World Journal of Pharmaceutical Science and Research

www.wjpsronline.com

Research Article

ISSN: 2583-6579
SJIF Impact Factor: 6.916
Year - 2023
Volume: 2; Issue: 6
Page: 315-324

NAPHTHALENE DEGRADATION STUDIES USING *BACILLUS LICHENIFORMIS* AND *ACINETOBACTER INDICUS* STRAIN ISOLATED FROM PETROLEUM CONTAMINATED SOIL OF CHHATTISGARH

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Article Received: 20 November 2023 | | ***Article Revised: 10 December 2023*** | | ***Article Accepted: 31 December 2023***

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DOI: https://doi.org/10.5281/zenodo.23417732

How to cite this Article: Sanjana Bhagat (2023) NAPHTHALENE DEGRADATION STUDIES USING BACILLUS LICHENIFORMIS AND ACINETOBACTER INDICUS STRAIN ISOLATED FROM PETROLEUM CONTAMINATED SOIL OF CHHATTISGARH. World Journal of Pharmaceutical Science and Research, 2(6), 315-324.

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ABSTRACT

Environmental contamination and serious health concerns have drawn a lot of attention to the remediation of "polycyclic aromatic hydrocarbons (PAHs)." However, native bacteria found at petroleum sites affected by PAHs may also be able to break down naphthalene. For effective naphthalene degradation, this study sought to isolate, characterize, and optimize process factors. Using naphthalene as a source of carbon and energy, six bacteria that break down naphthalene were isolated from contaminated soil in enrichment media. Strains NAP1 or NAP2, which show maximum growth on enrichment media, were selected for biodegradation assay. The selected isolates, Bacillus licheniformis (NAP1) and Acinetobacter indicus (NAP2) was found to degrade 0.8mg and 0.9mg of naphthalene in 5 days of incubation. According to the study, strains of Acinetobacter indicus and Bacillus licheniformis can be useful in cleaning up naphthalene pollution from petroleum-contaminated soil.

KEYWORDS: Polycyclic aromatic hydrocarbons, Naphthalene, Biodegradation, Petroleum, Bioremediation.

INTRODUCTION

"Polycyclic aromatic hydrocarbons (PAH)" are essentially made up of fused benzene rings arranged in linear, cluster, or angular patterns, ranging from the monocyclic molecule of benzene (molecular weight -78g) to nine-ringed structures (MW 1g up to 478g). PAHs are frequently employed as solvents in paints, insecticides, and several other industrial products. Both natural and man-made sources can produce PAHs. The use of petroleum products by humans and incomplete combustion of fossil fuels, biofuels, or other organic matter are the main sources of PAHs in the biosphere.[1,2,3]

Naphthalene, which is typically employed as a model compound to research PAH bioremediation, is classified as the first member of PAHs and consists of two fused aromatic rings. Significant amounts of PAHs are found in natural crude oil and coal reserves, which are produced when natural product molecules like steroids undergo chemical conversion. Naphthalene ($C_{10}H_8$), the bicyclic aromatic hydrocarbon, is the simplest PAH with lowest molecular weight (MW 128.16g). Naphthalene is mostly utilized as a step in the synthesis of phthalic anhydride. Surface active agents, resins, Carbamate insecticides, as a synthetic tanning agent, as a dye intermediate, as a moth repellent, and as a toilet and diaper pail deodorizer are some other uses. When coal, oil, and mothballs are burned, naphthalene is emitted into the environment. Small amounts are released by the manufacturing of coal tar, wood preservation, and other businesses. Tobacco smoke has also been found to contain naphthalene. Neurological impairment, Hemolytic anemia, and liver damage in neonates are linked to acute human exposure to naphthalene through ingestion, inhalation, and skin contact. Newborns born to mothers who "sniffed" and consumed naphthalene (as mothballs) during pregnancy have been documented to have hemolytic anemia. Retinal bleeding and cataracts result from long-term naphthalene exposure. Given that the Environmental Protection Agency (EPA) has designated naphthalene as a priority hazardous pollutant and Group C, a potential human carcinogen, research into naphthalene degradation is important.[9,20]

