Environment and Ecology 42 (4) : 1483—1490, October—December 2024
Article DOI: https://doi.org/10.60151/envec/ZEFM4711
ISSN 0970-0420

Comparative Study of Suspended and Immobilized Bacteria Isolated from Garage Soil on Vegetable Oil Degradation

Paramita Ghosh, Moksedul Hossain, Ananya Dhar, Nilanjana Mondal, Urmila Reang, Prashant Shukla

Received 5 June 2024, Accepted 13 August 2024, Published on 18 October 2024

ABSTRACT

As an essential part of the ecosystem, soil helps to support life by promoting interactions between bedrock, water, air, and biota. But the growing number of oil spills has raised serious environmental issues, presenting problems similar to petroleum hydrocarbon pollution. Vegetable oils are generally thought to be safe, but because of their toxicity and persistence, they may have negative environmental impacts. Bioremediation techniques have gained popularity as a sustainable method of cleaning up hydrocarbon-contaminated locations in response to this environmental problem. This study uses immobilized microbial technology to increase the bioremediation effectiveness by examining the ability of bacteria isolated from a garage oil spill region to degrade vegetable oil. The study compares the degradation rates with naturally occurring bacteria to assess the effectiveness of the detected bacterium's breakdown over time in both suspended and immobilized situations. The results add to our knowledge of microbial-mediated bioremediation in contaminated environments and highlight the promise of immobilized microbial technology as a workable method for reducing vegetable oil spills. After 45 days, immobilized bacteria showed a drastic reduction in hydrocarbon content compared to suspended bacteria, indicating increased efficacy. Moreover, the degradation rate of immobilized bacteria was significantly higher than that of suspended bacteria, as observed from the difference in hydrocarbon concentration between day 1 and day 45. Statistical analysis confirmed these findings, highlighting the effectiveness of immobilized bacteria in hydrocarbon degradation.

Keywords Oil-degrading bacteria, Two-way ANOVA, Immobilization, Bioremediation, Hydrocarbon.

Paramita Ghosh[1], Moksedul Hossain[2], Ananya Dhar[3], Nilanjana Mondal[4], Urmila Reang[5], Prashant Shukla[6]*

[1,6]Assistant Professor
[1,2,3,4,5,6]Department of Biotechnology, School of Bioscience and Biotechnology, Brainware University, Barasat, West Bengal 700125, India

Email : dps.bt@brainwareuniversity.ac.in
*Corresponding author

INTRODUCTION

An essential link between the components (air, bedrock, water and biota) that make up our environment is provided by the soil, which is an essential and irreplaceable natural resource (Adipah 2019). These elements interact to provide essential needs like food,

fuel, and fiber to support the organism. Petroleum hydrocarbon-contaminated soil is a severe global environmental issue that has drawn public attention in recent years. Vegetable oil spills are becoming more frequent and may provide greater challenges than hydrocarbon spills, despite the fact that petroleum has emerged as one of the most significant categories of organic pollutants (Mueller *et al.* 1996, Zhang 2018). Spills of vegetable oil can have detrimental effects on the environment, often resembling those of spills of petroleum-based oil (Mudge 1995, Mudge 1998).

Vegetable oils have always been seen as largely safe, non-toxic, and having little effect on the environment. Nevertheless, prior experience has proven that both acute and chronic pollution occurrences can have detrimental impacts, proving that this generalization is fallacious (Zhang 2018). Many vegetable oils have been reclassified as category Y (hazardous) items under Annex II of the MARPOL Convention, with accompanying carriage restrictions, as a result of this awareness. Unlike petroleum oils, which evaporate, disperse, dissolve, and emulsify when heated, vegetable oils react differently. They have the ability to polymerize on shorelines, enclosing the nearby area. They will either sink, slowly deteriorate, or disperse extensively without suffering from severe degradation (Fingas 2015). Hydrocarbon remediation using bioremediation techniques is currently gaining positive press as a viable environmentally acceptable treatment approach. Furthermore, because biological approaches enable the economical *in situ* biodegradation of oil fractions by microorganisms, they may be superior to physico-chemical treatment regimes in the removal of spills (Singh *et al.* 2008, Mueller *et al.* 1996, Joutey *et al.* 2013). Because they make up a large portion of both household and industrial waste, lipids—fats, oils, and greases—contribute fairly to environmental pollution. The breaking of the ester link to a fatty acid by an enzyme is the initial stage in the breakdown of vegetable-based oils. Numerous bacteria manufacture esterases and lipases, which are the enzymes that catalyze this biodegradation reaction (Broekhuizen *et al.* 2003). The primary components of plant, animal, and microbiological fats and oils are tri-, di-, and monoacylglycerols, which are broken down into carboxyl esterbonds by hydrophobic proteins called lipases (Saifudin and Chua 2006). Lipases are used in remediation attempts to break down lipid-rich water because of their influence on degradability. Nevertheless, the enzyme's heat instability and high cost for a single application are drawbacks (Saifudin and Chua 2006). Both saturated and unsaturated fatty acids biodegrade through the process of β-oxidation after the initial step of breakdown. Documents demonstrating that "vegetable oils and synthetic esters have a much better biodegradation capacity than mineral oil under aerobic as well as anaerobic conditions" (Broekhuizen *et al.* 2003) provide evidence that both natural and vegetable oils are biodegradable. Ghanavati *et al.* (2008) have reported use of microorganisms for cleaning of waste waters from steel factory.

