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AN APPROACH FOR THE BIODEGRADATION OF POLYCYCLIC AROMATIC HYDROCARBON

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ABSTRACT

Environmental pollution not only alters the environment but also changes the growth rate of various flora and fauna. Due to the irresponsible disposal of waste materials, the environment is going to be more and more polluted. Discharge of hydrocarbons in the water bodies is contaminating the water sources. These hydrocarbons are affecting the living organism. The solution to this problem has been found too expensive with little effects. To overcome this problem, some biological methods are introduced, in biological method; microbial degradation of hydrocarbons is the most promising method. The proposed study aimed to isolate and identify hydrocarbon-degrading bacterial species from the Mathura refinery, Mathura U.P. India. These species were isolated from petroleum-contaminated refinery water and grew on nutrient agar media, identified according to their distinct morphological, and biochemical characteristics, with16s rRNA sequencing. Results of this study confirmed the presence of various bacterial isolates such as *Brevibacillus nitrificans*, *Algoriphagus shivajiensis*, *Bacillus marisflavi*, *Acinetobacter junii*, *Pseudomonas pseudoalcaligenes*, and *Bacillus pumilus* from the collected samples based on the Bushnell Haas method and separation funnel method. Further, identified bacteria were tested for the maximum hydrocarbon degradation capacity in liquid culture, and results of the Gas Chromatography-Mass Spectrometry (GC-MS) suggested that only two bacterial species viz., *P. pseudoalcaligenes* and *B. pumilus* having the maximum hydrocarbon degradation capacity.

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1 Introduction

Mathura is located in Uttar Pradesh, the northern part of India at the bank of the Yamuna River. Heavy industrial development all over the world led to various unwarranted interruptions to the surrounding environment (Singh et al., 2020). This practice not only depletes the natural resources but also leads to drastic and unpredictable climatic changes. The harmful wastes that are released from industrial products remain a major cause of soil, air, and water pollution. Because of over-development and undesirable operations, many toxic pollutants discharged in the water, soil, and air cause various serious health problems but apart from these process, one of the major issues of water pollution in Mathura is due to the Mathura refinery (Yang et al., 2018) which discharges effluents in two ways i.e. one on the surface and another, in the atmosphere. Surface effluent like wastewater contains pollutants such as oil, sulphides, and phenol compounds (Hanafy et al., 2016; Ibrahim , 2016; Khan et al., 2020). Effluent treatment-plant reduces the pollution but cannot degrade the hydrocarbons completely and releases them into the Yamuna River. The effluents discharged in the atmosphere are petroleum vapours, fuel gases, and catalyst particles (Damian, 2013; Elshafie et al., 2015). Crude oil and its derivatives contain many aromatic and aliphatic hydrocarbons. To resolve this problem many microorganisms could be employed, these microorganism containing metabolic enzymes which have the capacity to degrade the hydrocarbons (Bento et al., 2005; Ting et al., 2011; Kumar et al., 2019). These kinds of bacteria are known as hydrocarbon clastic bacteria. The technique of bioremediation is an approving method for cleans up petroleum pollutants from water bodies (Adam, 2016; Mishra et al., 2019).

Previously reported bacterial species which are majorly responsible for hydrocarbon degradation are *Pseudomonas, Bacillus* (Ruchi et al., 2008; Wu et al., 2017), *Acinetobacter, Proteobacteria, Firmicutes, Alcaligenes* (Shahi et al., 2016), *Chryseobacterium*, *Micrococcus (*Wolińska et al., 2016, *Nitratireductor, Penicillium* and *Aspergillus* (Vanishree et al., 2014; Ameen et al., 2015).

For the identification of these bacterial strains, 16s rRNA sequencing has been employed to identify the bacterial species having hydrocarbon-degrading potential present in the refinery effluent. While the hydrocarbon-degrading potential of the isolated bacterial species have been evaluated by GC MS. Moreover, *a* phylogenetic neighbour-joining tree was also determined to explain the evolutionary interactions among various biological species of all the identified bacteria.

