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Research Article

Comparative Hydrocarbon-Degrading Efficiency of Wild and Mutant Strains of *Bacillus subtilis* and *Pseudomonas putida* Isolated from Refinery Effluents

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Abstract

Petroleum refinery effluents are characterized by the presence of pollutants such as hydrocarbons as such could be of serious environmental consequence if discharged into receiving sites without proper treatment to remove the pollutants. This study assessed the capacity of wild and mutant strains of *Bacillus subtilis* and P*seudomonas putida* isolated from refinery effluent in the degradation of hydrocarbons present in the effluent. The physicochemical parameters of the raw and treated effluent samples collected from Kaduna Refinery and Petrochemical Company (KRPC) were determined using standard guidelines. With the exception of turbidity (35.3 and 18.2 NTU), BOD (190 and 29.6 mg/L), COD (351.2 and 78.1 mg/L) and Oil and Grease (45.2 and 17.9 mg/L) for the raw and treated effluent respectively, and conductivity (695 µS/cm) for the raw effluent, all other parameters were within the permissible limits set by FMENV. Six (6) and eleven (11) isolates of Bacillus subtilis and Pseudomonas putida respectively were isolated from the effluent and screened for capacity to utilize and grow on mineral medium containing different concentrations (0.5%, 1%, 1.55% and 2%) of crude oil as the sole source of carbon. Isolates TE8 (*B. subtilis*) and TEC10 (*P. putida*) had luxuriant growth across the first three concentrations of crude oil and medium growth on medium containing 2% crude oil. Both isolates were treated with nitrous acid and UV- irradiation to generate mutants. Death rate of 59.67% (40.33% survival) and 66.33% (33.67% survival) were observed for *Bacillus subtilis* and *Pseudomonas putida* respectively on treatment with nitrous acid. Also death rate of 51.67% (48.33% survival)

and 40% (60%) were observed for Bacillus subtilis and Pseudomonas putida respectively on exposure to UV- irradiation. The relative efficiency of hydrocarbon degradation by the wild and mutant strains was assessed by evaluating the Hydrocarbon utilizing bacterial (HUB) counts and changes in concentration of oil and grease at intervals of 3 days for 15 days. The UV-mutant strains had slightly higher HUB counts (2.73×108, 1.5×108 and 2.391×108) than the wild strains (2.73×108, 1.32×108 and 2.39×108) while the nitrous acid mutant strains had the lowest HUB counts (8.35×107, 8.13×107 and 5.07×107) for P. putida, B. subtilis and their co-culture respectively. Also, higher oil and grease degradation was observed in the UV-mutant strains with 96.23%, 92.60% and 99.38%, followed by the wild strains with 87.25%, 80.25% and 88.89% and the nitrous acid mutant strains with 80.25%, 68.52% and 81.48% oil and grease degradation for *P. putida, B. subtilis* and their co-culture respectively. It was therefore concluded that while wild strains of *B. subtilis* and *P. putida* are good hydrocarbon degrading agents, irradiation with UV results in increased efficiency and the co-culture of the bacteria were more efficient than the individual culture.

Keywords: Biodegradation; Pseudomonas Putida; *Bacillus subtilis*

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 [1-3]. Wastewater (effluents) 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, sulphides, naphthylenic acids, and other chemicals [4-6], most of which are known to be mutagenic, carcinogenic, and growth inhibitory and, by extension, can have adverse effects on the ecology of the receiving sites and public health [1,7].

Petroleum refinery effluents contain organics and have a characteristic oily nature; hence, when discharged into water bodies, they cause depletion of dissolved oxygen (due to the transformation of the organic components into inorganic compounds by microorganisms), prevent reaeration, and cause the loss of biodiversity through a decrease in amphipod populations important in food chains and eutrophication [1,8,9].

Effluents are usually treated before discharge into the receiving sites [10]. 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 [11-14]. 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 [15,16]. Furthermore, the impact of these toxicants on the quality of the effluent-receiving water bodies and soils is rarely investigated [10,17].

The high demand for petroleum as a source of energy and as a primary raw material for chemical industries in recent years has resulted in an increase in its production worldwide. This dramatic increase in production, refining, and distribution of crude oil has brought with it an ever-increasing problem of environmental pollution [18-20]. However, the persistence of petroleum pollution depends on the quantity and characteristics of the hydrocarbon mixture and on the properties of the affected ecosystem [21,22].

Several techniques, including physical, chemical, and biological methods, have been developed to resolve the problem of petroleum pollution [21,23]. However, the most promising approach so far is microbial degradation since the physico-chemical methods are rather too expensive, not environmentally friendly, and do not result in total elimination of the pollutant [24,25]. Microbial degradation (biodegradation) involves the use of microbes to break down potentially harmful pollutants into harmless products [26,27]. Hence, the ability to isolate high numbers of certain microorganisms from petroleum-polluted environments is commonly taken as evidence that such microorganisms are active petroleum degraders [28,29]. Several researchers have recorded successes in the use of bacteria, especially members of the genus *Bacillus* and *Pseudomonas*, in the degradation of petroleum hydrocarbon [30-34].

Improvement in the ability of microorganisms to degrade a pollutant could be achieved through modification of the environment or the organism. The organism can be modified through mutagenesis. Various mutagens abound, and the exposure of organisms to ultraviolet (UV) light and treatment with nitrous acid has been employed with relative success [35-37]. Such mutants, under optimal growth conditions, could possess enhanced petroleum degradation potential than their parents [38,39].

Liquid wastes generated by refineries consist of chemical components such as oil and grease, phenols, benzene, toluene, ethylbenzene, and xylene (BTEX), ammonia, suspended solids, cyanide, sulphide, nitrogen compounds, and heavy metals such as Fe, Cd, Ni, Cr, Cu, Mo, Se, V, and Zn—most of which are known to be toxic [40-42]. It has been reported that adherence to legally set toxicant levels for effluents by refineries and petrochemical plants in Nigeria has always been very low [15,43]. This is because most of the methods employed for the treatment of wastewater from such plants have been shown to be inefficient [11,12,44]. As such, wastewaters may still pose serious dangers, leading to the accumulation of toxic materials at the receiving sites (i.e., bodies of water and soils) with far-reaching consequences on the ecosystem [11,12,45].

The pollutants (hydrocarbons) persist in the environment as the resident microorganisms handle or remedy the problem very slowly [28,46]. Such polluted habitats lose their capability to support both plant and animal life and thus constitute public health and socioeconomic hazards [7,28]. The organic content of the effluent exerts a very high level of biological oxygen demand while its oily nature prevents reaeration of contaminated water bodies. Thus, such contaminated water bodies could be rendered anaerobic, resulting in massive fish kills and loss of many aquatic lives, thereby distorting the natural equilibrium that exists in such aquatic environments [47-51]. In the same vein, discharge of such effluent into the soil could drastically alter the physicochemical properties of the soil, thereby reducing its productivity [27,52].

The physicochemical methods used to remove hydrocarbon pollutants in general are too complex, expensive, frequently result in the introduction of new toxic substances into the environment, and seldom ensure total elimination of the pollutants [44,53]. There is therefore the need to develop an efficient, cost-effective, sustainable, and eco-friendly approach for removing hydrocarbons from refinery effluent before discharge into receiving sites [2,54].

There are several reports to the effect that wild strains of *Bacillus* spp. and *Pseudomonas* spp. are very active in the degradation of petroleum hydrocarbons [30,55,56]. Other bacteria capable of utilizing hydrocarbon as the only source of carbon and energy, thereby degrading it to harmless products, have been reported [46,57]. However, dependence on the capacity of the wild strains in clean-up processes has been shown to require an extended period of time. There is therefore the need to conduct investigations with a view to augmenting the degradative capacity of wild strains for faster removal of petroleum contaminants from polluted sites [38,58].

It is very necessary to develop an effective means of degrading hydrocarbons in effluents generated from indigenous refineries and petrochemical plants as compliance with the legally set toxicant levels for refineries and petrochemical plants in Nigeria has always been very low [15,59].

The aim of this study was to assess the capacity of wild and mutant strains of *Bacillus subtilis* and *Pseudomonas putida* isolated from refinery effluent in the degradation of hydrocarbons.

Materials and Methods

Collection of refinery effluent samples

Raw and treated refinery effluent samples were collected from Kaduna Refining and Petrochemical Company (KRPC), Kaduna Nigeria, by using a clean cup to draw the samples from the effluent ponds into new clean 1 litre plastic containers [60]. The samples were transported at ambient temperature to the Department of Microbiology, Ahmadu Bello University Zaria for analysis.

Already Profiled Escravos light crude oil was obtained from the Department of Microbiology, Ahmadu Bello University Zaria.

Determination of the Physico-chemical Properties of the Raw and Treated Refinery Effluents

The physicochemical parameters that were assayed included: pH and temperature

The pH and temperature of the effluents were determined at the point of collection using the HANNA combo tester (H198130, Den-

ver, USA); a water proof tester that accurately measures pH and temperature in a single test. Briefly, following the manufacturer's instructions, the electrodes connected to each meter were submerged in a clean bucket containing the sample. The values read for each parameter by the tester were observed and recorded.

Electrical conductivity

The conductivity of the sample was determined using a conductivity cell. The temperature of the sample was first adjusted to about 20ºC and a portion of the sample was used to wash the conductivity cell and then the sample was filled completely ensuring that no air bubbles adhered to the electrode and the readings were recorded.

