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Bioremediation of Contaminated Soil with Hydrocarbons in Gas Stations with Alkane Hydroxylase Purified from *Pseudomonas florescens*

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Abstract

Petroleum product pollution of soil is a worldwide issue that threatens human and environmental health and destroys natural ecosystems. It also interferes with the ecosystem's ability to function properly. Thus, the necessity to clean up polluted locations is vital. Bioremediation is an economical and ecologically friendly approach. The ability of certain bacteria to proliferate in situations contaminated with oil makes bacterial alkane hydroxylases highly desirable for bioremediation applications. Additionally, this enzyme facilitates specific hydroxylation of chemically inert alkanes, which is useful for synthesizing expensive compounds like medicines. In this study with a high level of alkane hydroxylase at 40°C after 72 hours of incubation, *Pseudomonas fluorescens* was found to be the most effective alkane degrader, followed by *Pseudomonas aeruginosa*, which had a lower productivity for alkane hydroxylase. Upon reaching a peak after 96 hours with 67% degradation of the hydrocarbons, alkane hydroxylase at a concentration of 150 µg/ml removed 43% of the hydrophobic pollutant from the polluted soil following a 24-hour incubation period.

Keywords: alkane hydroxylase, *Pseudomonas* spp., bioremediation.

المعالجة الحيوية للتربة الملوثة بالهيدروكربونات في محطات الوقود باستخدام إنزيم ألكان هيدروكسيلاز المُنقّى من بكتيريا *Pseudomonas Fluorescens*

الخلاصة:

إن قدرة بعض البكتيريا على التكاثر في المواقع الملوثة بالزيت تجعل هيدروكسيد الألكان البكتيري مرغوبًا للغاية في تطبيقات المعالجة الحيوية. بالإضافة إلى ذلك، يسهل هذا الإنزيم عملية الهدرجة المحددة للألكانات الخاملة كيميائيًا، وهو أمر مفيد في تصنيع مركبات باهظة الثمن مثل الأدوية. في هذه الدراسة مع مستوى عالٍ من هيدروكسيد الألكان عند 40 درجة مئوية بعد 72 ساعة من الحضانة، وجد أن الزائفة المتألقة هي أكثر مزيلات الألكان فعالية، تليها الزائفة الزنجارية، التي كانت لديها إنتاجية أقل

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لهيدروكسيد الألكان. عند الوصول إلى الذروة بعد 96 ساعة مع تحلل الهيدروكربونات بنسبة 67%، قام هيدروكسيلاز الألكان بتركيز 150 ميكروغرام/مل بإزالة 43 % من الملوثات الكارهة للماء من التربة الملوثة بعد فترة حضانة مدتها 24 ساعة.

1. Introduction

Petroleum and natural gas both include large amounts of alkanes [1]. Alkanes are widely distributed and can infiltrate the environment through biological production, oil spills, and natural seepages [2]. Petroleum-based goods contain a wide spectrum of hazardous chemicals, including aromatic hydrocarbons, cycloalkane, and n-alkane [3]. Because of operating procedures and mishaps, contaminated areas may arise during extensive petroleum exploration, transportation, processing, storage, and use [4]. Fuel spills at gas stations can mostly be caused by subterranean storage tank leaks, which can contaminate the ground and soil. To protect human health and the equilibrium of ecosystems, contaminated water and soil should be cleaned up and restored [4, 5].

In oil-contaminated areas, removing long-chain hydrocarbons and n-alkanes is crucial to minimizing ecological harm. Bio-augmentation, on the other hand, is a very promising technique that necessitates very effective bacteria [6]. Both aerobic and anaerobic circumstances can result in biodegradation; however, aerobic processes proceed much more quickly and are more energy-efficient [7]. When considering the metabolic profile, two groups of hydrocarbon-degrading bacteria are identified: the first group includes members that have extended catabolic preferences and can use both aromatic and n-alkane hydrocarbons; the second group's members have limited metabolic capabilities and can only break down alkanes [8, 9]. Microorganisms that break down hydrocarbons can be found in a variety of settings, including pure ones. However, communities exposed to hydrocarbons become acclimated, and earlier exposure to hydrocarbons determines their abundance in varied habitats [10]. It has been determined that among microorganisms, bacteria are the primary and active agents in the hydrocarbon breakdown process [10, 11]. Hydrocarbon-degrading bacterial species are found in nature in large quantities and are not limited to a small number of taxa. Numerous bacterial taxa, including *Acinetobacter*, *Pseudomonas*, *Gordonia*, and *Rhodococcus*, have been identified as hydrocarbon degraders [12, 13].