Bioremediation, which employ biodegradation potential of microorganisms, is an effective technology towards detoxification and volume reduction. It is believed to be a non-invasive, cost-effective technology and shows minimal negative impact on environment compared to other physicochemical methods of PAH degradation. Although certain fungi and algae can change these chemicals, high molecular weight PAHs are often resistant to microbial attack. Microorganisms growing on contaminated site are more capable of degrading oil than those microorganisms from non contaminated site. Naphthalene is rapidly mineralized by pure bacterial culture, bacterial consortium in sediment, and aquatic ecosystem. Bacterial genera *Mycobacterium, Corynebacterium, Aeromonas, Rhodococcus,* and *Bacillus* have been the subject of recent research on napthalene degradation.[8,9,10] Popular bacterial genus that uses hydrocarbons as a source of carbon and energy is Pseudomonas, which produces a bio-surfactant that increases the absorption of such immiscible “hydrophobic compounds.” Both molecular oxygen atoms are incorporated into the aromatic molecule to generate cis-1,2-dihydroxy-1,2-dihydronaphthalene, which is then dehydrogenated to form 1,2-dihyoxynaphthalene, starting the oxidative route of naphthalene[18]. Ring fission enzymes use the dihydoxylated derivative as a substrate, which is oxygenatively cleaved to produce salicylate, catechol, and CO2 as an intermediary.[14,15]

Microorganisms like bacteria, fungi, yeast, and microalgae can break down petroleum hydrocarbons like naphthalene. Physiochemical remediation techniques are not economical for cleaning up petroleum-contaminated sites. Because it appears to be a promising solution for cleaning up polluted areas, research is being done on the use of microorganisms for bioremediation of PAH-contaminated environments. The goal of the current study was to identify and isolate naphthalene-degrading bacteria from petroleum-contaminated locations that can break down naphthalene more effectively.

MATERIALS AND METHOD

Sample collection

Samples of petroleum oil contaminated soil from three separate locations of the Hindustan Petroleum Depot, Mandir Hasaud, Rawabhata, and Pachpedinaka, Raipur, Chhattisgarh, India, were gathered. Soil was collected randomly 5-10 cm beneath the surface using sterile spatula and were packed in sterile polybags. Used soil pH= 11, Color: Blackish Brown.

Enrichment and isolation of naphthalene-degrading bacteria

Using the enrichment culture approach, pure bacterial cultures were extracted from soil contaminated by petroleum oil. 50ml of mineral salt media ($FeSO_4$ $7H_2O$ 0.01g/L, $MgSO_4$ $7H_2O$ 0.2g/L, K_2HPO_4 1.8g/L, NH_4Cl 4.0g/L, and NaCL 0.1g/L) was mixed with 3grams of contaminated soil, and 0.016g/ml of naphthalene was added as an enrichment substrate. The mixture was shaken at 200rpm and 37°C for five days. 5ml aliquot was transferred to a new 50ml mineral salt medium with the same naphthalene content after growth, and the same conditions were maintained during incubation. Following three successive enrichments, 30 microliters of culture suspension were applied to MSM plates that had been treated with 0.2mg/ml of naphthalene. a single source of carbon and energy. Mixed cultures were obtained after two days of incubation at 37°C. Streaking on MSM plates was used to aseptically select and purify morphologically different colonies. After that, pure culture is kept on MSM broth. After that, broth is smeared on MSM plates with higher naphthalene content. The best-growing isolates from this group were chosen, kept in MSM medium, and kept at 4°C for future research.

Morphological and biochemical characterization

Biochemical testing and Gram staining were used to tentatively describe the selected isolates. Gram staining was performed on the selected colony from the plate incubated as well as of the broth. Biochemical test was performed for taxonomical characterization, which includes endospore staining, gelatin liquefaction, H_2S production, starch hydrolase, catalase, methyl red-voges proskauer (MR-VP), glucose, sucrose, oxidase, and citrate utilization test.

