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Enhancement of Kerosene Biodegradation by a Biosurfactant-Producing Strain of *Klebsiella pneumoniae* Isolated from Hydrocarbon-Contaminated Soil

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Abstract: Kerosene pollution, stemming from its widespread use as a fuel and solvent, poses significant health and environmental risks. This study aimed to isolate biosurfactant-producing *Klebsiella pneumoniae* from petroleum-contaminated soil and apply the biosurfactant to enhance kerosene biodegradation. Among twelve isolates screened, seven produced biosurfactants, with *K. pneumoniae* S9 exhibiting the highest emulsification index (E24 = 45%). The biosurfactant was extracted, purified, and characterized as a lipopeptide via Thin-Layer Chromatography (TLC) and Fourier Transform Infrared (FT-IR) spectroscopy. Supplementation with the biosurfactant significantly accelerated kerosene degradation, achieving 64% efficiency within an 11-day incubation period. These results demonstrate the potential of this biosurfactant as an effective agent for the bioremediation of kerosene-contaminated environments.

Keywords: Bioremediation; Kerosene degradation; Biosurfactant; *Klebsiella pneumoniae*; Lipopeptide; Emulsification index; Hydrocarbon-contaminated soil

1 Introduction

Environmental contamination by petroleum hydrocarbons and their byproducts remains a serious global challenge [1, 2]. Kerosene, a refined product obtained through the fractional distillation of crude oil (150–275 ℃), is a significant pollutant due to its widespread use as a domestic fuel in developing nations, as well as an industrial solvent, lubricant, and pesticide [3]. Intentional or accidental release of kerosene into ecosystems leads to moderate-to-high acute toxicity, negatively impacting soil fertility, agricultural productivity, and the health of local biota [4, 5].

In response to stringent environmental regulations, bioremediation has emerged as a highly promising and sustainable remediation technology. Its advantages over physicochemical methods include lower cost, the potential for in situ application, and environmental friendliness [6, 7]. This process primarily relies on the metabolic activities of bacteria and fungi, which can utilize petroleum hydrocarbons as a carbon source for growth. Bacteria are considered the most active and primary agents in this process due to their diverse enzymatic systems and degradative genetic repertoire [2, 6, 8, 9].

A critical mechanism enhancing bacterial hydrocarbon degradation is the production of biosurfactants. These surface-active biomolecules, which include glycolipids, lipopeptides, and polymeric surfactants—significantly increase the bioavailability of hydrophobic compounds by reducing surface tension and facilitating emulsification [8, 10]. Furthermore, biosurfactants are biodegradable, less toxic, and exhibit various biological activities, making them superior to synthetic surfactants for environmental applications [11, 12]. Numerous bacterial genera, including *Pseudomonas*, *Bacillus*, *Acinetobacter* and others, have been identified as potent producers of biosurfactants with high hydrocarbon-degrading efficacy [13–15].

While many studies have focused on crude oil degradation, targeted research on kerosene-specific bioremediation strategies is crucial [16–18]. Therefore, the objectives of this study were twofold: (i) to isolate and characterize biosurfactant-producing bacteria from petroleum-contaminated soil, and (ii) to screen, apply, and evaluate the efficacy of the produced biosurfactant in enhancing the kerosene degradation process.

2 Methodology

2.1 Sample Collection and Bacterial Isolation

Fifteen petroleum-contaminated soil samples were collected and serially diluted (10^{-2} to 10^{-8}) with distilled water. All samples were promptly cultured on MacConkey and Blood agar plates and incubated aerobically at 37 ℃ for 24 hours [19].

2.2 Identification of Bacterial Isolates

Presumptive *Klebsiella pneumoniae* colonies were selected based on morphology and subculture for purity.

• Initial identification was based on Gram staining and standard biochemical tests including lactose fermentation, indole production, citrate utilization, and urease activity [20, 21].

• Final confirmation was obtained using the API 20E system (bioMérieux, France), according to the manufacturer's instructions. Pure isolates were stored short-term on MacConkey agar at 4 ℃ and long-term in Brain Heart Infusion (BHI) broth with 20% glycerol at -20 ℃ [22, 23].