Total dissolved Solids

One hundred millilitre (100 ml) of the sample was filtered using 0.45mm filter paper and placed in a pre-weighed crucible and dried in an oven at 103^{0}C for 2 hours. The dish was allowed cool briefly and dried in a desicator for 1hour 30 minutes. The weight of empty crucible was subtracted from the weight of crucible after drying to give weight of total residue. The total dissolved solids were calculated using the formula APHA [60]:

$$\text{Total dissolved solid (mg/l)} = \frac{\text{Weight of total residue}}{\text{Volume of sample}} \times 1000$$

Total suspended solids

An evaporating dish was weighed empty and 100 ml of the sample was dispensed into it, evaporated to dryness in a drying oven at 103^{o}C for 3hours, allowed to cool and dried in a desicator. The dish was reweighed and the total suspended solids were obtained using the formula:

$$\text{Total suspended solids (mg/l)} = \frac{\text{Weight of suspended solid}}{\text{Volume of sample analyzed}} \times 1000$$

Dissolved oxygen and biochemical oxygen demand

Dissolved oxygen and biological oxygen demand was determined using HANNA instrument (HANNA 3200, Denver USA). Dissolved oxygen was determined by inserting the instrument into the sample at the point of collection while the BOD was carried out by transferring the sample into a BOD bottle and incubated for five days at 25^{0}C. The cell of the instrument was inserted into the incubated sample and the level of residual oxygen was recorded. The value of residual DO was then subtracted from the initial DO value to obtain the BOD of the samples [61].

Nitrates

The sample was adjusted to pH 7.0 and 100 ml of the sample was placed in a beaker and allowed to evaporate to dryness in a water bath. The resulting residue was dissolved using a glass rod with 2 ml disulphonic acid reagent and transferred to Nessler's tube. Six millilitres (6ml) of ammonium hydroxide (NH_4OH) and EDTA was added drop-wise wise till the residue dissolved. A blank was also prepared the same way using distilled water instead of the sample. The absorbance of the colour developed was then read at 410nm with a light path of 1cm using spectrophotometer. The nitrate concentration was estimated using a standard curve. The nitrate concentration in the samples was calculated using the formula APHA [60].

$$\text{Nitrate (mg/l)} = \frac{\text{Nitrate (N)} \times 1000}{\text{Volume of sample analyzed.}}$$

Sulphates

One hundred millilitres (100ml) of the sample was dispensed into 250 ml Erlenmeyer flask containing 5 ml of conditioning reagent and mixed using a magnetic stirrer. Ten grams of barium chloride crystals was added while the solution was still being stirred. The turbidity was measured for 30seconds after stirring at interval of 4 minutes using turbidity meter (Hach 2100N, USA). A blank was run with no barium chloride added. The sulphate concentration was calculated using the formula below and sulphate concentration was estimated by reading off the standard curve APHA [60].

Mg /l of sulphate (SO_4) = $MgSO_4$ × **1000**/ml of sample taken

Phosphates

Preliminary sample treatment

About 0.1 ml of phenolphthalein indicator was added to 100 ml of the sample and concentrated hydrochloric acid solution was added drop wise until the colour turns pink.

Colour development

One hundred millilitres (100ml) of treated sample was dispensed into a flask and 4.0 ml of molybdate reagent was added

and mixed thoroughly. One millilitre (1ml) of stannous chloride reagent was added drop-wise. Distilled water was used to prepare a blank in the same way. After 10 minutes, the absorbance of the colour developed was measured photometrically at 690 nm using spectrophotometer. The phosphate content of the samples was read off the standard curve and the concentration was obtained using the formula APHA [60]:

$$\text{Mg/l } PO_4 = \frac{PO_4 \times 1000}{\text{Volume of sample analyzed.}}$$

Microbiological analysis

Isolation of Bacillus subtilis and *Pseudomonas putida* from the raw refinery effluent

Preparation of mineral salt medium

Hydrocarbon utilizing strains of *Bacillus subtilis* and *Pseudomonas putida* were isolated from the effluent sample using mineral salt medium (MSM) with the following composition per litre: KH_2PO_4 (2.0g), $NaNO_3$ (2.0g), NaCl (0.8g), KCl (0.8g), $Na_2HPO_4.12H_2O$ (2.0g), $MgSO_4$ (0.2g), $FeSO_4.7H_2O$ (0.001g). The salts above were dissolved in 1(one) litre of distilled water and sterilized by autoclaving at 121^{0}C for 15 minutes [62,63].

Inoculation of sample

This was carried out by following the method reported by Naga., *et al*. [64] with slight modification. The effluent sample was shaken vigorously and 200 ml was dispensed into a beaker and allowed to set at room temperature on a thoroughly disinfected laboratory work bench for 4 hours to concentrate the sample by sedimentation. The supernatant was discarded leaving the sediment to about 20 ml volume followed by vigorous shaking to resuspend the sediment. Ten (10) ml of the concentrated effluent sample was dispensed into 250 ml conical flask containing 90 ml of mineral salt medium supplemented with 1% (v/v) of crude oil. The preparation was incubated at ambient temperature on a shaker at 150 rpm for 14 days. About 50 ml of the culture was dispensed into a sterile 100 ml conical flask and heat shocked in a water bath at 80^{0}C for 1 hour to eliminate all non-spore formers. Both the heat shocked and non-heat shocked samples were serially diluted to a factor of five and 0.1 ml from each dilutions of the heat shocked and non-heat shocked culture were respectively inoculated unto the surface of nutrient agar (for B. *subtilis*) and centrimide agar (for P. putida) prepared according to manufacturer's instruction. The resulting bacterial colonies were examined for size, shape, margin consistency and pigmentation. Distinctive colonies were sub-cultured unto nutrient agar plates by streaking for purification. The resulting pure colonies were subcultured unto a nutrient agar slant medium for further studies and preserved at 4-5^{0}C [64].

Identification of the isolates

The isolates were identified with a combination of microscopic and biochemical techniques. For the microscopic identification, the isolates were gram stained and examined microscopically to determine their gram reaction and cellular morphology. Spore staining test was carried out on isolates showing gram positive reaction and rod morphology. Then the spore forming gram positive rod isolates and those that showed gram negative reaction and rod morphology were transferred unto nutrient agar slant medium and preserved in the refrigerator at 4-5^{0}C for further identification tests.

Biochemical identification

The isolates were biochemically identified following the scheme described by Cowan and Steel [65] which include.

Motility test

This test was carried out by inoculating the motility medium with the colonies of the gram positive and negative isolates in separate test tubes. A stab was made with a straight wire to a depth of about one third the total volume of the medium. The culture was then incubated at 35^{o}C for 24hours. If the culture turns cloudy (turbid) after incubation, it means the organism is motile but if growth is restricted to the line of inoculation and the rest of the culture remains clear, then the organism is non-motile [65].

Citrate utilization test

This test was carried out by inoculating the colonies of the gram positive and negative rod isolates on Simmons' citrate agar slant and the inoculated slants were incubated at 35^{o}C for 48-72 hours and examined for development of a deep blue colour which indicated a positive reaction [65].

Urease test

Urease test was carried out by inoculating urea agar slant with the colonies of the gram positive and negative isolates and the slant was incubated at 35^{o}C for 48-72 hours and examined for the development of bright pink or red colour which indicated a positive reaction [65].

Nitrate reduction test

This was carried out by inoculating Nitrate Broth lightly with colonies of the gram positive and negative rod isolates and incubated at 37°C for 24-48hrs. One (1 ml) of reagent A (sulphanilic acid) was added followed by 1 ml of reagent B (α-napthol amine) and examined. A deep red colour showed the presence of nitrite and thus, indicating that nitrate has been reduced.

To tubes didn't show a red colour within 5 min, powdered zinc (up to 5 mg ml of culture) was added and allowed to stand and then examined for red colour which indicated that nitrate was present in the medium (i.e. not reduced by the organism) and absence of red colour indicated nitrate was absent in the medium (i.e. reduced by the organism to nitrite, which in turn was itself reduced) [65].

Starch hydrolysis test

This was carried out by inoculating colonies of the gram positive and negative rod isolates on nutrient agar containing 0.2% soluble starch and the plates were incubated at 30°C for 24 hours. After incubation, the plates were flooded with Lugol's iodine and examined for zone of hydrolysis [65].

O-nitro-phenyl-D-galactopyranoside (ONPG) test

This was carried out by inoculating separate tubes of ONPG broth with colonies of the gram positive and negative rod isolates and incubated at 37°C for 48 hours and examined for β-galactosidase activity which was indicated by the appearance of a yellow colour due to the production of o-nitrophenol [65].

Carbohydrate fermentation test

A series of test tubes containing sterile nutrient broth with 1% each of membrane-filter-sterilized (0.45µm) single fermentable sugar was inoculated with the test organisms. The sugar fermentation tubes were incubated at 35°C for 24 hours. At the end of the incubation period, all tubes were examined for acid following the addition of methyl red indicator and then compared with the control for interpretation [65]. A total of eight (8) sugars were used for the fermentation tests, these include; Glucose, Cellobiose, Galactose, Mannose, Melibiose, Raffinose, Salicin and Xylose. This was done for only the gram positive rod isolates.