MTC (Maximum tolerance concentration)

MTC of isolates was determined, which is the maximum concentration of naphthalene at which growth of the microorganisms is inhibited. To obtain MTC, broth containing pure culture of naphthalene-degrading isolates was streaked on MSM plates with different concentrations of naphthalene (0.001 gm to 1 gm) and incubated.

Identification of bacterial isolates NAP 1 and NAP 2 by 16s rRNA sequence

PCR amplification

Molecular identification of NAP1 and NAP2 bacterial isolates was done using 16s rRNA sequence. The Himedia Kit was used to separate the sample's genomic DNA. High-quality PCR was used to amplify the 1.5 kb 16s-rDNA fragment. The following 16s primers were used in PCR to amplify the 16S rRNA gene. Primer for Forward: 5' – AGHGTBTGHTCMTGNCTCAS–3') and 16s Reverse Primer:(5'– TRCGGYTMCCTTGTWHCGACTH – 3'). 35 cycles of 95°C for 5minutes, 94°C for 30seconds, 50°C for 30seconds, 72°C for 1.30minutes, and 72°C for 7minutes each made up the thermocycling conditions.

16sRNA Sequencing

ABI 12500 Genetic Analyzer was then used to sequence the PCR results. BLAST was used to obtain closed relative sequences of the 16S-rRNA genes of naphthalene-degrading bacteria in the gene bank database using the data analysis program "Seq Scape_v 5.2." 10µl of sequencing mix is included. The Sequencing Reaction Reaction Mix for "Big Dye Terminator Ready": 4µl, Template (100ng/ul): 1µl, Primer (10pmol/λ): 2µl, Milli Q 12µl of water. Conditions of PCR (25 cycles): Initial Denaturation: 5 mins at 96°C; Denaturation: 30 sec at 96°C; Hybridization: 30 sec at 50°C; Elongation: 1.30 mins at 60°C.

Determination of naphthalene degradation efficiency of isolates

Spectrophotometric analysis was used to track the isolates' capacity to degrade naphthalene. Naphthalene, the only source of carbon in MSM medium, was present in concentrations of 0.2-1mg/ml. The inoculums was 1milliliter of the enhanced cultures (24 hours) from the isolates' enriched broth. Using absorbance at 365nm against uninoculated media as a control, the isolate's capacity to break down naphthalene was measured spectrophotometrically.

RESULT

Isolation and characterization

The enriched soil sample obtained from contaminated sites of petrol pump contained two morphologically distinct colonies. They were designated as NAP1 and NAP2. Isolate NAP1 is gram-positive, non- motile, rod-shaped bacterium, whereas NAP2 is gram negative bacteria. They form circular and milky white colonies on MSM (Mineral salt media). They appeared as circular, raised, and dark-colored colonies on MSM plates.

Biochemical characterization

Isolate NAP1 and NAP2 show positive results for endospore staining, Indole test, and Methyl red test. NAP1gave negative results for catalase, oxidase, sucrose utilization, H2S production, and starch hydrolase test, whereas NAP2 gave negative results in catalase, oxidase, glucose, sucrose utilization, H2S production, starchhydrolase test, Voges-Proskauer, gelatin liquefaction, and citrate utilization test (Table 1).

MTC (Maximum Tolerance Concentration)

Both isolates' MTCs were found in different concentrations of naphthalene. MTC of NAP1 isolatewas 0.8 gram of naphthalene, whereas that of NAP2 isolate was 0.9gram, as shown in figures 1 A & B.

