

BIOREMEDIATION OF PHENOL BY USING *PAENIBACILLUS LACTIS* STRAIN FROM PETROLEUM CONTAMINATED SOIL

Shreya Gupta, Sanjana Bhagat*

Department of Biotechnology, Govt Nagarjuna PG College of Science, Raipur.

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*Corresponding Author: Sanjana Bhagat

Department of Biotechnology, Govt Nagarjuna PG College of Science, Raipur.

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ABSTRACT

Phenol is a toxic aromatic compound commonly found in industrial effluents generated by petroleum refineries, petrochemical industries, pharmaceutical manufacturing units, and other chemical processing facilities. Owing to its persistence and harmful effects on human health and the environment, the development of sustainable methods for phenol removal is of considerable importance. In the present study, indigenous phenol-degrading bacteria were isolated from petroleum-contaminated soil collected from the Hindustan Petroleum Corporation Limited (HPCL), Atal Nagar, Nava Raipur, Chhattisgarh, India. Isolation was carried out using Bushnell-Haas (BH) medium supplemented with phenol as the sole carbon source. The degradation efficiency of the isolates was evaluated by the 4-aminoantipyrine (4-AAP) assay, while the most efficient isolate was identified by 16S rRNA gene sequencing. The selected isolate was identified as *Paenibacillus lactis* and demonstrated remarkable tolerance to phenol, exhibiting growth at concentrations up to 1000 mg/L. Phenol degradation was confirmed by the disappearance of the characteristic color in the 4-AAP assay after 20 days of incubation. High-performance liquid chromatography (HPLC) revealed a substantial reduction in phenol concentration together with the formation of degradation intermediates, whereas Fourier transform infrared spectroscopy (FTIR) demonstrated changes in characteristic functional groups, indicating structural transformation of phenol during microbial metabolism. The results demonstrate that the isolated *Paenibacillus lactis* possesses excellent phenol degradation capability and high tolerance to elevated phenol concentrations, highlighting its potential application in the bioremediation of phenol-contaminated soil and industrial wastewater. The study further emphasizes the significance of indigenous bacterial strains as environmentally sustainable and cost-effective agents for the remediation of phenolic pollutants.

KEYWORDS: Phenol biodegradation, *Paenibacillus lactis*, Bioremediation, Petroleum-contaminated soil, 16S rRNA, HPLC, FTIR, 4-aminoantipyrine assay.

INTRODUCTION

Phenol and its derivatives are among the most hazardous organic pollutants released into the environment through the activities of petroleum refineries, petrochemical industries, pharmaceutical manufacturing units, resin and plastic production facilities, pulp and paper mills, textile industries, and coal processing plants. Due to their high toxicity, persistence, and poor biodegradability under natural conditions, these compounds pose significant risks to aquatic ecosystems, soil quality, and human health. Exposure to phenol has been associated with skin and eye irritation, damage to the liver and kidneys, neurological disorders, and adverse effects on the respiratory system.^[1,2]

Conventional methods for phenol removal, including adsorption, chemical oxidation, membrane filtration, solvent extraction, and incineration, are often expensive, energy-intensive, and may generate secondary pollutants requiring further treatment. In contrast, microbial biodegradation has emerged as an environmentally friendly, sustainable, and cost-effective alternative. Phenol-degrading microorganisms utilize phenol as a carbon and energy source, converting it into simpler and less toxic compounds through enzymatic metabolic pathways. This approach minimizes secondary pollution while providing an efficient strategy for the remediation of contaminated environments.^[3,4]

Numerous bacterial genera, including *Pseudomonas*, *Bacillus*, *Acinetobacter*, *Rhodococcus*, *Citrobacter*, and *Paenibacillus*, have been reported to possess the ability to degrade phenolic compounds. However, the biodegradation efficiency of these microorganisms varies depending on factors such as phenol concentration, pH, temperature, nutrient availability, oxygen supply, and the genetic characteristics of the microbial strain.^[5,6,7]