Oxidase test

This was carried out by placing a piece of filter paper in a clean Petri dish and 2-3 drops of freshly prepared oxidase reagent was added. Using a glass rod, a colony of the test organism was smeared on the filter paper and examined for the development of a blue-purple colour within a few seconds which indicated a positive test and absence of blue-purple colour within 10 seconds indicated a negative test.

Indole test

Indole test was carried out by inoculating colonies of the gram positive rod isolates into 1% peptone water and then the inoculated peptone was incubated at 37°C for 24 hours. After 24 hours of incubation, 3 drops of Kovac's reagent was added and shaken and examined. A positive reaction was indicated by the development of a red colour in the reagent layer above the broth while a negative reaction was indicated by a yellow colour [65].

Methyl Red - Voges-Proskauer (MR-VP) test

This test was carried out by inoculating 5 ml of MR-VP broth with colonies of the gram positive rod isolates and then the inoculated broth was incubated at 35°C for 48-72 hours. After 24 or 48 hours of incubation, about 1 ml of the cultured broth was transferred to a small test tube to which 2-3 drops of methyl red indicator was added and examined. Formation of red colour on addition of the indicator signified a positive methyl red test and a yellow colour signified a negative test.

To the rest of the broth, 2 drops of 40% potassium hydroxide was added followed by 2 drops of 5% α-Naphthol in ethanol. The tube was shaken and placed in a slope and examined. Development of a red colour starting from the liquid – air interface within 1 hour indicated a VP positive test while no colour change indicated VP negative test [65].

Isolates confirmed to be *Bacillus subtilis* and *Pseudomonas putida* on the basis of biochemical characteristics were authenticated using the Microgen kits; *Bacillus*-ID kit for *Bacillus subtilis* and GNA and GNB Enterobacteriaceae-ID kit for *Pseudomonas putida*. The kits were obtained from Microgen Bioproducts Company, United Kingdom (UK). Isolates thus authenticated were stored in the refrigerator for further studies.

Standardization of inoculum

A suspension of the wild strains of *Bacillus subtilis* and *Pseudomonas putida* isolated was prepared separately in normal saline and compared with a 1.0 McFarland standard. Colonies were added to the normal saline until it was as turbid as the McFarland standard to obtain a bacterial population density of 3.0×10^8cfu ml [64].

Screening the Capacity of *Bacillus subtilis* and *Pseudomonas putida* to Degrade Hydrocarbons

Mineral salt agar medium was prepared as previously described into different conical flasks and enriched separately with 0.5%, 1%, 1.5% and 2% crude oil and sterilized by autoclaving. The medium were dispensed into petri dishes and allowed to solidify after which they were inoculated with 18hours culture of the *Bacillus subtilis* and *Pseudomonas putida* isolates separately by streaking. The preparation was incubated at ambient temperature for 48 hours. The capacity of the isolates to degrade hydrocarbons was determined by the ability of the organisms to grow on the medium which were categorized as luxuriant growth, medium growth, scanty growth and no growth [64]. Isolates that grew well on the various concentration of the crude oil media was subjected to mutation.

Induction of mutation

Induction of mutation using ultraviolet (UV) irradiation

Five (5) ml of the standard suspensions (in phosphate buffer) of the wild strains were aseptically transferred into different sterile Petri plates and exposed to the light of a UV at 254 nm wavelength in a dark room for 10, 20 and 30 minutes at a distance 6cm from the UV light source. The irradiated cells were transferred into a sterile centrifuge tube and treated with caffeine (0.2w/v) and stored in the dark overnight to avoid photo-reactivation. The tubes were centrifuged at 3000rpm, the supernatant was discarded and the sediment resuspended in normal saline and washed thrice. Then, 1 ml of the irradiated cell suspension was serially diluted in normal saline and plated by transferring 0.1 ml unto the surface of a sterile nutrient agar plate using spread plate method and incubated at 37^0C for 24 hours [66].

Induction of mutation using nitrous acid

Standard suspension of the isolates was prepared and acetate buffer (0.2M, pH 4.5) was also prepared in accordance with the procedure reported by Idise., *et al*. [66]. To 50 ml of the standard suspension in 150 ml conical flask, 50 ml of acetate buffer was added and treated with 1.5 ml of 2.0M nitrous acid. This was allowed to stand at ambient temperature for 20 minutes. The reaction was terminated by serial dilution with Tris HCl. The treated cells were centrifuged at 1500rpm for 5 minutes and resuspended in acetate buffer and washed thrice to remove all traces of nitrous acid. The treated cells were then inoculated using spread plate technique on nutrient agar and incubated at 37^0C for 24 hours [66,67].

Plates with about 50% survival rate after treatment were used for further studies. The resulting mutant strains of *Bacillus subtilis* and *Pseudomonas putida* were stored in nutrient agar slant. Then, hydrocarbon degradation capacity test was carried out for the selected wild and mutant isolates of *Bacillus subtilis* and *Pseudomonas putida*.

Determination of Hydrocarbon Degrading Capacity of the Wild and Mutant *Bacillus subtilis* and *Pseudomonas putida*

Mineral salt medium was prepared as previously described but using raw (untreated) effluent as the solvent instead of distilled water. About 99 ml of the effluent medium was dispensed into four (4) 250 ml Erlenmeyer flask and sterilized by autoclaving. Two of the flasks were inoculated separately with 1 ml (1.5×10^8cfu/ml) suspension of the selected isolates of *Bacillus subtilis* and *Pseudomonas putida*, the third flask was inoculated with a co-culture of the both isolates while the fourth was not inoculated, serving as a control [68]. This was done for both the wild and mutant strains of the isolates. The set-up was incubated at ambient temperature on a rotary shaker at 150rpm for 3, 6, 9, 12 and 15 days respectively. After each interval of incubation, a set of the experiment was analysed for growth and residual hydrocarbons [69].

The bacterial growth was determined by measuring the optical density using spectrophotometer [64], while the residual oil and grease (hydrocarbons) was determined using gravimetric method [70]. Here, the oil and grease was extracted by mixing 50 ml of culture broth with 50 ml of petroleum ether in a separating funnel and shaken vigorously to get a single emulsified layer. Acetone was added to it and shaken gently to break the emulsification, which resulted in three layers. Top layer was a mixture of petroleum ether, oil and acetone; clumping cells made up the middle layer and the bottom aqueous layer contained acetone, water and biosurfactant

in soluble form. The two lower layers were discarded while the top layer containing a mixture of petroleum ether, residual hydrocarbons and acetone was collected in a clean pre-weighed vial. The extracted oil was passed through anhydrous sodium sulphate to remove moisture. The resulting petroleum ether and acetone was evaporated on a water bath and dried briefly in a desiccator and weighed. The residual oil and grease after biodegradation was determined using the formula [69].

$$\text{Oil and Grease (mg/l)} = \frac{\text{A-B} \times 1000}{\text{Volume of sample analyzed}}$$

Where;

A = Weight of vial + residual oil and grease

B = Weight of empty vial

Statistical analysis of data

Data obtained in this study were subjected to analysis of variance (ANOVA) test at 95% confidence interval (0.05 level of significance) using Statistical Package for Social Science (SPSS) version 17 and were presented in tables charts and graphs where necessary.

Results and Discussion

Results

Physicochemical parameters of raw and treated refinery effluent

Improvement in quality was observed between the raw effluent and that undergoing treatment (biologically treated). The pH, Temperature, Total Dissolved Solids (TDS), Nitrate, Sulphate and Phosphate of both effluent samples were within the permissible limits stipulated by the Federal Ministry of Environment (FMENV) for refinery effluents in Nigeria while other parameters analyzed for the raw effluent were above the permissible limits. The Turbidity, Biochemical Oxygen Demand (BOD), Chemical Oxygen Demand (COD) and oil and grease content of the effluent undergoing treatment were above the maximum permissible limits as shown in Table 1.

Table 1: Physicochemical Properties of Raw and Treated Petroleum Refinery Effluent.

Physicochemical Parameters	Raw Effluent	Biologically Treated Effluent	Percentage Reduction (%)	FMENV Standard
pH	6.86	6.58	4.08	6.0–9.0
Temperature (∘C)	29.9	20.7	30.77	≤40
Conductivity (µS/cm)	695	374	46.19	≤400
Dissolved Solids (mg/L)	354	216	38.98	≤500
Suspended Solids (mg/L)	35	14	60	≤50
Turbidity (NTU)	35.3	18.2	48.44	≤10
Dissolved Oxygen (mg/L)	160	5.7	96.44	≥10
Biochemical Oxygen Demand (mg/L)	190.5	29.6	84.46	≤10
Chemical Oxygen Demand (mg/L)	351.2	78.1	77.76	≤10
Oil and Grease (mg/L)	45.2	17.9	60.4	≤10
Nitrate (mg/L)	0.04	0.03	25	≤10
Sulphate (mg/L)	18.06	9.169	49.23	≤50
Phosphate (mg/L)	2.8	0.01	99.64	(Missing)

BOD = Biological Oxygen Demand, TDS = Total Dissolved solids, COD = Chemical Oxygen Demand, FEMNV = Federal Ministry of Environment, Nigeria, µS/cm = Micro siemens per centimetre, mg/l = Milligram per litre.

