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BIODEGRADATION OF DIESEL AND CRUDE OIL USING *Corynebacterium* sp AND *Lysinobacillus fusiformis* 5B STIMULATED WITH BIOSURFACTANT, BIOCHAR AND IRON OXIDE NANOPARTICLES

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Abstract: Petroleum oil and its processing product are the most common anthropogenic contaminants. It contains many compounds that pose a significant risk for the environment and human health and have cytotoxic, mutagenic, and carcinogenic effects. This study was conducted to determine diesel biodegradation using Corynebacterium xerosis and crude oil using Lysinibacillus fusiformis 5B stimulated with biochar, biosurfactant, and iron oxide nanoparticle. The isolate was grown on a minimal salt medium (MSM) with diesel and crude oil as the carbon source. The test organisms were screened for the ability to utilise diesel and crude oil as sole carbon and energy source by inoculation into MSM containing 0.4 g/L of NH_4Cl, 1.2 g/L of KH_2PO_4, 1.8 g/L of $K_2HPO_4.3H_2O$, and trace amounts of 0.2 g/L of $MgSO_4.7H_2O$, 0.01 g/L of $FeSO_4.7H_2O$ and 0.01 g/L of NaCl. The biodegradation studies were done using five conical flasks collectively with MSM (18 mL), isolate (2 mL), biochar (100 mg), crude oil (2 mL), biosurfactant (100 mg), and Iron oxide nanoparticles (FeONp)7 (100 mg). A control was set up without the isolate. The biodegradation was observed for 20 days, and the rate of biodegradation was determined by the weight loss method at 5 days interval. The colony-forming unit also determined microbial growth. The test organisms could utilise diesel and crude oil as their carbon source. The highest percentage biodegradation for Corynebacterium species was obtained on day 20 (86%). The un-inoculated control had 12 %. There was a gradual increase in the counts of the isolate from 4 × 106 CFU ml-1 (day 0) to 192 ×106 CFU ml-1 (day 20), Lysinobacillus fusiformis 5B gave an increase in biodegradation from 15.25 % at day 0 to 36.95 % at day 20. At the same time, the control had 12.15 % and 35.05 %, respectively. The microbial count also increased from 1.8x105 CFU/mL at day 0 to 3.8x105 CFU/mL at day 20. This study showed that Corynebacterium sp could utilise diesel, and Lysinibacillus fusiformis 5B utilised crude oil, which can be used to biodegrade petroleum-contaminated environments.

Keywords: Biodegradation, Biochar, Biosurfactant, Iron oxide nanoparticles, Diesel, crude oil

1.0 Introduction

Petroleum hydrocarbon is the most

important source of energy globally; nevertheless, ordinary extraction and drilling activities of this fossil energy supply cause major environmental problems [1]. Petroleum contains a wide spectrum of cytotoxic, mutagenic, and carcinogenic chemicals, posing a major risk to the environment and human health [2]; because of their complex structure, extensive toxicity, and durability, petroleum hydrocarbons are among the most important organic pollutants. Oily pollutants have invaded the environment through oil exploration, mining, transportation, and storage as industrial production has increased. Oil contamination is primarily absorbed by soil [3,4]. Bacteria found in soil that can break down various petroleum compounds, such as petroleum hydrocarbons, have gotten much interest.

Diesel oil is a fraction of crude oil distilled around 200 °C to 350 °C. It consists of alkane, aromatic compound, cyclic, and branched alkanes that contain carbon-9 to carbon-25 per molecule. Environmental pollution can result from accidental spills or leakage of pipelines and storage tanks. Diesel fuel is highly toxic and can also cause cancer [5]. Diesel fuels differ in respect to their source and production method. Oils contain heavy residues from distillation and thermal cracking along with a variety of additives such as amines, phenol, polymeric substance and organic nitrate, these additives enhance the performance of the engine and delivery system, fuel handling, stability and contaminant control [6].

Crude oil and its derivatives are among the most common contaminants in the environment, and due to their widespread, they pose severe risks to human health and water bodies. Hence, intense remediation practices are required at the contaminated sites. Environmental contamination problems from petroleum extraction, transportation, and storage are becoming more widely recognised worldwide. Nigeria's crude oil production has increased to approximately 2.8 million barrels per day. Nigeria is presently Africa's top oil producer and the world's sixth-largest [7].