2 Materials and methods

2.1 Sample collection and strain isolation

The effluent sample was collected from the Mathura refinery and transferred to the laboratory for bacterial growth. Initial isolation was performed using serial dilution. Primarily incubated on the nutrient agar media overnight then the single morphological colony of bacteria transferred on separate nutrient agar media (NAM) plate and incubated overnight at 37° C and finally aseptically transferred to the NAM. For the identification of bacteria, morphological analysis was carried out as suggested by Mishra et al. (2019) and Sagheer et al. (2017). Biochemical tests were performed with the help of VITEK2; this is an automated system for microbial growth analysis which provides accurate data for microbial growth. A total of 64 tests were performed in 64 wells plates to determine the various metabolic activities in the presence of the inhibitory substance. With the help of four reagents cards identified the different classes of the organism. By using the DensiChek turbidity meter turbidity was measured (Mishra et al., 2019). For estimation of the hydrocarbon degradation capacity, all six isolated bacterial strains cultured on Bushnell Haas media determines the utilization of hydrocarbons and another one is by separation funnel method (Bushnell & Haas, 1941; Sagheer et al., 2017). For this, fix the number of bacteria, crude oil, and the nutrient broth was mixed and incubated for 35 to 45 minutes (Mishra et al., 2019).

2.2 Gene-ontological Analysis

For Gene-ontological analysis, we used interPro scan tool and found information about protein family and biological process, molecular function, and cellular component about 16S rRNA sequence. Further, for the Annotation of the bacterial genome BaSys tool was used. This tool supports automated, in-depth annotation of bacterial genomic sequences. It accepts raw nucleotide sequence data and an optional list of gene identification information (Glimmer) and provides extensive textual annotation and hyperlinked image output. BASys uses >30 programs to determine 60 annotation subfields for each gene, including gene/protein name, GO function, possible paralogues, and orthologues, molecular weight, isoelectric point, operon structure, subcellular localization, signal peptides, transmembrane regions, secondary structure, 3D structure, reactions, and pathways.

2.3 Characterization and identification of bacteria- 16s RNA sequencing

After the DNA extraction PCR analysis is performed and purifies the PCR product by removing the unincorporated PCR primer and dNTPs by using a Montage PCR clean-up kit. The PCR product sequenced using the 27 forward and 1492 reverse primer for

sequencing reaction ABI PRISM BigDyeTM Terminal cycle sequencing Kit with AmpliTag DNA polymerase (FS enzymes) used. For sequencing protocol, single-pass sequencing was performed on each template, and the fluorescent-labeled fragment was purified. Sample suspended in distilled water are subjected to electrophoresis in an ABI3730xl. For the alignment search, the BLAST tool for the 16s RNA sequence by using NCBI blast similarity search was used. Cure the aligned sequence using the program G blocks 0.9lb.G blocks removed the noise of alignments. For phylogeny analysis, PhyML 3.0 aLRT was used and HKY85 was used as the submission model. For Tree rendering Tree Dyn 198.3 was used.

2.4 Analysis of hydrocarbon degradation GC MS

Crude oil in Bushnell Haas media with the isolates *Pseudomonas pseudoalcaligenes* and *Bacillus pumilus* were analyzed for hydrocarbon degradation ability. For this, a fractional (100 µl) of bacterial cells were moved to a 250 ml volumetric flask containing 100 ml sterile Bushnell Haas media supplemented with 1% crude oil. An Un-inoculated Bushnell Haas media flask was kept as a control (Hassanshahian et al., 2014). During the degradation process (cultivating for 0, 5, and 10), the crude oil broths of the *P. pseudoalcaligenes* and *B. pumilus* strains were analyzed using GC-MS (Thermo scientific TRACE 1300GC, TSQ DUO MS; the chromatographic column: 5MS - 30m×0.25 ID). For the analysis of GC MS first, the mixture was centrifuged at 8000*g for 20 minutes at 4^{o} C and the hexane supernatant (3mL) was extracted from the media. This liquid was filtered with the help of 0.45 µm. The operation temperature of the detector and injector were 280^{o}C and 250^{o} C respectively. The oven temperature program was as follows: 60 °C held for 0.5 min and raised to 280 °C for 10 min, with an isothermal period of 32 min at the end. The ionization energy was 70 eV and the inlet velocity was 1.5 mL min^{-1}.

3 Results and Discussion

3.1 Isolation and screening of hydrocarbon-degrading bacteria

The enrichment culture method was used for the isolation as well as for screening of the hydrocarbon-degrading bacteria. Six bacterial strains were isolated from the effluent sample and identified based on morphological characterization, biochemical analysis, and 16s RNA sequencing and these were identified as *Brevibacillus nitrificans*, *Algoriphagus shivajiensis*, *Bacillus marisflavi*, *Acinetobacter junii*, *Pseudomonas pseudoalcaligenes*, and *B. pumilus*.