Isolation and Characterization of *Bacillus subtilis* and *Pseudomonas putida* from the Raw and Treated Refinery Effluent

The isolates were identified using a combination of cultural characteristics, microscopy and biochemical reactions as presented in Tables 2 and 3 for *Bacillus subtilis* and *Pseudomonas putida* respectively.

All the isolates (B. *subtilis*) were Gram positive, rod shaped and endospore formers. On the basis of cultural characteristics, all the isolates had creamy colonies and were circular in shape. With the exception of the isolates TE3, RE12 and RE17 that had convex shaped morphology, all the other isolates were flat. Isolates TE4, RE12 and RE20 formed large colonies on nutrient agar while isolates TE6, TE10 and RE19 formed tiny colonies on the same medium (Table 2).

Table 2: Cultural, Microscopic and Biochemical Characteristics of Gram Positive Isolates.

Isolate Code	Colonial Morphology	Gram Reaction	ES	Biochemical characteristics													Tentative Identity
				SH	M	C	U	I	Cat	MR	VP	NR	G	Sal	Gal	Xyl	Ara	
TE1	SCCF	G+ve rods	+(oval)	+	+	+	-	-	+	-	+	-	+	-	-	+	+	*B.* species
TE2	SCCF	G+ve rods	+(oval)	+	+	+	-	-	+	-	+	+	+	-	+	+	+	*B. subtilis*
TE3	SCCC	G+ve rods	+(oval)	+	+	+	-	-	+	-	+	+	+	+	+	+	+	*B. subtilis*
TE4	LCCF	G+ve rods	+(oval)	+	+	+	-	-	+	-	+	+	+	-	+	+	+	*B. subtilis*
TE6	TCCF	G+ve rods	+(oval)	+	+	+	-	-	+	+	+	-	-	+	-	+	+	*B.* species
TE7	SCCF	G+ve rods	+(oval)	+	+	+	-	-	+	-	+	+	+	-	+	+	+	*B. subtilis*
TE8	SCCF	G+ve rods	+(oval)	+	+	+	-	-	+	-	+	+	+	+	-	+	+	*B. subtilis*
TE9	SCCF	G+ve rods	+(oval)	+	+	+	-	-	+	-	+	+	+	+	+	+	+	*B. subtilis*
TE10	TCCF	G+ve rods	+(oval)	+	+	+	+	-	+	-	+	-	+	+	+	+	+	*B.* species
RE12	LCCC	G+ve rods	+(oval)	+	+	+	-	-	+	-	+	+	+	-	-	+	+	*B. subtilis*
RE13	SCCF	G+ve rods	+(oval)	+	+	+	-	-	+	-	+	+	+	-	-	+	+	*B.* subtilis
RE14	SCCF	G+ve rods	+(oval)	+	+	+	-	-	+	-	+	+	+	+	-	+	+	*B. subtilis*
RE15	SCCF	G+ve rods	+(oval)	+	+	+	-	-	+	-	+	+	-	-	-	+	+	*B. subtilis*
RE16	SCCF	G+ve rods	+(oval)	+	+	+	-	-	+	-	+	+	+	-	+	+	+	*B. subtilis*
RE17	SCCC	G+ve rods	+(oval)	-	+	-	+	-	+	-	+	+	-	+	+	+	+	*B.* species
RE18	SCCF	G+ve rods	+(oval)	-	+	-	+	-	+	-	+	+	-	-	-	+	+	*B.* species
RE19	TCCF	G+ve rods	+(oval)	-	+	+	-	-	+	-	+	+	-	+	+	+	+	*B.* species
RE20	LCCF	G+ve rods	+(oval)	-	+	+	+	-	+	-	+	+	-	+	+	+	+	*B.* species

KEY: TE; Treated Effluent, RE: Raw Effluent, SCCF: Small Cream Circular Flat, SCCC; Small Cream Circular Convex, LCCF; Large Cream Circular Convex, TCCF, Tiny Cream Circular Flat, LCCC; Large Cream Circular Convex, ES; Endospore, SH; Starch Hydrolysis, M; Motility, C; Citrate, U; Urease, I; Indole, Cat; Catalase, MR; Methyl Red, VP; Vogues Proskeur, NR; Nitrate Reduction, Sal; Salicin, Gal; Galactose, Xyl; Xylose, Ara; Arabinose.

All the isolates reacted positively to starch hydrolysis, motility, citrate, VP, nitrate reduction Catalase test and fermented all the sugars tested, with the exception of RE17 and RE18 that were both negative to starch hydrolysis and citrate tests, RE19 and RE20 negative to starch hydrolysis, TE1, TE6 and TE10 negative to nitrate reduction test, R15 and R18 negative to glucose, Salicin and galactose fermentation, TE1, RE12 and RE13 to salicin and galactose fermentation, TE6 to glucose and Galactose, TE2 and TE4 to Salicin, TE8 to galactose and RE17, RE19 and RE20 to glucose fermentation. All the isolates were negative to indole, methyl red and urease tests with the exception of isolates TE6 and TE10, RE17, RE18, RE20 which reacted positively to methyl red and urease respectively.

Table 3 depicts the gram negative bacteria isolated from the sample. All the isolates were rod shaped and had small, greenish, and circular colonies on centrimide agar. With the exception of isolates TEC3, TEC11, TEC12, REC13, REC19 and REC20 that were flat all the other isolates were convex-shaped.

Table 3: Cultural, Microscopic and Biochemical Characteristics of Gram Negative Isolates.

Isolate Code	Colonial Morphology	Gram Reaction	Biochemical characteristics										Tentative Identity
			M	C	U	OX	NR	Glu	Ara	Fruc	Xyl	Suc	
TEC1	SGCC	G-ve rods	+	+	+	+	-	+	+	-	-	-	*Pseudomonas* spp.
TEC2	SGCC	G-ve rods	+	+	+	+	-	+	+	-	-	-	*Pseudomonas* spp.
TEC3	SGCF	G-ve rods	+	+	+	+	+	+	+	+	+	-	*Pseudomonas* spp.
TEC4	SGCC	G-ve rods	+	+	-	+	+	+	+	+	+	-	*Pseudomonas* spp.
TEC5	SGCC	G-ve rods	+	+	-	+	+	+	+	+	+	-	*Pseudomonas* spp.
TEC6	SGCC	G-ve rods	+	+	-	+	-	+	+	-	-	-	*Pseudomonas* spp.
TEC7	SGCC	G-ve rods	+	+	+	+	-	+	+	-	-	-	*Pseudomonas* spp.
TEC8	SGCC	G-ve rods	+	+	+	+	-	+	+	-	-	-	*Pseudomonas* spp.
TEC9	SGCC	G-ve rods	+	+	+	+	-	+	+	-	-	-	*Pseudomonas* spp.
TEC10	SGCC	G-ve rods	+	+	-	+	-	+	+	-	-	-	*Pseudomonas* spp.
TEC11	SGCF	G-ve rods	+	+	+	+	+	+	+	+	+	-	*Pseudomonas* spp.
TEC12	SGCF	G-ve rods	+	+	+	+	+	+	+	+	+	-	*Pseudomonas* spp.
REC13	SGCC	G-ve rods	+	+	-	+	+	+	+	+	+	-	*Pseudomonas* spp.
REC14	SGCC	G-ve rods	+	+	+	+	+	+	+	+	+	-	*Pseudomonas* spp.
REC15	SGCC	G-ve rods	+	+	-	+	+	+	+	+	+	-	*Pseudomonas* spp.
REC16	SGCC	G-ve rods	+	+	-	+	+	+	+	+	+	-	*Pseudomonas* spp.
REC17	SGCC	G-ve rods	+	+	+	+	-	+	+	+	-	-	*Pseudomonas* spp.
REC18	SGCF	G-ve rods	+	+	+	+	-	+	+	-	-	-	*Pseudomonas* spp.
REC19	SGCF	G-ve rods	+	+	+	+	-	+	+	-	-	-	*Pseudomonas* spp.
REC20	SGCF	G-ve rods	+	+	+	+	-	+	+	-	-	-	*Pseudomonas* spp.

KEY: TE; Treated Effluent, RE: Raw Effluent, SGCF: Small Green Circular Flat, SGCC; Small Green Circular Convex, M; Motility, C; Citrate, U; Urease, NR; Nitrate Reduction, Glu; Glucose Ara; Arabinose, Fruc; Fructose, Xyl; Xylose, Suc; Sucrose

All the isolates were motile, citrate and oxidase positive and fermented glucose and arabinose but not sucrose. TEC4, TEC5, TEC6, TEC10, REC13, REC15 and REC16 were urease negative while the others were positive. TEC3, TEC4, TEC5, TEC11, TEC12, REC13, REC14, REC15, and REC16 were positive to nitrate reduction, fructose and Xylose fermentation test while others were negative except REC17 that is positive to only fructose fermentation.

Identification of Isolates using Microgen® *Bacillus and Enterobacteriaceae* (GNA+GNB) Kits

The Microgen® kit identification for *Bacillus subtilis* and *Pseudomonas putida* is presented in Table 4. Six (6) and eleven (11) isolates were identified as *Bacillus subtilis* and *Pseudomonas putida* respectively.

Table 4: Identity of *Bacillus subtilis* and *Pseudomonas putida* Isolates Based on Microgen Identification Kit.