Vegetable oils biodegrade between 70% and 100% in 28 days, according to multiple tests (Aluyor *et al.* 2009). Even while they decompose naturally, recent studies indicate that the toxicity of vegetable oils makes them potentially more hazardous than hydrocarbon spills, and they are also growing more frequent. This study aims to explore the effectiveness of bacteria found in a garage oil spill area in breaking down vegetable oil. We utilized immobilized microbial technology to enhance bioremediation efficiency. This method offers several advantages, including increased microbial density, consistent biological activity, reduced biomass loss, resistance to harmful chemicals, and superior environmental tolerance. Alginate was employed as a support for microorganisms to maintain their attachment and enhance oil absorption, thereby promoting bacterial interaction and accelerating biodegradation. We evaluated the efficacy of the identified isolate in both suspended and immobilized conditions for hydrocarbon biodegradation. Comparisons were made by analyzing the degradation rate percentage over the incubation period, assessing the degree of hydrocarbon degradation relative to naturally occurring bacteria and immobilized bacteria.

MATERIALS AND METHODS

Bacterial isolation

In a sterile container, soil samples were taken from the gasoline and diesel-spilt garage on the brainware

University campus in Barasat, India. Using serial dilution-agar plating technique on nutrient agar medium and mineral salts medium with coconut oil, respectively, the enrichment and isolation of oil-degrading bacterial cultures were carried out. The morphological and biochemical properties of the isolated bacterial cultures were identified.

Screening of strains

A mineral salts medium supplemented with one percent coconut oil was employed as the carbon source for isolated bacterial cultures. These cultures were incubated in a shaker set at thirty degrees Celsius. The growth of bacteria was monitored by measuring culture densities using spectrophotometric absorption. Strains demonstrating faster degradation of coconut oil were further identified. Sub-culturing was conducted repeatedly for selected pure colonies, followed by transfer onto Nutrient agar slants for morphological and biochemical identification.

Identification and characterization of bacterial isolates

Morphological characterization

According to Cheesbrough (1981), color, form, transparency, and margin were investigated and noted as colony morphological traits. All isolates had their microscopic characteristics documented using the gram stain procedure.

Biochemical characterization

Oxidase assay : The filter paper was first saturated with freshly made oxidase reagent before the tested bacterial colony was applied. The appearance of a blue-purple color within 10 seconds was indicative of a positive oxidase test (Cheesbrough 1981).

Catalase examination : A positive catalase test is defined as the detection of gas bubbles within 10 seconds of adding pure bacterial culture to 5 milliliters of hydrogen peroxide solution (Cheesbrough 1981).

Test of indole : When 0.5 ml of Kovac's reagent was given to an incubated bacterial culture on SIM media at 35°C for 24 hrs, the resultant appearance of vivid red and yellow hue indicated a positive and negative result, respectively (Cheesbrough 1981).

Test of Voges–Proskauer (VP) **:** The formation of acetylmethyl carbinol from glucose fermentation is indicated by the appearance of a pink color at the surface following the addition of 5% Voges-Proskauer (VP) reagent A and 40% Voges-Proskauer.

Lactose fermentation test **:** Lactose and phenol red were used as the pH indicators throughout an 18–24 hour incubation period at 35–37°C for the lactose fermentation test. The lactose has begun to ferment when it becomes yellow. An orange hue could result from a postponed fermentation process. Results of gas production the Durham tube's trapped bubbles showed that lactose fermentation is producing gas.

