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Diesel-Degrading Potential of *Pseudomonas putida* Isolated from Effluent of a Petroleum Refinery in Nigeria

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Abstract

The contamination of soil and groundwater by hazardous chemicals has become a major concern due to the associated risks to human health and the environment. The ability of Pseudomonas putida isolated from a petroleum refinery effluent to degrade diesel was assessed in this study. The effluent sample was collected from the treatment plant in the Kaduna Refining and Petrochemical Company (K.R.P.C), Nigeria. The physicochemical properties and heavy metal content of the effluent was determined, and three strains of Pseudomonas putida were identified among the bacteria isolated using conventional biochemical and phenotypic tests. The strain showing the highest degradation potential after screening was selected for the final biodegradation studies. The ability of the selected strain of Pseudomonas putida (C15a) to utilize the hydrocarbons in diesel was assessed over a period of eighteen days, and monitored on a three-day interval by evaluating the pH, hydrocarbon utilizing bacterial count and oil and grease content. It was observed that the organism was able to utilize diesel for its metabolic needs as shown by the increase in hydrocarbon utilizing bacterial (HUB) count and corresponding decrease in oil and grease content as well as pH. The highest hydrocarbon utilizing bacterial count was observed at day 15 (1.85 x 10^7 CFU/mL) with highest hydrocarbon degradation occurring at day 18 (98.3%). The strain of Pseudomonas putida (C15a) isolated in this study can be used as a candidate for further bioremediation studies on petroleum.

Keywords: *Petroleum, biodegradation, Pseudomonas putida, diesel, effluent*

INTRODUCTION

Petroleum refinery and petrochemical industries play an immense role in national development and improved quality of life. However, pollution effects of the wastes from these industries are causes for worry (Nwaichi *et al.*, 2013). Soil and ground water are often contaminated due to inevitable spillage during oil exploration, transportation, extraction, refining and also leakages from underground storage tanks and pipelines (Xiong *et al.*, 2015). Effluents (wastewater) released from petroleum refineries are characterized by the presence of large quantities of petroleum products, polycyclic and aromatic hydrocarbons, phenols, metal derivatives, surface active substances, sulfides, naphthylenic acids and other chemicals (Musa *et al.*, 2015), most of which are known to be mutagenic, carcinogenic and growth inhibitory and by extension can have adverse effect on the ecology of the receiving sites and public health (Nwaichi *et al.*, 2013).

Effluents are usually treated before discharge to the receiving site. But due to inefficient treatment systems, the pollutants may not be completely eliminated, consequently polluting water bodies, soils and underground water with potentially serious consequences on the ecosystem (Otukunefor and Obiukwu, 2005). Though the compositions of effluents are set by various regulatory agencies, compliance with the legally set toxicant levels for refineries and petrochemical plants in Nigeria has always been very low (Obot *et al.*, 2007). Furthermore, the impact of these toxicants on the quality of the effluent receiving water bodies and soils is rarely investigated (Otukunefor and Obiukwu, 2005).

Several techniques including physical, chemical and biological techniques have been developed to resolve the problem of petroleum pollution (Darsa *et al.*, 2014). However, the most promising approach being researched so far is microbial degradation since the physico-chemical methods are rather too expensive, not environmentally friendly and does not give a total elimination of the pollutant (Umanu *et al.*, 2013). Microbial degradation (biodegradation) involves the use of microbes to break down potentially harmful pollutants into harmless products (Hamza *et al.*, 2012).

Crude oil degrading bacteria such as *Pseudomonas* sp, *Micrococcus* sp. and *Bacillus* sp. could metabolize the toxic components of crude oil, leading to its degradation (Onwurah, 2003).

