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Diesel Biodegradation by Selected Bacterial Species isolated from Contaminated Soils

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Abstract. Diesel soil contamination is considered a serious environmental problem with widespread negative effects on the environment, human health, and living organisms. The aim of this study is to investigate the efficiency of some local bacterial species to degrade diesel. Bacterial species were isolated from soils contaminated with hydrocarbons from areas south of Babylon Governorate . To estimate the efficiency of biodegradation of diesel, gravimetric analysis was used. The results showed that: *Enterobacter cloacae* complex degraded 44.44% (1%, 10 day) and 57.14% (1%, 15 day); 50% at 3% for both periods. *Escherichia hermannii*: 11.11% (1%, 10 day), 14.28% (1%, 15 day); 13–20% at 3%. *Citrobacter sadlakii*: 28.57% (1%, 10 day), 66.70% (1%, 15 day); 30–62.50% at 3%. *Staphylococcus lentus*: 66.70% (1%, 10) day, 71.42% (1%, 15 day); 30–37.50% at 3%. *Aeromonas salmonicida*: 33.33% (1%, 10 day), 42.85% (1%, 15 day); 40–50% at 3%. *Sphingomonas paucimobilis*: 22.22% (1%, 10 day), 28.57% (1%, 15 day); 20–25% at 3%. All six bacterial species possessed the *alk* gene (confirmed by PCR), responsible for petroleum hydrocarbon degradation. These strains show strong potential for bioremediation of diesel-polluted sites.

Keywords. Diesel Biodegradation, bacteria, *alk* gene

1. Introduction

Diesel soil contamination is considered a serious environmental problem with widespread negative effects on the environment, human health, and living organisms. This contamination often arises from fuel spills during transportation, storage, or usage, whether caused by accidents, leaks, or improper practices [1,2]. Diesel contamination alters the physical properties of the soil, impeding the infiltration of water and air, which negatively affects root aeration and plant growth. It also changes the soil texture and forms an oily layer. Additionally, there are chemical changes, as the increased concentration of hydrocarbons leads to altered pH levels and a reduction in essential nutrients for plants[3,4]. therefore, it has become essential to resort to bioremediation, as it is a modern, promising, and environmentally friendly technology that is effectively used to remove a wide variety of organic pollutants, including diesel fuel, from both soil and water. The concept of

bioremediation is based on the natural ability of microorganisms to exploit these pollutants as a primary source of energy and carbon necessary for their growth and reproduction. These microorganisms break down the pollutants through a series of successive metabolic reactions, where the chemical bonds of the pollutants are gradually broken, enabling the removal of a wide range of hydrocarbons present in diesel [5,6]. Many previous studies in addition to our current study, have proven that there are certain types of bacteria that have the ability to decompose diesel and use it as a sole source of carbon due to their genetic ability and the fact that these types of bacteria possess the akl gene responsible for decomposing diesel[7,8]. Among the most important microorganisms are: *Sphingomonas paucimoblis bacteria, Escherichia hermannii bacteria Pseudomonas ،Acinetobacter ،Rhodococcus Staphylococcus lentus Alcanivorax, Bacillus, and fungal species, Aspergillus ،Penicillium* [9].The main objective of this study is to use microorganisms to treat diesel polluted environments in a modern, environmentally friendly and inexpensive way [10].

2. Materials and Methods

2.1. Bacterial Strains

Enterobacter cloacae complex ,Escherichia hermannii, Citrobacter sedlakii,Staphylococcus lentus , Aeromonas salmonicida ,Sphingomonas paucimobilis, These bacterial species were isolated from diesel-contaminated soil as mentioned[11,12]. and identified using a VITEK2 compact system.