2.3 Screening for Biosurfactant Production

Bacterial isolates were screened for biosurfactant production using two methods:

• Emulsification index (E24): Cell-free supernatant was mixed with toluene (1:1, v/v), vortexed for 2 minutes, and left for 24 hours. E24 was calculated as the height of the emulsified layer divided by the total height of the liquid column, multiplied by one hundred [24].

• Oil-Spreading Technique: ten μL of cell-free supernatant was added to the center of a Petri dish containing 40 mL of distilled water and twenty μL of olive oil. The diameter of the clear zone formed was measured after 30 seconds [24].

2.4 Biosurfactant Extraction and Purification

Biosurfactant from the most potent producer (isolate S4) was precipitated from the cell-free supernatant by acidification to pH 2.0 using 6M HCl and overnight incubation at 4 ℃. The precipitate was extracted using chloroform: methanol (2:1, v/v) solvent system. The organic solvent was evaporated using a rotary evaporator. The crude biosurfactant was further purified via silica gel column chromatography (60 mesh) using chloroform–methanol–water (65:25:4, v/v/v) as the mobile phase. Active fractions, identified by the E24 assay, were pooled for characterization [25, 26].

2.5 Characterization of Biosurfactant

The purified biosurfactant was characterized by Thin-Layer Chromatography (TLC): performed on silica gel plates with a chloroform–methanol–water–acetic acid (65:25:2:1.5, v/v/v/v) solvent system. Lipopeptides were detected using 0.2% ninhydrin reagent. Fourier Transform Infrared Spectroscopy (FT-IR): The functional groups of the biosurfactant were identified using an FT-IR spectrometer with a scanning range of 400–4000 cm^{-1} [27].

2.6 Kerosene Degradation Assay

• The efficacy of the purified biosurfactant in enhancing kerosene degradation was evaluated. The bacterial isolate was incubated with kerosene as the sole carbon source for 15 days at 30 ℃. Residual kerosene was extracted with a mixture of 50% H_2SO_4 and NaCl, and its concentration was determined by measuring absorbance at 256 nm using a UV spectrophotometer. A standard curve ($Y = 0.0022X + 0.0012$, $R^2 = 0.9999$) was used for quantification.

• The degradation efficiency was calculated as follows [28]: Degradation efficacy (%) = (1 – [Residual kerosene concentration / Initial kerosene concentration]) × 100

3 Results and Discussion

Results are divided into subsections that concisely and precisely interpret the findings and draw clearer conclusions.

3.1 Identification of *Klebsiella pneumoniae*

Presumptive *K. pneumoniae* isolates from petroleum-contaminated soil samples were identified based on characteristic colony morphology: large, mucoid, pink (lactose-fermenting) colonies on MacConkey agar and large, mucoid, non-hemolytic colonies on Blood agar (Figure 1) [29]. Microscopic examination revealed Gram-negative, short bacilli, occurring singly, in pairs, or short chains [30].

Biochemical profiling confirmed the identity of the isolates. All were catalase-positive, oxidase-negative, and produced negative results for indole and positive results for citrate utilization and urease activity. On Kligler Iron Agar (KIA), isolates produced an acidic butt and slant (yellow/yellow) with gas production, indicative of glucose and lactose fermentation without H_2S production [19–21, 31]. Final confirmation was obtained using the API 20E system,

which identified eight isolates from soil as *K. pneumoniae*. However, the IMVC results distinguish *K. pneumoniae* from other lactose-fermenting taxa. An indole test revealed no evidence of *K. pneumoniae*. According to the indole test, some intestinal bacteria that have enzyme tryptophanase, which breaks down the amino acid tryptophan into indole, pyruvic acid, and water, can hydrolyze tryptophan to indole. When Kovac's reagent is introduced to a broth that is empty of indole, a red ring will not form on top of the broth because indole-negative bacteria, including *Klebsiella*, don't make tryptophanase [31] see Figure 2.