Bioremediation is a natural, efficient, and cost-effective method. In addition, it converts hydrocarbons into less toxic compounds through metabolic and enzymatic reactions. Biodegradation is mainly carried out by bacteria, yeast and fungi [7]. Different types of bacteria, fungi, and yeasts have been shown to degrade hydrocarbons, with bacteria being the best group of microorganisms for hydrocarbon biodegradation [8]. Compared to other physical and chemical procedures, bioremediation is cost-efficient and environmentally friendly. However, human-associated risk, properties of the contaminated site and type of pollution must be evaluated before treatment can be carried out. In situ and ex, situ contaminated site remediation methods are distinguished depending on its location.

The most active agents in petroleum hydrocarbon degradation are bacteria, which act as the primary degraders of spilt oil in the environment. Several bacteria are even known to feed exclusively on hydrocarbons as their source of carbon. They include the following bacteria genera, *Bacillus* sp,

Pseudomonas sp, *Streptomyces* sp, *Vibrio* sp, *Mycobacterium* sp, *Marinobacter sp, Alcaligenes sp, Acinetobactersp, Flavobacterium sp, Micrococcus sp, Corynebacterium sp, Sphingomonas sp., Micrococcus sp,* etc.fungi such as *Penicillium, Candida sp, Fusarium sp, Aspergillus* sp, *Articulosporium* sp, *Trametes versicolor, Pleurotustu berregium,* algae such as *Monoraphidium braunii, Amphoracoffe aeformis* and plants such as *Brassica juncea* and *Helianthus* [9-11].

Lysinibacillus and other bacteria from related genera have previously been reported to biodegrade petroleum hydrocarbons [12-13]. These organisms are known to be efficient producers of biosurfactants used to degrade petroleum hydrocarbon [14]. Because *Lysinibacillus* is a spore-forming bacteria resistant to physical and chemical impacts, it may thrive in various contaminated environments, including oil-polluted soil. Although there have been various studies on the biodegradation of crude oil, most of these have used different *Bacillus* species; the utilisation of *Lysinibacillus fusiformis* for this purpose has been comparatively understudied.

Surfactant is one of the technological factors that affect the biodegradation of petroleum hydrocarbons. In order to improve the rate of biodegradation of pollutants, it is necessary to improve their bioavailability for microorganisms. Surfactants can modify the interfacial tension between contaminated particles and soil, allowing hydrocarbons to be transferred to the mobile phase. This can result in the dispersion of non-aqueous phase liquid droplets in the soil solubilisation and oil solubilisation into the core of the aggregate of surfactant molecule and the stabilisation emulsions [15]. One of the setbacks of bioremediation is reaching the contaminant in the subsurface, which can be solved using iron-nanoparticle (FeONp). Remediation of contaminated soils with engineered nanomaterial provides more effective and cheaper approaches than other conventional methods because of the increased reactivity of nanoparticles and the possibility of in situ treatment [16].

This study aimed to biodegrade diesel using *Corynebacterium xerosis* and Crude oil using *Lactobacillus fursiformis* stimulated with biochar, biosurfactant and iron-oxide nanoparticle composites.

2.0 Materials and Methods

2.1 Collection of Bacteria isolate

Corynebacterium sp and Lysinibacillus *fusiformis* 5B isolates utilised in this study were obtained from the Federal University of Technology Minna's Microbiology Laboratory. Yakubu (2019) identified the *Lysinobacillus fursiformis* 5B from cement samples.

2.2 Petroleum hydrocarbon collection

Diesel fuel was collected with a 750 mL container from filling station, Minna, Niger State, Nigeria. In contrast, Crude oil was collected from the Federal University of Technology Minna Microbiology Laboratory.

