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Comparative analysis of the efficacy of free and immobilized bacteria in degradation of diesel oil

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Abstract
In this study, bacterial strain D-4 was isolated from petroleum contaminated sites and was considered as efficient diesel oil degrader, based on its ability to survive up-to 14% concentration of diesel oil. D-4 was morphologically and biochemically characterized as gram (+) bacteria rough colony surface. Isolate D-4 was immobilized in sodium alginate beads and subjected to viability test by storing at 25 °C and 4 °C for 60 days. It was discovered that the bacteria immobilized in alginate beads was most viable when stored at 25 °C, while they were also reasonably viable at 4 °C. Free and immobilized cells of D-4 were also analyzed for their ability to degrade diesel oil in Bushnell Hass broth supplemented with 1% (v/v) diesel oil for a period of 35 days. Significantly higher degradation rates were observed when diesel oil was treated with immobilized cells of D-4 as compared to free cells and control. This study suggests that immobilization can be a more efficient option for remediation of contaminated sites as compared to employing bacteria in freely suspended state.

Keywords: Immobilization, bacteria, bioremediation, petroleum hydrocarbon, sodium alginate

1. Introduction
Unintentional and purposeful introduction of hazardous organic pollutants including petroleum hydrocarbons into the environment is a matter of serious concern. Numerous anthrophogenic activities allow petroleum hydrocarbons to enter the environment frequently and in substantial quantities [1]. Notably, the process of oil extraction, transportation, storage, and consumption could potentially contaminate soil or water, posing a risk to human health and the environment [2, 3]. Environmental contamination due to petroleum products has now a widespread issue in both industrialized and developing nations. Therefore, it is crucial to identify environmentally safe environmental remediation techniques that can manage the complications caused by petroleum contamination [4, 5].

Degradation of oil pollutants by employing microbes has become the most popular approach for treating contaminates sites because of its advantages of low cost, complete mineralization and no secondary pollution [6, 7]. However, maintaining a high biomass of bacterial populations is essential for bioremediation since freely suspended bacteria frequently engage in competition with local microbes, neutralizing the benefits of the dominating bacteria [8, 9]. In addition, certain exogenous cultivated microorganisms are tested in soft environments and may find it challenging to adapt to natural environmental circumstances [10, 11]. Bacterial cells need to be immobilized in order to increase the survival and retention of the bioremediation agents in the contaminated locations [12]. Immobilisation is a method of entrapping bacteria in polymeric matrices, which increases the bioremediation efficiency through higher microbial density and makes them more resistant to the harsh environmental conditions and other microbes [13, 14, 15]. For immobilization, a range of natural and synthetic carrier materials have been employed, including inorganic, polymeric, and composite materials [16]. Because of their increased stability and longevity, immobilized cells have been widely exploited in the synthesis of valuable compounds, wastewater treatment, and bioremediation of pollution [12].

Currently, agar, gluten, polyacrylamide (PVA) and sodium alginate (SA) are among the immobilization materials that have been documented in the literature. But while selecting the carrier materials, its characteristics must be taken into account. SA are superior to other porous materials in terms of mechanical strength, microbial toxicity, mass transfer efficiency, ease of microbial decomposition, and cost. In the present investigation, sodium alginate (SA) was chosen as the carrier material for entrapping bacterial cells because they are inexpensive and conveniently accessible porous hydrophilic gels. The study was conducted with following objectives: (i) investigate the effect of immobilization viability of diesel degrading bacterial

isolates. (ii) Explore the difference in diesel degradation efficiency between immobilized bacteria and free suspended bacteria

2. Materials and methods

2.1 Oil sample

The diesel oil was purchased from an oil filling station located on the campus of G. B. Pant University of Agriculture & Technology, Pantnagar, Uttarakhand. It was sterilized using 0.2 m membrane filters, the diesel oil was stored at 4 °C in a conical flask for further research [17, 18].