Identification of bacterial isolate by 16s-rRNA sequence

Potential naphthalene degrading bacterial isolates were selected for molecular identification. Genomic DNA of the bacterial isolates was isolated by the standard method (Fig. 2 B) and the PCR amplification was done using 16S rRNA gene primer (Fig. 2 C). Molecular identification of NAP1 and NAP2 bacterial isolates was done using16srRNA sequencing. Strain NAP1 formed a phyletic lineage within the genus *Bacillus licheniformis* (Fig.3 & 4), according to a phylogenetic analysis on the basis of "16S rRNA gene sequences." A phylogenetic tree was then obtained, whereas NAP2 belonged to the *Acinetobacter indicus* (Fig. 5& 6).

Determination of naphthalene degradation efficiency of isolates

Breakdown of naphthalene by strains NAP 1 and NAP 2 was also assessed in the contaminated soil environment. The degradation experiments showed that after five days of incubation, naphthalene chemicals in the soil degraded. Nevertheless, the addition of nutrients greatly enhanced the biodegradation of naphthalene compounds. The degradation tests unequivocally showed that naphthalene could be broken down by both bacterial strains, "NAP 1 and NAP 2." NAP 1 as well as NAP 2 both isolate show maximum growth at day 4 at absorbance 0.12 and 0.14, respectively, at 365nm (Fig. 7). The selected isolates, *Bacillus licheniformis* (NAP1) and *Acinetobacter indicus* (NAP2) was found to degrade 0.8mg and 0.9mg of naphthalene in five days of incubation.

DISCUSSION

Removal of toxic substances from environment through microorganisms and biological degradation is a current trend in decontamination of polluted areas. PAH is a ubiquitous pollutant that could be deleterious to human health, since some PAH are potent mutagenic and carcinogenic. Depending on the type and quantity of hydrocarbons present, the biodegradation of petroleum hydrocarbons is a complicated process. Hydrocarbons can be broken down by bacteria. *Alcaligenes, Aeromonas, Bacillus, Acinetobacter, Burkholderia, Berjerinckia, Flavobacterium, Corynebacterium, Pseudomonas, Micrococcus, Mycobacterium, Lutibacte-rium, Rhodococcus, Streptomyces, Sphingomonas, Stenotrophomonas, Tistrella,* and *Vibrio* are among the microorganisms that have been reported to be able to break down PAH.[4,5] There have also been reports of certain rhizobial bacteria using PAH. Naphthalene-degrading bacteria are easy to isolate, and they are mostly Gram-negative bacteria. Recently biodegradative pathway of naphthalene has been reported in genera *Corynebacterium, Mycobacterium, Bacillus, Aeromonas, and Rhodococcus.*[19,21,22]

NAP1 and NAP2, two naphthalene-degrading isolates, were used in this research, enriched and isolated from petroleum contaminated site of Hindustan Petroleum depot of Raipur, Chhattisgarh, India. Both naphthalene-degrading microbial isolate is screened and chosen on the basis of their capacity to use naphthalene as their only energy and carbon source. NAP 1 is closely related to *Bacillus licheniformis* based on its molecular characterization by 16s-rRNA sequence. It is a Gram-positive bacterium and capable of naphthalene degradation, whereas NAP2 is closely related to *Acinetobacter indicus*. Both strains are capable of naphthalene degradation. Environmental concerns and regulatory restrictions have slowed field testing of genetically engineered bacteria for pollution biodegradation and biotechnological research. Therefore, increasing awareness of the advantages of using engineered microorganisms, popularizing accomplishments, and improving the distribution of knowledge must be top priorities. In addition to glucose in MSM medium containing naphthalene, affecting the growth of *Bacillus licheniformis* and *Acinetobacter indicus.* Growth of Bacillus licheniformis increases in the presence of 10mg/50 ml of glucose concentration, whereas *Acinetobacter indicus* shows increased growth at 20mg/50 ml of glucose concentration. Recently found that growth of *Pseudomonas extremaustralis, Pseudomonas veronii,* and *Pseudomonas Antarctica* was enhanced by adding 50mg/l of glucose, while *Pseudomonas trivialis, Pseudomonas reactans* can grow at any concentration of glucose. Bacteria catabolize PAH either by Salicyclate pathway or Pthalate pathway. *Bacillus licheniformis* and *Acinetobacter indicus* degrade naphthalene via Pthalate and Salicylate *pathway*. Both can grow when toluene and phenol are present. Most bacteria that degrade naphthalene are members of the Pseudomonas genus. NPL1, pND13, and NAH7 are plasmids that contain the gene encoding naphthalene oxidation in Pseudomonas, although NAH7 is the one that has been examined foremost.[6,7,23]