Immobilization of bacteria

A 3% Sodium Alginate solution was made by mixing 3 g of Sodium Alginate with 100 ml of distilled water, then autoclaved. A 5% Calcium Carbonate solution was created by adding 5 g of calcium carbonate to 100 ml of hot distilled water, and a 4% Calcium Chloride solution was formed by shaking 4 g of calcium chloride with 100 ml of distilled water. For a 0.1 M HCl solution, 4.15 mL of concentrated HCl (37%) was added to 500 ml of distilled water. To prepare the embedding material, the Sodium Alginate solution was warmed and mixed with the Calcium Carbonate solution. For immobilization, mineral salts medium supplemented with one percent coconut oil medium containing cells and embedding material were mixed in a ratio of 1:10 (weight/volume) and added to a 4% Calcium Chloride solution to form immobilized granules. These granules were then transferred into the 0.1 M HCl solution to remove the calcium carbonate. Subsequently, the degradation activity was conducted using the immobilized granules.

Degradation studies

The selected cultures were grown in Luria Bertani media and are immobilized using Sodium Alginate. The suspended and immobilized bacterial in conical flask having LB medium with 1% coconut oil were

incubated at room temperature. On day 1, 15, 30 and 45 the hydrocarbon content of both flasks was determined and the number of bacterial populations was measured by pour plate technique. The total hydrocarbons in the both sets were determined spectrophotometrically. 5 mL samples from the treatments were mixed with equal volume of xylene to extract hydrocarbons from the samples. The extracted hydrocarbons were detected spectrophotometrically at 420 nm. Degradation was determined as the difference between the day 1 and final concentrations of total hydrocarbons.

Statistical analysis

Representative data from three independent experiments are presented as the mean value ± standard deviation (SD). To assess the statistical significance between the control group and alternative test regimes, as well as among different test sets for the three isolated bacteria (S1, S2 and S3), we conducted a one-way Analysis of Variance (ANOVA) followed by Tukey's multiple comparison test. A Two-way Analysis of Variance (ANOVA) was performed, followed by posthoc comparisons utilizing the Bonferroni test, to discern significant differences in the percentage of extracted hydrocarbons under both suspended and immobilized conditions across various time intervals. A significance level of $p<0.05$ was considered statistically significant. Statistical analyses were carried out using GraphPad Prism 5 software.

RESULTS

With 1% coconut oil as the carbon source, a total of 11 pure cultivated bacteria were able to grow in the specified conditions. Three of the bacteria were chosen because of their notable capacity to break down other microorganisms. After a standard methodology was followed for their physical and biological evaluation, they were assigned the designations S1, S2, and S3 (Table 1).

The total hydrocarbon from both suspended bacteria and immobilized bacteria were extracted and assessed by spectrophotomter and were compared with the control for 15, 30 and 45 days (Fig. 1). Further the total hydrocarbon percentage of each bacteria S1, S2 and S3 were compared for their activity. Result showed the percentage of extracted hydrocarbon in suspended culture in 15th day is very high as compared to control and immobilized set.15, 30 and 45 days and was statistically compared with the control between the different days of S1, S2 and S3 bacteria. The percentage of suspended S1 and S2 bacteria in 15 days was not statistically significant as compared to the control whereas suspended S3 bacteria showed a significant decrease in the percentage of hydrocarbon in first 15 days. However, in immobilized bacteria there is a significant decrease in the total hydrocarbon