2.2 Isolation of Bacteria

Soil samples were collected from sites contaminated with diesel oil. Further, the techniques of enrichment and serial dilution were used for isolating bacteria from the soil samples. 10 g of soil sample was dissolved in 100ml Bushnell Hass broth supplemented with 1% (v/v) diesel oil and incubated at 30 °C and 120 rpm for 7 days. After 7 days of incubation, enriched sample was serially diluted from 10^{-1} to 10^{-6} dilutions. 100 µl of sample from 10^{-4}, 10^{-5} & 10^{-6} dilutions was subsequently taken and spread into petri plates containing nutrient agar and plates were incubated for 24 hours at 28 °C. Morphologically different bacterial colonies were chosen and purified. For subsequent use, purified bacterial isolates were stored in slants and glycerol stock.

2.3 Screening of potential bacterial strain for diesel degradation

Bacterial cultures purified on nutrient agar plates were inoculated in BH broth media supplemented with 1% (v/v) diesel and kept at 28 °C, 120 rpm for 7 days and screened by recording their OD at 600 nm after 7 days of incubation. 12 bacterial isolates with maximum growth were screened by determining Minimum Inhibitory Concentration (MIC) of diesel oil which is diesel concentration at which no growth occurs. In order to determine MIC, selected diesel oil-degrading bacterial isolates were cultured on BH agar plates with progressively higher concentrations of diesel oil until no growth occurred. 24-48 hours old bacterial colonies were streaked in freshly made BH agar plates over-layered with gradually increasing concentration of diesel oil 2%, 4%, 6%, 8%, 10%, 12%, 14%, 16% & 18%. The plates were subsequently incubated for 3 days at 30 °C in an upright position such that the diesel oil remained in contact with the agar surface. Bacterial isolate (D-4) exhibiting maximum MIC was finally selected and regarded as efficient diesel oil degrader.

2.4 Morphological and Biochemical Characterization

Bacterial isolate (D-4) was streaked on nutrient agar plates, and after 24 hours of incubation at 30 °C, its morphological & cultural characteristics, including the margin, cell shape, size, elevation, surface, and Gram's staining, were noted. Additionally, for its biochemical characterization, gelatin liquefaction, catalase, oxidase, casein hydrolysis, carbohydrate utilization pattern, and IMViC test were carried out.

2.4.1. Gelatin hydrolysis

10% gelatin was added to nutrient broth (NB) to create the gelatin tubes, which were then autoclaved. The tubes were inoculated with a live, growing bacterial culture and kept at 30 °C for one to two days. The tubes were then placed in ice, and their liquefaction or solidification was observed. Tube liquefaction is regarded as positive and tube solidification as negative.

2.4.2. Catalase

To assess catalase activity, actively growing culture was transferred to clean glass slide with a sterile loop. A drop of 3% H_2O_2 was added to it and observed for evolution of oxygen bubbles. The generation of bubbles denotes a successful outcome.

2.4.3. Oxidase test

When a drop of actively growing culture from NB was added to an oxidase disc, the disc's colour changed from white to purple, signalling positive outcomes.

2.4.4. Casein hydrolysis

In separate flasks, nutrient agar and skim milk agar were prepared and autoclaved. Just before pouring onto plates, the contents of both flasks were combined. Plates were poured, allowed to solidify, and then inoculated with short, thick streaks in the centre. Plates were incubated for 48–72 hours at 30 °C. Observations were made to see if distinct zones appeared which would indicate a favourable reaction.

2.4.5. IMViC & Carbohydrate utilization

With the help of a standardized colorimetric identification KB001 HIIMViCTM biochemical test kit (bought from (Hi Media Laboratories Pvt. Limited., India), selected bacterial isolates were subjected to IMViC and carbohydrate utilization test.