An increase of metal concentration adversely affects soil microbial properties e.g. respiration rate, enzyme activity, which appears to be very useful indicators of soil pollutions. Heavy metals of specific concern to surface water systems are cadmium, chromium, mercury, lead and arsenic. These heavy metals can bind to the surface of microorganisms and may even breach inside the cells (Gupta *et al.*, 2016). Heavy metals are the potent inhibitors of biodegradation (Kratochvil and Volesky, 1998). These metals cannot be degraded, and eventually persists in the environment. The toxic properties of heavy metals result mainly from the interaction of metals with proteins (enzymes) and inhibition of metabolic processes (Kumar *et al.*, 2014).

The objectives of the study were to: determine the physicochemical properties and heavy metal content of the refinery effluent, isolate and characterize *Pseudomonas putida* strains from the refinery effluent, screen the strains of *Pseudomonasputida* for the ability to degrade diesel and monitor the extent of hydrocarbon biodegradation by the selected isolates using Hydrocarbon Utilizing Bacteria (HUB) count, oil and grease content, and pH.

MATERIALS AND METHODS

Sample collection

The effluent sample was collected in a sterile amber-coloured glass bottle (500 mL), from the treatment plant in the Kaduna Refining and Petrochemical Company (KRPC), Kaduna State, Nigeria.

Determination of the physico-chemical properties of the petroleum refinery effluents

The physicochemical parameters that were analyzed include: pH, temperature, turbidity, Electrical Conductivity (EC), Total Dissolved Solids (TDS), Dissolved Oxygen (DO), Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), Nitrate, Phosphate, and Sulphate, Organic carbon, Total Nitrogen using the Standard methods as described by APHA (1995).

Heavy metal analyses

The determination of heavy metals (Chromium, Manganese, Lead, Cadmium, and Zinc) was performed with a bulk scientific 205 atomic absorption spectrophotometer. The instrument's setting and operational conditions were done in accordance with manufacturer's specifications. The instrument was calibrated with analytical-grade metal standard stock solutions (1 mg/L) in replicate. One hundred and fifty millilitre (150 mL) of sample was transferred to a beaker, 5 mL concentrated HNO_3 was added and the mixture was evaporated almost to dryness on a hot plate. Two millilitres of concentrated HNO_3 was added to dissolve the residues on the walls of the beaker. The distilled digested sample was filtered and made up to 50 mL and analyzed using Atomic Absorption Spectroscopy (AAS). A blank was prepared in the same manner using distilled water instead of the sample (Radojevic and Bashkin, 1999).

Isolation and identification of *Pseudomonas putida*from petroleum refinery effluent

Two hundred milliliters of the prepared Mineral salt media (MSM) contained in a 500mL Erlenmeyer flask was inoculated with 1% (v/v) of the effluent sample. The flask was incubated for 7 days at room temperature on a shaker. Ten-fold serial dilutions were carried out for all suspensions of enrichment samples. Each dilution (0.1ml) was inoculated unto the surface of freshly prepared cetrimide agar plates (prepared according to manufacturer's instruction) using spread plate method. The plates were incubated at 37°C for 24 hours. The resulting bacterial colonies were examined for size, shape, margin consistency and pigmentation. Distinctive colonies were then sub-cultured unto nutrient agar plates by streaking for purification. The resulting pure isolates were sub-cultured unto a nutrient agar slant medium and kept in a refrigerator until characterization and identification (Manal, 2011). Morphological and biochemical studies such as Gram's reaction, motility, citrate, Urease, nitrate reduction, Starch hydrolysis, ONPG, Carbohydrate fermentation, Oxidase tests were performed as described by Cowan and Steel, (2003).

Further identification of the isolates were carried out using Microgen kits; GNA and GNB Enterobacteriacae-ID kit for oxidase positive gram negative bacteria.

Screening of isolates of *Pseudomonas putida* for diesel-degrading potential

Standardization of inoculum

Suspension of *Pseudomonas putida* corresponding to $1.8x10^9$ CFU/mLisolated were prepared separately in normal saline and compared with a McFarland standard (6.0). Colonies were added to the normal saline until it was as turbid as the McFarland standard to obtain a bacterial population density of $1.8x10^9$ CFU/ml (Atta, 2009).

