Indian Journal of Ecology (2022) 49(3): 748-751
DOI: https://doi.org/10.55362/IJE/2022/3589

Manuscript Number: 3589
NAAS Rating: 5.79

Evaluation of Diesel Degradation Potential of *Klebsiella pneumoniae* Strain VM18 Isolated from Petroleum Contaminated Soil of Mathura

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***Abstract*:** The present investigation was conducted to isolate, characterize and identify one of the best bio degrader bacterial isolate and to measure its biodegradation capacity. *Klebsiella pneumoniae* strain VM18 was the isolated bacteria which showed the degradation efficacy of 62.84% after 35th day of incubation in Bushnell Hass medium. GC-MS analysis reveals that, *Klebsiella pneumoniae* strain VM18 degraded C_{51}-C_{60} fraction from 2.54% to 1.49% and converted them into C_{31}-C_{50} after 21 days of incubation. This can be concluded that bacterial strains have potential to degrade the petroleum hydrocarbons present in contaminated soil environment.

***Keywords*:** *Klebsiella pneumoniae,* Bushnell Hass medium, Contaminated, Biodegradation, Hydrocarbon

The petroleum industry has played a significant role in global economy but as usual it also has some demerits on our ecosystem as well. The occurrence of pollution through refining, storage and distribution of petroleum products are a universal problem across the globe (Nogales et al 2011). As petroleum refineries are magnificently large industrial complexes which connected with large chemical processing and producing units via inland and outland incorporated pipelines, carrying various streams of fluids. These pipeline are underground so they passed through the agricultural land and sometimes crossing the water bodies. During transportation of oil from the source of production to various consuming sites, there is a huge risk of accidental spillage (Chang et al 2014). If accidental spillage occurs, it affects the food chain and food web of marine ecosystem drastically. Analysis of soil health is the most important step to maintain or improve the quality of the soil (Schoenholtz 2000). The effect of toxicity mainly depends on their concentration, composition and environmental factors (Ekhaise and Nkwelle 2011). According to the Germany, Federal Environment Agency in 2012, incomplete combustion of oil at a lower temperature and less oxygen supply is the main sources to produce PAHs. Generally, PAHs are considered to be potent atmospheric pollutants that consist of fused aromatic rings. According to behaviour they considered as a carcinogenic, mutagenic, and teratogenic compounds.There are some technologies used for restoring the soil fertility, which was rigorously contaminated with PAHs. Bioremediation is one of the natural approaches that based on microorganisms to remove the toxic pollutants from the environments. Microbial ranges are available to clean up contaminated sites occur due to the presence of petroleum hydrocarbons. Microorganisms have a tendency to convert these chemical compounds into energy, cell mass and biological waste products (Rahman et al. 2002). The present investigation was designed to isolate, identify the petroleum hydrocarbons degrading microbial population. Evaluation of biodegradation potential of each microbial isolates is the major objective of the investigation.

MATERIAL AND METHODS

Soil sample was collected from automobiles workshops sites around the Mathura. The samples were dug from trowel from 0 to 20 cm below the topsoil surface. In order to remove plants or other waste residues the soil get air dried and sieved through 200 mesh sieves. The soil samples then stored in sample storage bags and kept in refrigerator till further analysis.

Isolation and Screening of hydrocarbon utilizing bacterial strain: Enrichment culture technique was used to isolate petroleum hydrocarbon degrading microbes. Bushnell Haas Media was used for the isolation of pure culture and 1% (v/v) diesel was used as a carbon and energy sources (Bushnell and Haas 1941). For screening, 1 gm of contaminated soil samples was suspended and vortexes in 10ml of sterile distilled water. After it 1ml of solution was taken out and used as inoculums for isolation of oil degrading microorganisms. 100ml of BHM broth medium was transferred to each flask and sterilized. Flask was kept in rotary shaker at 150 rpm and 28ºC for 7 days. After one week of incubation, 10ml of sample from primary enrichment was transferred to a fresh BHM broth. Unless otherwise stated, after 2nd enrichment 1 ml of medium was plated after appropriate dilution on BHM agar plate and incubated at 28ºC. After 48-hour incubation, pure colonies were isolated using streak plate method. All isolated microorganisms were stored at 4ºC for further uses. The screening of biodegradation potential was performed using DCPIP method (Montagnolli et al 2015). The organisms were cultivated on Bushnell Hass Broth for 48 h and exposed to different concentrations of Diesel oil in test tubes having a constant amount (0.5 g L^{-1}) of DCPIP at pH 7. The experiments were