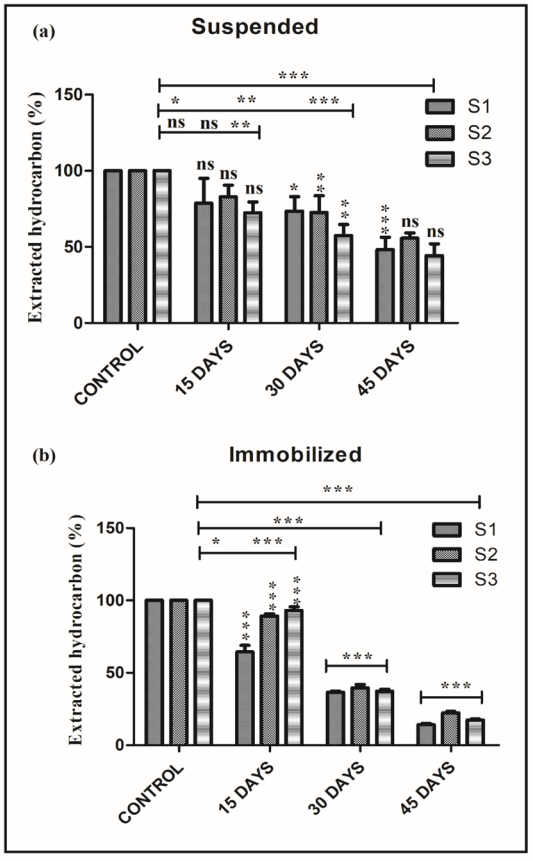

Fig. 1. (a) Percentage of extracted hydrocarbon by suspended bacteria. (b) Percentage of extracted hydrocarbon by immobilized bacteria. Statistical significance of each bacterial group with untreated control is analyzed by One Way ANOVA test (P ANOVA< 0.0001) followed by a post hoc Tukey's test. Data is represented as mean ± SD of triplicates determinations from their independent experiments with **p value <0.01 and ***p value <0.0001versus untreated control.NS: Not significant.

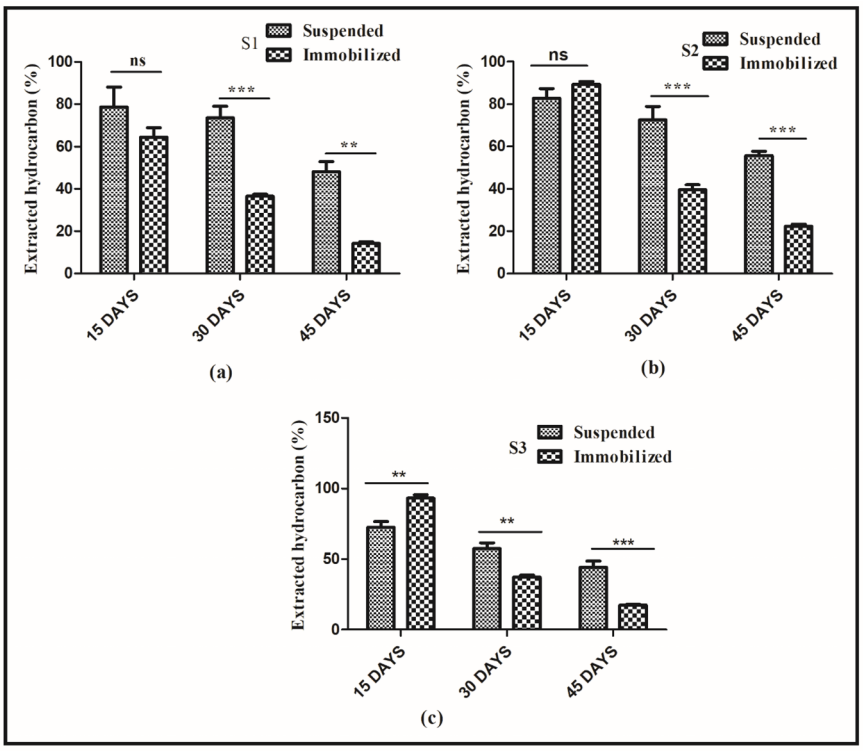

Fig. 2. Comparison of percentage of extracted hydrocarbon in suspended and immobilized bacteria of each S1, S2 and S3 for 15, 30 and 45 days. Statistical significance of each bacterial group with untreated control is analyzed by Two Way ANOVA test (P ANOVA< 0.0001) followed by a post hoc bonferroni test. Data is represented as mean ± SD of triplicates determinations from their independent experiments with **p value <0.01 and ***p value <0.0001versus untreated control.NS: Not significant.

from day 15 to day 45 and strikingly decrease on 45th day. The one way ANOVA and Tukey's multiple comparison test (with all the experimental regime) was shown in Table 2 for S1, S2 and S3.

In the next phase of the study (Fig. 2), the percentage of total hydrocarbons in S1, S2 and S3 for each set of suspended and immobilized was compared in different time interval (15, 30, 45 days). Results revealed that the bacteria S1 and S2 in both suspended and immobilized sets showed no significant difference on the 15th day whereas S3 bacteria showed a significant decrease in percentage of extracted hydrocarbons in immobilized condition as compared to suspended condition. Together, the above experiment revealed that after 45 days there was a drastic reduction in the hydrocarbon content in immobilized bacteria showing its increased efficacy as compared to the suspended.

Table 1. Biochemical characterization of isolated bacteria.