2.3Iron-oxide nanoparticle,

Biosurfactant and Biochar

This was obtained from the microbiology laboratory, Federal University of Technology, Minna

2.4 Screening of Isolate for the Potential to Utilise Diesel and Crude Oil

This was done by inoculating an overnight culture into a minimal salt medium (MSM) plate, which contains Diesel and Crude Oil as the sole source of carbon. The isolate was tested for its capacity to utilise the crude oil. 0.4 g/L NH_4Cl, 1.2 g/L KH_2PO_4, 1.8 g/L $K_2HPO_4.3H_2O$, and trace amounts of 0.2 g/L $MgSO_4.7H_2O$, 0.01 g/L $FeSO_4.7H_2O$, and 0.01 g/L NaCl were used to make the minimal salt medium. The composition was sterilised at 121 ºC for 15 minutes. After sterilisation, the composition was allowed to cool and poured aseptically into a petri-dish. When it was gelled, *Corynebacterium* sp and Lysinibacillus *fusiformis* were inoculated using the streak method of inoculation on the plate containing diesel and crude oil as carbon sources. The plates were incubated for 5 days at 37°C. The growths of the bacteria species were monitored at regular intervals.

2.5 Experimental design

The biodegradation studies were done by inoculating 2 mL of 24 hours culture broth of the isolate into 18 mL of a sterile mineral salt medium that contained 2 g of petroleum hydrocarbon, 100 mg of biosurfactant, 100 mg of biochar, 100 mg of iron-oxide nanoparticle in a conical flask. The experiment setup was in duplicate with a control flask, which contained 18 mL of a sterile mineral salt medium that contained 2 g of petroleum hydrocarbon, 100 mg of biosurfactant, 100 mg of biochar, 100 mg of iron-oxide nanoparticle without the isolate. The flasks were incubated at 30 °C for 20 days. At five day's intervals, duplicate flasks were removed.

2.6 Determination of Biodegradation activities

The extent of diesel fuel biodegradation in the mixture was determined by emptying the mixture with diethyl ether in a separating funnel. After shaking vigorously, the separating funnel was put on the retort stand and allowed to separate the organic phase from the liquid phase, the tap was open to removing the MSM while the solvent oil-mixture was collected into a beaker of a known weight. The solvent was evaporated using a water bath. The new weight of the beaker consisting of residual oil was recorded [17]. The percentage of degradation of diesel fuel was calculated using the formula of [18].

$$\%\ Biodegradation = \frac{weight\ of\ initial\ oil - weight\ of\ residual\ oil}{weight\ of\ initial\ oil} X\ 100$$

3.0 Results

3.1 Screening of the test isolate

Corynebacterium sp was able to utilise diesel as a sole carbon source, and *Lysinibacillus fusiformis* was able to utilise crude oil as the sole carbon and energy source, thereby showing a visible growth after 5 days of incubation. Figure 1 shows the visible growth of *Lysinibacillus fusiformis* 5B on the MSM and nutrient agar plate.

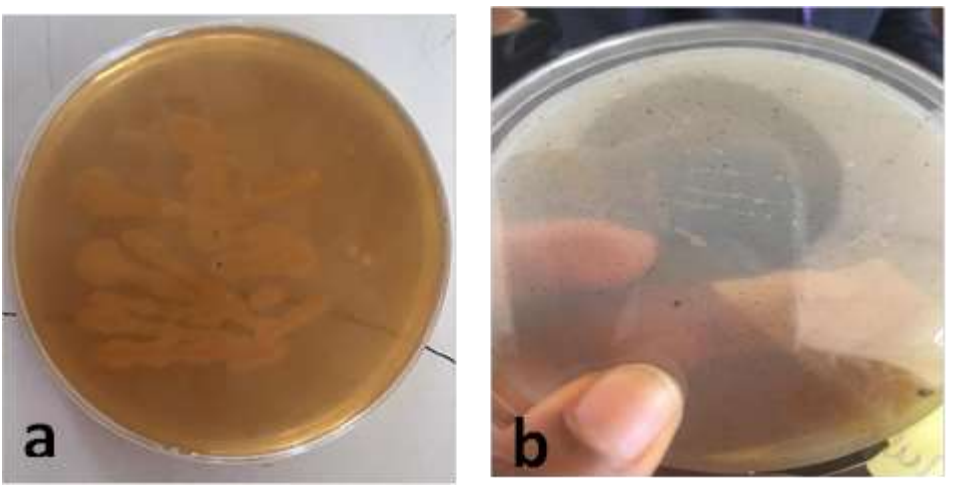

Figure 1. *Lysinibacillus fusiformis* 5 B on nutrient agar plate (A) and MSM plate (B)