Fifty millilitre of MSM prepared as previously described was dispensed into five (5) 100mL

Erlenmeyer flasks and autoclaved at 121^{0}C at The inoculum for each isolate was introduced to the sterile medium at 10% (v/v) and diesel was added at 0.5% (v/v). The fourth flask and the fifth flasks served as control containing only MSM and MSM + diesel, respectively. The flasks were incubated at room temperature on an orbital shaker for seven days. Absorbance readings (at 540nm) were obtained on the first and the seventh day of incubation respectively. Increase in absorbance was used as a measure of increase in bacterial growth and their corresponding ability to degrade diesel (Atta, 2009).

Biodegradation studies

*Pseudomonas putida*C15a was observed to be the most efficient strain and as such, was selected for further studies. The most efficient One hundred milliliters (100) mL of mineral salt media (MSM) prepared as previously described was dispensed into two (2) 250mL Erlenmeyer flask and isolate of *Pseudomonas putida* (McFarland standard 6 was used to standardise the inoculums size) was added to the sterile medium at 10% (v/v) and enriched with 0.5% (v/v) diesel as source of hydrocarbon. The second flask which served as the control contains MSM and diesel.

The set-up was incubated on a rotary shaker at 150 rpm at ambient temperature for 18days. At 3 days intervals, the ability of the isolates to degrade hydrocarbons was studied by determining the hydrocarbon utilizing bacterial (HUB) count, optical density and changes in pH of the culture (Okerentugba and Ezeronye, 2003).

Hydrocarbon utilising bacteria (HUB) count

An aliquot (1mL) of the broth culture from each flask would be introduced into test tubes containing 9 mL of sterile distilled water, then a ten-fold dilution was carried out up to dilution 10^{-5}, and then 0.1mL of the dilution 10^5 was aseptically inoculated on nutrient agar 15atm for 15 minutes. plates using the spread plate method and incubated at 35^{0}C for 24 hours, the colonies obtained were counted (Atta, 2009).

Optical density

Five milliliter (5mL) of the broth culture was aseptically transferred from each flask into test tubes, and the residual hydrocarbons extracted by adding 5mL dichloromethane (DCM) and centrifuged at 5000 rpm for 5minutes. The resulting supernatant is read at a wavelength of 250nm using a UV- Visible spectrophotometer (Nwankwegu *et al.*, 2016). The residual hydrocarbon was calculated after determining the amount of hydrocarbon from a prepared standard using known amounts of hydrocarbon (Manal, 2011); using the formula below:

Percentage degradation= $\left[\frac{\text{RESIDUAL HYDROCARBON control} - \text{RESIDUAL HYDROCARBON treatment}}{\text{RESIDUAL HYDROCARBON control}}\right] \times 100$

Determination of pH

The pH was determined at 3 days intervals using a pH meter and the readings were recorded.

RESULTS

The physicochemical properties are as shown in table 1. The effluent had pH value of 6.8 and temperature of 26.2°C which were taken at the site of sample collection.

The concentration of each heavy metal in ppm for the refinery effluent is shown in Table 2. The concentration of the metals in the effluent in decreasing order is, Zn >Pb> Cr > Cd >Mn.

Three isolates were tentatively identified as strains of *Pseudomonas putida* on the basis of biochemical characteristics (Table 3), and subsequent identification using Microgen kit (Table 4).

Table 1: Physico-chemical properties of the refinery effluent sample

Physicochemical Property	Value
DO (mg/L)	200
BOD (mg/L)	100
COD (mg/L)	1200
EC (mg/L)	620
TDS (mg/L)	950
Turbidity (NTU)	179
pH	6.8
Temperature (°C)	26.2
Nitrate (mg/L)	6.81
Phosphate (mg/L)	27.12
Sulphate (mg/L)	4.82
Organic carbon (mg/L)	10.77
Total carbon (mg/kg)	1.54

Table 2: Heavy metal concentration of petroleum refinery effluent

Heavy metal	Amount (mg/L)	USEPA Standard (mg/L)
Manganese	0.000	0.05
Lead	0.456	0.015
Cadmium	0.007	0.005
Zinc	1.034	5.0
Chromium	0.009	0.1

Table 3: Biochemical and characterization of strains of *Pseudomonasputida*

Test	**Reaction**
Gram staining	-
Motility	+
Citrate	+
Oxidase	+
Catalase	+
Starch hydrolysis	-
Inference	*Pseudomonas putida*

+: Positive, -: Negative

Table 4: Phenotypic identification of isolates of *Pseudomonas putida* based on Microgen identification kit.