Isolate's Code	Prophile Number	Probability (%)	Identity
TE2	73710427	99.68	*B. subtilis*
TE3	74370622	95.75	*B. subtilis*
TE7	74370622	95.75	*B. subtilis*
TE8	74370622	95.75	*B. subtilis*
TE9	74370624	75.75	*B. subtilis*
RE12	74370622	95.75	*B. subtilis*
TEC1	640722001	90.5	*P. putida*
TEC2	640522001	90.5	*P. putida*
TEC6	640522001	90.5	*P. putida*
TEC7	640522001	90.5	*P. putida*
TEC8	640722001	90.5	*P. putida*
TEC9	640522001	90.5	*P. putida*
TEC10	640522001	90.5	*P. putida*
REC17	640522001	90.5	*P. putida*
REC18	640722001	90.5	*P. putida*
REC19	640722001	90.5	*P. putida*
REC20	640522001	90.5	*P. putida*

KEY: TE, Treated Effluent, RE; Raw Effluent, TEC; Treated Effluent (Inoculated on centrimide agar), REC; Raw Effluent (Inoculated on centrimide agar).

Crude Oil Utilization by the Isolates of *Bacillus subtilis* and *Pseudomonas putida*

The ability of wild strains of *Bacillus subtilis* and *Pseudomonas putida* isolates to grow on mineral medium containing varying concentrations of crude oil as the sole carbon source is presented in Table 5.

Bacillus subtilis isolate TE8 and *Pseudomonas putida* TEC10 showed luxuriant growth in Mineral medium containing 0.5%, 1% and 1.5% concentrations of crude oil but moderate growth on that containing 2% crude oil. All the isolates of *Bacillus subtilis* and *Pseudomonas putida* showed luxuriant growth on medium containing 0.5% crude oil with the exception of isolate TEC2 which

Table 5: Growth of *Bacillus subtilis* and *Pseudomonas putida* Isolates on Mineral Medium Containing Crude Oil.

Isolate's code	Organism	Crude Oil Concentration (%)			
		0.5	1.0	1.5	2.0
TE2	*B. subtilis*	+++	++	+	+
TE3	*B. subtilis*	+++	++	+	+
TE7	*B. subtilis*	+++	++	+	+
TE8	*B. subtilis*	+++	+++	+++	++
TE9	*B. subtilis*	+++	++	+	+
RE14	*B. subtilis*	+++	++	+	-
TEC1	*P. putida*	+++	++	+	+
TEC2	*P. putida*	+++	+++	+	+
TEC6	*P. putida*	+++	++	+	+
TEC7	*P. putida*	++	++	+	+
TEC8	*P. putida*	++	++	+	+
TEC9	*P. putida*	+++	++	+	+
TEC10	*P. putida*	+++	+++	+++	++
REC19	*P. putida*	+++	++	+	+
REC20	*P. putida*	+++	++	+	+
REC17	*P. putida*	++	++	+	-
REC18	*P. putida*	++	++	+	-

KEY: TE, Treated Effluent, RE; Raw Effluent, TEC; Treated Effluent, REC; Raw Effluent

+ = Scanty growth, ++ = Medium growth, +++ = Luxuriant growth, - = No growth.

had luxuriant growth on 1% crude oil medium, all the other isolates medium and scanty growth on medium containing 1% and 1.5% crude oil respectively. Also, isolate RE14, REC17 and REC18 showed no evidence of growth on the mineral medium containing 2% crude oil while all the other isolates of both *Bacillus subtilis* and *Pseudomonas putida* showed scanty growth.

Nitrous Acid and UV- Light Modification of Selected of Bacillus subtilis and Pseudomonas putida Strains

The effects of treatment with nitrous acid and UV-Irradiation on the selected strains (strains which had best growth in all the concentrations of crude oil tested) of *Bacillus subtilis* and *Pseudomonas putida* are presented in Table 6 and 7 respectively. Treatment with nitrous acid for 20 minutes caused the death of 59.67% of the *Bacillus subtilis* with 40.33% survival thus recording a higher survival rate than the *Pseudomonas putida* where the treatment caused the death of 66.33% of the cell population with a survival rate of 33.67% (Table 6).

Exposure to UV-radiation for 30 minutes resulted in the death of 51.67% of the population of *Bacillus subtilis* exposed with 48.33% of the population surviving the treatment. The population of *Pseudomonas putida* exposed to similar treatment recorded a 40% percentage death rate while 60% of the cell population survived (Table 7).

Evaluation of the Hydrocarbon Degradation potential of the Wild and Mutant Bacillus subtilis and Pseudomonas putida

Biodegradation potential of wild and mutant strains of *Bacillus subtilis* and *Pseudomonas putida* was evaluated by assessing the growth pattern of the isolates and measurement of residual oil and grease in effluents inoculated with the test isolates.

Table 6: Effects of 20 minutes Nitrous Acid Treatment on *Bacillus subtilis and Pseudomonas putida.*

Organism	ITVC (cfu/ml)	FTVC (cfu/ml)	% Kill	% Survival
B. subtilis	3.00×10^8	1.21×10^8	59.67	40.33
P. putida	3.00×10^8	1.01×10^8	66.33	33.67

KEY: ITVC; Initial Total Viable Count, FTVC; Final Total Viable Count.

Table 7: Effects of 30 minutes UV-Irradiation on *Bacillus subtilis and Pseudomonas putida.*

Organism	TVC (cfu/ml)	FTVC (cfu/ml)	% Kill	% Survival
B. subtilis	3.00×10^8	1.45×10^8	51.67	48.33
P. putida	3.00×10^8	1.80×10^8	40.00	60.00

KEY: ITVC; Initial Total Viable Count, FTVC; Final Total Viable Counts.

Growth pattern

The wild strain of *B. subtilis* (B), nitrous acid mutant (BNA) and UV mutant (BUV) strains of *B. subtilis* exhibited different growth patterns as shown in Figure 1. The highest population densities of 1.634×10^8cfu ml and 1.33×10^8cfu ml were recorded for B and BUV respectively, at day 12. While the population density of B remained relatively stationary to day 15, BUV declined to 1.492×10^8cfu/ml. Growth rate was slowest in BNA which attained highest population density of 9.03×10^7cfu ml at day 12 followed by a slight decline in population to 8.13×10^7cfu/ml.

The growth patterns of the wild strain of *P. putida* (P), UV-mutant strain (PUV) and nitrous acid mutant strain (PNA) are presented in Figure 2. Highest population density was observed in PUV followed by P while PNA had the lowest population density. P and PNA attained highest population density of 2.93×10^8cfu ml and 9.03×10^8cfu ml respectively at day 12 which was followed by a death phase. Highest population density (3.77×10^8cfu/ml) was observed in PUV at day 9 after which a steady decrease in population was observed in the remaining days of incubation.

Different growth patterns were observed in the mixed cultures of the wild strains of *B. subtilis* and *P. putida* (B+P), UV-mutants (BUV+PUV) and nitrous acid mutants (BNA+PNA) during the degradation of hydrocarbons in raw refinery effluent as shown in Figure 3. Though, the bacterial population of the cultures increased gradually from day 0 to 9, faster growth rate was observed in BUV+PUV. B+P and BUV+PUV attained highest population densities (3.181×10^8 and 2.192×10^8cfu ml respectively) at day 9 while BNA+PNA attained its highest population density (1.15×10^8cfu/ml) at day 12. A decline in population was observed in all the cultures after day 9. B+P showed a sharp decline fron days 9 to 12 and remained stationary. BUV+PUV and BNA+PNA showed a sharp and steady death phase in the remaining period of incubation.

Oil and grease degradation

The oil and grease degradation capacity of the wild and mutant strains of *B. subtilis and P. putida* are presented in figures 4 - 6. Figure 4 shows the percentage amount of oil and grease degraded by the wild strain of *B. subtilis* (B), UV-mutant strain (BUV) and nitrous acid mutant strain (BNA). Consistent pattern in oil and grease degradation was observed in BNA through out the period of incubation. B and BUV showed higher but similar degradation pattern with BNA from day 0 to 6 which was followed by a sharp increase in rate of degradation between day 6 and 9 while slower rate of degradation was observed in the remaining period of incubation. BUV showed highest percentage amount of oil and grease degraded followed by B while BNA showed the least degradation capacity.

The nitrous acid strain of *P. putida* (PNA), showed a steady degradation rate through the period of incubation. The wild strain (P) showed similar rate from day 0 to day 6 after which a sharp increase in degradation rate was observed between day 6 and 9 followed by

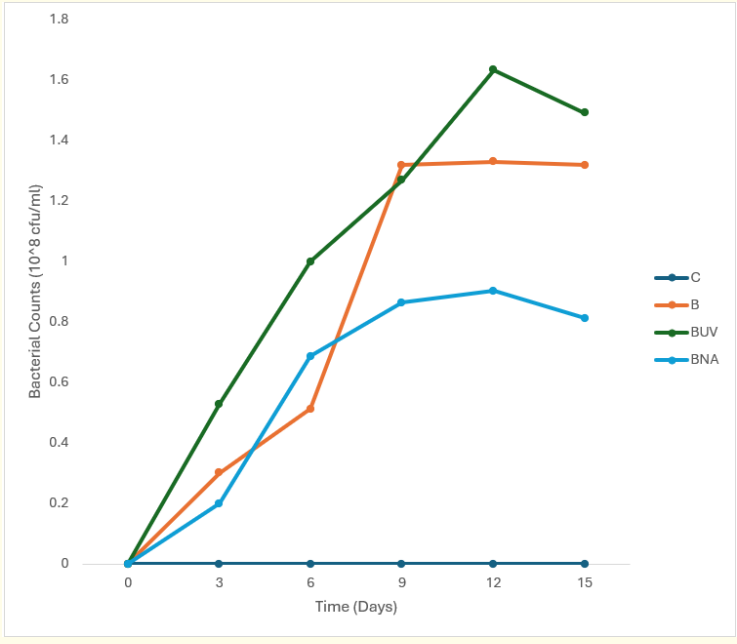

Figure 1: Changes in Bacterial Counts during Hydrocarbon Degradation by Wild and Mutant Strains of *Bacillus subtilis*. C: uninoculated, B: *Bacillus subtilis*, BUV: UV-Irradiated *Bacillus subtilis*, BNA: Nitrous Acid Treated *Bacillus subtilis*.