2.6 Immobilization using Alginate Beads

For immobilization using sodium alginate, 300 mL of 3% Sodium alginate and 100 mL of 0.2 M $CaCl_2$ were made and autoclaved separately. Bacterial culture was allowed to grow in nutrient broth for 48 hours and centrifuged, from which pellets were obtained. The pellet so obtained was mixed with 50 mL of 3% sodium alginate (1:1 w/v) and stirred completely. The mixture was then filled into a sterile syringe and dispensed drop-wise into chilled solution of 0.2 M $CaCl_2$ where formation of beads took place. Then, beads were kept at room temperature for 60 min to allow their solidification. The beads were incubated with 2mL $CaCl_2$ in sterile glass tubes with caps and maintained at 4 °C and 25 °C for 60 days [19].

2.6 Determination of viability of immobilized cells

Furthermore, in order to check viability of bacterial isolate encapsulated in sodium alginate beads, 1 g of beads stored at 25 °C and 4 °C were finely grounded using a sterile pestle & mortar. Serial dilution was then performed to determine bacterial CFU on agar plates using spread plate method. The experiments were repeated at interval of 10 days up-to 60 days in order to check the viability of bacterial isolates under different storage materials and at different temperatures [19].

2.7 Effect of immobilization on diesel oil degradation

Diesel oil degradation efficiency of free and immobilized cells of D-12 was analyzed by studying degradation of diesel oil in BH broth medium at every 7 days intervals up-to 35 days. In order to determine the biodegradation efficiency of

free cells, 24-48 hours old cultures of isolated bacterial strains was inoculated into 100 mL flasks containing 50 mL nutrient broth supplemented with 5 g diesel oil. While for determination of biodegradation efficiency of immobilized cells, 15 immobilized beads were inoculated in 50 mL of nutrient broth medium supplemented with diesel oil (1% v/v). A control containing only nutrient broth and diesel oil (and no bacterial isolate) was also maintained. Incubation of flasks was done at 30 °C & 120 rpm for 7 days. After incubation, samples were taken and subjected to gravimetric analysis for the assessment of diesel oil degradation. The diesel degradation efficiency of the free cells and immobilized cells was then compared.

2.7.1. Gravimetric analysis

Broth containing free and immobilized bacterial cells was centrifuged for 30 minutes at 10000 *g*. While the bacterial biomass pellet was discarded, the spent broth was used to measure the amount of oil degradation. Additionally, solvent extraction procedure utilizing a separating funnel is employed to separate the residual diesel oil from the spent broth [20]. The supernatant was placed in a separating funnel, 10 mL of n-hexane was added, and the mixture was forcefully shaken. Then, by keeping the separating funnel upright on a stand, it was left undisturbed for 5 minutes resulting in the formation of two different layers: upper organic layer containing n-hexane and oil mixture; lower solvent layer containing spent broth. Following that, the solvent layer was separated by gently passing through a funnel lined with filter paper and collecting in a 50-mL flask. To guarantee that all of the leftover diesel oil was removed, this process was performed three times. The extractant solvent (n-hexane) was allowed to evaporate at 60 °C by setting the flask containing the extract (diesel and n-hexane) on a hot plate. Following that, remaining diesel oil from the upper layer was gathered in the pre-weighed beaker. The weight of a beaker containing remaining diesel oil was also noted. The percentage of degradation for residual diesel was then determined using the formula below. (Liu *et al*., 2016; Bekele *et al*., 2022; Sihag and Pathak, 2022) [17, 22, 23].

Weight of Residual diesel oil= Weight of beaker containing extracted diesel oil – Weight of empty beaker.
Amount of diesel oil degraded = Weight of diesel oil added in the media – Weight of residual diesel

$$\% \text{ degradation} = \frac{\text{Amount of diesel oil degraded}}{\text{Amount of diesel oil added in the media}} \times 100$$

3. Results & Discussion

3.1. Isolation and Screening

A total of 30 bacterial cultures were isolated from enriched soil and were analyzed for their growth in BH broth supplemented with diesel oil observed by recording OD at 600 nm (Figure 1). Further, 12 isolates showing maximum OD at 600 nm wavelengths were selected and considered as potential diesel oil degraders. These 12 selected strains were designated as D-1, D-2,D-3,….,D-12.