2.2 Diesel degradation assay

The bacterial species were activated by nutrient broth medium for 24 hours, Then the samples were placed in a centrifuge at 3600 rpm for 10 minutes, the activation medium was discarded, the cells were washed 3 times with saline solution, then 10 ml of liquid Bushnell has mineral salt was added , and an inoculum OD600 = 0.5-0.8 was obtained Then the bacterial inoculum was added to 90 ml of medium containing 1% and 3% diesel in 250 ml flasks and placed in the shaking incubator 150 rpm at 30 degrees for 10 and 15 days, after the end of the incubation period, Add 1% 1N HCL To inhibit the action of bacteria on the growing culture, the remaining diesel is then extracted by adding petroleum ether and aceton with a ratio of 1:1 volume/volume ,the flask is then placed in the shaker at 120 for 20 minutes, after which the solvent containing the diesel is collected from the top of the flask and poured into a pre-weighed Petri dish. the extracted components are allowed to evaporate by placing the Petri dish containing them in oven at a temperature of 72°C, the dish is then weighed with the remaining sediment, and then the weight of the dish is subtracted to obtain the weight of the remaining diesel, and the percentage of decomposition is calculated according to equation

$$\text{Percentage of decomposition \%} = \frac{\text{Diesel initial concentration - final concentration}}{\text{Initial concentration}} \times 100 \quad [13]$$

2.3 Detection of the gene alkB through PCR testing

DNA extraction: activate all bacterial species in nutrient broth for 24 hours at 37°C. Place the samples in bendorf tubes in a centrifuge for 5 minutes[14], then discard the activation medium. Wash the cells with saline solution and discard it. add 200 µL Tris-EDTA and phenol solution. Place the collection tubes on a vortex for 1 minute, then in a centrifuge for 5 minutes. Then, take 160 µL then we add Tris-EDTA, put the sample on a vortex and then in a centrifuge for 5 minutes, again we take 160 µL and repeat adding chloroform, put the sample on a vortex and then in a centrifuge for 5 minutes and then take 150 µL for the purpose of DNA examination. Polymerase chain reactions (PCR) were performed using the primers listed in

Table number (1), The contents of the PCR mixture were as follows: master mix, volume 25 µL, template DNA, volume 5 µL, forward primer volume 3 µL, reverse primer 3 µL, nuclease free water 14 µL, where the final total volume was 50 µL[15]. The thermal cycler program for the polymerase chain reaction (PCR) was set to an initial denaturation step of 5 minutes at 94°C, followed by 30 cycles, each of which included a denaturation step of 1 minute, followed by an annealing step at 58°C for 1 minute, an extension step at 72°C for 1 minute, and a final extension step at 72°C for 10 minutes. The resulting bands were detected by 1.5% agarose gel, separated by electrophoresis, and visualized by a Bio-Rad ChemiDoc MP after staining with ethidium bromide.

Table 1. Primer sequences used to detect the gene responsible for diesel degradation [16]

Primer	sequence	length
alk-2F	GAGACAAATCGTCTAAAACGTAA	22
alk-2R	TTGTTATTATTCCAACTATGCTC	22
alk-3R	CCG TAGTGCTCGACGTAGTT	20
alk-3F	TCGAGCACATCCGCGGCCACCA	22

3. Results and Discussion

3.1. Diesel Degradation Ability by the tested bacterial strains

Results showed that the *Enterobacter cloacae complex* was able to degrade diesel by 44.44% during 10 days at a diesel concentration of 1% and 57.14% period 15 days at the same concentration, while at a diesel concentration of 3% the average degradatioin rate through 10 days was 50% and also at 15 days the degradation rate was 50%, In a previous study[17]. these bacteria recorded a very high rate of decomposition of phenolic compounds at low concentrations, as shown in Figure 1. As for *Escherichia hermannii bacteria*, a low degradation rate was recorded at a 1% diesel concentration at in 10-day period, reaching 11.11%. during 15-day , the degradation rate increased slightly, reaching 14.28%. at a 3% diesel concentration, the degradation rate period 10 days reached 13%, and at 15 days, the degradation rate also increased slightly, reaching 20%, However, in a previous study[18]. these bacteria recorded a high rate of degradation of phenolic compounds at low concentrations. as shown in Figure 2. As for the results of *Citrobacter sadlakii bacteria* at a concentration of 1%, decomposition rate was recorded at 28.57% , period 10 days, and increase in the degradation rate was recorded over a period of 15 days, reaching 66.70%. As for the concentration of 3% and through 10 days, it was recorded at 30%, and the decomposition rate also increased over a period of 15 days, reaching 62.50% This bacteria was also mentioned in a previous study to decompose phenolic compounds[19][20]. as shown in Figure 3 , *Staphylococcus lentus bacteria* showed a 66.70% degradation rate after 10 days at a 1% diesel concentration. The highest degradation rate among the six bacterial species was recorded over 15 days at the same concentration, reaching 71.42%. However, this rate decreased at a 3% concentration, reaching 30% after 10 days and 37.50% over 15 days as in a previous study [21]. As shown in Figure 4. While the results for *Aeromonas salmonicida bacteria* showed a 33.33% degradation rate after 10 days at a 1% diesel concentration, the degradation rate increased slightly over a 15-day period, reaching 42.85% at the same