Several approaches have been developed to enhance phenol biodegradation, including microbial acclimatization, consortium-based degradation, immobilized cell systems, process optimization, and molecular characterization of efficient degraders. Despite these advances, the discovery and characterization of native bacterial strains capable of tolerating and degrading high concentrations of phenol remain essential for developing effective large-scale bioremediation technologies.^[8,9,10]

In the present study, soil samples collected from the Hindustan Petroleum Corporation Limited (HPCL), Atal Nagar, Nava Raipur, Chhattisgarh, India, were investigated for the isolation of indigenous phenol-degrading bacteria. The isolated strain was evaluated for its phenol degradation efficiency using the 4-aminoantipyrine (4-AAP) assay, while its maximum tolerance concentration (MTC) toward phenol was determined under laboratory conditions. Molecular identification was carried out using 16S rRNA gene sequencing, and the biodegradation process was further confirmed through Fourier Transform Infrared Spectroscopy (FTIR) and High-Performance Liquid Chromatography (HPLC). The findings of this study provide valuable insights into the potential application of indigenous *Paenibacillus lactis* for the bioremediation of phenol-contaminated soils and industrial wastewater.

MATERIALS AND METHODS

Sample Collection

The soil sample were collected from Hindustan petroleum corporation limited (HPCL) situated in Atal Nagar, Nava Raipur, Chhattisgarh, India. Samples were collected aseptically from contaminated site using sterile zip-lock pouch and transported to the laboratory for analysis.

Enrichment and Isolation of Phenol Degrading Bacteria

To isolate specialized microbial strains capable of metabolizing phenol, a 1g soil sample was suspended in 10 mL of sterile phosphate buffer solution maintained at a neutral pH of 7.0. This mixture was agitated thoroughly and allowed to stand undisturbed for 30 minutes. The primary enrichment phase was initiated by inoculating a 2 mL aliquot of this supernatant into 100 mL of Bushnell-Haas (BH) broth medium containing an initial baseline concentration of 200 mg/L of phenol. The enrichment cultures were incubated continuously at a controlled temperature of 30°C and occasionally kept on shaker at 130 rpm for 4 days. Following this initial cultivation period, isolation was performed by streaking loops of the enriched media onto fresh BH agar plates supplemented with 200 mg/L of phenol. These selective agar plates were incubated under identical conditions at 30°C for 48 hours to screen for discrete colonial growth of phenol-tolerant bacteria. Individual target colonies were sequentially re-isolated onto fresh BH agar plates spiked with 200 mg/L of phenol to guarantee strain purity and establish standalone single-culture lines. Once purity was confirmed, the isolated monocultures were stored inside a laboratory refrigerator at a constant temperature of 4°C.

Estimation of Phenol via 4-Aminoantipyrine Assay

The isolated bacterial strains screened during this study were inoculated into independent test tubes containing BH broth media spiked with varying phenol concentrations ranging between 50-500 mg/L. This step was carried out to identify highly resilient candidate strains displaying superior degradation capacities. The 4-AAP analytical protocol offers a straightforward, highly sensitive, and widely validated methodology for detecting trace phenolic complexes in aqueous solutions. In this analytical framework, the collected phenolic samples were coupled with the 4-AAP reagent under alkaline conditions, and color development was driven via oxidative coupling using potassium ferricyanide as the primary oxidizing agent. The formation of a characteristic reddish-pink dye indicated the presence of un-degraded residual phenol in the matrix. A measurable decrease in color intensity following the incubation window, when compared against uninoculated control samples, provided direct confirmation of successful phenol breakdown by the bacterial isolate.^[11,12]

Molecular Characterization and Identification of Selected Bacteria

The isolated bacterial candidate exhibiting the peak maximum degradation threshold for phenol was selected for taxonomic identification and validated via 16S rRNA gene sequence analysis. Genomic extraction was followed by Polymerase Chain Reaction (PCR)-driven amplification of the diagnostic 16S rRNA gene locus. The resulting amplicon fragments were purified and sequenced, with the laboratory sequencing workflow executed by Biokart India Private Limited, located in Bengaluru, India.