Isolate code	Octal code	Probability (%)	Identity
C2b	640522001	90.5	*Pseudomonas putida*
C11b	640522001	90.5	*Pseudomonas putida*
C15a	640722001	90.5	*Pseudomonas putida*

The isolates of *Pseudomonas putida* were tested for their ability to degrade diesel using the absorbance value as shown in Figure 1. Among the strains of *Pseudomonas putida* isolated, C15a showed the highest increase in optical density (69.08%) while, C11b had the lowest (-27.45%).

The percentage of hydrocarbon removal increased during the experimental period (Figure 2), as exhibited from the increase in optical density, which implies increase in cell growth. This validated the hydrocarbon utilizing bacterial (HUB) count, which was observed to increase of the sample during the experiment (figure 3). The control had no growth all through the period of the experiment. In the first 3 days of the biodegradation experiment, a low bacterial count was recorded, followed by an increase in growth before dropping on the 18th day (3.5 x 10^6 CFU/g).

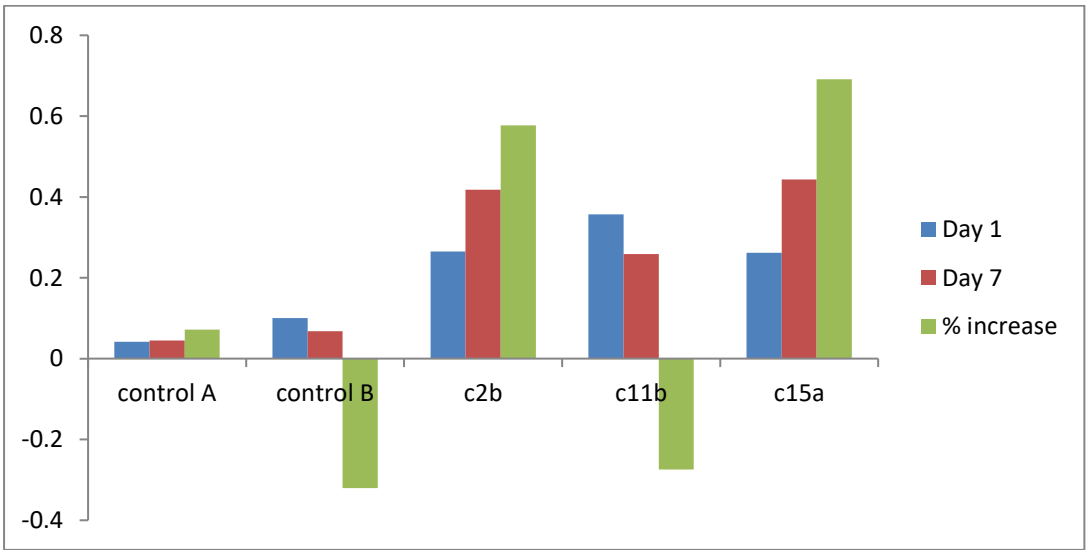

Fig. 1: Diesel-degrading potential of *Pseudomonas putida* strains based on optical density