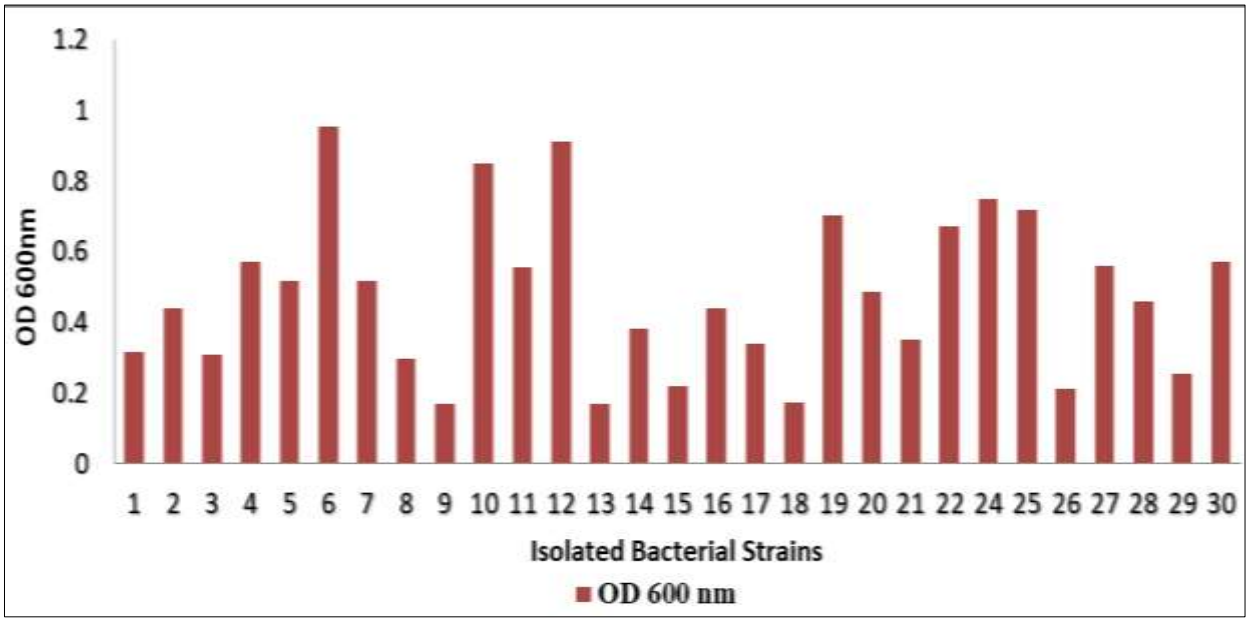

Fig 1: OD_{600} of 30 isolated bacterial strains after 7 days of incubation

Among 12 selected isolates showing maximum growth, D-4 exhibits survival up-to 16% concentration of diesel oil while other isolates were not able to survive at 14% (v/v) diesel oil concentration (Table 1). Therefore, D-4 exhibiting growth in maximum concentration of diesel oil was finally selected for further study and considered as potential diesel oil degrader.

Table 1: Minimum inhibitory concentration of diesel oil for selected bacterial strains.

Bacterial Strains	Diesel Oil Concentration (%)								
	2%	4%	6%	8%	10%	12%	14%	16%	18%
D-1	+++	+++	+++	++	++	+	-	-	-
D-2	+++	+++	+++	++	+	_	-	-	-
D-3	+++	+++	++	++	+	-	-	-	-
D-4	+++	+++	+++	+++	++	++	+	-	-
D-5	+++	+++	++	+	+	-	-	-	-
D-6	+++	+++	+++	+++	+	+	-	-	-
D-7	+++	+++	+++	++	+	-	-	-	-
D-8	+++	+++	+++	+++	+	+	-	-	-
D-9	+++	+++	++	+	+	-	-	-	-
D-10	+++	+++	+++	++	++	+	-	-	-
D-11	+++	+++	++	+	+	-	-	-	-
D-12	+++	+++	+++	++	+	+	-	-	-