Further, confirmatory tests were performed for the estimation of hydrocarbon degradation. Among the tested bacterial strains, maximum oil degradation capacity was reported in *P. pseudoalcaligenes* and *B. nitrificans*. Morphological characteristics of the isolated bacteria are a yellow, pink, white, milky white, green, and cloudy white colony. Comparison of sequence demonstrated that the isolated bacteria are *B. nitrificans*, *A. shivajiensis*, *B. marisflavi*, *A. junii*, *P. pseudoalcaligenes*, and *B. pumilus*.

3.2 16s RNA sequencing

16s RNA sequencing is important for the study of phylogeny and taxonomy analysis. Results of 16s RNA analysis revealed that (i) all the bacteria present in the effluent sample is responsible for oil degradation but after the Bushnell Haas method and separation funnel method identified that two bacteria having maximum hydrogen degradation capacity (ii) the function of 16s RNA gene over time will not change (iii) 16s RNA gene sequence contains sufficient information for observation (Table 1 & 2).

3.3 Gene ontological analysis

Gene ontological analysis was carried out to identify length, cell location, gene position, GC Content complexity, molecular weight, the metabolic function of the isolated bacteria (Table 3).

3.4 GC-MS analysis

For hydrocarbon degradation confirmation GC MS analysis was performed. It was performed on the crude oil sample containing various hydrocarbons and advance it performed on oil extracts from inoculum media. The assessment of hydrocarbon degradation was carried out by *B. pumilus*, *P. pseudoalcaligenes* during day1, day 5, and day 10. The result showed that the formation of new compounds after the 5^{th}day was reported because of the biodegradation of hydrocarbon degradation (Figure 1 & 2).

On day one GC MS results identified 74 compounds in the crude oil and almost all the compounds are toxic for the human health and environment and it creates aspiration pneumonitis problems. After the microbial treatment (treatment with *P. pseudoalcaligenes* and *B. pumilus*) for the 5^{th} and 10^{th} days suggested that out of 74 compounds, 52 show degradation in different ranges, and 10 compounds show complete degradation because of *P. pseudoalcaligenes* (Figure 1). Compounds that are showing the complete degradation after the treatment of *P. pseudoalcaligenes* are Naphthalene, 1, 3-dimethyl-, 5-Ethyldecane, Naphthalene,1, 6, 7-trimethyl-, 1-Ethyl-4-methoxy-9H-pyrido [3, 4-b] indole, on the Retention Time of respectively 10.75, 10.87, 12.85, 17.90. Other than these hydrocarbons some hydrocarbons are produced by the microorganism like 10-Methylnonadecane this type of hydrocarbon is not very toxic but they are also increasing their area of toxicity (Figure 4).

Table 2 Phylogenetic tree neighbor-joining based on 16s rDNA gene sequence showing the relationship between bacterial and other relatives within the genus