Determination of Maximum Tolerance Concentration (MTC)

The selected bacterial isolates were evaluated to determine their Maximum Tolerance Concentration (MTC) limits toward phenol. This assessment was conducted by inoculating the cultures onto Bushnell-Haas agar plates formulated with an escalating concentration gradient of phenol ranging from 200-1000 mg/L. The inoculated plates were transferred to an incubator and held at 30°C for a 48-hour observation window. Following incubation, the plates were inspected for visible colonial development.

Fourier Transform Infrared Spectroscopy (FTIR) Analysis

To characterize the underlying functional groups present in the phenol sample matrices following a 7-day incubation period, Fourier Transform Infrared Spectroscopy (FTIR) was implemented. The chemical spectral analysis was carried

out using a Nicolet Summit X FTIR spectrophotometer platform configured with an Everest Attenuated Total Reflection (ATR) accessory and a deuterated triglycine sulfate (DTGS) KBr beam detector. To ensure accuracy and baseline reproducibility, data collection utilized 32 individual sample scans per matrix, evaluated at standard room temperature conditions. The resulting infrared interferograms were evaluated based on characteristic vibrational absorption peaks to identify structural changes corresponding to the functional groups of phenol and its intermediates.

High-Performance Liquid Chromatography (HPLC) Analysis

High-Performance Liquid Chromatography (HPLC) analysis was utilized to quantitatively monitor the exact degradation pathways and monitor residual phenol concentrations within the treated volumes. The analytical verification of the liquid samples was carried out using an integrated HPLC system configured with a Photodiode Array (PDA) detector set to a primary monitoring wavelength of 254 nm. The sample injection volume was fixed precisely at 10 μ L for each automated analytical run. The specific retention times and target peak area values generated during the runs were systematically documented to identify and calculate the concentration of phenolic molecules. The resulting chromatograms were interpreted to map out the overall breakdown efficiency and establish the final degradation fingerprint of the parent phenol compound.^[14,15]

RESULTS AND DISCUSSION

Enrichment & Isolation of Phenol degrading microorganisms

The soil samples collected from the petroleum depot were enriched in BH Broth supplemented with phenol as the sole carbon source. After incubation, bacterial growth was observed, in the form of turbidity indicating the presence of microorganisms capable of utilizing phenol. Distinct bacterial colonies were isolated on BH agar plates containing phenol and purified by repeated streaking.

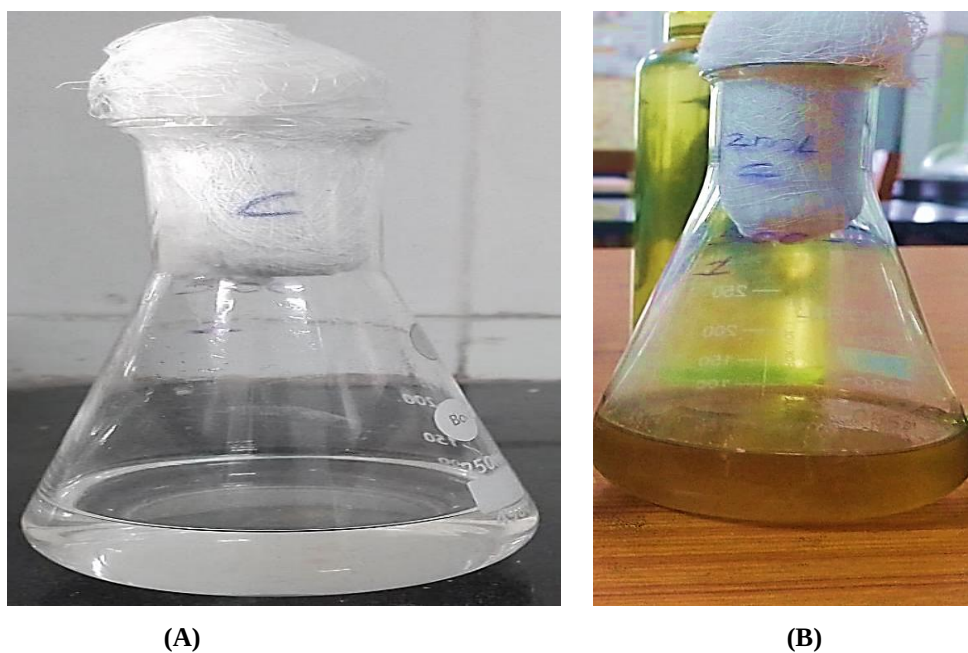


Fig. 1: Enriched media, (A) Before & (B) After incubation.