Figure 1. Colonial morphology of *Klebsiella pneumoniae* on (a) Blood agar and (b) MacConkey agar

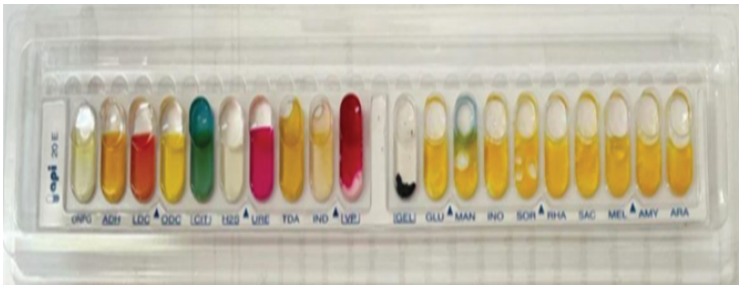

Figure 2. API 20E test strip result for confirmed *Klebsiella pneumoniae* isolate

3.2 Screening and Selection of Biosurfactant Producers

Isolates were screened for biosurfactant production using the emulsification index (E24) and oil-spreading techniques. Seven of the twelve *K. pneumoniae* isolates tested positive, with isolate S9 exhibiting the highest activity (E24 = 45%, oil-spreading diameter = 10.5 mm) (see Figure 3 and Table 1). This screening is a reliable and rapid method for identifying potent biosurfactant producers [24, 32, 33].

Figure 3. Emulsification test showed a positive result

Kerosene and hexadecane were not emulsified by the cell-free broth of *Ochrobactrum anthropi* and *Citrobacter freundii* strains, but crude oil, diesel, motor oil, sunflower oil, and olive oil were [34]. An indirect technique for evaluating the generation of biosurfactants is an emulsification index. This test for determining the amount of biosurfactant is thought to be quick, easy and accurate [35]. While log phase biomass slowed production, an inoculum of stationary phase bacterial biomass at a percentage of 0.05% (w/v) was shown to be sufficient in initiating maximum biosurfactant production. At pH 2, 7 g/L of biosurfactant was produced using the acid precipitation method. FTIR spectroscopy was used to assign the surfactant to the glycolipopeptide class [32]. The biosurfactant was created as a main metabolite that accompanied the creation of cellular biomass (growth-associated kinetics), as evidenced by the fact that the biosurfactant biosynthesis utilizing olive oil mostly took place during the exponential growth phase [36].

Table 1. Biosurfactant production by *Klebsiella pneumoniae* isolates

Isolate	Biosurfactant Activity	
	Emulsification Index (E24)	Oil-Spreading (mm)
K. pneumoniae U1	41	9.1
K. pneumoniae U2	-	-
K. pneumoniae U3	33	7.5
K. pneumoniae U4	35	8.2
K. pneumoniae S5	-	-
K. pneumoniae S6	-	-
K. pneumoniae S7	-	-
K. pneumoniae S8	37	8.8
K. pneumoniae S9	45	10.5
K. pneumoniae S10	-	-
K. pneumoniae S11	39	8.2
K. pneumoniae S12	42	9.7

3.3 Extraction, Purification, and Characterization of Biosurfactant

Biosurfactant from the selected isolate (S9) was optimally extracted using a 2:1 chloroform-methanol ratio, yielding 8.13 g/L with an E24 of 52% (Figure 4). Subsequent purification via silica gel column chromatography yielded a fraction with an E24 of 59% (Figure 5). Acid precipitation prior to extraction proved effective, as biosurfactants become less soluble and precipitate at low pH, facilitating recovery [25, 37].

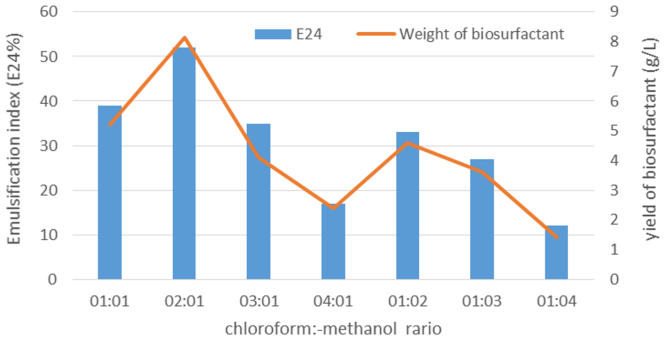

Figure 4. Biosurfactant yield extracted using different chloroform-methanol ratios