performed in sealed lid test tubes to sustain carbon dioxide saturation and block further aeration. The uninoculated tube serves as control for the study. Test tubes then incubated at 37°C at 180 rpm in digital shaking incubator. Continuous shaking ensured proper oxygen availability to maintain aerobic conditions. The reaction was observed visually till the end of incubation and also spectrophotometrically (600 nm) at an interval of 24 hrs using Elico SL-159 model UV visible spectrophotometer. Active bacterial culture able to reduce DCPIP results colour less cell solution which ultimately indicates 100 % cell biodegrading activity.

Identification of Bacterial strain by 16S rRNA nucleotide sequencing: Genomic DNA was extracted by Himedia Bacterial genomic DNA isolation kit. The quality of DNA was analysed by 1% agarose gel electrophoresis stained with ethidium bromide. Moreover, quantity of extracted DNA was measured in µg/µl by using ND-1000, USA, Nanodrop spectrophotometer at 260 nm and 280 nm. The purity of DNA was determined by calculating ratio of absorbance at 260-280nm. The purity of a particular DNA was assessed by considering the 260nm/280nm ratio comes near 1.8 and then DNA were diluted up to the concentration 50ng/µl. Afterwards, DNA was amplified through polymerase chain reaction (PCR), thermal cycler using the primers 16S rDNA (SENSE) 5'TAGGGAGGAAAGGTGTGAA3' (Tm: 54.5ºC) and 16S rDNA (ANTISENSE) 5'CTCTAGCTTGCCAGTCTT3' (Tm: 53.7ºC). PCR run at programme of 30 cycles and amplification was carried out as follows: 94ºC for 5 min, 94ºC for 30sec, 54ºC for 30 sec, 72ºC for 1:30 sec, 72ºC for 10 min and 4ºC incubation at the end of the final cycle. 1% of agarose gel electrophoresis was performed again with loaded DNA ladder (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500, 2000 and 3000 base pairs Bangalore Genei, India) for the analysis of yield of amplified product. The amplified DNA bands were then visualized in UV Trans illuminator and photograph was taken into gel documentation system (Alpha-Innotech, USA). The amplified fragment was then sequenced into Applied Bio systems 3500 genetic analyser, USA. The sequence was subjected to BLAST at NCBI (www.ncbi.nlm.nih.gov/blast). The estimation of sequence composition and phylogenetic tree construction was done by using MEGA software version 6.0.

Gas chromatography/Mass spectrometry analysis: Analysis of hydrocarbons present in diesel oil was carried out via GC/MS technique. 1 ml of extracted residual oil, after 7, 14, 21, 28, 35 days of incubation was used for the analysis of Gas Chromatography and Mass Spectrometry (GC-MS) (Shimadzu QP-2010 Plus with Thermal desorption system TD20).Volatile compounds present in a mixture are separated by Gas Chromatography. The separated compounds can be identified and quantified through Mass spectrometry. The gas chromatograph equipped with a split–split less injector (split ratios of 50:1) was used for the GC-MS analysis. The oven temperature was initially at 40°C and then programmed to 270°C at a rate of 8°C/min where it was held for 5 min. The temperatures of injector, transfer line and ionization source were all 250°C. The electron impact ionization was tuned at 70 eV and Helium was used as carrier gas with an average linear velocity of 1.0 mL/min.