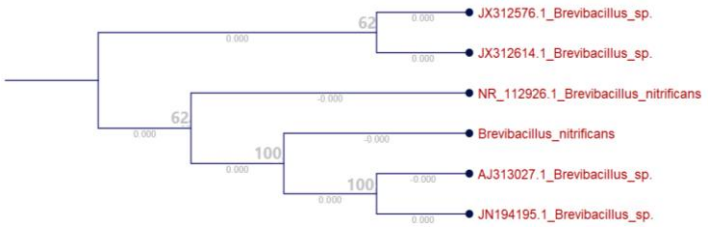
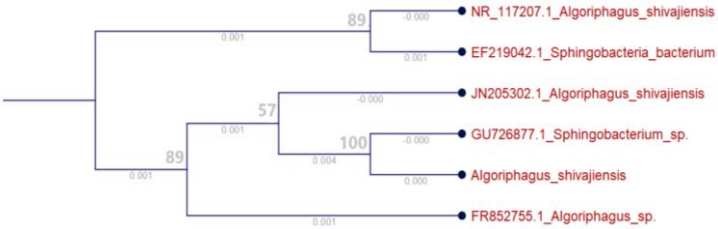
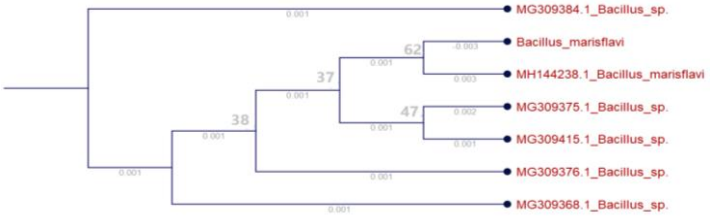
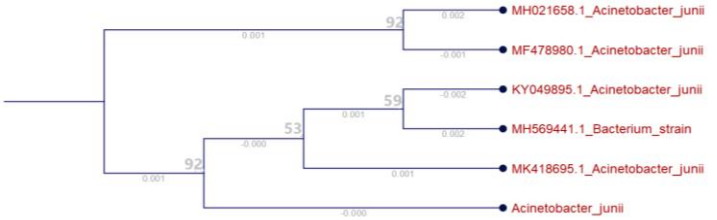
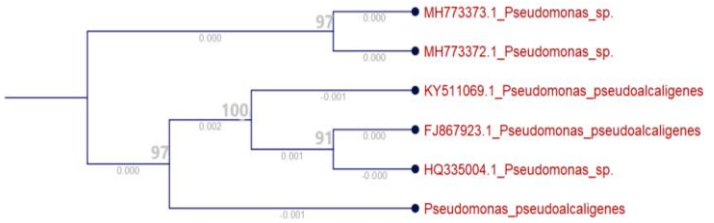
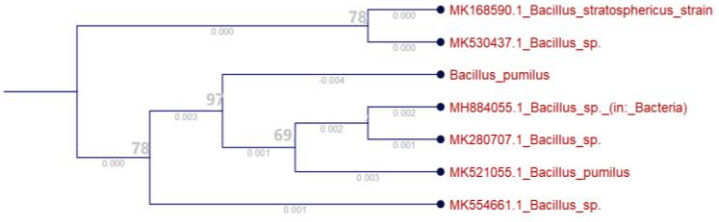
S. No.	Bacteria	Phylogenetic tree
1.	*Brevicillius nitrificans*	
2.	*Algoriphagus shivajiensis*	
3.	*Bacillus marisflavi*	
4.	*Acinetobacter junii*	
5.	*Pseudomoas pseudoalcaligenes*	
6.	*Bacillus pumilus*	

Table 1 Showing the accession numbers and 16s RNA sequencing generated data description of all the six bacteria

S.No.	Accession Number	Query Id	Description	Molecule Type	Query Length	Program
1.	MN581665	lcl|Query_39621	Nucleotide collection (nt)	nucleic acid	1415	BLASTN 2.8.1+
2.	MN581666	lcl|Query_176021	Nucleotide collection (nt)	nucleic acid	1393	BLASTN 2.8.1+
3.	MN581667	lcl|Query_223641	Nucleotide collection (nt)	nucleic acid	1421	BLASTN 2.8.1+
4.	MN581668	lcl|Query_11583	Nucleotide collection (nt)	nucleic acid	1395	BLASTN 2.8.1+
5.	MN581669	lcl|Query_166675	Nucleotide collection (nt)	nucleic acid	1405	BLASTN 2.8.1+
6.	MN581670	lcl|Query_233099	Nucleotide collection (nt)	nucleic acid	1424	BLASTN 2.8.1+

Table 3 Gene ontological analysis of isolated bacteria using interpro scan tool and found information about protein family and biological process, molecular function and cellular component about 16S r RNA sequences