Fig. 2: Enriched media kept on Shaker at 130 rpm.



(A)



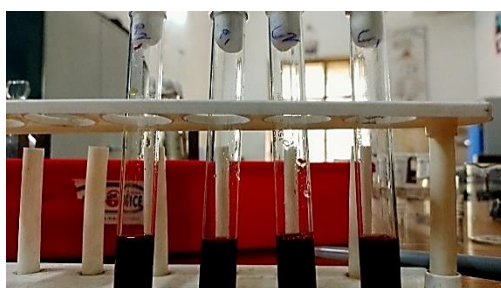
(B)



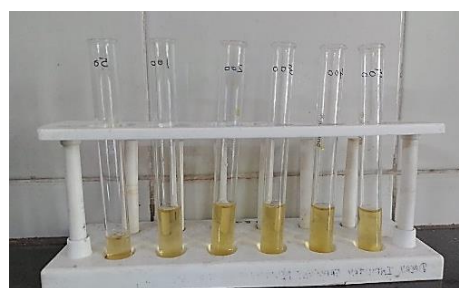
(C)

Fig. 3: (A) Bacterial Culture Isolated from Enriched Media (B) & (C) Pure Culture of Phenol-Degrading bacteria Degradation Assay.

The degradation of phenol by the isolate was confirmed using the 4-AAP assay. During the post-incubation analysis, the characteristics reddish-pink color intensity associated with unreacted phenol progressively faded. By the 20th day of incubation, the reaction mixture turned completely clear, demonstrating the exhaustive degradation of phenol by the inoculated by bacterial strain.



(A)



(B)

Fig. 4: 4-AAP Assay (A)Day-1, (B)Day- 20.

Identification of phenol degrading bacteria

Genetic sequencing and molecular characterization identified the isolated bacterium as a strain of *Paenibacillus lactis*, specifically designated as strain UNIJAG.PL.OP217. The analysis targeted the 16S ribosomal RNA gene, with the resulting sequence officially cataloged under the Sequence ID OP891057.1. Phylogenetic alignment and BLAST analysis indicated that the next closest genetic homologue corresponds to the complete genome sequence of *Paenibacillus lactis* strain FW14 chromosome, which is cataloged under the Sequence ID CP139098.1.

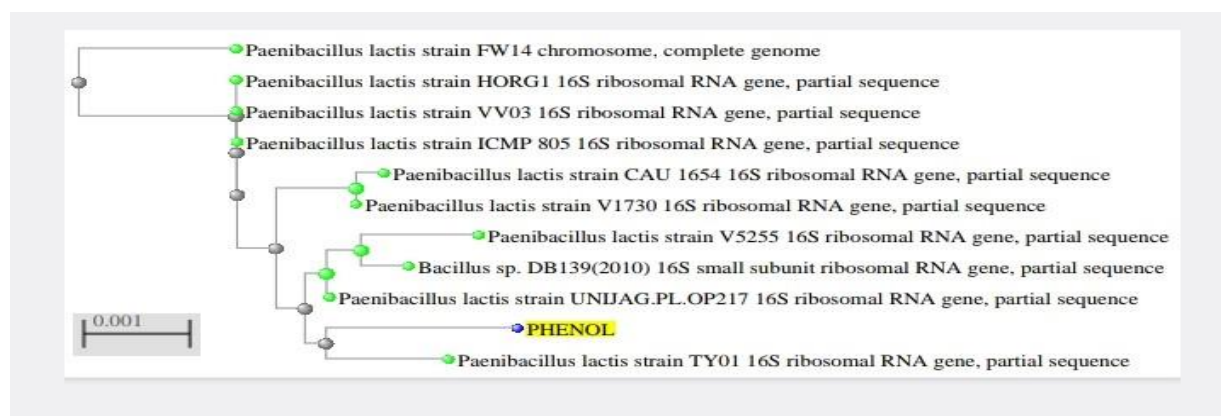


Fig. 5: Phylogenetic tree.