Environmental microorganisms employ four distinct degradation pathways to oxidize n-alkanes. Although the enzymes metabolizing n-alkanes in different bacteria may change, all of the enzymes engaged in the first stage of the aerobic breakdown of alkanes belong to the alkane hydroxylase family [13,14]. The class of enzymes known as alkane hydroxylases, depending on the type of enzyme, adds atoms of oxygen produced from molecular oxygen to various locations of the alkane

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terminal [12, 15]. The current study's objectives were to isolate bacteria that break down hydrocarbons and separate alkane hydroxylase from polluted soils in a gas station and purify it for use as a bioremediation.

2. Materials and Methods

2.1. Soil samples sources

From polluted locations around the gas station, twelve soil samples were taken. From the top 0–20 cm of a plot next to a petrol station, a polluted soil sample was taken. After being moved to a laboratory and sealed in sterile vials, the soil sample was kept at -20°C until it was analyzed further.

2.2. Enrichment and screening of alkane hydroxylase producers

The soil samples were grown in Bushnell Hass broth with the addition of tetracotane (C40) as a carbon source. At 30, 35, 40, 45, and 50 °C for 24, 48, 72, and 96 hours, the following ingredients make up Bushnell Hass broth (g/ L):0.2 $MgSO_4$, 0.02 $CaCl_2$, KPO4 1.0; 1.0 K_2PO_4, 1.0 NH_4NO_3 and 0.05 $FeCl_3$ [16]. Following the incubation time, Centrifugation was used to extract each sample for 15 minutes at 4°C and 10,000 rpm. Following this, the samples were cleaned twice in a pH 7.0 potassium phosphate buffer. Using the same buffer, the pellet was sonicated for five minutes at 0°C. It was then centrifuged for twenty-five minutes at 20,000 rpm and 4°C. The supernatant was then maintained at 4°C.

2.3. Protein Determination

Using bovine serum albumin as a standard, the protein content was determined using the Bradford method for protein measurement depending on the standard curve of bovine serum albumin [17].

2.4. Alkane hydroxylase assay

The alkane hydroxylase assay was performed on the produced supernatant. 0.12% CHAPS buffer (pH 7.5), 10 mM Tris–HCl, 0.2 mM NADH, 20 µL of tetracotane solution (2% hexadecane in 80% DMSO), and 30 µL of crude enzyme were all present in the reaction mixture. 340 nm absorbance was used to measure enzyme activity. The activity of alkane hydroxylase activity was determined by applying the usual slope method for n-hexadecane [16].

2.5. Bacteriological characteristics

Depending on Bergey's Manual of Determinative Bacteriology the diagnosis and identification of bacterial isolates was performed using physiological analysis and biochemical testing [18].

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2.6. Alkane hydroxylase purification

Alkane hydroxylase was purified with some modifications as described in [16]. Ammonium sulfate precipitation was used to purify alkane hydroxylase, and saturation ratios between 30 and 75% were attained by continuously swirling the mixture in an ice bath for 30 minutes. After that, the enzyme was dissolved in phosphate buffer saline (PH 7.5) and centrifuged for 30 minutes using cooling centrifugation to measure crude enzyme activity. After being dialyzed against the same buffer for the entire night at 4^{o}C in a dialysis tube, the resultant ammonium sulfate precipitates were concentrated with sucrose. The sample was loaded onto a 2.5 x 60 cm Sephadex G-200 column that had been pre-calibrated with the same buffer after the dialysis procedure, and the same buffer was also used for the elution. For additional research, the concentrated active fractions were used. They were also analyzed for protein content and enzyme activity.

2.7. Biodegradation efficacy of the purified alkane hydroxylase

Remaining concentrations resulting from the aerobic n-alkane breakdown process in polluted soils with the method that described by Aboulkacem and Maroui [14] was modified to assess the residual concentrations resulting from the aerobic breakdown of n-alkanes by alkane hydroxylase in polluted soils containing hydrocarbons. The following treatments were applied to 5 g of each contaminated soil sample: For the treatment, 10 mL of purified alkane hydroxylase solution at concentrations of 25, 50, 100, 150, and 200 µg/mL was used as the control, and it was incubated for 7 days at 30°C. Centrifugation at 5000 g for 20 minutes was used to separate the sediment and supernatant. Using separating funnels, a double volume of chloroform was then added to the supernatant. After that, 2g of anhydrous sodium sulfate was added to the extracted solution in order to remove any remaining moisture. The remaining hydrocarbon-containing chloroform phase was then cautiously decanted and given time to air dry. The amount of residual oil that remained after the chloroform evaporated was measured.

$$\text{B}i\text{o}de\text{g}ra\text{d}a\text{tion percentage (\%)} = (Pi/P\text{r}–Pe\text{v}) \text{ x}100$$

Where; Pi was the original n-alkane quantity, Pev was the removed n-alkane quantity, and Pr was the residual n-alkane quantity.