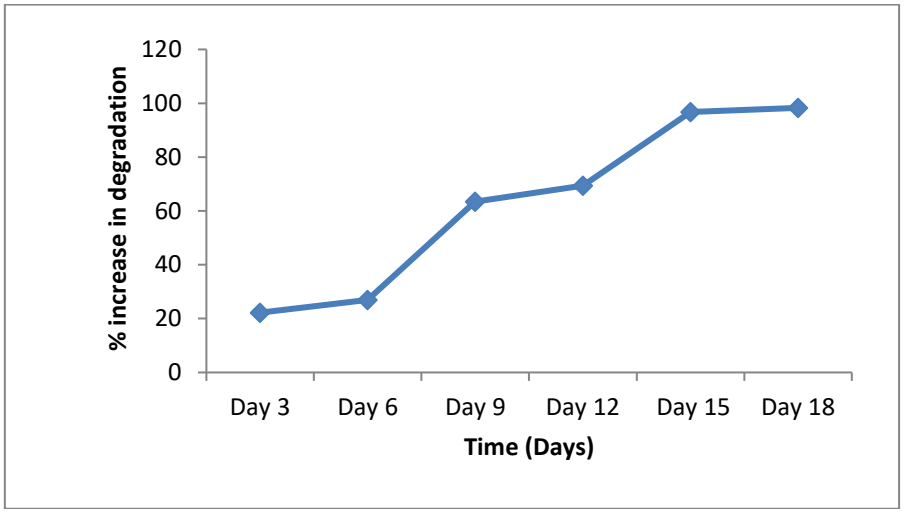

Fig 2: Percentage degradation of diesel by *Pseudomonas putida*C15a

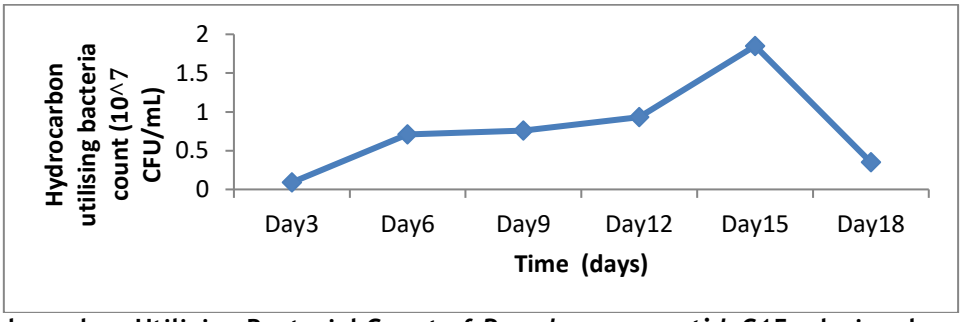

Fig. 3: Hydrocarbon Utilizing Bacterial Count of *Pseudomonas putida*C15a during degradation of diesel

DISCUSSION

The effluent sample used in the isolation of the target bacteria is rich in hydrocarbons since it is a product of the refining of petroleum, thus the bacteria and fungi present in the effluent are adapted to the presence of these compounds and can actively utilize them for their metabolic needs. Hydrocarbon-degrading microorganisms from refinery effluent have been isolated and these isolates have successfully been subjected to biodegradation studies on a laboratory scale. Species of either *Bacillus* or *Pseudomonas* or both have been consistently isolated from oil polluted soil and implicated in crude oil biodegradation (Satishkumar *et al.*, 2008; Idise *et al.*, 2010; Agarry*et al.*, 2012; Vinothini *et al.*, 2015; Riskuwa-Shehu and Ijah, 2016). The turbidity of the effluent sample was very high (179 NTU) and it is an indication of high microbial contamination as well as the presence of other compounds released as a result of the refining process. However, in a study conducted in Warri, Delta State, in the southern part of Nigeria, the effect of refinery and petrochemical effluent on the quality of a creek (Uzoekwe and Oghosanine, 2011) was analysed. In that study, the turbidity values recorded at the discharge point, upstream and downstream were 50.17NTU, 21.65NTU and 21.67NTU respectively. These values are much lower than the value obtained in this study. The pH of the effluent is in the acidic range which is expected due to the presence of bye products from the refining process. The high organic carbon content is an indication of the presence of hydrocarbons in the sample; the nitrogen (nitrate) content is very optimal for growth of microorganisms. A proportion of Carbon and nitrogen shows a nutrient rich medium for the proliferation and growth of the microorganisms in the effluent. The value of BOD is considerably high, indicating that bacteria in the effluent are rapidly depleting the oxygen as the organic waste is being degraded. This is consistent with other studies on the isolation of hydrocarbon degrading microbes from refinery effluent (Machido *et al.*, 2014; Stanley *et al.*, 2017).