concentration. The degradation rate continued to increase at a 3% concentration, reaching 40% over a 10-day period and 50% after a 15-day period , as in a previous study [22] . As shown in Figure 5, The last type of bacterial strain used in this study was *Sphingomonas paucimoblis bacteria.* Which recorded relatively low decomposition rates at a concentration of 1%, reaching 22.22% over a period of 10 days, and 28.57% over a period of 15 days. At a concentration of 3% diesel, the rate reached 20% over a period of 10 days, and 25% over a period of 15 days, as shown in Figure 6. The results of the current study are similar to previous studies [23]

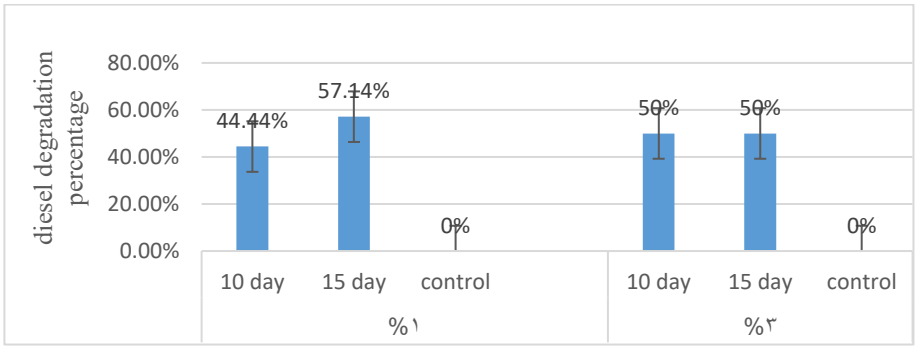

Figure 1: The percentage of diesel degradation by *Enterobacter cloacae complex*

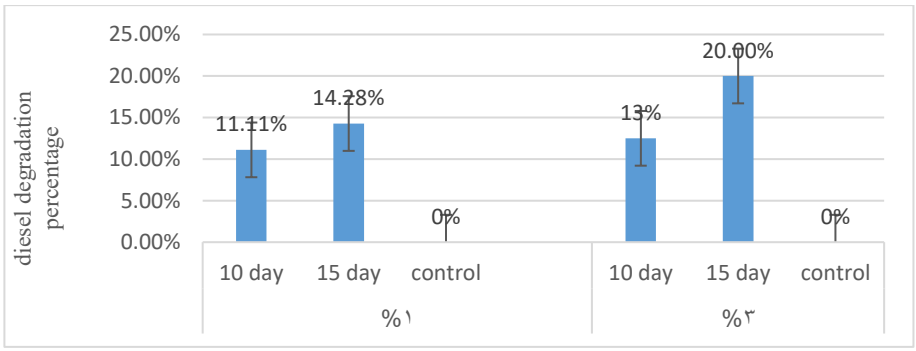

Figure 2: The percentage of diesel degradation by *Escherichia hermannii bacteria*

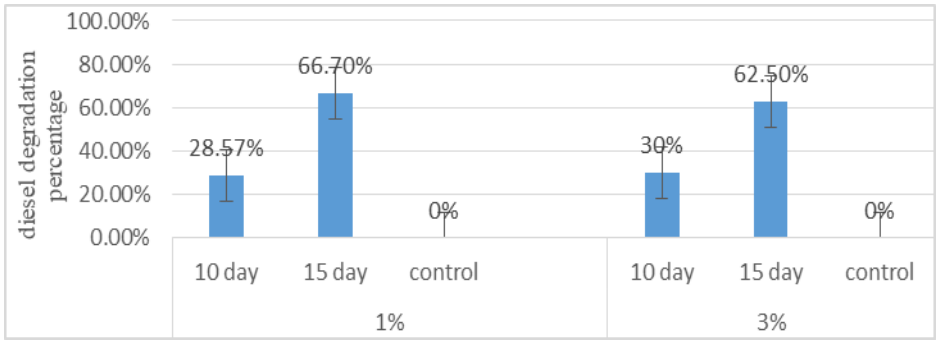

Figure 3: The percentage of diesel degradation by *Citrobacter sadlakii bacteria*

