTGATCGGCTCAG-3' and Reverse primer: 5'-ATCGYTACCTTGTTACGACTT-3' The PCR reaction mixture comprises 0.5µl of each primer, 10µl of 10X Taq DNA polymerase assay buffer, 4µl (2.5mM) of each dNTP (deoxynucleotide), 1µl (3U/µl) of Taq DNA polymerase enzyme, 1µl of DNA and 71µl of water for final volume of 100µl.
7. Terms of PCR Amplification
8. The initial denaturation was performed at 94oC for 5min followed by 35 denaturation cycles at 94oC for 30 seconds. Primer annealing was performed at 55 °C for 30 seconds and the extension was performed at 72 °C for 1 min. The final extension took place at 72 °C for 5 min. Electrophoresis and visual analysis of the PCR product obtained by UV transilluminator.
9. Nucleotide sequencing and alignment

The sequences obtained from 16S rDNA sequences were compared to the non-duty nucleotide database at the National Center for Biotechnology Information (NCBI) using their world wide website and BLAST (Basic Local Alignment Search Tool) algorithm and multiple sequence alignment by cluster W. A phylogenetic tree was built using version 3.695 of PHYLIP. Evolutionary distance matrices for the neighbor-joint/UPGMA approach have been computed using the DNADIST algorithm software. This program reads nucleotide sequences and writes a remote matrix output file[20].

Results and Discussion:-

Colony characteristics:

Of the 10 samples of each milk waste soil sample, six of the milk waste samples were selected for studies based on the shape, quality and color of the samples. Observations on the colony characteristics of the isolates were presented in Table 1. Colonies of isolates is circular to irregular in milk samples[21]. Colonies were usually yellow to white. The shape ranged from regular to irregular, with entire to undulating margins. Bacterial isolates have been stained to observe their morphological characteristics and the findings are shown in Table 1. In milk waste samples, both are gram-positive with cocci and bacillus. The structure of most of the cells was in pairs and chains (Table 1).

Table 1:- Colony characterization and microscopic examination of Isolated Bacteria.

Sr. No.	Sample collection sites	Isolate No.	Colony colour	Shape	Pigmentation	Colony size and texture	Gram's staining
1	Dairy waste	HW1	White	Circular	yellow	Smooth	Gram +bacilli
		HW2	yellow	Irregular	white	smooth	Gram + Cocci
		HW3	white	Circular	Yellow	smooth	Gram + bacilli
		HW4	Yellowish White	Irregular	white	Smooth	Gram + cocci
		HW5	yellow	Irregular	White	smooth	Gram + bacilli
		HW6	White	Irregular	yellow	smooth	Gram + Cocci

Isolation of Indigenous Bacterial Species:

Phenanthrene tolerant bacteria were isolated after the dairy waste soil samples was serially diluted and transferred onto M9 minimal agar medium amended with 10mg/l of phenanthrene. A concentration of 10 mg/l was chosen based on the prevailing concentration [22-24]. Four Phenanthrene tolerant bacteria from dairy waste (HW2, HW1, HW3, HW5) were isolated. The enumeration of phenanthrene bacteria was done in M9 minimal agar medium amended 10mg/l of phenenanthrene by serial dilution and spread plate technique.

Tolerance Concentration Study:

The resistance to phenanthrene by the isolate was tested with concentrations of 10mg/l of phenanthrene. The isolates showed tolerance was tabulated in table2.

Table 2:- Phenanthrene tolerance studies of dairy waste isolates.

Isolate	Concentration of Phenanthrene	Tolerance to phenanthrene
HW1	10-1000mg/l	450mg/l
HW2	10-1000mg/l	450mg/l
HW3	10-1000mg/l	420mg/l
HW5	10-1000mg/l	420mg/l

Morphological Characterization:

Gram staining was performed for all the isolates selected based on tolerance and observed under light microscope at 1000X magnification (Fig. 2).

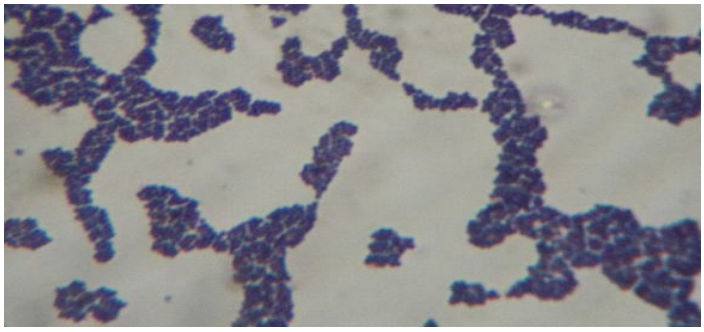

Fig. 1:- Morphological characterization of the isolates.

Biochemical Characterization:
The biochemical tests such as catalase, oxidase, IMViC, phenylalanine deamination, urease, H2S production and nitrate reduction were performed for the phenanthrene tolerant indigenous mine water isolates [25]. The results are listed in the table 3.

Table 3:- Biochemical characterization of the bacterial isolates.