According to the current investigation, naphthalene degradation in both strains proceeds via distinct intermediates from naphthalene to salicylate and subsequently to catechol. Pseudomonas sp.32 has been found to produce catechol as a significant intermediate product during the biodegradation of naphthalene. Additionally, research has demonstrated that a number of Pseudomonas strains (P. putida AK5 and ND6) were capable of breaking down naphthalene through a pathway that produced phthalic acid and catechol.

Hydrocarbons are significant pollutants in the environment that are hard to break down. Certain *Bacillus* species are quite good at decomposing these pollutants, and they can thrive in environments contaminated with hydrocarbons. Different *Bacillus* strains have distinct genes that produce enzymes and biosurfactants required for hydrocarbon

breakdown, according to their genetic makeup. Additionally, additional *Bacillus*-related taxa, like *Geobacillus* and *Paenibacillus*, have demonstrated the ability to biodegrade.[6,11,13]

Recent study found that the genetically modified *B. licheniformis* strain (GEM-NY2) biodegraded benzo(a)pyrene by 78.21-80.16% compared to the wild strain.[15] Therefore, more research and development are needed to create and produce these enzymes in large quantities so that *Bacillus* species can become a viable group for removing hydrocarbon pollutants from the environment. Numerous bacteria from the genera *Pseudomonas, Acinetobacter, Bacillus, Staphylococcus, Arthrobacter, Alcaligenes, Janibacter, Micrococcus, Enterobacter, Sphingobacterium*, and *Clostrodium* have been identified as decomposing naphthalene when found in petroleum-contaminated locations.[12,16,17]

Table 1: A result shows biochemical characterization of bacterial isolate.

S.N	Tests	NAP1	NAP2
1	Endospore staining	**+**	**+**
2	Gelatin liquefaction	+	**-**
3	Indole test	+	**+**
4	Methyl red test	+	**+**
5	Voges- Proskauer test	+	**-**
6	Citrate utilization test	+	**-**
7	Starch hydrolase test	-	**-**
8	Catalase test	-	**-**
9	Oxidase test	-	**-**
10	Fermentation test		
11	Glucose	+	**-**
12	Sucrose	-	**-**
13	Motility test	**-**	**-**