Table 1: Blast result of isolated bacteria

Description	Per. Ident	Accession
Paenibacillus lactis strain UNIJAG.PL.OP217 16S ribosomal RNA gene.	99.78	OP891057.1
Paenibacillus lactis strain FW14 chromosome, complete genome	99.78	CP139098.1
Paenibacillus lactis strain HORG1 16S ribosomal RNA gene.	99.7	MZ674207.1
Paenibacillus lactis strain CAU 1654 16S ribosomal RNA gene.	99.7	OK287048.1
Paenibacillus lactis strain V5255 16S ribosomal RNA gene.	99.7	PP256757.1
Paenibacillus lactis strain TY01 16S ribosomal RNA gene.	99.7	MG837028.1
Paenibacillus lactis strain VV03 16S ribosomal RNA gene.	99.7	KT160169.1
Paenibacillus lactis strain ICMP 805 16S ribosomal RNA gene.	99.7	PP103238.1
Paenibacillus lactis strain V1730 16S ribosomal RNA gene.	99.7	OR453741.1
Bacillus sp. DB139(2010) 16S small subunit ribosomal RNA gene.	99.69	HM566852.

MTC Test

The isolate showed growth at phenol concentrations up to 900 mg/L, after 2 days of incubation while no growth was observed at higher concentrations (1000 mg/L), but keeping the agar plates in incubator for 8 days it showed slight growth. Therefore, the MTC of the isolates was determined to be 1000 mg/L.

Table 2: Empirical growth observations across the phenol concentration gradient.

Applied Phenol Concentration (mg/L)	Growth
200	+++
300	+++
400	++
500	++
600	++
700	++
800	+
900	*
1000	-- (No growth detected after 4 days of incubation) * (Slight growth after 8 days)

Where: +++ = heavy, uninhibited colony expansion, ++ = Moderate, stable growth, * = Minimal, highly constrained colony formation, - = No growth