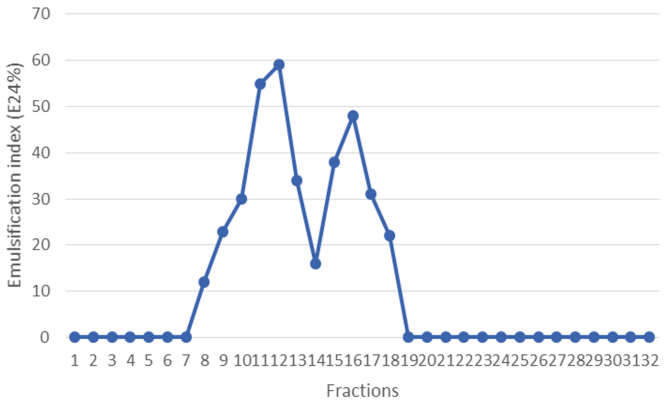

Figure 5. Elution profile of the biosurfactant from silica gel chromatography

Characterization via FT-IR spectroscopy indicated a lipopeptide structure. Key peaks included broad stretches at 3420–3282 cm^{-1} (N–H bond), 1642 cm^{-1} (C=O stretch, amide I), 1529 cm^{-1} (N–H bend, amide II), and 1220–1128 cm^{-1} (C–N stretch), consistent with peptide functional groups [26, 37] (Figure 6). TLC further confirmed the lipopeptide nature, with ninhydrin reagent revealing amino acid spots (Rf = 0.81) and iodine vapor detecting lipids

(*Rf* = 0.79) [2] (Figure 7).

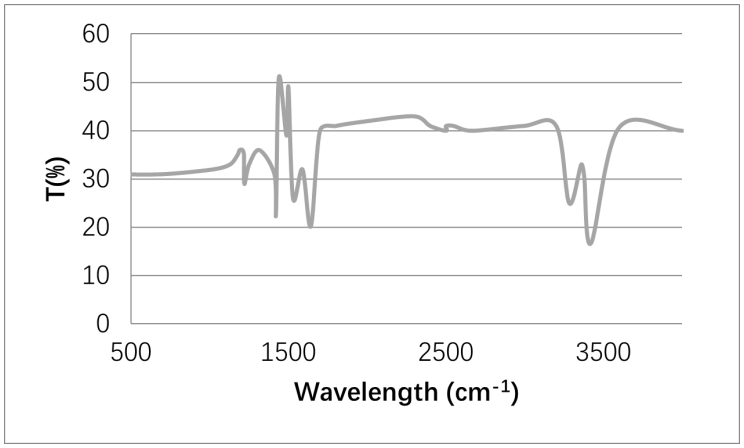

Figure 6. Fourier Transform Infrared Spectroscopy (FT-IR) spectrum of the purified biosurfactant

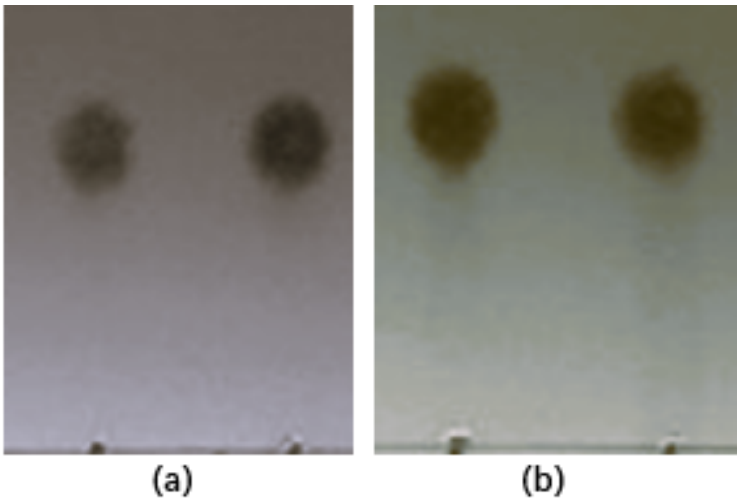

Figure 7. Thin-Layer Chromatography (TLC) analysis of the biosurfactant: (a) lipid detection with iodine; (b) amino acid detection with ninhydrin