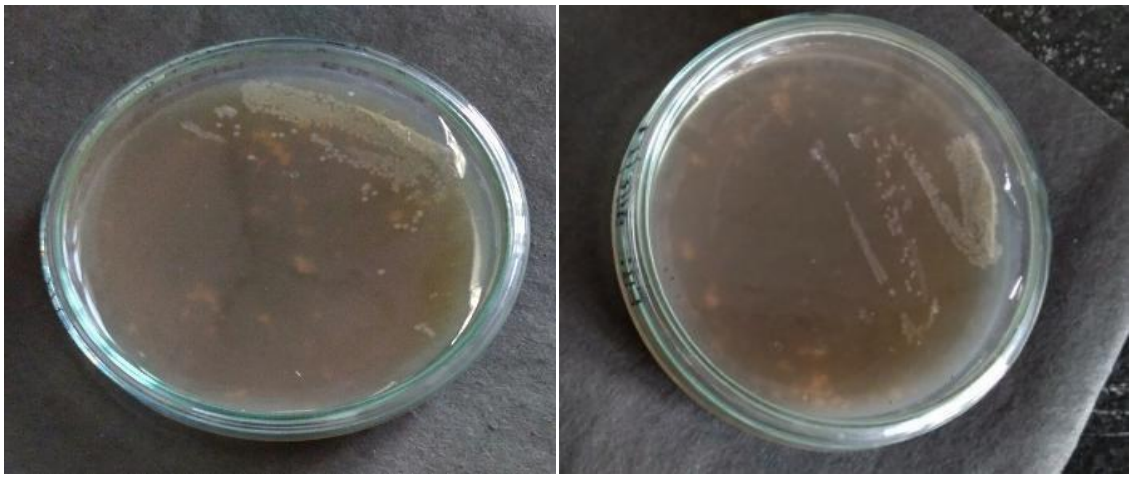


Fig 1A: MTC PLATE OF NAP 1. **Fig 1B: MTC PLATE OF NAP 2.**

Fig. 1A & B: Showing maximum tolerance concentration of NAP1 and NAP2 strain.

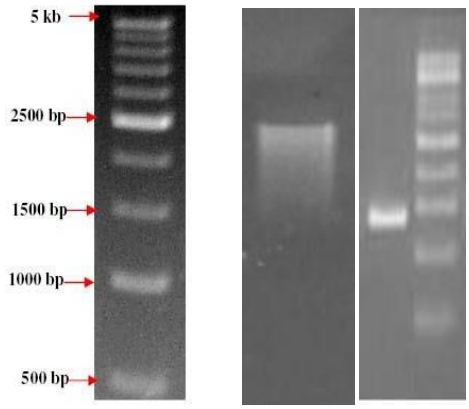


Fig. 2: A- L – 500 bp DNA ladder; B- Genomic DNA from Bacterial sample loaded in 1% agarose gel; C- PCR amplification of 16s rDNA fragment from Bacterial sample. The size of PCR amplified product is ~1.12kb.

TCCTGATGTCAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAAC
CGGGGCTAATACCGGATGCTTGATTGAACCGCATGGGTCGATTATAAAAGGTGGCTTTTAGCTACCACTTACAAA
TGGACCCGCGGGGCATTACCTAGTTGGTGAGGAAACGGTTCACCAAGGCAACGATGCGTACCCAACCTGAAAGG
GGGATCGGCCACACTGGGACTGAAACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATG
GACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGAAAA
AACAAGTACCGTTCAAATAGGGCGGAACCTTAACGGAACCTAACCAAAAATCTTCGGTTAACTACGTGCCACCAC
CCGGGTTATTACTTAGGTGGGAACCGTTGCCCGGAATTATTGGCTGTAAATGCCGCCCAGGCGATTGCTTAAATCT
GATGTGAAATCCCCCGGCTCACCCGTGGGAGGGTGATTGAAAAGTGGGGGTATTTGAGTGCAGACCACCCGATTG
AATTTCCCCGTGTACCGTTGAAATGAGAAAAAATGGCGTCGAAAACCGGGGGCCTACGGGTATTTTCTGGTTTAA
CCTGACGCTTAGGAATTAAATCTCCTCTACCGAACACAATTACCCCCCTGCCAATCGACCCCCCCCAGCCTGAGG
CGGGGGGTTTTTCTGGGATTTTGGCCTTTCAGGCGTGGCGGGCATTTTCTTCACAAAAATTTCCCGGGGGGAAGTT
TGGCCCCCCAACATTTGAACCCCGGGGGGATTGGCCGGGAGTTACCCCAGGGCTT

Fig. 3: Aligned sequence of Bacillus licheniformis strain (NAP 1).