	S1	S2	S3	Flower colony	Hazard colony	Small colony	Smooth colony
Gram staining	–Ve	–Ve	–Ve	+Ve	+Ve	+Ve	+Ve
Catalase test	–Ve	–Ve	–Ve	–Ve	–Ve	–Ve	–Ve
Oxidase test	+Ve	+Ve	+Ve	+Ve	+Ve	+Ve	+Ve
Indole test	+Ve	+Ve	+Ve	+Ve	+Ve	+Ve	+Ve
MR-VP test	–Ve	–Ve	–Ve	–Ve	–Ve	–Ve	–Ve
Lactose fermentation test	+Ve	–Ve	+Ve	+Ve	–Ve	–Ve	+Ve

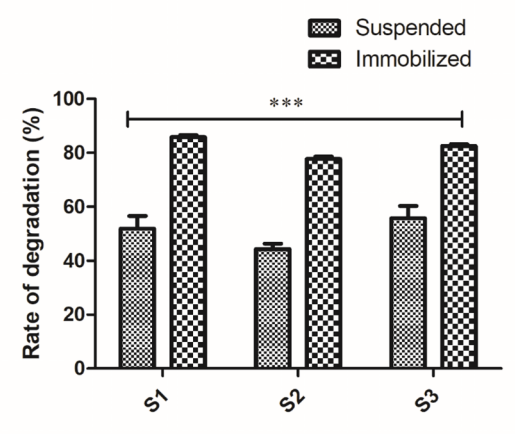

Fig. 3. Rate of degradation of suspended and immobilized bacteria in percentage. Statistical significance of each bacterial group with untreated control is analyzed by Two Way ANOVA test (P ANOVA< 0.0001) followed by a post hoc Bonferroni test. Data is represented as mean ± SD of triplicates determinations from their independent experiments with **p value <0.01 and ***p value <0.0001versus untreated control. NS : Not significant.

Next the degradation rate of suspended and immobilized bacteria was statistically compared by the difference between the total concentration of hydrocarbons on day 1 and day 45 (Fig. 3). It was observed that there was a significant increase in rate of degradation of the immobilized bacteria as compared to the suspended bacteria.

DISCUSSION

A wide range of hazardous organic chemicals are being purposefully or unintentionally releazed into the environment, which is causing increasing public concern (Hassanshahian *et al.* 2013, 2014). Pollution from oil has spread over industrialized and developing nations. It is among the most hazardous known sources of pollution at the moment. It might put the environment in danger. Environmentalists dread it much because it can be difficult to contain if it leaks out (Tebyanian *et al.* 2013). Vegetable oils, like mineral oils, can differ greatly and will operate differently depending on their distinctive characteristics when releazed into the marine environment (Popoola and Onilude 2017). These attributes are contingent upon various elements at the time of the feed stock's cultivation, such as the climate, level of processing, specific nature of the oil, sea level, and meteorological conditions during the spill (Santhini *et al.* 2009).Vege-

Table 2. Tukey's multiple comparison test for S1, S2 and S3.

Tukey's multiple comparison test	Mean difference	Q	Significant p < 0.05	Summary	95% CI of diff
Immobilized S1					
Control vs 15 days	0.5757	15.08	Yes	***	0.4028 to 0.7485
Control vs 30 days	1.027	26.90	Yes	***	0.8538 to 1.200
Control vs 45 days	1.388	36.37	Yes	***	1.215 to 1.561
15 days vs 30 days	0.4510	11.82	Yes	***	0.2782 to 0.6238
15 days vs 45 days	0.8123	21.29	Yes	***	0.6395 to 0.9852
30 days vs 45 days	0.3613	9.468	Yes	***	0.1885 to 0.5342
Immobilized S2					
Control vs 15 days	0.1693	8.301	Yes	***	0.08364 to 0.2549
Control vs 30 days	0.9915	48.63	Yes	***	0.9059 to 1.077
Control vs 45 days	1.277	62.62	Yes	***	1.191 to 1.362
15 days vs 30 days	0.8223	40.33	Yes	***	0.7366 to 0.9079
15 days vs 45 days	1.108	54.32	Yes	***	1.022 to 1.193
30 days vs 45 days	0.2853	13.99	Yes	***	0.1996 to 0.3709
Immobilized S3					
Control vs 15 days	0.1120	5.229	Yes	*	0.01499 to 0.2090
Control vs 30 days	1.006	46.97	Yes	***	0.9090 to 1.103

Table 2. Continued.