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3. Results and Discussion

3.1. Enrichment and screening of alkane hydroxylase producers

Twelve soil samples were cultured for 24 hours at 30°C in Bushnell Hass broth supplemented with tetracotane. Sample 7 showed more alkane hydroxylase activity than the other samples, as shown in Figure (1). From wastewaters contaminated with hydrocarbons, It was discovered that certain bacterial strains can grow on discarded oil and provide both carbon and energy It was discovered that certain bacterial strains can grow on discarded oil and provide both carbon and energy [19]. The bioremediation of oil-polluted sites may employ *Pseudomonas* spp. and *Micrococcus* spp. A crucial requirement for the effective bioremediation of severely polluted areas is the ability of these hydrocarbon-degrading bacteria to withstand high concentrations of petroleum [20, 21].

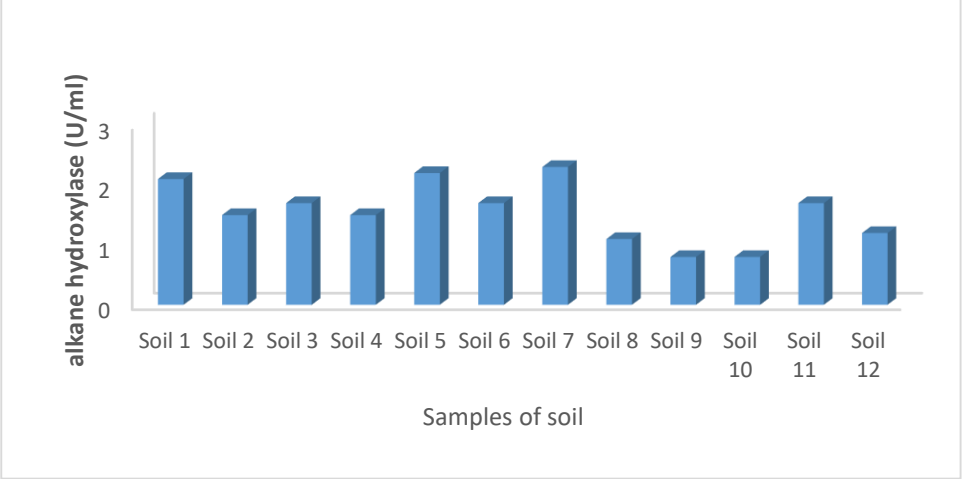

Fig. (1): Detemination of alkane hydroxylase production in hydrocarbon polluted soils

When tetracotane was used as a carbon source, as shown in Figure (2), the influence of temperature on alkane hydroxylase production was investigated. The findings indicated that 40°C was the ideal temperature for alkane hydroxylase synthesis.

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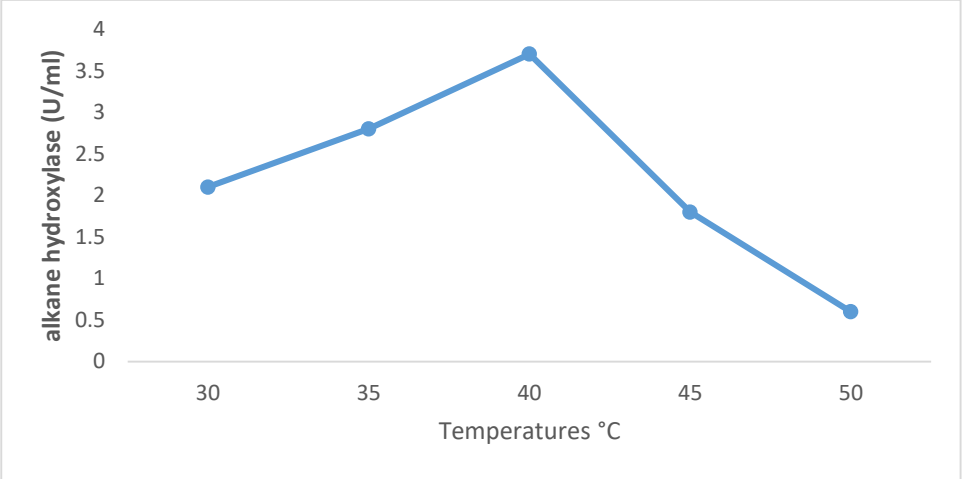