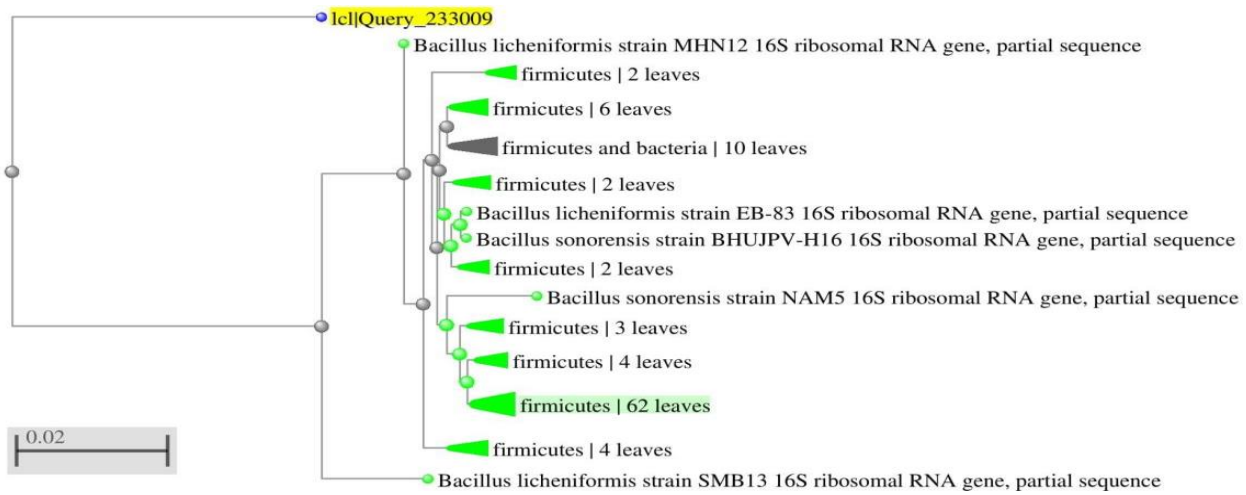


Fig. 4: Phylogenetic tree analysis of Bacillus licheniformis strain (NAP 1)

CTGCAGTCGAGCGGACCGGGTGGGTTCGCTCCCTGACTTAGCGGCGGACGGGTGAGTAATGCTTACGAATCTGC
CTATTACTGGGGGACAACATTCCGAAAGGGATGCTAATACCGCATACGCCCTACGGGGGAAAGCAGGGGATCTT
TTGCGCCCTTGCTCTTATATATGACTCTTTCCCATTTTATCTATGGGGTGGTATATAGGTCTACCAAGGACACTCTC
TGTAGCGGCTCAGAGAAGATGCCCCCCCACTCTGGCTCTGACACACTGCCCATACTCCTACGAGAGGACTCAGTG
GGGAATATTGCACAGTGGGGAACCCCCTGATCCCCATGTGCCGCGTGTGAGAAGAGGGTCTTGTGTGTAAAGAT
CTCTTAACGAGGAGAAGAGGCGCTCTGAGATAACCCCCTAGAGGCGGGCGCTTCTCTCGGAAAATAAACCCCGT
ATAACTCTGCCCCCACACCCGGGGAAATACAGAGGGGGAGAGCGTTTCGCGTATTTTGTGGGCGAAAAGCGCGC
ATGTGGGTCTTATTAACTCATGTGTAAAATCCCCGATTTTAATTTGAGAATTGTTTTCTATTGTGTATATATAGAA
TGTGAGAGAAGGTTGATAAAATTTCCAGGGGGAAGCGGAGATGTGCATAGATATCTGGGAGAATAGCGGTGGA
AAGCGCACGTCTGTGCTCTAACTCTGACTCT

Fig. 5: Aligned sequence of Acinetobacter indicus strain (NAP 2).