Tukey's multiple comparison test	Mean difference	Q	Significant p < 0.05	Summary	95% CI of diff
Control vs 45 days	1.325	61.86	Yes	***	1.228 to 1.422
15 days vs 30 days	0.8940	41.74	Yes	***	0.7970 to 0.9910
15 days vs 45 days	1.213	56.63	Yes	***	1.116 to 1.310
30 days vs 45 days	0.3190	14.89	Yes	***	0.2220 to 0.4160
Suspended S1					
Control vs 15 days	0.3447	3.724	No	ns	-0.07451 to 0.7638
Control vs 30 days	0.4243	4.585	Yes	*	0.005154 to 0.8435
Control vs 45 days	0.8320	8.989	Yes	***	0.4128 to 1.251
15 days vs 30 days	0.07967	0.8608	No	ns	-0.3395 to 0.4988
15 days vs 45 days	0.4873	5.265	Yes	*	0.06815 to 0.9065
30 days vs 45 days	0.4077	4.405	No	ns	-0.01151 to 0.8268
Suspended S2					
Control vs 15 days	0.2777	4.401	No	ns	-0.008100 to 0.5634
Control vs 30 days	0.4420	7.005	Yes	**	0.1562 to 0.7278
Control vs 45 days	0.7153	11.34	Yes	***	0.4296 to 1.001
15 days vs 30 days	0.1643	2.604	No	ns	-0.1214 to 0.4501
15 days vs 45 days	0.4377	6.936	Yes	**	0.1519 to 0.7234
30 days vs 45 days	0.2733	4.332	No	ns	-0.01243 to 0.5591
Suspended S3					
Control vs 15 days	0.4433	7.353	Yes	**	0.1703 to 0.7164
Control vs 30 days	0.6840	11.34	Yes	***	0.4109 to 0.9571
Control vs 45 days	0.8945	14.84	Yes	***	0.6214 to 1.168
15 days vs 30 days	0.2407	3.992	No	ns	-0.03240 to 0.5137
15 days vs 45 days	0.4511	7.482	Yes	**	0.1781 to 0.7242
30 days vs 45 days	0.2105	3.491	No	ns	-0.06260 to 0.4835

table oil properties frequently have an impact on how it behaves in the environment, although this influence is poorly researched and understood. As a result, it is somewhat harder to anticipate the behavior and destiny of certain vegetable oils than it is for mineral oils. Thus, our study demonstrated the potential of bacteria isolated from a hydrocarbon-rich location to degrade vegetable oil. Tamothran *et al.* (2022) have described importance of pollution caused by vegetable oil spills in aquatic environment. The microbial degradation is a better prospect for degrading vegetable oils in most environments. In a system with vegetable oil as only external carbon source, our investigation showed that the bacteria isolated from a mineral-rich site significantly degrade vegetable oil. The use of biotechnological procedures involving microorganisms to address issues with environmental contamination has been expanding quickly in the last several years. Researchers have demonstrated the great stability, adaptability, and wide range of applications of biological approach for petroleum cleanup (Scott 1987). It is also cost-effective and efficient. Keeping bacterial populations' biomass high is essential for bioremediation. Bacterial cells need to be immobilized in order to increase the bioremediation agents' survival and retention in the contaminated locations. Because immobilized cells have a longer lifetime and better cell stability and survival, they are widely used in the bioremediation of pollutants, wastewater treatment, and synthesis of valuable compounds (Margaritis and Merchant 1984, Fu *et al.* 2019). For environmental applications, the use of immobilized cells has been studied as an alternative method. These biocatalysts may provide a more extensive and cost-effective application in waste management, industry, health, and the creation of bioprocess and monitoring instruments such as biosensors (Scott 1987). Base on this assumption, we found that the isolated immobilized bacteria were statistically more efficient in degrading vegetable oil than the suspended ones in 15-days laps. There was a 2 fold increase in the degradation percentage of immobilized bacteria improving its capacity as compared to the suspended form. Immobilization hydrocarbon degrader's bacteria have high potential

to clean up oil contamination and can be facilitating oil biodegradation in polluted environments (Cassidy *et al.* 1996).

CONCLUSION

The most compelling argument for bioremediation is the ability of microorganisms isolated from a petroleum-contaminated site to biodegrade vegetable oils. As per the results obtained we can conclude that this bacteria is capable of breaking down both vegetable and mineral oils. With more study being done in these areas, improvements are both inevitable and currently being seen. The bacteria could be employed as substitute biodegrading agents in light of the health of the soil and surrounding environment. These cultures can be employed to actively break down pollutants and purify the environment in response to biodegradation.

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