S. No.	Bacteria	Accession Number	Protein Name	Length	Cell Location	Gene Position	Complexity (Gc-Content)	Cys/Met_Content	Molecular Weight	Metabolic Function
1.	*B. nitrificans*	NR_112926	Low-quality protein conserved hypothetical protein	1491bp	Cytoplasm	476-219 (Counter clockwise)	55.43	2.4 % Cys (Translated Protein) 1.2 % Met (Translated Protein) 3.5 % Cys+Met (Translated Protein) 2.4 % Cys (Mature Protein) 0.0 % Met (Mature Protein) 2.4 % Cys+Met (Mature Protein)	Translated: 9199, Mature: 9068	Secondary metabolite biosynthesis, transport, and Catabolism
2.	*A. shivajiensis*	JN205302.1	Hypothetical protein	---------	Cytoplasm	293-186 (Counter clockwise)	52.78	0.0 % Cys (Translated) 5.7 % Met (Translated) 5.7 % Cys+Met (Translated) 0.0 % Cys (Mature) 2.9 % Met (Mature) 2.9 % Cys+Met (Mature)	Translated: 3942, Mature: 3810	Translation, ribosomal structure and biogenesis
3.	*B. marisflavi*	----------	Low quality conserved hypothetical protein	---------	Cytoplasm	508-251 (Counter-clockwise)	56.20	2.4% Cys (Translated Protein) 1.2 % Met (Translated Protein) 3.5 % Cys+Met (Translated Protein) 2.4 % Cys (Mature Protein) 0.0 % Met (Mature Protein) 2.4 % Cys+Met (Mature Protein)	Translated: 9474, Mature: 9343	Transport and catabolism
4.	*A. junii*	----------	Conserved hypothetical protein	----------	Cytoplasm	891-685 (Counter-clockwise)	53.04	2.9 % Cys (Translated Protein) 1.5 % Met (Translated Protein) 4.4 % Cys+Met (Translated Protein) 2.9 % Cys (Mature Protein) 1.5 % Met (Mature Protein) 4.4 % Cys+Met (Mature Protein)	Translated: 7639, Mature: 7639	Transport and catabolism
5.	*Pseudomoas*	----------	Conserved hypothetical protein	1426 bp	Cytoplasm	497-198 (Counter clockwise)	52.17	1.5 % Cys (Translated) 2.9 % Met (Translated) 4.4 % Cys+Met (Translated) 0.0 % Cys (Mature) 2.9 % Met (Mature) 2.9 % Cys+Met (Mature)	Translated: 7734, Mature: 4032	Function unknown
6.	*B. pumilus*	----------	Low quality conserved hypothetical protein	1434 bp	Cytoplasm	789-583 (Counter-clockwise)	56.33	3.0 % Cys (Translated) 1.0 % Met (Translated) 4.0 % Cys+Met (Translated) 3.1 % Cys (Mature) 0.0 % Met (Mature) 3.1 % Cys+Met (Mature)	Translated: 11232, Mature: 11101	Secondary metabolite biosynthesi, catabolism

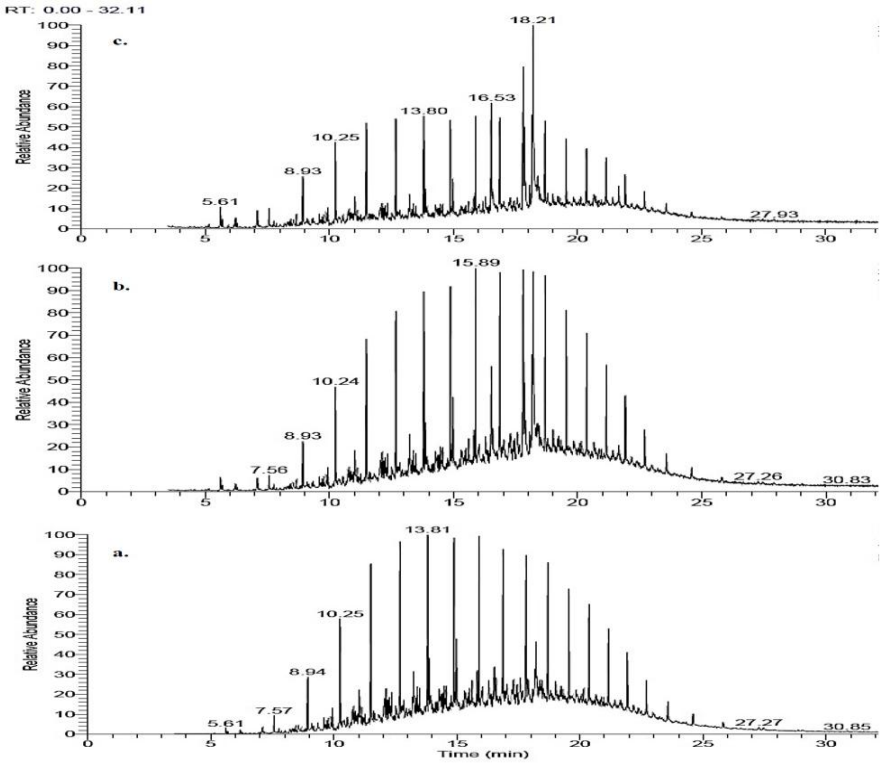


Figure 2 *Pseudomonas pseudoalcaligenes* GC MS analysis 1st day (a), 5th day (b), and 10th day (c)