RESULTS AND DISCUSSIONS

Isolation and characterization of *Klebsiella pneumoniae* strain VM18: The isolate grew well on Bushnell Hass medium with 1% (v/v) diesel oil. The pure culture of microbe was obtained after successful enrichments to the medium. The microbial isolates are characterized morphologically on the basis of their size, shape, opacity, and pigmentation. *Klebsiella pneumoniae* rhizoid in shape having smooth texture. The opacity of the microbes was opaque with flat elevation and white pigmentation. 50µl of DNA stocks were prepared for further molecular analysis which was then diluted after determining the DNA concentrations. Integrity checking of extracted genomic DNA was high intensities of bands compared with Lambda DNA marker. Presence of highly resolved, high molecular weight band indicates good quality of the DNA. Quantity of the DNA was determined by measuring the absorbance at 260nm using spectrophotometer. 50ng/µl was considered as enough concentration for DNA amplification. The concentration of DNA obtained was 288.7, which is then diluted to attain the desired concentration by DNase RNase free water. The absorbance of protein was checked at 280nm, so the A_{260}/A_{280} confirms the purity of the DNA. The ratio of absorbance at 260nm/280nm was found to be 1.67 signifies slight protein contamination which was purified later to acquire the desired value. Molecular sequencing of the conserved part of 16S rRNA gene reveals the most potent diesel oil degrading bacteria is *Klebsiella pneumoniae* strain VM18 which shows remarkable catabolic activity for petroleum hydrocarbons. Sequence similarities were found by aligning sequences through BLAST and identified the closest strains from NCBI database. The partial sequence of 16S rRNA gene of identified bacterial strain, has been deposited into Gene bank with the accession number MG928409 (Fig. 1).

Preliminary Screening and Biodegradation Potential of *Klebsiella pneumoniae*: The strain showed the decent decolourization activity. The ODs taken at 600nm from 0 to 216 hrs with the interval of 24 hrs was 1.661 to 1.437 (Fig. 2). Growth rate of *Klebsiella pneumoniae* strain VM18 was also determined by taken ODs from 7 to 35 days of incubation at 620 nm through turbidometric

>MG928409.1 *Klebsiella pneumoniae* strain VM18 16S ribosomal RNA gene, partial sequence
ATGCAGTCGAGCGGTAGCACAGAGAGCTTGCTCTCGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGG
GAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAATGTCGCAAGACCAAA
GTGGGGGACCTTCGGGCCTCATGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGC
TCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAG
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGT
GTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCCGGGAG

Fig. 1. Nucleotide sequence of *Klebsiella pneumoniae* strain VM18 submitted in NCBI with Accession No. MG928409

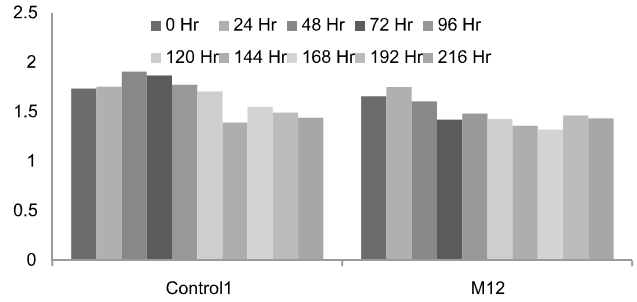

Fig. 2. Microbial growth at 620nm in DCPIP assay for *Klebsiella pneumoniae* strain VM18

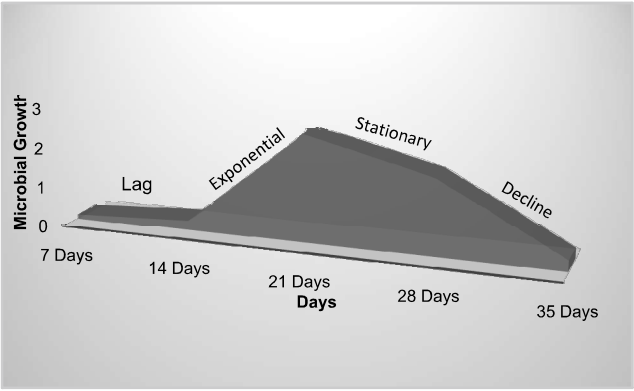

Fig. 3. Microbial growth curve of 14 potent Microbial isolates from 7 to 35 days of incubation at 620nm