Degree of growth on NA plates, +++ Good, ++ Moderate, + Survival, - No growth

3.2 Morphological and Biochemical Characterization

Bacterial strain D-4 was reported to be small sized, spherical shaped gram (+) bacteria having rough colony surface. Morphological and cultural characteristics of the selected bacterial strain D-4 is listed in Table 2. Outcomes of IMViC and carbohydrate utilization tests as well as enzyme test *viz.* catalase, casein hydrolysis, gelatin hydrolase and oxidase that were conducted on the bacterial isolates D-4 are presented in Table 3.

Table 2: Morphological & cultural characteristics of strain D-4

Morphological Characteristics	D-4	Cultural Characteristics	D-4
Gram reaction	+ve	Colony size	Small
Cell size	Small	Colony shape	Circular
Cell shape	Spherical	Margin of colony	Iregular
Arrangement of cells	Streptococci	Elevation of colony	Convex
Endospore formation	-ve	Surface & color of colony	Rough, Yellow

Table 3: Biochemical characteristics of D-4

Test	Results
Catalase	+
Oxidase	+
Casein hydrolysis	+
Gelatin liquefaction	-
Starch hydrolyses	-
Indole	+
Methyl red	-
Voges Proskauer	+
Citrate	+

Table 4: Carbohydrate utilization test for bacterial strain D-4.

IMViC & Carbohydrate utilization test	Results
Mannitol	-
Rhamnose	+
Sucrose	-
Glucose	+
Adonitol	-
Arabinose	-
Lactose	-
Sorbitol	-

3.3. Determination of viability of immobilized cells

Viability was calculated as CFU mL^{-1} by counting CFU on nutrient agar plates at intervals of 10 days during the course of 60 days. Moreover, viability of bacterial cells was examined at two different storage temperatures i.e. 4 °C and 25 °C. Results were demonstrated in the Table 5. Immobilized bacterial cells stored at 25 °C showed greater viability as compared to cells stored at 4 °C

Table 5: CFU count of sodium alginate immobilized D-4 at subsequent time interval

S. No.	Immobilized cells	CFU mL^{-1} at subsequent time interval (days)					
		10^{th} day	20^{th} day	30^{th} day	40^{th} day	50^{th} day	60^{th} day
1.	Sodium alginate beads (4 °C)	$11.96x10^6$	$7.82x10^6$	$3.33x10^6$	$9.84x10^5$	$6.58x10^5$	$4.58x10^5$
2.	Sodium alginate beads (25 °C)	$13.44x10^6$	$9.75x10^6$	$5.91x10^6$	$2.33x10^6$	$1.02x10^6$	$8.64x10^5$

Microbes are utilized to breakdown oil during the bioremediation process. The low survival rate and poor environmental adaptability of microorganisms make bioremediation challenging to implement. In the bioremediation process, microorganisms are used to degrade oil. In addition, oil's limited solubility, non-polarity, and

hydrophobicity impose further restrictions on oil breakdown [24]. Immobilised cells can help to resolve the aforementioned issues as they promote the development of biofilms [25]. Moreover, immobilized systems offer more consistent living environment to the microorganisms as compared to freely suspended cells [26, 27]. According to Ali and Naeimpoor (2013), immobilized cells were able to break down larger amounts of phenanthrene because the carrier shielded the microbial cells from soluble harmful intermediates formed during pollutant intake [28].

Since, carrier material used for immobilization system is a crucial component; free cells should be immobilized on a biodegradable carrier to obtain increased cell density and activity [29]. In addition to supplying nutrients to the bacteria, appropriate carrier can also have the additional benefit of enhancing the bioavailability of contaminants. Numerous carrier materials, including activated carbon, plant waste, vermiculite, etc., have been shown to immobilize microorganisms through adsorption, trapping, or chemical bonding. Alginate is a biodegradable polysaccharide that is mostly extracted from phaeophyta, and it has been discovered that cells immobilised with alginate are more effective at degrading ethylbenzene [30]. Alginate beads have beneficial physicochemical characteristics include high biocompatibility, the existence of a microenvironment, and the capacity to inhibit cell growth.