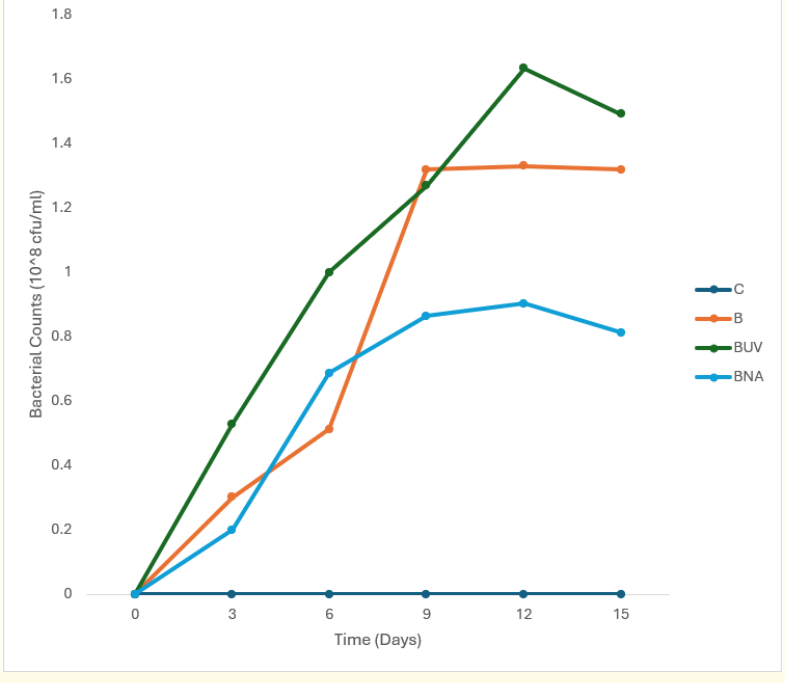

Figure 2: Changes in Bacterial Counts during Hydrocarbon Degradation by Wild and Mutant Strains of *Pseudomonas putida*. C: uninoculated, P: *Pseudomonas putida*, PUV: UV-Irradiated *Pseudomonas putida*, PNA: Nitrous Acid Treated *Pseudomonas putida*.

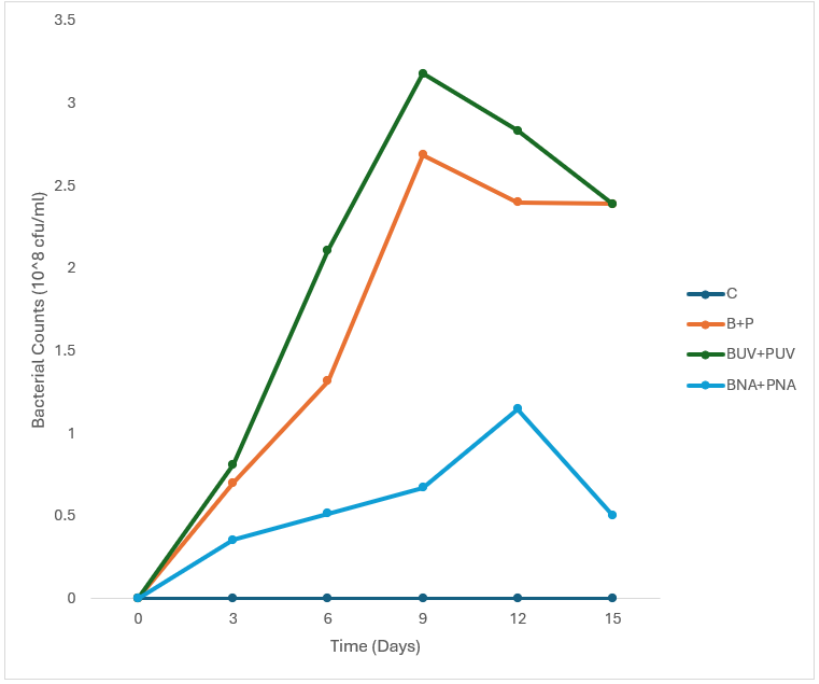

Figure 3: Changes in Bacterial Counts during Hydrocarbon Degradation by the Co-culture of Wild and Mutant Strains of *Bacillus subtilis* and *Pseudomonas putida.*

C: uninoculated, B+P: *Bacillus subtilis* + *Pseudomonas putida*, BUV+PUV: UV-Irradiated *Bacillus subtilis* + UV-Irradiated *Pseudomonas putida*, BNA+PNA: Nitrous Acid Treated *Bacillus subtilis* + Nitrous Acid Treated *Pseudomonas putida.*

a lower rate which remained steady through the remaining incubation period. Much lower rate was observed in the UV- mutant strain (PUV) from day 0 to day 6 after which a very sharp increase was observed between day 6 and 9. PUV showed the highest degradation capacity followed by P as shown in Figure 5.

The rate of oil and grease degradation by co-culture of the nitrous acid mutants of *B. subtilis* and *P. putida* (BNA+PNA) was relatively steady all through the period of incubation. The co-culture of the wild strains (B+P) showed a higher degradation rate from day 0 to day 9 but a reduction in rate of degradation was observed and was steady to the end of the incubation period. The co-culture of the UV-mutants (BUV+PUV) started with a lower rate which greatly increased between day 6 and 9. BUV+PUV showed the highest degradation capacity followed by B+P as shown in Figure 6.

Discussion

Physicochemical parameters of the refinery effluent

The raw refinery effluent was grossly polluted as most of the physicochemical parameters analysed were found in concentrations considered to be deleterious. Therefore, it will be unsafe to discharge such effluent into receiving sites without adequate treatment to reduce the pollutant levels. However, some of the parameters (pH, temperature, suspended solids, nitrate, sulphate and phosphate) were found to be within the permissible limits set by the Federal Ministry of Environment for (FEMNV) refinery effluent in Nigeria.

The occurrence of certain physicochemical parameters such as temperature, total suspended solids, total dissolved solids, dissolved oxygen, nitrate, sulphate and phosphate at levels consid-

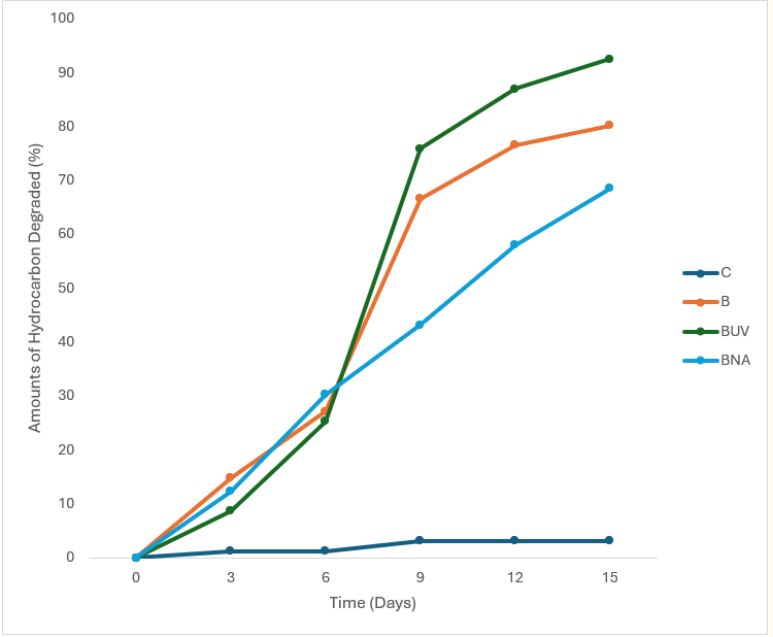

Figure 4: Percentage Amount of Hydrocarbons Degraded by Wild and Mutant Strains of *Bacillus subtilis*.
C: uninoculated, B: *Bacillus subtilis*, BUV: UV-Irradiated *Bacillus subtilis*, BNA: Nitrous Acid Treated *Bacillus subtilis*.