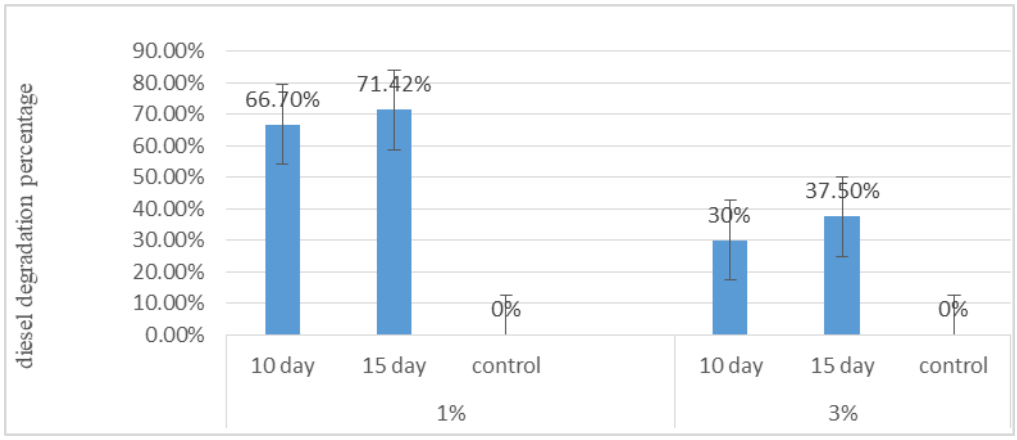

Figure 4: The percentage of diesel degradation by *Staphylococcus lentus bacteria*

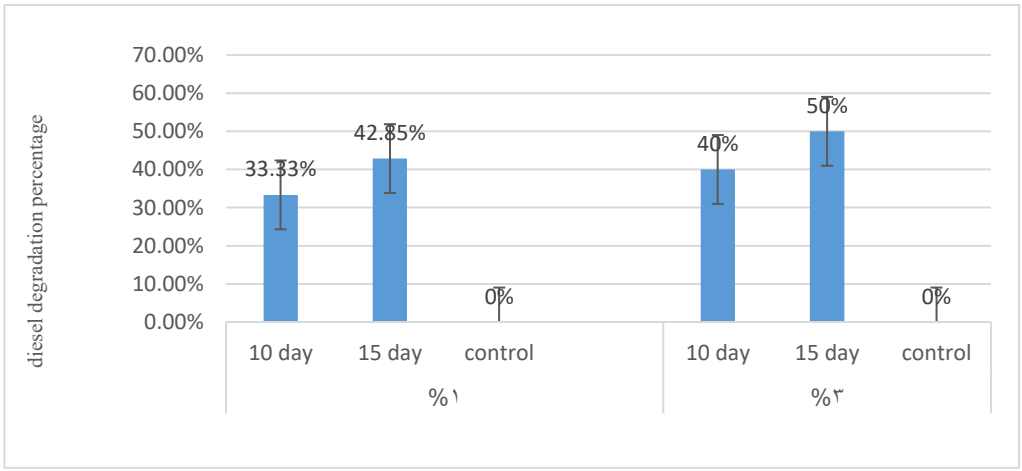

Figure 5: The percentage of diesel degradation by *Aeromonas salmonicida bacteria*