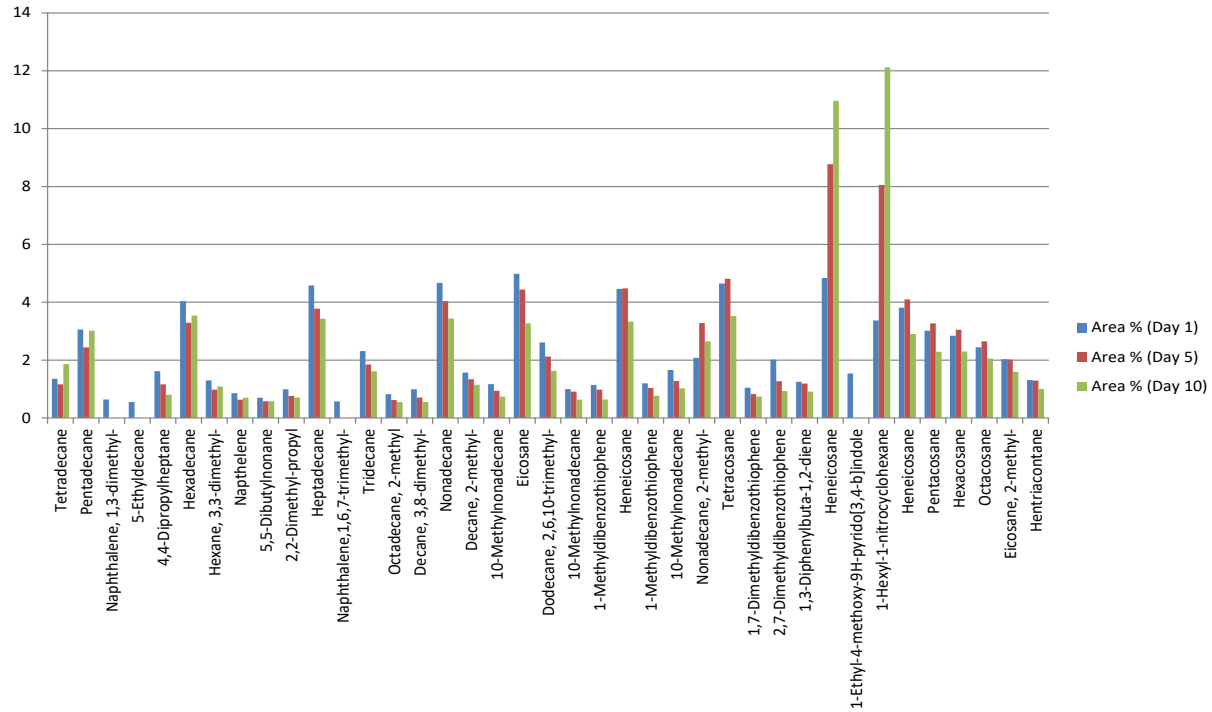


Figure 4 *Pseudomonas pseudoalcaligenes* GC MS analysis day 1(a), 5th day (b), and 10th day (c) in reference to degradation area and retention time.