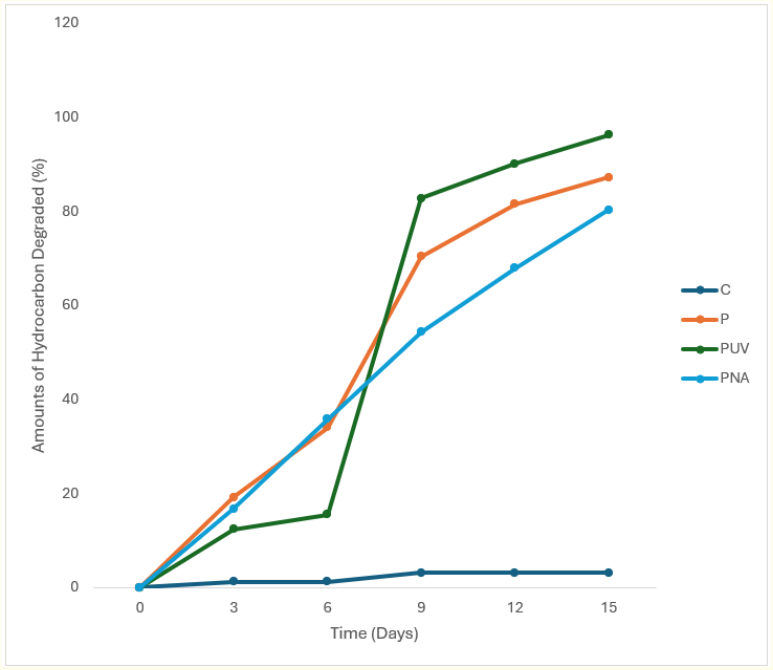

Figure 5: Percentage Amount of Hydrocarbons Degraded by Wild and Mutant Strains of *Pseudomonas putida.*
C: uninoculated, P: *Pseudomonas putida,* PUV: UV-Irradiated *Pseudomonas putida*, PNA: Nitrous Acid Treated *Pseudomonas putida*.

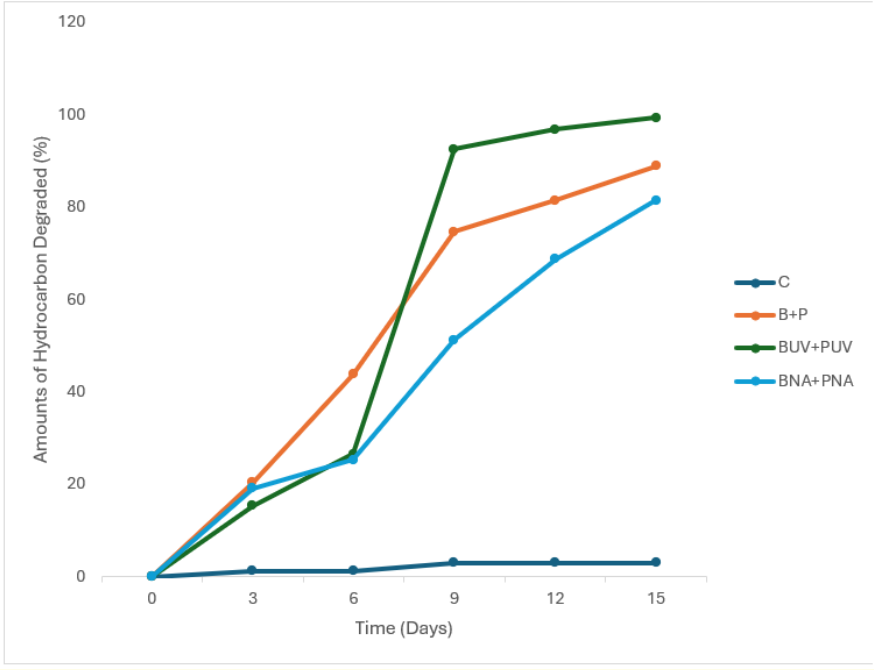

Figure 5: Percentage Amount of Hydrocarbons Degraded by the Co-culture of Wild and Mutant Strains of *Bacillus subtilis* and *Pseudomonas putida*.

C: uninoculated, B+P: *Bacillus subtilis* + *Pseudomonas putida*, BUV+PUV: UV-Irradiated *Bacillus subtilis* + UV-Irradiated *Pseudomonas putida*, BNA+PNA: Nitrous Acid Treated *Bacillus subtilis* + Nitrous Acid Treated *Pseudomonas putida.*

ered not hazardous to the environment based on FEMNV standard could be attributed to several factors which includes season of the year and time of sampling, process water used and other chemicals used in the refining process [71].

The high amounts of dissolved oxygen observed in the raw effluent could be as a result of the use of highly oxygenated process water. The fact that the effluents were coming fresh from the refining tower could also suggest that active microbial degradation of the organics that would have drastically reduced the level of the dissolved oxygen had not set in [72]. The time of sampling (morning hours during dry and cold season) and the process water used could be the reason for the low amounts of suspended solids, dissolved solids and low temperature recorded. This is because rainfall plays an important role in increasing the suspended and dissolved solids of wastewater due to constant washing of top soil materials into the effluent pond while temperature of wastewater is usually a function of the prevailing environmental temperature [73-75].

Though the concentrations of these parameters appear to be low and within the acceptable limits, the need to treat prior to discharge into receiving sites cannot be overemphasized as continuous discharge could result in accumulation of these substances which may become hazardous especially if the receiving sites are soils or water bodies that are stagnant or with a very low flowrate.

These findings agree with the findings of Otukunefor and Obiukwu (2005); Obot., *et al*. (2007) and Marcus and Ekpete (2014) who reported values of temperature, TDS, phosphate, nitrate and sulphate similar to that obtained in this work and within the FEMNV standard [71,74,76]. However, this finding disagrees with the report of Uzoekwe and Oghosanine (2011) and Ajao., *et al*. (2013) who reported a much higher value of TDS and DO (higher than the FEMNV standard) respectively [73,77].

The turbidity, BOD, COD and Oil and Grease content of the effluent samples were very high and well above the permissible limits set by FMENV for refinery effluents in Nigeria. The high turbidity could be attributed to the cumulative effects of the suspended and dissolved solids contained in the effluent. The high BOD value could be as a result of the presence of pollutants of organic origin especially hydrocarbons. The COD value obtained, in the same vein is a measure of oxygen demand exerted by both the organic component and reduced inorganic component of the effluent while the high value of oil and grease could have resulted from aggregate of oil that escaped the refining process into the effluent receiving ponds [73,76,78].

The reduction in the values of these parameters in the biologically treated effluent (effluent undergoing treatment) (Table 1) could be attributed to the activity of the autochthonous microorganisms in the biological treatment plant in a bid to digest the organic materials to obtain energy.

Based on the results obtained, the effluent is a potential source of environmental hazard and as such must be treated thoroughly before discharge to avoid drastic alteration of the physicochemical properties of the receiving sites. High or increased turbidity of the effluent receiving water body could reduce the rate of aeration from the atmosphere and penetration of sunlight which can contribute to a continuous build up of oxygen deficit with consequent devastating effect on the aquatic organisms. Also, proliferation of aquatic organisms that require sunlight to thrive could be reduced drastically as penetration of sunlight become very minimal [72,75].

BOD test is useful in determining the relative organic waste loading of water. High value therefore indicates the presence of large amount of organic pollutants and relatively higher level of microbial activities with consequent depletion of oxygen content [73]. The high values of BOD observed in this study are causes for worry because the continuous discharge of waste water containing organic matter into water bodies could result in depletion of oxygen as microorganisms engage in biochemical decomposition of the organic pollutants [72]. This problem leads to the inadequate maintenance of higher life forms. In addition, oxygen availability is important because the end products of chemical and biochemical reactions in anaerobic systems often produce aesthetically displeasing colours, tastes and odours in water [72]. The findings in this work are in agreement with those of Otukunefor and Obiukwu (2005); Uzoekwe and Oghosanine (2011); Ajao., *et al*. (2014) and Marcus and Ekpete (2014) who also reported high values of BOD (much higher than the FEMNV standard) in raw petroleum refinery effluents [71,73,74,79].

Joel and Amajuoyi (2009) reported that high level of contamination with oil and grease poses a great concern and long term threat to all forms of life [80]. Oil and grease are sticky in nature; they tend to aggregate, clogging drain pipes and sewer lines, causing unpleasant odours and corroding sewer lines under anaerobic conditions [81,82]. They also interfere with unit operations in municipal wastewater treatment plants because they float as a layer on top of the water. They also stick onto pipes and walls consequently blocking strainers and filters [82]. Though, the amount of oil and grease observed in the treated effluent was within the permissible limit it can still pose serious threat to receiving environments as such amount (8.9 mg/l) is capable of exerting a BOD that may stress the environment. This result is in agreement with the of findings of Ajao., *et al*. (2014) who reported oil and grease content of raw effluent to be 43.2mg/l in Kaduna refinery but disagree with the work of Otukunefor and Obiukwu (2005) who reported the oil and grease content of treated effluent from Port-Harcourt refinery to be 13.52 mg/l [71,79].