Fig. (2): Effect of tempertures on alkane hydroxylase production

It was also tested how different incubation times affected the synthesis of alkane hydroxylase from soil samples. The results show that after 72 hours, alkane hydroxylase productivity was at its peak, Figure (3).

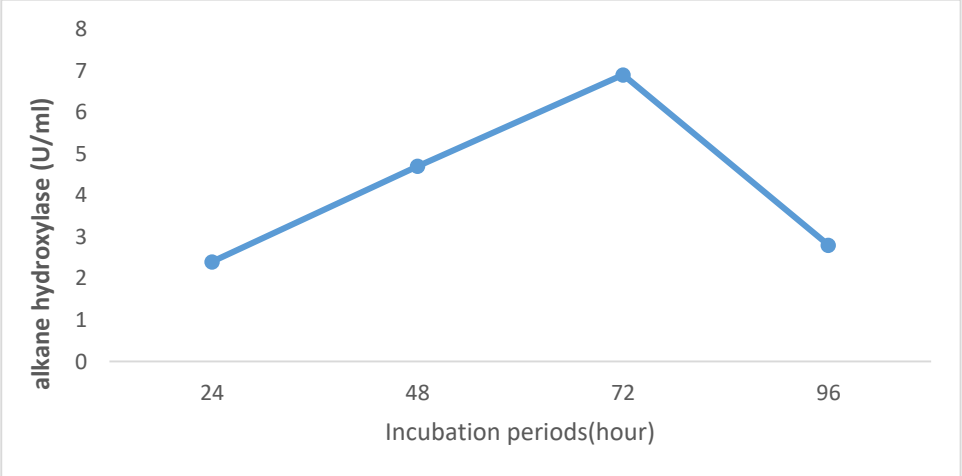

Fig. (3): Effect of incubation periods on alkane hydroxylase production

3.2. Morphological and biochemical characteristics

The producing isolates were identified as *Pseudomonas* spp. based on their biochemical, morphological, and cultural characteristics. Of these isolates, four were identified as *Pseudomonas aeruginosa* and eight as *Pseudomonas fluorescens*, with the latter having lower productivity of alkane hydroxylase. Petroleum hydrocarbon-contaminated soil samples that contained the separated microorganisms. According to [22] these isolates may be able to break down contaminated soils, but how well they do so would depend on the biological, chemical, and

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physical factors present. One typical hydrocarbon degrader is *Pseudomonas*. *Pseudomonas* strains should be kept separate from locations where petroleum waste is discharged due to their capacity to break down petroleum hydrocarbons [20, 23].

3.3. Alkane hydroxylase purification

Following the precipitation of ammonium sulfate saturation at 55% using 13.6 U/ml, alkane hydroxylase was put onto a Sephadex G-200 column. Two protein peaks emerged when the phosphate buffer saline was used for elution; the first peak included alkane hydroxylase activity, leading to the purification of alkane hydroxylase with a final yield of 41% and 18.3 U/ml as enzymatic activity, as shown in Table (1) and Figure (4).

Table (1): Purification of alkane hydroxylase from *Pseudomonas fluorescens*

Purification step	Size (ml)	alkane hydroxylase activity (U/ ml)	Protein conc. (mg/ml)	Specific activity (U/ mg)	Yield (%)
Crude extract	53	9.9	2.6	3.80	100
$(NH_4)_2SO_4$ precipitation	18	13.6	1.4	9.71	46
Sephadex G-200	12	18.3	1.1	16.6	41