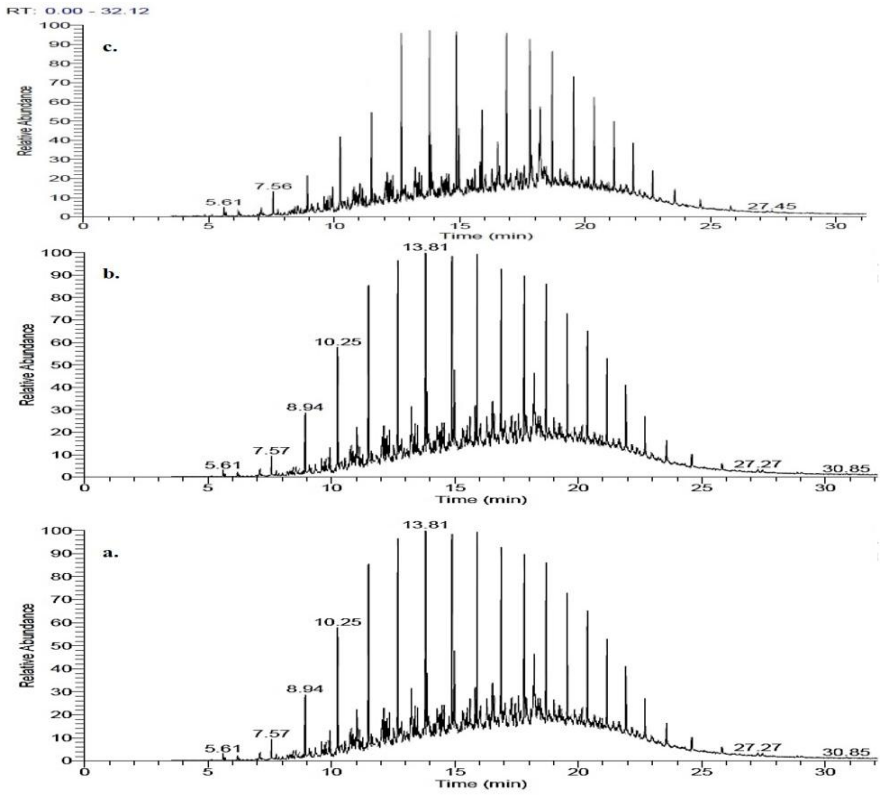


Figure 1 *Bacillus pumilus* GC MS analysis at 1 day (a), 5th day (b) and 10th day (c)

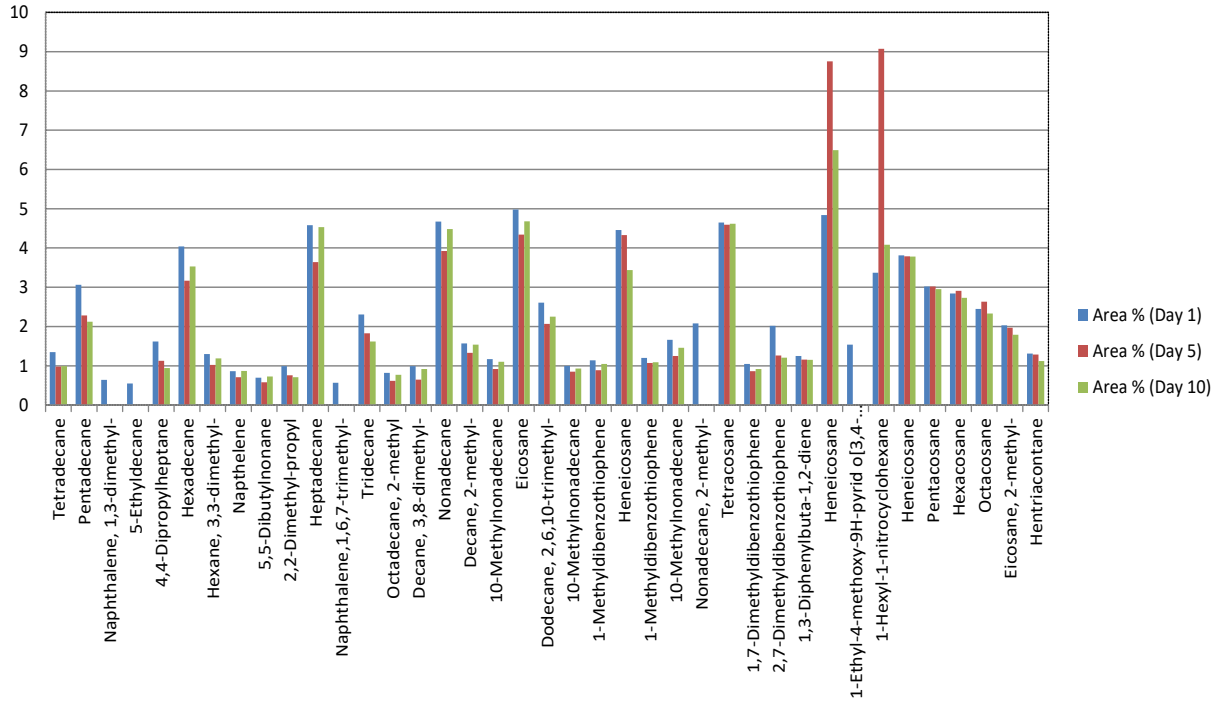


Figure 3 *Bacillus pumilus* GC MS analysis Day 1(a), 5th Day (b), and 10th Day (c) in reference to degradation area and retention time.

Some hydrocarbon is degrading from high range to low range like alkane or paraffin compounds like eicosane, Eicosane, 2-methyl-,Dodecane, 2,6,10-, Tridecane, Octadecane, Decane, Nonadecane, Hexadecane, Hexane, 3,3-dimethyl-, Naphthalene, are also present in crude oil and microbial treatment reducing the rang of toxicity. Few compounds like Heneicosane, Pentacosane, Hexacosane, Octacosane increasing their range from low to high after microbial treatment but they don't have any harmful effects on human health.