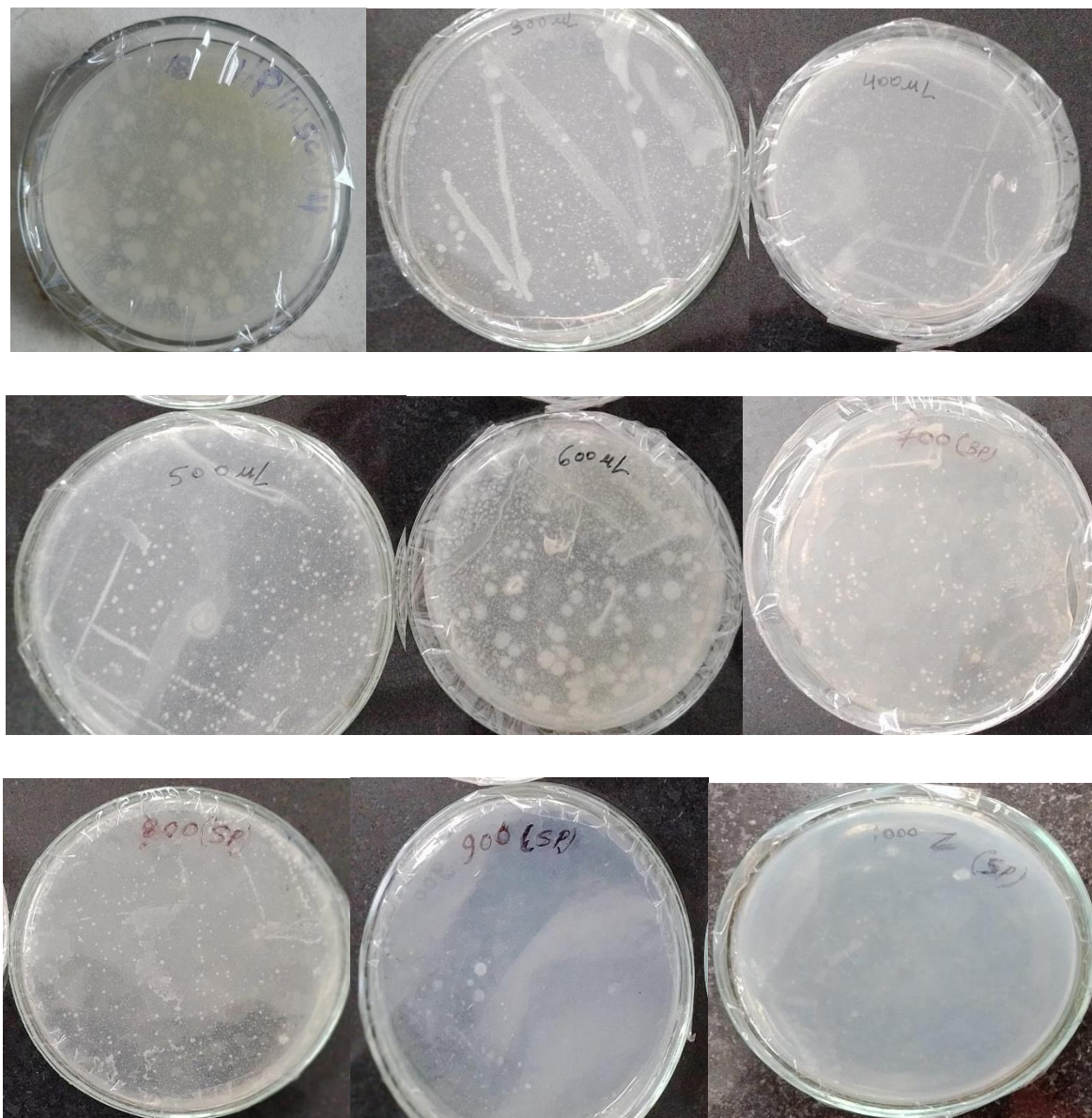


Fig. 6: MTC Test of phenol degrading bacteria at different concentration of phenol from 200-1000mg/L.

FTIR Analysis

FTIR analysis confirmed the presence of characteristic functional groups of phenol in the sample. A broad absorption peak observed at 3307 cm^{-1} indicated O–H stretching vibration of the phenolic hydroxyl group. The peak at 1660 cm^{-1} corresponded to aromatic C=C stretching vibrations. Absorption bands at 1384 cm^{-1} and 1135 cm^{-1} were attributed to O–H bending and C–O stretching vibrations respectively. The peak observed near 836 cm^{-1} represented aromatic C–H bending vibrations. These characteristic peaks confirmed the presence of phenolic compounds in the analyzed sample.

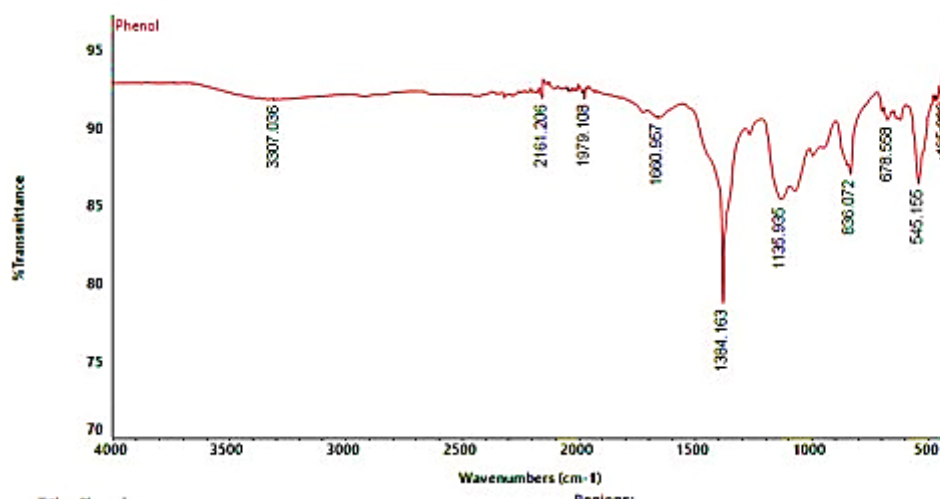


Fig. 7: FTIR Graph.