Among the analyzed heavy metals, Pb and Cd exceeded the recommended levels set by the

United State Environmental Protection Agency (USEPA). The value for cadmium is only slightly higher than the standard however; the concentration of lead is much higher. Lead is commonly released in effluents from petroleum, textile, tannery, and chemical fertilizer industries.

Based on the conventional biochemical tests carried out, three (3) strains of *Pseudomonas putida*were isolated. *Pseudomonas putida*is a known degrader of hydrocarbons, and has been the subject of many studies on biodegradation of petroleum hydrocarbons (Saidu *et al.*, 2018; Agbo, 2019). The bacterium is known to possess genes necessary for the utilization of monoaromatic and polycyclic aromatic hydrocarbons (Gomes *et al.*, 2005; Higashioka *et al.*, 2009; Isaac *et al.*, 2013).

During screening for biodegradation of diesel, the three strains of *Pseudomonas putida* performed to varying capacities (Fig. 1). This might be due to differences in the genetic makeup of the individual strains thus resulting in varying metabolic and functional abilities between members of the same species. One of the strains (C11b) even showed a decrease in the percentage of diesel reduced after the period of incubation. It's possible this strain does not possess the same functional capabilities as the other two strains; this could be the absence of the key catabolic genes either on its chromosome or plasmid.

The biodegradation experiment of diesel using the selected strain, *Pseudomonas putida* C15a, showed a constant increase in the percentage of hydrocarbons utilized. The highest percentage biodegradation of the diesel was observed on day 18 (Fig. 2). The reason for the continuous biodegradation in the sample might be due to reduction in the concentration of hydrocarbons in the sample as the enzymes responsible for the aerobic breakdown of the hydrocarbons act on the resulting intermediates at every stage of the metabolic process. This is in agreement with the findings of Rahman*et al.* (2002) who reported increase in the rate of biodegradation of crude oil, as the concentration of oil reduces.

It was observed that there was a steady increase in the HUB count (Fig. 3) of *Pseudomonas putida* C15a, reaching its peak on the day 15 of incubation (1.85 X 10^7 CFU/mL). Increase in growth of inoculum during biodegradation experiments is usually linked to nutrient availability, thus as increasing amounts of hydrocarbon are utilized it leads to the proliferation of the hydrocarbon degrading bacteria. By the end of the experiment (day 18), the count was observed to fall, probably as a result of depletion of the carbon source (diesel). Thus, the number of the hydrocarbon utilizing bacteria and the rate of degradation can be said to be linked. From this it can be understood that the efficiency of the organism and its stage of growth should be taken into consideration in clean up or removal of oil from the environment (Darsa*et al.*, 2014).

The utilization of the crude oil as sole carbon and energy source by these microorganisms resulted in their growth with a concomitant production of acid. These acidic metabolic products might account for the decrease in pH of the culture medium (Okerentugba and Ezeronye, 2003).

CONCLUSION

Contamination of water with hydrocarbon wastes stimulates indigenous microbial populations, which are capable of utilizing the hydrocarbon substrates as their sole carbon and energy sources thereby degrading the contaminants. Several bacterial species have been identified as having the ability for oil degradation. These organisms carry out their normal life processes using these contaminants as their source of nutrients. Metabolic processes of these organisms are capable of using chemical contaminants as energy source, rendering the contaminants harmless or less toxic in most cases. This study proposed a better solution for bioremediation of spilled petroleum hydrocarbons in soil and water ecosystems. Hence, *Pseudomonas putida* could be effectively utilized as a form of bioremediation in the removal of hydrocarbons from contaminated environments.

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