Compounds that are showing complete degradation after the treatment of *B. pumilus* are almost similar to the compounds that are responsible in the case of *P. pseudoalcaligenes*. Approximately 32 compounds are showing the degradation of hydrocarbons after the treatment of *B. pumilus.* Heneicosane, 1-Hexyl-1-nitrocyclohexane, Hexacosane, Octacosane, 5, 5-Dibutylnonane increasing their range from low to high after microbial treatment no data is available which showing toxicity of these compounds on human health (Figure 3).

4 Discussion

The chemical and physical methods such as bioaccumulation, chemical oxidation, photo-oxidation as well as volatilization are hardly successful in cleaning up and rapid removal of polycyclic aromatic hydrocarbons, also these methodologies are not cost effective and safe than the microbial bioremediation. Moreover, bacteria were considered as important hydrocarbon degrading-agents

in an environment that are ubiquitous and free living organisms. Moreover, petroleum hydrocarbons are considered as predominant resources of energy utilized by industries as well as in our day to day life. Meanwhile, petroleum products are considered to be the main pollutants of the environment. Because of the complex compositions of petroleum, it can cause various toxic impacts. Also, it can cause sub-lethal chronic toxicity, lethal toxicity, or even both based on the organism exposed, dosage, and exposure (Ruberto et al., 2003; Rajaei et al., 2013).

Six bacterial strain were isolated from Mathura refinery outlet effluent by nutrient agar medium, and all the six bacteria are capable of utilizing the hydrocarbon, out of six two bacterial species having the maximum capacity of utilizing the hydrocarbon, confirmed by Bushnell media and separation funnel method (Ivanova et al., 2012; Sagheer el al., 2017).

Results interpreted by biochemical test for all the six bacteria and 16 sRNA sequencing confirmed that these six bacteria are *Brevibacillus nitrificans*, *Algoriphagus shivajiensis*, *Bacillus marisflavi*, *Acinetobacter junii*, *Pseudomonas pseudoalcaligenes*, and *Bacillus pumilus*. Among these, two bacteria that having the maximum ability of PAH degradation were identified as *P. pseudoalcaligenes* and *B. pumilus* (Patel, 2001; Janda & Abbott, 2007). GC MS analysis was done on these two bacteria for three different days intervals viz., day1 day 5, and day 10 (Douglas et al., 1992), revealed the presence of a total of 74 compounds in crude oil and after the treatment with *P. pseudoalcaligenes* and *B. pumilus* 52 showed degradation in different ranges, while 10 compounds showed complete degradation of toxic material (PAH) present in crude oil (Parthipan et al. 2018). The attached graph of 39 compounds showed the degradation of PAH by different days. After the GC MS analysis, it can be occluded that the *P. pseudoalcaligenes was* having the maximum hydrocarbon degradation capacity.

The study finally concluded that *P. pseudoalcaligenes* possess better ability in the degradation of PAH than the selected microorganisms. Microbial degradation remains a promising mechanism for the ecological recovery of PAH contaminated sediments and the removal of PAH chemical degradation. The main aim of this research is to provide recent knowledge in PAH degradation concerning the previous analysis. This process enables the optimization, monitoring, and overcoming of the prevailing limitations in the application of microbial flora in PAH degradation. Ultimately it can be suggested that *P. pseudoalcaligenes* is the batter biological source for bioremediation (Safari et al. 2019).

Conclusion

This study is focusing on the isolated bacteria from the effluent sample of Mathura refinery, and these bacteria are identified as *B. nitrificans*, *A. shivajiensis*, *B. marisflavi*, *A. junii*, *P. pseudoalcaligenes*, *B. pumilus*, and have a direct relationship between the crude oil degradation and isolated bacteria. All the isolated strain from Mathura refinery effluent has a high level of hydrocarbons degradation ability. After the Bushnell Haas media analysis and separation funnel method out of six, two strains were identified as a maximum crude oil-degrading strain. In this study of GC MS analysis of isolated 2 strains showed the degradation of aromatic hydrocarbon. The GC MS analysis showed the presence of hydrocarbon in the crude oil and these hydrocarbons are contaminating the environment. Thus, this study concludes that the successful bioremediation approach using microorganism is one of the best techniques for hydrocarbon degradation.

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Conflict of interest

The authors declare no conflict of interest

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