3.2 Rate of Biodegradation

The percentage biodegradation of diesel oil treated by the *Corynebacterium* sp stimulated with biosurfactant, biochar and iron-oxide nanoparticle for 25 days is shown in Table 1. The percentage of diesel degradation by the bacteria on day 20 had the highest percentage (91.00%) of biodegradation in comparison with the un-inoculated control 17 %, while the degradation of crude oil by *Lysinibacillus fusiformis* 5 B stimulated with biosurfactant, biochar and iron-oxide nanoparticle for 20 days are shown in Table 2. The highest degradation (37.95 %) was observed after 20 days compared to control with 35.52 %. While the least was observed on day 0 (16.25 %)

Table 1 Percentage of diesel biodegradation by the *Corynebacterium* sp

Microbial isolate(days)	0	5	10	15	20
Bacterial isolate	31.00±7.07	72.00± 7.07	73.00±7.07	75.00± 7.07	91.00±7.07
Control (uninoculated)	15.00±7.07	29.00±7.07	16.00±7.07	17.00±7.07	17.00± 7.07

Table 2 Percentage of crude oil biodegradation by the *Lysinibacillus fusiformis*

Microbial isolate(days)	0	5	10	15	20
Bacterial isolate (%)	16.25±1.41	20.35± 1.41	33.65±1.41	35.65± 1.41	37.95±1.41
Control(uninoculated)(%)	12.57±0.60	19.52±0.67	31.50±0.70	33.07±0.10	35.52± 0.67

3.3 Growth of Isolate During the Biodegradation

The growth of the *Corynebacterium* sp during diesel biodegradation is shown in Figure 1. Day 0 had a count of 4 $\times 10^6$, and the maximal count was on day 20 had 192 $\times 10^6$ and the growth of *Lysinibacillus fusiformis* during biodegradation of crude oil is shown in Figure 2. The highest colony count was seen on day 20, with a value of $3.8x10^5$. The lowest colony count was seen on day 10, with a value of $1.8x10^5$.

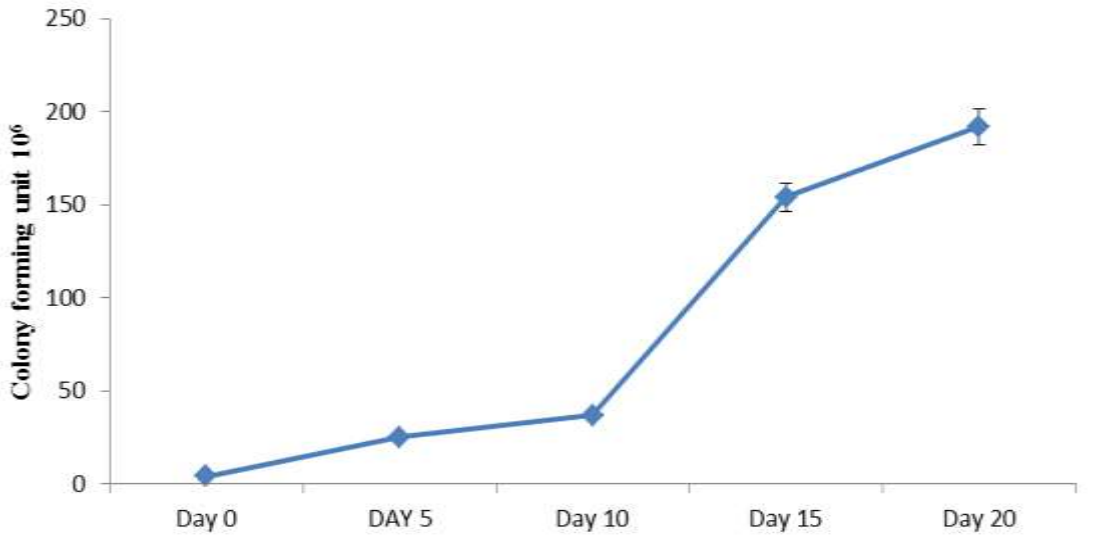

Figure 1 Growth profile of the Corynebacterium sp during biodegradation of diesel for 20 days