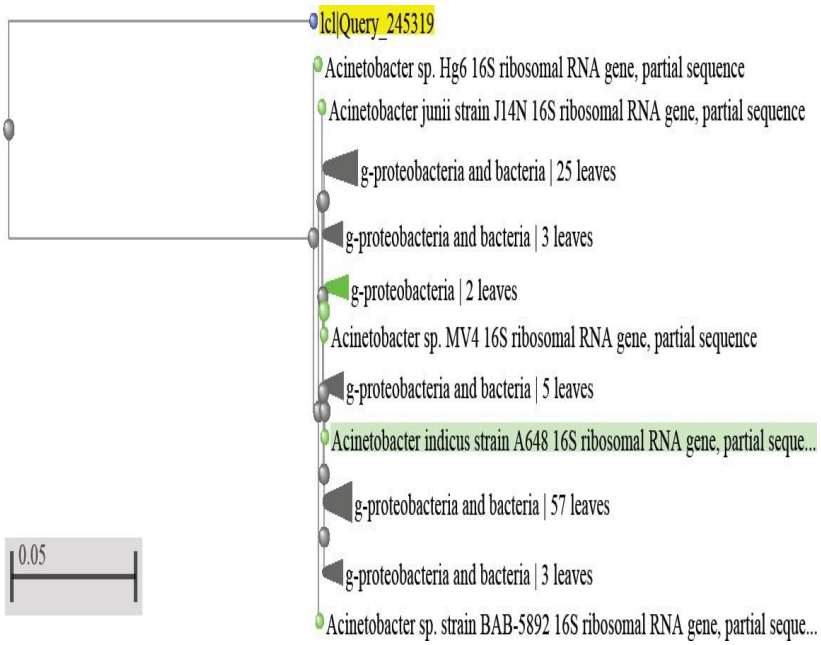


Fig. 6: Phylogenetic tree analysis of Acinetobacter indicus strain (NAP 2).

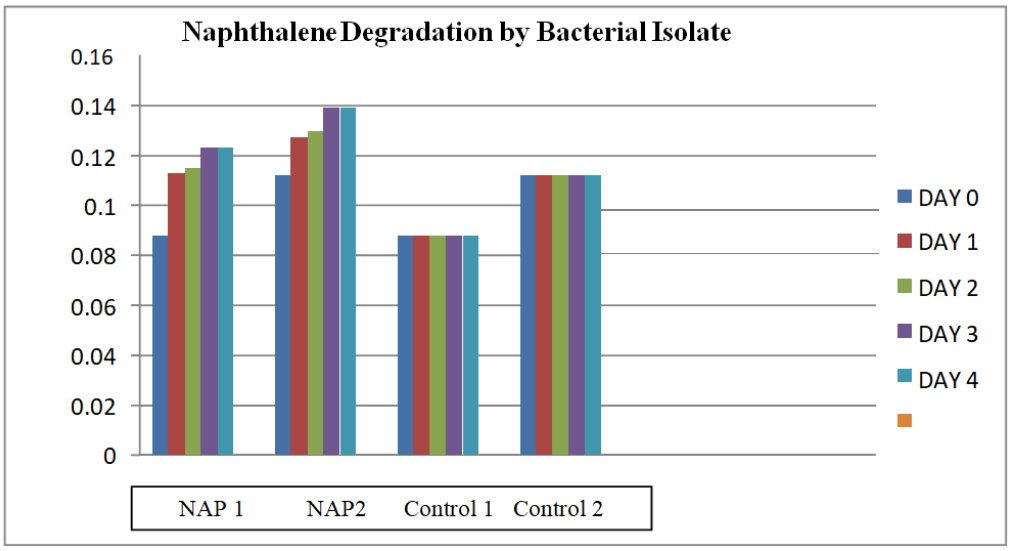


Fig. 7: Graph showing naphthalene degradation by bacterial isolate.

CONCLUSION

The potential of bacteria isolated from petroleum-contaminated soils to degrade naphthalene was examined in the current investigation. *Acinetobacter indicus* (NAP2) and *Bacillus licheniformis* (NAP1) were the two chosen bacterial strains that were found to exhibit effective degradation. Nevertheless, it also depends on the source of isolation, the presence of a carbon source, the PAH content, and the optimization of other factors. The microbial consortium's strains could be helpful in the cleanup process since they can break down the primary component of soils contaminated by petroleum. Research demonstrated that using the enrichment approach to break down PAHs in a liquid medium could be possible and that strain improvement could speed up this process. Naphthalene and other organic pollutants can be bioremediated using the new strains that were produced.

ACKNOWLEDGEMENT

The Department of Biotechnology assistance in providing the lab facility to conduct present work is appreciated by author.

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