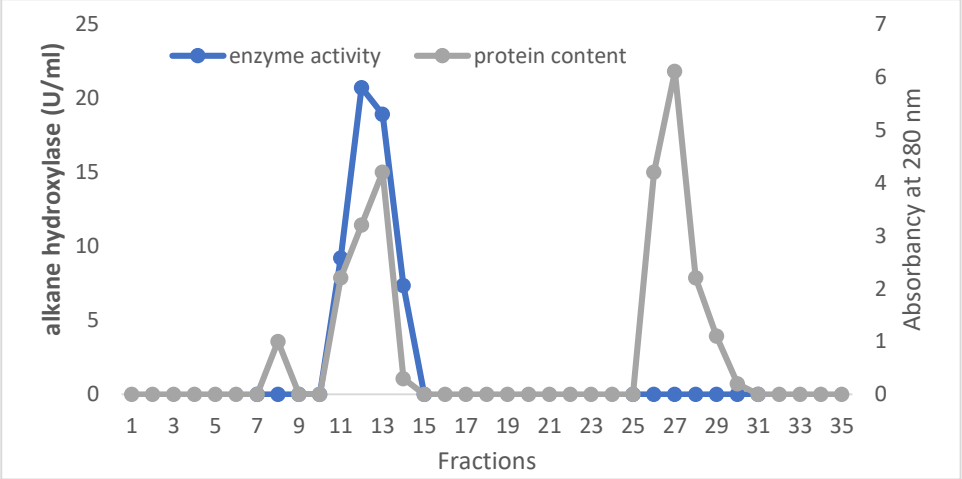

Fig. (4): Gel filtration for Purification of alkane hydroxylase from *Pseudomonas fluorescens*

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3.4. Biodegradation efficacy of the purified alkane hydroxylase

Figure (5) shows the percentage of n-alkanes that were broken down by the purified alkane hydroxylase from the contaminated soil containing hydrocarbons. After incubating for 24 hours, the data unambiguously shows that 150 µg/ml of alkane hydroxylase improved the elimination of hydrophobic pollutant (43%), from polluted soil. The hydrocarbons also broke down over time as the incubation period increased, peaking at 96 hours with a degradation rate of 67% before progressively falling. Consequently, bioremediation has gained recognition as an alternate technique for clearing oil-contaminated environments. Microbial colonies are vital to this process because they either directly destroy pollutants or interact with other additional microbes.

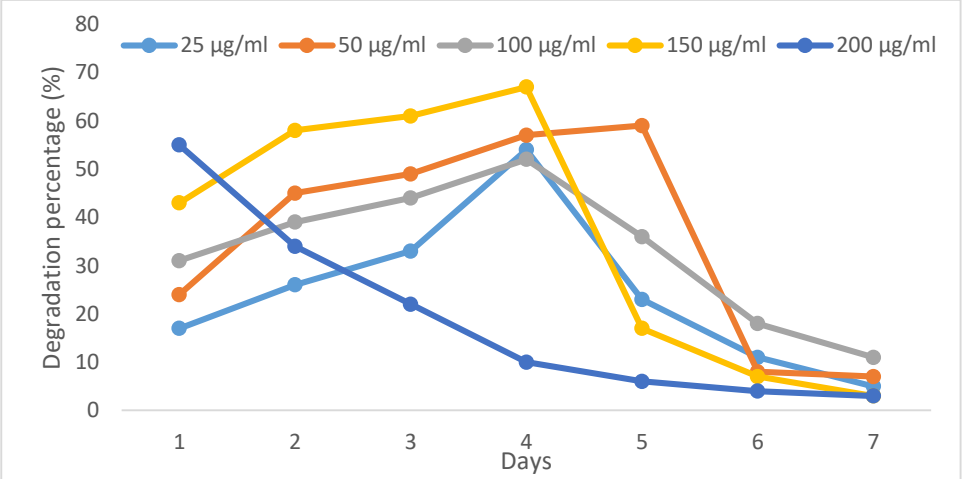

Fig. (5): Removal of hydrophobic pollutant from polluted soils with different concentrations of alkane hydroxylase and different incubation periods

One of the main ways in removal of petroleum hydrocarbons from environment is by natural populations of microorganism's biodegrading them [3]. The alkB gene was present in the degrading strains, which was supported by their strong alkane degradation capabilities. The capacity of these strains to break down hydrocarbons suggests that they could be useful in the bioremediation of areas contaminated by hydrocarbons. [12]

4. Conclusions

An alkane hydroxylase produced by *Pseudomonas fluorescens* considered as an important tool for bioremediation for soil contaminated with hydrocarbons when it used at limited concentration ant at certain temperature and incubation period. Therefore, the bioremediation of oil-contaminated environments with enzymes such as alkane hydroxylase considered as an alternate technique for clearing for cleaning up hydrocarbon-contaminated areas.

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