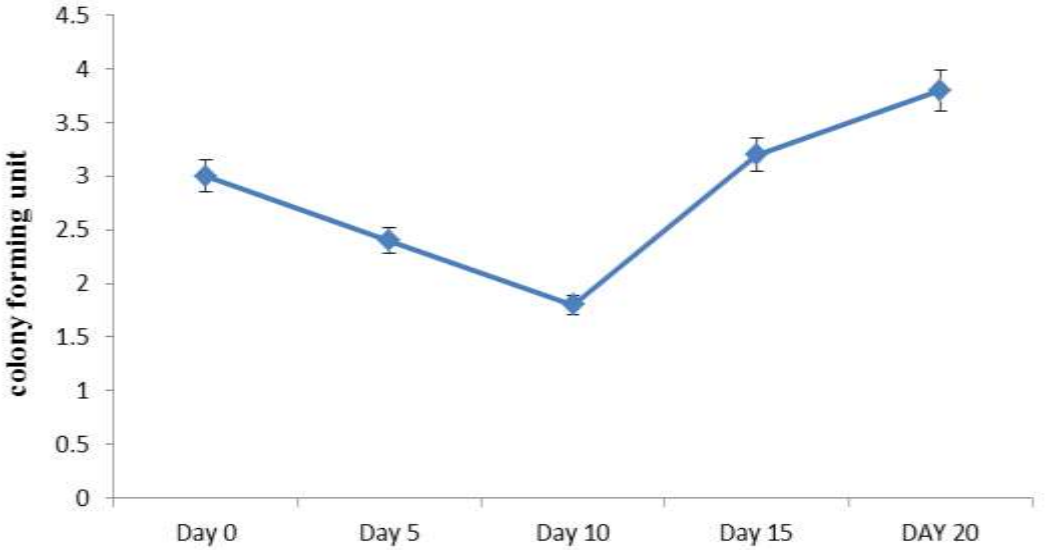

Figure 2. Growth profile of the *Lysinibacillus fusiformis* during biodegradation of diesel for 20 days

4.0 Discussion

Enrichment with target compound substrates is one of the most important strategies to isolate microbes with unusual metabolic abilities. This method has successfully isolated, screened, and characterised the required microorganism [19]. The ability of the two bacteria isolates to grow on the mineral salt medium with 1% of the petroleum hydrocarbon showed they are both capable of utilising the petroleum fuel as their sole source of carbon. This is similar to the report of [20], who stated that based on growth ability, three aliphatic hydrocarbon-degrading microbial strains, *Yarrowia* sp, *Corynebacterium* sp and *Acinetobacter* sp.

[7] also, use a similar approach to screen fungi isolate for diesel oil-degrading potential. This could be due to the presence of hydrocarbon-degrading enzymes such as laccases, manganese peroxidases and lignin peroxidases. *Corynebacterium* sp was able to grow on the mineral salt medium supplemented with 1% diesel after 5 days of incubation, suggesting that the isolate can resist the toxic effect of diesel and can utilise diesel as a carbon and energy source, hence having the potential to degrade contaminated diesel environment, the biodegrading potential of *Corynebacterium* sp have been reported by previous studies [21].

Lysinibacillus fusiformis was able to thrive on MSM supplemented with crude oil. This may be due to the organism's ability to use crude oil as a carbon source. The previous report of [22] also stated that *L. fusiformis* was able

to tolerate heavy metals and was able to biosorp heavy metals up to 99 %.

The percentage biodegradation of diesel and Crude Oil by the bacteria isolates and colony-forming unit increased with an increase in incubation period from 0 to 20 days. This revealed the potential of *Corynebacterium* species to use diesel oil and *Lysinibacillus fursiformis* to utilise crude oil as a sole carbon source. This might be due to the ability to produce biosurfactant, efficient hydrocarbon assimilation by receptor site for binding hydrocarbon and might have a feature that enhances the emulsification and transport of hydrocarbon into the cell and the presence of enzymes such as oxygenase and peroxidase that introduce molecular oxygen into the hydrocarbon and produce intermediate that will later enter common energy-yielding catabolic pathway [18]. The rate of degradation observed in the control flask might be due to photo-oxidation, abiotic loss, and evaporation.