Isolation of Hydrocarbon Utilizing *Bacillus subtilis* and *Pseudomonas putida*

Eleven (11) and six (6) isolates of *P. putida* and *B. subtilis* were isolated from the refinery effluent samples after 14 days of enrichment in mineral medium containing 1% (v/v) of crude oil (Table 4). The occurrence of *B. subtilis* and *P. putida* from refinery effluent portrays that these organisms were able to adapt and thrive in environments polluted with petroleum hydrocarbons. Also, their ability to survive in mineral medium enriched with 1% (v/v) of crude oil as the sole source of carbon and energy during two weeks of enrichment technique is important and suggests the synthesis of enzymes involved in crude oil metabolism [83]. These findings showed that *B. subtilis* and *P. putida* were able to adapt to hydrocarbon polluted sites and that enrichment technique is useful in isolation of various culturable oil degrading bacteria from contaminated sites. The findings are also in agreement with the work of other researchers such as Okerentug-

ba and Ezeronye (2003); Bako., *et al*. (2008); Idise., *et al*. (2010); Moneke and Nwangwu (2011); Salam and Obayori (2013); Chikere and Ekwuaba (2014) and Liyange and Manage (2016) who isolated either *Bacillus* or *Pseudomonas* species or both from petroleum polluted sites [66,68,84-88]. The mechanisms employed by the autochthonous microorganisms to achieve this feat includes synthesis of inducible enzymes, mutations such as single nucleotide change or DNA re-arrangement that results in degradation of the compound and acquisition of genetic information from closely related or phylogenetically distinct population within the hydrocarbon-challenged community [89].

Higher numbers of hydrocarbon utilizing *B. subtilis* and *P. putida* were isolated from the treated effluent than the raw effluent. This could suggest that the bacteria community in the treated effluent was more adapted to petroleum hydrocarbons probably due to longer exposure which enhanced better adaptation and colonization of the biological treatment plant unlike the raw effluent that came fresh from the refining process as a result, the bacterial community may not have completely adapted to the environments.

Utilization of Crude Oil as Source of Carbon by the Wild Strains of *B. subtilis* and *P. putida*

The isolated strains of *B. subtilis* and *P. putida* were tested for their ability to grow on solid mineral salts medium (MSM) with crude oil as the sole carbon source. Visual detection of bacterial growth was taken as an indication of ability to utilize crude oil. This measurement is rapid and simple being used by several workers [80-82]. It gives a preliminary screening of the degradation ability of all isolates within a short time. The results presented in Table 4.5 show different levels of growth as luxuriant, medium and scanty on mineral salt agar medium enriched with different concentrations of crude oil. The difference in the ability of the various strains of *Bacillus subtilis* and *Pseudomonas putida* to grow on mineral medium containing varying concentrations of crude oil may be attributed to better adaptation with corresponding better developed enzyme system for hydrocarbon metabolism of certain strains than others and the presence of different catabolic genes involved in hydrocarbon degradation in the bacterial species [83,84].

Evaluation of Hydrocarbon Degradation by the Wild and Mutant of *B. subtilis* and *P. putida*

The present findings revealed that both the wild and mutant strains of the isolates (*B. subtilis* and *P. putida*) have varying hydrocarbon degradation capacity. *P. putida* showed higher ability to degrade hydrocarbons than *Bacillus subtilis* and such difference in the rate of hydrocarbon degradation may be due to the presence of different catabolic genes and better developed enzyme systems involved in hydrocarbon degradation in the bacterial species [93,94]. This is in agreement with the findings of Anene and Chika (2011); Moneke and Nwangwu (2011); Nwanyawu and Abu (2012) and Vinothini., *et al*. (2015) who working independently observed that *Pseudomonas* species had higher hydrocarbon degradation efficiency than *Bacillus* species [85-87].

While all the strains tested degraded considerable amounts of hydrocarbons, highest hydrocarbon degradation was observed in the UV-mutant strains of both isolates. The nitrous acid mutant strains showed lower degradation capacity compared to the wild strains. The observed difference in the degradative capability of the wild and mutant strains could be attributed to the changes in the genetic make-up or catabolic genes of the isolates that may have occurred in a bid to survive the exposure to mutagens. While the UV-irradiation resulted in increased hydrocarbon degradation, treatment with nitrous acid resulted in decreased ability to degrade hydrocarbons. This finding agrees with the findings by Idise., *et al*. (2010) who reported that UV-irradiated nitrous acid treated strains of *Bacillus cereus* and *Pseudomonas putida* showed higher hydrocarbon degradation than the wild strains [66]. Similarly, the findings agree with that of Ajao., *et al*. (2013) who reported higher activity of cathecol-2,3-dioxygenase enzyme in UV-irradiated strains of *Pseudomonas* species than their parent counterparts [77].

Mutation does not always result in improved efficiency of organisms. The phenotypic expression of mutated genes could be either on the positive (favourable) or negative (unfavourable) note depending on the specific changes exerted on the nucleotide sequence of the exposed organism [66]. This could however be seen as the reason the nitrous acid mutant strains of both isolates had lower hydrocarbon degradation capacity than the wild strains.

The co-culture of both the wild and mutant strains of the isolates exhibited higher capacity to degrade hydrocarbons than the individual organisms. This could suggest that individual organisms could only metabolize limited range of hydrocarbons [88]. Many researchers such as [89] also reported higher hydrocarbon degradation efficiency in bacterial consortium than individual cultures. This has led to the assertion that mixed culture exhibited superior degradative potential than pure culture strains [88]. In a culture, some species utilize intermediates of degradation of the original hydrocarbon produced by other members of the culture leading to a complete degradation of the oil [89]. Several reports have confirmed microbial consortia as better degraders than the individual organism. Thus, a mixed culture is a better inoculum for bioremediation. The observation of diauxic growth phenomenon in the complex mixture of petroleum is expected as petroleum consists of linear as well as aromatic compounds which usually required different enzymes and biodegradation pathways [66].

These findings showed that the isolates achieved a great feat in hydrocarbon degradation as the percentage degradation ranged from 68.52% to 99.38% after incubation of samples for 15 days. With the exception of the nitrous acid mutant strain of *Bacillus subtilis*, all the other strains tested brought the concentration of oil and grease (46mg/l) in the raw refinery effluent to within the permissible limit set by the Federal Ministry of Environment (FMENV) with the co-culture of the UV-mutant strains of the *Bacillus subtilis* and *Pseudomonas putida* reducing the concentration of oil and grease from 46mg/l to as low as 0.29mg/l. In similar studies, Ajao., *et al*. (2014) reported 90% oil and grease degradation after 20 days of incubation, Idise., *et al*. (2010) reported 98.25 oil degradation, Rahman., *et al*. (2002) also showed degradation of oil up to 78% after incubation of samples for 20 days inoculated with the bacterial consortium [66,79]. Singh., *et al*. (2013) also demonstrated 70% degradation of oil from the wastewater after 10 days of incubation [89].

Biodegradation of petroleum hydrocarbon generally relies upon the cooperation of more than a single bacterial species. This is particularly true when complete mineralization of the hydrocarbons to CO_2 and H_20 is desired. A pure bacterial culture may not have the metabolic capability to readily degrade certain compounds or to have the biomass necessary to degrade the compounds rapidly enough. Consortium or mixed populations with overall broad enzymatic capacities may be required to achieve total degradation of the petroleum. The higher biodegradation rate and microbial activity observed in the mixed culture may be related to shift in importance of one metabolic pathway over another possibly as a result of microbial synergism. The assumption that, more than one hydrocarbon compound degradation pathway exists in different microbial species and therefore, it is possible that individual bacteria able to degrade more than one aromatic substrate will have more than one pathway for their metabolism [77].

The utilization of the oil resulted in increase in the population densities of the various bacterial strains. The growth rate of the organisms correlated with the rate of oil degradation, suggesting that the break down of the hydrocarbons resulted in the provision of utilizable compounds required for their growth. Ajao., *et al*. (2013) also reported an increase in log number of cells with increase in degradation efficiency [77]. The trends observed in the growth of the various isolates were similar with each attaining its highest population density either at day 9 or day 12 of the incubation period and this coincides with the period during which highest amount of oil was degraded. Though the highest hydrocarbon degradation capacity was observed in the co-culture of the UV-mutant strains, the UV-mutant strain of *P. putida* had the highest population density of 3.77×10^8cfu/ml. A decrease in population occurred between day 12 and 15 in all the strains. This could be attributed to decrease in the amount of utilizable hydrocarbons consequently resulting in competition, build-up of toxic substances such as organic acids and other metabolic products that may be unfavourable to the growth of the organisms. Other researchers such as Nwanyanwu and Abu (2012); Esedafe., *et al*. (2015) and Asadirad., *et al*. (2016) reported that that bacterial isolates growing in mineral medium containing various hydrocarbons as the carbon source increased in population with increase in the rate of degradation but decreased progressively as the incubation continued [86].

The observation of diauxic growth phenomenon in the complex mixture of petroleum is expected as petroleum consists of linear as well as aromatic compounds which usually require different enzymes and biodegradation pathways [79].

Conclusion

Based on the observations made in this study, the following conclusions were drawn, the raw petroleum refinery effluent was grossly polluted especially with pollutants of organic origin. The petroleum refinery effluent is a good source for Bacillus subtilis and Pseudomonas putida with hydrocarbon degrading capacity. Isolates of Bacillus subtilis and Pseudomonas putida from refinery effluent undergoing biological treatment were better developed for hydrocarbon degradation than those from raw refinery effluents. Mutants of Bacillus subtilis and Pseudomonas putida with improved hydrocarbon degradation can be generated by treatment with UV-irradiation. While wild strains of Bacillus subtilis and Pseudomonas putida had a good hydrocarbon degrading efficiency, modification by irradiation with UV- light at 254 nm for 30 minutes resulted in increased efficiency.

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