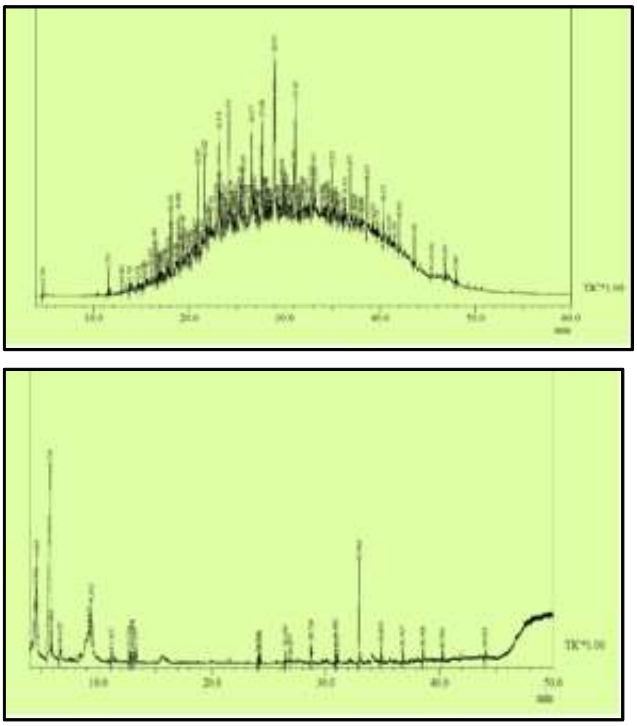

Fig. 4. GC-MS Chromatogram of Isolate *Klebsiella pneumoniae* after A) 7th day B) 35th day of incubation

method (Fig. 3). The degradation efficacy of *Klebsiella pneumoniae* strain VM18 is 62.84% after 35th day of incubation (Fig. 4). The major peaks of the isolate were observed of tetrapentacontane, tetratetracontane, tetracontane, pentriacontane at their retention times of 37.210, 34.160, 33.885 and 35.290 respectively. All these compounds present in diesel oil were degraded into butane, pentane, undecane, phenol, nonane, cyclobutanol and docosane.

The microbial strain was identified as *Klebsiella pneumoniae* strain VM18, through 16sRNA sequencing. It is a gram-negative, nonmotile, rod-shaped bacterium with a prominent polysaccharide-based capsule, belongs to family *Enterobacteriaceae*. There are number of strains of *Klebsiella pneumoniae* involved in biodegradation of petroleum hydrocarbons (Zafra et al 2016, Wokem et al 2017, Rajkumari et al 2018). *Klebsiella pneumoniae* strain VM18 degrades diesel oil into compound like butane, pentane, nonane. Similar results were obtained by Rodrigues et al (2009). *Klebsiella pneumoniae* strain VM18 had degraded 62.84% hydrocarbons. However in previous results, *Klebsiella pneumoniae* CR23 degrade 58% of engine oil and 59.7% of petroleum refinery effluent (Oaikhena et al. 2016, Adeleye et al 2018). This might be due to petroleum composition of engine oil, diesel oil and petroleum effluent, environmental conditions or the metabolic activity of microbial strain (Ozyurek and Bilkay (2017) GC-MS analysis reveals that, *Klebsiella pneumoniae* strain VM18 degraded C_{51}-C_{60} fraction from 2.54 to 1.49% and converted them into C_{31}-C_{50} after 21 days of incubation. It completely removed carbon chain length from C_{31}-C_{50} after 35 days of incubation. These results revealed that higher molecular weight hydrocarbons take longer time to degrade and persist in environment (Kanaly and Harayama 2000). *Klebsiella* Sp. considered as a potent biodegrader of petroleum hydrocarbons but it takes longer time to degrade higher molecular weight hydrocarbons. This might be due to physical properties of long hydrophobic carbon chain. As the chain increase their solubility decrease due to increase in hydrophobicity (Zander 1983). The results of GC-MS analysis also confirmed that, *K. pneumoniae* strain VM18 was efficiently degraded C_{11}-C30 up to 46% to 50% after 28 days of incubation.

CONCLUSION

Klebsiella pneumoniae strain VM18 is capable to utilize the diesel oil as an energy source. The capability to degrade diesel oil makes it a potential degrader and can be considered as bioremediation tool. However, individual organisms often prefer to metabolize a limited range of hydrocarbon substrates. Several microorganisms are not able to function when cultured in environment so it required more research particular in this field.

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Received 12 December, 2021; Accepted 23 May, 2022