3.4 Effect of immobilization on diesel oil degradation:

The diesel degradation capacity of free and immobilized cells of bacterial isolate D-4 in Bushnell Hass media supplemented with 1% (v/v) diesel oil were studied by gravimetric analysis at an interval of 7 days for 35 days. Results are demonstrated in Table 6. The results show that there is a notable difference in the rate of diesel degradation, with the maximum degradation percentage being recorded on the 35th day of incubation. It is also clear that the degradation of diesel oil improves with the increase in incubation time. It is also clear that rate of degradation of diesel oil by immobilized cells was comparatively higher than that of free cell (Figure 2).

Table 6: Diesel oil degradation using free cells & immobilized cells of bacterial isolate D-4

	(%) Percent Biodegradation				
Bacterial Isolate	**7th day**	**14th day**	**21st day**	**28th day**	**35th day**
Control	12.28±0.23	16.12±0.25	20.56±0.71	21.56±0.23	22.78±0.14
Free D-4 cells	14.25±0.63	30.23±0.01	42.96±0.75	56.47±0.17	68.21±0.28
Immobilized D-4 cells	24.41±0.63	39.94±0.46	56.28±0.47	71.28±0.71	80.39±0.11

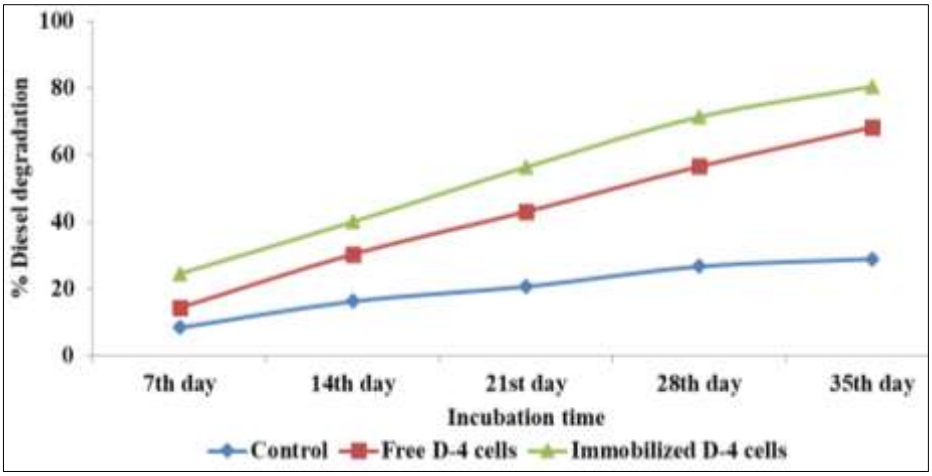

Fig 2: Comparative degradation of diesel oil by free & immobilized cells of D-4 at different time intervals.

Conclusion

The findings of this study demonstrated that cell immobilization enhanced the ability of the bacterial system to break down diesel oil. Diesel degradation rates by the immobilized cells were accelerated due to the aggregation effect and increase in number of adsorption sites. The current work shows that bacteria can be utilized at contaminated areas for degradation in immobilized forms. A significant difference was noticed in degradation ability of free and immobilized cells with the best degradation rate obtained for immobilized cells in alginate beads 80.39% as compared to free cell i.e. 68.21%. However in control only 22.78% degradation was achieved. Bacteria have the benefit of a longer shelf life when delivered in these entrapped forms rather than in freely suspended form. Alginate beads have shown to be efficient supportive carriers in the current investigation, increasing the viability and diesel-degrading capacity of strain D-4. Therefore, immobilization of microorganisms is a reusable technique that immobilizes free microorganisms or enzymes in a fixed space while maintaining high activity, which includes prevention of cell loss, strong tolerance to adverse environments, high biological stability, and ease of storage and use.

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