The gradual increase in the Corynebacterium sp during the course of biodegradation might be due to the ability of Corynebacterium sp to use diesel as a source of carbon and energy and might also be due to the supplement added to improve the activities of the microorganism. [23] reported that the periodic supplement of bacteria and nutrients helped maintain the bacteria numbers and enhanced the remediating activities in the hydrocarbon contaminated soil until the treatment period.

According to [24], the *Lysinibacillus fusiformis* species has a maximum potential of 89 per cent, and these bacteria can be used to bioremediate polluted hydrocarbon environments efficiently. According to [25], *Lysinibacillus fusiformis* has a higher potential to digest crude oil, and the bacterial species can be harnessed and used to clean up crude oil-contaminated environments.

The maximal rate of degradation by *Corynebacterium* sp (91%) could be due to the addition of biochar, biosurfactant and iron-nanoparticle, as biochar serves as a nutrient that stimulates the growth of the isolate, biosurfactant reduces surface tension while iron-nanoparticle enhance penetration [26] reported that the addition of nutrient enhances the biodegradation of oil pollutant and biosurfactant also enhance solubilisation and removal of the pollutant. Thus, biochar is an absorbent for hydrocarbon degradation and stimulates the microbial population in contaminated soil. It also neutralises the toxic nature of crude oil [27]. Biosurfactants are advantageous due to their low toxicity and biodegradation potential [28]. [30] tested a biosurfactant from *Pseudomonas aeruginosa* for its ability to remove oil from contaminated Alaskan gravel samples under various conditions, including the concentration of the surfactant, time of contact, the temperature of the wash and presence or absence of gum, they reported increased oil displacement of of about 2-3 folds in comparison to water alone.

Biosurfactant, iron oxide nanoparticles and biochar formulation can be effective, cheap and eco-friendly agents for stimulating remediation of

diesel oil-polluted soil. A higher rate of degradation was observed in diesel oil compared to crude oil. This might be due to the nature of the hydrocarbon present in crude oil, suggesting that crude oil contains more complex hydrocarbon compounds than diesel. Diesel oil is composed of a large proportion of aliphatic hydrocarbons, which are prevalent in the soil as a consequence of spills. The aliphatic hydrocarbon is usually degradable compared to aromatic hydrocarbon, although the different fractions of aliphatic hydrocarbon varies in their degradability pattern. [23] in agreement with the findings of [30], whose G.C.- Analysis revealed that alkenes were generally the most biodegraded compound. Aromatic hydrocarbons were particularly less degraded than alkenes. The degradation rates of organic compounds in petroleum mixtures vary widely. The n-alkanes biodegradation is more rapid, followed by simple aromatics, whereas cycloalkanes and aromatics degrade more slowly [26].

5.0 Conclusion

Corynebacterium sp was able to thrive on mineral salt medium supplemented with diesel, and *Lysinibacillus fusiformis* was able to thrive on MSM supplemented with crude oil, indicating that both organisms have petroleum oil-degrading potential. Corynebacterium sp's highest count and percentage degradation was obtained on day 20 (91%); the un-inoculated control had 17 %. There was a gradual increase in the counts of the isolate from 4 10^6 CFU ml^{-1} (day 0) to 270 10^6 CFU ml^{-1} (day 25), and *Lysinibacillus fusiformis* had maximum degradation (37.95 %) after 20 days compared to control, with 35.52 %. The highest bacterial count during biodegradation was 3.8 x 10^5 CFU ml^{-1}. The highest bacterial count during biodegradation was 3.8 x 10^5 CFU ml^{-1}. This study revealed that *Corynebacterium* sp and *Lysinibacillus fusiformis* 5B with biosurfactant-iron-oxide-biochar nano-composite can be developed for bioremediation of environments contaminated by petroleum hydrocarbon.

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