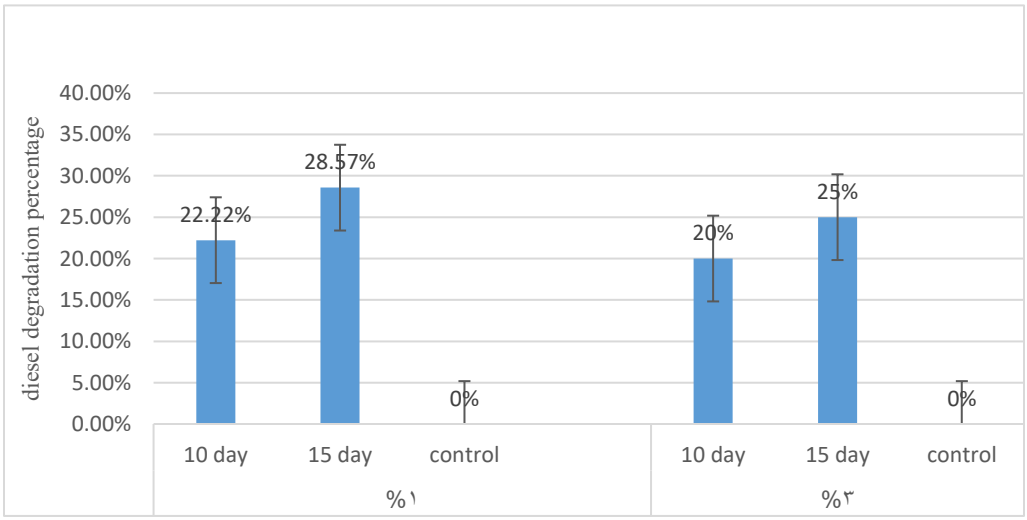

Figure 6: The percentage of diesel degradation by *Sphingomonas paucimoblis bacteria*

3.2 detection of alk gene responsible of diesel degradation

PCR results showed that (1) enterobacter cloacae complex was only positive for alkM gene Since this gene analyzes long-chain alkanes, diesel is considered a long-chain alkanes, as mentioned in the study [24]. while other bacterial species were only negative and did not contain this gene. On the other hand, (2) Escherichia hermannii,(3) citrobacter sadlakii,(4) staphylococcus lentus, (5)Aeromonas salmonicida, and (6) sphingomonas paucimoblis contained the second gene alkB. The presence of this gene in these bacteria means that they are able to decompose diesel because it contains long-chain alkanes[25].

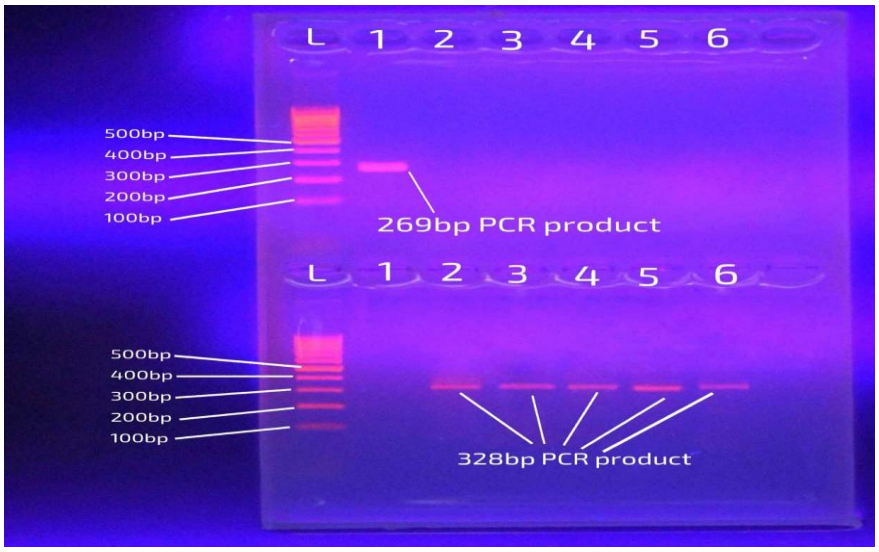

Figure 7. Agarose gel electrophoresis of PCR products The expected size of gene alk1was (269bp) , while the expected size of gene alk2 was (328bp).

Conclusion

Strains of *Enterobacter cloacae complex* , *Escherichia hermannii* ,*Citrobacter sadlakii* ,*Staphylococcus lentus,Aeromonas salmonicida* ,and *Sphingomonas paucimoblis* may be considered as potential means for the treatment of various diesel contaminated environments. The most efficient isolate in diesel degradation in the current study was *Staphylococcus lentus* followed by *Citrobacter sedlakii*.

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