Sr. No.	Isolate	Name of Biochemical Tests										Bacterial species Identified
		Indo	MR	VP	Citra	Urea	Nitra	Gluc	Fruc	Man	Lact os	
1	HW3HW5	-	-	+	+	_	-	+	+	+	Variable	Bacilus cereus
2	HW2 HW1	-	+	-	-	_	D	A/G	A/G	A/G	A/G	Micrococcus spp.

+ - (positive), - (negative), D- doubtful, A/G – Acid **&** Gas

Maximum Tolerance Concentration (Mtc) Study:
The resistance to Phenanthrene by the isolate was tested with various concentrations of Phenanthrene from 0 to 10mM. The Micrococcus luteus showed a MTC value of 28Cfu/ml at 8mM.

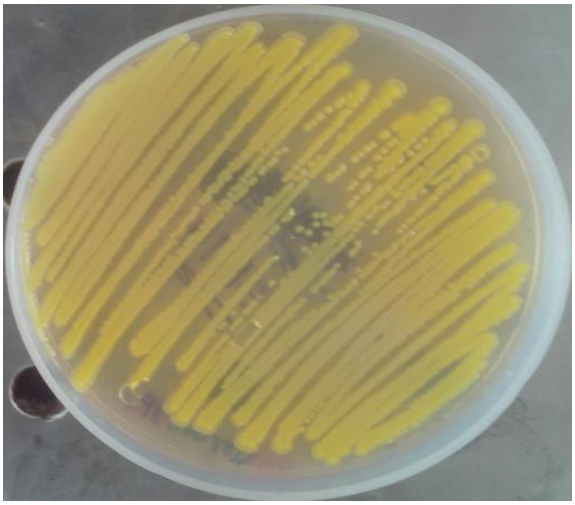

Fig 2:- Pure culture of Micrococcus.

Table 4:- The mean value of Cfu/ml for Micrococcus spp Inoculated in nutrient agar medium at different concentration of heavy metal after 48 hrs.

Micrococcus spp						
Name of heavy metal	Conc. Of heavy metal (mM)	1st experiment	2nd experiment	3rd experiment	Mean	Standard deviation
Phenanthrene	0	90	90	90	90	0
	2	72	74	72	73	0.57735
	4	55	53	52	53	0.57735
	6	47	47	47	47	0.57735
	8	28	28	28	28	0.57735
	10	0	0	0	0	0

Isolation of Genomic DNA:
The Genomic DNA was isolated and can be confirmed by the 1% running agarose gel electrophoresis and visualized the strong bands under UV Tran's illuminator (Fig 3).

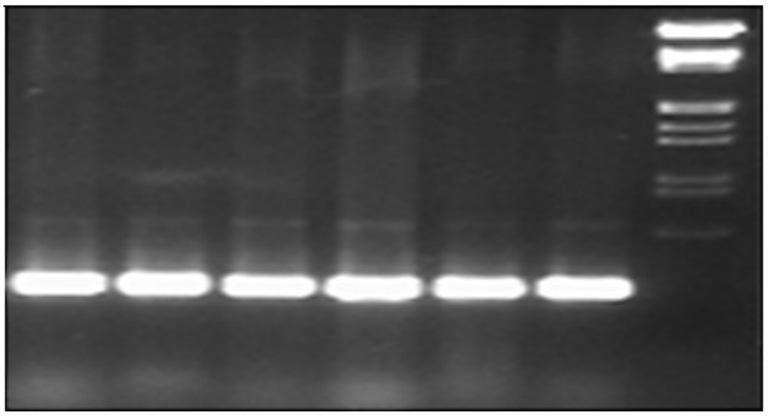

Fig 3:- DNA isolation of Micrococcus luteus.

PCR amplification:
The amplified DNA was isolated and can be confirmed by the 1% running agarose gel electrophoresis and visualized the bands under UV Tran's illuminator (Fig 4).

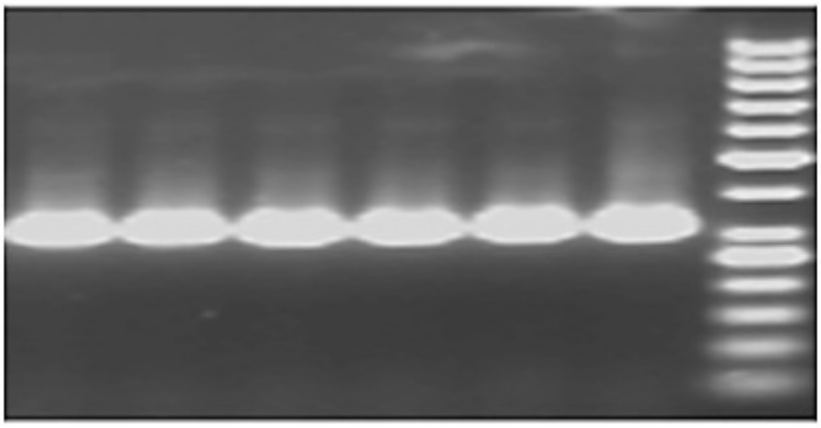

Fig 4:- PCR amplification of Micrococcus luteus.