The efficacy of the purified biosurfactant in enhancing kerosene degradation was evaluated over 14 days. Degradation efficiency increased with incubation time, reaching a maximum of 64% after 11 days, with no significant increase thereafter (Figure 8). The rate at which kerosene broke down in the environment went up steadily until it hit a plateau on day 11. This stability phase indicates that the most bioavailable and readily degradable constituents of kerosene were utilized within the initial 11 days. The following plateauing may be due to the remaining stubborn hydrocarbon fractions, the lack of important nutrients in the culture medium, or the buildup of metabolic intermediates that could slow down microbial activity under the current batch circumstances. *K. pneumoniae* S9's 64% kerosene degrading efficiency in just 11 days is an extremely competitive performance when compared to what has been published before. For example, Borah and Yadav [13] found that a comparable strain that breaks down hydrocarbons had a degradation rate of 55–60% over a longer period of 14 days. Moreover, although many investigations attained marginally superior cumulative degradation, they necessitated extended incubation durations; for instance, Meena et al. [37] reported a 70% degradation of hydrocarbons over 20 days. Our results also support the work of You et al. [28], which showed that *K. pneumoniae* is more effective in breaking down petroleum hydrocarbons. The high daily degradation rate in this study (approximately 5.8% per day) underscores the enhanced efficacy of the produced lipopeptide biosurfactant in increasing the bioavailability of kerosene, thereby expediting metabolic uptake in comparison to previously reported benchmarks [13, 28, 37–40]. The results underscore the potential of microbial biosurfactants as effective, eco-friendly agents for the bioremediation of petroleum-hydrocarbon-contaminated environments.

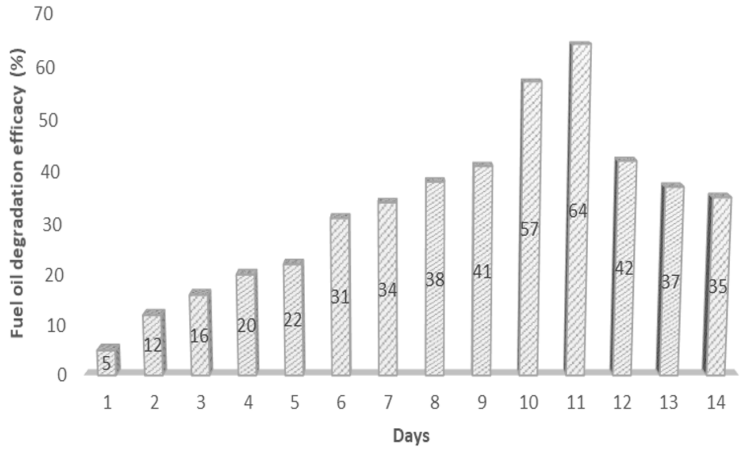

Figure 8. Time-course of kerosene degradation efficiency by the biosurfactant

4 Conclusions

This study demonstrates the potential of a native strain of *K. pneumoniae* (S9) as an effective agent for the bioremediation of kerosene-contaminated environments. The isolate was shown to produce a potent lipopeptide biosurfactant that significantly enhanced the degradation efficiency of kerosene, achieving 64% degradation within 11 days. The findings highlight a sustainable and promising strategy for mitigating petroleum hydrocarbon pollution. The use of bacteria isolated from contaminated sites ensures their adaptation and efficacy in such environments. Furthermore, the concomitant production of biosurfactants presents a key advantage by increasing the bioavailability of hydrophobic pollutants, thereby accelerating their breakdown. This research contributes to the development of inexpensive, efficient, and environmentally secures biological solutions for the restoration of ecosystems impacted by kerosene and other petroleum hydrocarbons. Future work should focus on optimizing large-scale biosurfactant production and evaluating the efficacy of this approach in field-scale applications. It is essential to acknowledge that the results of this study were derived from controlled laboratory batch conditions. These results show that *K. pneumoniae* S9 has a high potential for degradation, but in real-world situations or soil microcosms, it may not work as well because of things like soil heterogeneity, changing temperatures, and competition with native microbial communities. Hence, future research is necessary to assess the efficacy of this strain and its biosurfactant in actual contaminated environments to connect laboratory success with field application.

Author Contributions

Conceptualization, W.H.M. and S.N.M.; methodology, S.A.T.; validation, S.A.T. and H.M.A.; formal analysis, W.H.M.; investigation, S.A.T.; resources, H.M.A.; data curation, S.N.M.; writing—original draft preparation, S.A.T.; writing—review and editing, W.H.M.; supervision, S.N.M.; project administration, W.H.M.All authors have read and agreed to the published version of the manuscript.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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