Table 3: FTIR Result.

Peak (cm-1)	Functional group	Interpretation
3307	O-H Stretching	Phenolic hydroxyl group
1660	C=C Stretching	Aromatic ring
1384	O-H bending	Phenolic compound
1135	C-O Stretching	Alcohol/Phenol group
836	C-H bending	Aromatic compound

HPLC Analysis

HPLC analysis of the phenol sample showed multiple chromatographic peaks, indicating the presence of phenol and its possible degradation intermediates. A total of six peaks were observed at retention times of 4.482, 4.721, 5.724, 6.456, 6.636, and 7.147 minutes. The major peak was detected at 4.721 min, contributing 56.116% of the total peak area, suggesting it as the predominant compound in the sample. Additional peaks with lower area percentages indicated formation of intermediate metabolites during phenol degradation. The presence of multiple peaks suggests partial transformation of phenol by bacterial activity and supports biodegradation of the compound.

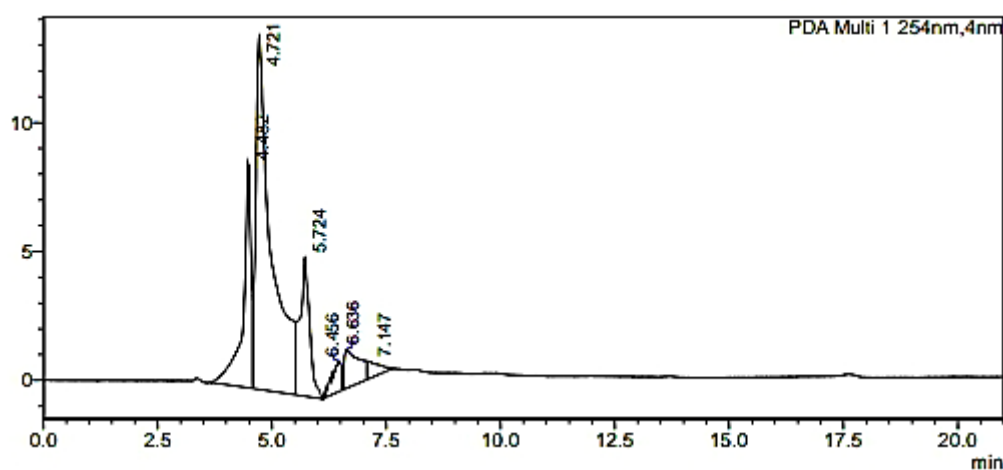


Fig. 8: HPLC Graph.

Table 4: HPLC Report Analysis.

Peak No.	Retention time	Area %	Interpretation
1	4.482	18.656	Possible degradation product
2	4.721	56.116	Major compound
3	5.724	14.089	Intermediate
4	6.456	3.221	Minor product
5	6.636	5.916	Minor product
6	7.147	2.003	Minor product

DISCUSSION

In the present study, phenol-degrading bacteria were successfully isolated from soil collected from petroleum depot through enrichment culture using phenol as the sole carbon source. The successful isolation of phenol degraders from hydrocarbon-contaminated soil is consistent with the findings of Patil et al., who reported the isolation of phenol-degrading bacteria from petrochemical-contaminated sites due to the natural adaptation of indigenous microorganisms to phenolic pollutants. The occurrence of phenol-degrading microorganisms in contaminated environments indicates selective pressure favoring organisms capable of utilizing phenol as a carbon and energy source.^[16,17,18]

The isolated strain exhibited growth and phenol degradation up to 1000 mg/L phenol, demonstrating significant tolerance toward phenolic stress. Similar observations were reported by Patil et al., where *Rhodococcus biphenylivorans* tolerated 1100 mg/L phenol. Although the tolerance observed in the present study is slightly lower, it still indicates substantial adaptation to phenol-contaminated environments. High phenol tolerance is an important characteristic for bioremediation applications because elevated phenol concentrations are often inhibitory to microbial growth.^[19, 20,21]