16S rRNA nucleotide amplification sequence and phylogenetic relationship:
Potential isolate showing interesting recognition outcomes. On the basis of the 16S rRNA gene nucleotide homology and phylogenetic analysis, the sequences were identified and submitted to the NCBI [26-28].

Phylogenetic analysis:
In general, the sequencing of 16 S rRNA genes was used as an important identification tool for bacterial classification. Reasons include its existence in almost all bacteria; its function has not changed over time and the 16 S rRNA gene is broad enough to provide a gene and species recognition for isolates. DNA samples of all bacterial isolates were run on the agarose gel and the bands were visualized under the Gel doc[29-32]. Sequencing of the 16 S rRNA gene has been completed. On the basis of the 16 sequences of S rRNA, phylogenetic dendrograms were constructed to classify the genetic relationship between bacterial isolates [33-36]. Identification of the isolates was shown in Table 4 and their phylogenetic dendrograms were shown in the table 4 (Fig 5).

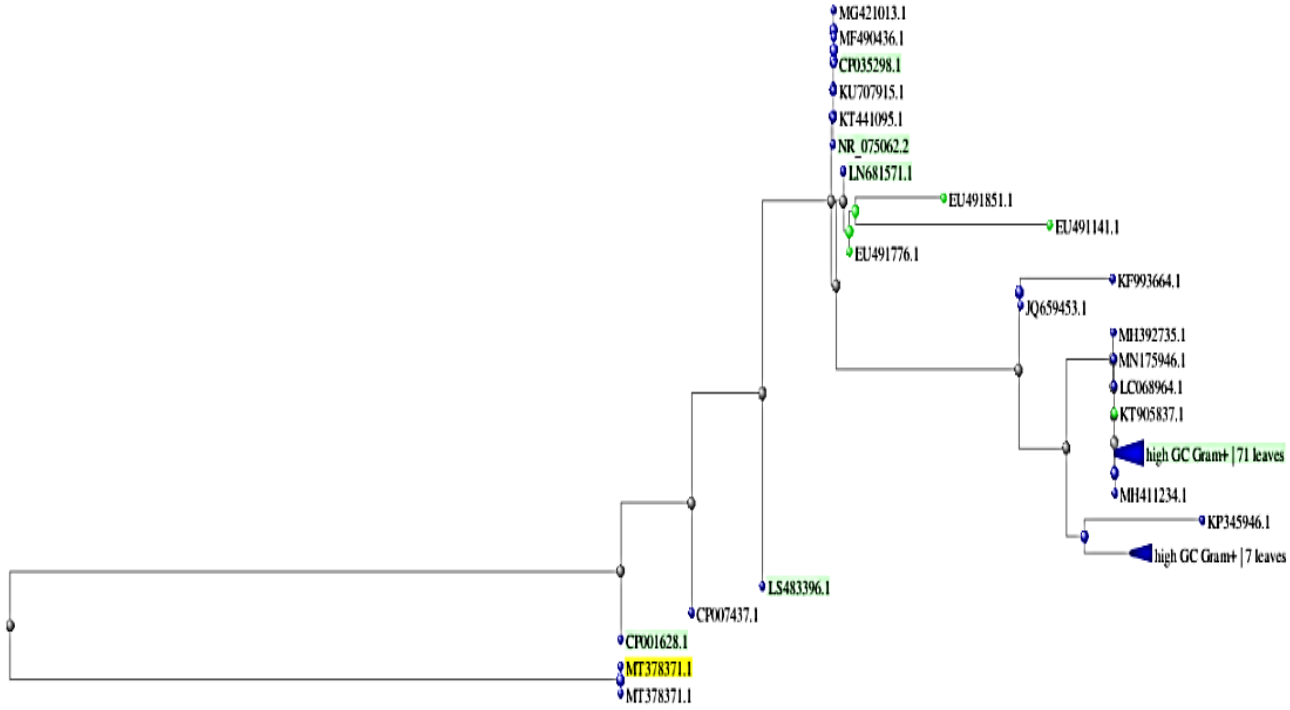

Fig 5:- Phylogenetic tree showing close homologs to Micrococcus luteus.

The 16S-rRNA sequences of the strains, Micrococcus luteus ANUES01 were submitted to GenBank and the accession numbers (GenBank MT378371) were obtained. The phylogenetic trees of all three isolates were obtained.

Conclusion:-

In this study, Phenanthrene degrading bacterium was isolated from dairy samples and characterized by morphological, biochemical and molecular methods. In the morphological tests it was identified as Gram-positive, nonmotile, coccus, tetrad-arranging, pigmented, bacterium. Biochemical tests showed MR positive and Catalase, VP, indole negative results. The 16s rRNA sequencing of the bacterium confirmed that it was Micrococcus luteus and tolerance of this bacteria to phenanthrene was upto a MTC value of 28Cfu/ml at 8mM. The novel bacterial strain sequence was deposited in NCBI with the Accession number MT378371 in the name of Micrococcus luteus ANUES01.

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