The 4-aminoantipyrine assay showed a gradual reduction in phenol concentration, evidenced by the disappearance of the characteristic red coloration and reduced absorbance values after incubation. This observation indicates active utilization and degradation of phenol by the isolate. Similar methodology was employed by Patil et al. for quantifying residual phenol during degradation studies. The colorless appearance of the culture after incubation further confirms efficient phenol removal by the bacterial isolate.^[22,23]

16S rRNA gene sequencing identified the isolate as a member of the genus *Paenibacillus*. Several species belonging to this genus have been reported to possess biodegradation capabilities against aromatic compounds and environmental pollutants. The identification of a phenol-degrading *Paenibacillus* strain suggests that this genus can contribute significantly to the biodegradation of phenolic contaminants in polluted soils.^[24-30]

HPLC analysis revealed a marked reduction or disappearance of the phenol peak after bacterial treatment compared with the initial sample. This result confirms the degradation of phenol and supports the observations obtained from the 4-AAP assay. Similar chromatographic reductions in phenol concentration have been reported in studies involving efficient phenol-degrading bacteria. The decrease in peak intensity and changes in chromatographic profile indicate metabolic transformation of phenol into intermediate or end products.^[31-34]

FTIR spectra demonstrated noticeable changes in functional groups after bacterial treatment. The reduction or disappearance of characteristic phenolic O–H stretching vibrations and alterations in aromatic ring-associated peaks suggest biodegradation of phenol. These spectral modifications indicate structural transformation of the phenolic

molecule during microbial metabolism. Similar analytical approaches have been widely used to confirm degradation of aromatic pollutants by bacterial isolates.

The results collectively demonstrate that the isolated *Paenibacillus* sp. possesses considerable potential for phenol biodegradation. The isolate tolerated phenol concentrations up to 1000 mg/L, showed positive degradation in the 4-AAP assay, and the degradation process was further confirmed through HPLC and FTIR analyses.

CONCLUSION

Phenol represents a highly detrimental and persistent ecological contaminant that is frequently released in the wastewater streams of oil refineries, chemical processing plants, pharmaceutical factories, and diverse manufacturing facilities. Its accumulation within natural environments presents severe hazards to public health and aquatic ecosystems. To address this, microbial biodegradation has gained widespread recognition as an efficient, cost-effective, and ecologically sustainable method for neutralizing and extracting phenol from impacted sites.

In the investigative work, soil samples collected from petroleum depot were used for the isolation of phenol-degrading bacteria. Enrichment and screening techniques were employed to obtain bacterial isolates capable of utilizing phenol as a sole carbon source. The most efficient isolate was selected and subjected to morphological, biochemical, and molecular characterization. Based on 16S rRNA gene sequencing and phylogenetic analysis, the isolate was identified as a member of the genus *Paenibacillus*.

The phenol degradation potential of the isolate was assessed through Maximum Tolerance Concentration (MTC) studies, where the bacterium exhibited growth and tolerance up to 1000 mg/L phenol. Phenol degradation was monitored using the 4-aminoantipyrine (4-AAP) assay, which showed a gradual decrease in phenol concentration during incubation. The assay mixture became colorless after 20 days of incubation, indicating substantial degradation of phenol by the bacterial isolate. High Performance Liquid Chromatography (HPLC) analysis further confirmed the reduction of phenol concentration in the treated samples. Fourier Transform Infrared Spectroscopy (FTIR) analysis revealed changes in characteristic functional groups, suggesting the biotransformation of phenol into intermediate and less toxic compounds.

The results demonstrate that the isolated *Paenibacillus* strain possesses remarkable phenol degradation efficiency and can tolerate high phenol concentrations. Therefore, this bacterium holds significant potential for application in the bioremediation of phenol-contaminated soils and industrial wastewater. The study highlights the importance of indigenous microorganisms as sustainable tools for environmental pollution control and remediation.

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