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Research Article

Hydrocarbon degradation by autochthonous species of *Bacillus cereus* and *Pseudomonas aeruginosa* Isolated from Kaduna Refinery Effluents

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Abstract

The present work was undertaken to assess the extent of crude oil degradation by Bacillus cereus and Pseudomonas aeruginosa isolated from effluents collected from Kaduna Refining and Petrochemical Company, Kaduna, Nigeria. The samples were analyzed microbiologically using standard microbiological techniques. The isolates of Bacillus cereus and Pseudomonas aeruginosa were studied to determine their biodegrading capacities on varying concentrations of crude oil as the sole carbon source using minimal medium. The bacterial growth (increase in cell number cfu/ml) was used as indices of biodegradation. The test on the degrading activity of isolates on crude oil from effluent samples revealed that Bacillus cereus and Pseudomonas aeruginosa were the potent degraders of crude oil. There was statistically significant association between time and the increase in bacterial cell numbers ($P = 0.00 < 0.05$). The implication of the study in relation to biodegradative activity has been discussed.

Key words: *Hydrocarbons, exploration, pollution, physicochemical, Effluents, Bacillus cereus and Pseudomonas aeruginosa.*

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Introduction

Crude oil is composed of a mixture of aliphatic paraffin, alicyclic and aromatic hydrocarbons which differ in their susceptibility to microbial attack (Chikere and Azubuike, 2013).

Crude oil is currently the principal source of energy on which all modern industrial production systems depend (Barathi and Vasudevan, 2016). According to Okey (2003), it will remain the major source of energy for all industrial processes for the next several decades while reliable alternative energy sources are being found.

Current technologies employed in the refining of crude petroleum into the diverse petro-products, usually produce large quantities of effluents that are toxic and often resulting in pollution of water bodies and soils into which they are discharged (Asmae *et al*., 2014). Since water bodies and soils contaminated by refinery effluents often lose their capacity to support plant and animal life, such effluents constitute very serious public health and socio-economic hazards (Olukunle *et al*., 2015). Though only some researches have been conducted on the effluents of Kaduna Refining and Petrochemical Company (KRPC), evidences from studies of similar effluents in the country have shown that, the toxic effects of refinery effluents can be recorded at 1.5 km distance from the point of discharge (Otokunefor and Obiukwu, 2005).

Traditionally, several physical and chemical methods have been employed in the remediation of environments polluted by refinery effluents. But such methods are fraud with many problems. For instance, they are often costly, time consuming and above all, unfriendly to the environment being cleaned. For these reasons, attention is currently centered on microbial degradation (biodegradation) by the natural micro flora as a possible mechanism for the elimination of pollutants thrown into the environment inform of refinery effluents. For this purpose, either autochthonous or allochthonous microorganisms or both can be used (Ajayi *et al.,* 2008; Bryan and Voskanyan, 2012). Biodegradation of hydrocarbons by natural population of microorganisms represent one of the primary mechanisms of eliminating petroleum from the environment (Malik and Ahmed, 2012).

A wide range of studies have dealt with biotransformation and bioremediation of petroleum hydrocarbons and interest in exploiting crude oil-degrading organisms for

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environmental clean-up has become central to petroleum microbiology. There are so many bacteria and fungi with this ability (biodegradation of oil pollution) and these organisms are widely distributed in marine, freshwater and soil habitats (Wake, 2005), but scientists reported that indigenous microorganisms are more efficient for biodegradation of oil pollutant.

The ability to degrade and/or utilize hydrocarbon substrates is exhibited by a wide range of bacteria and fungi (Das and Chandran, 2011). Prior exposure of a microbial community to hydrocarbons is important in determining how rapidly subsequent hydrocarbon inputs can be degraded, a phenomenon known as adaptation which is brought about either by induction, depression of enzymes, genetic changes or selective enrichment (Wuyep, *et al*., 2006). This work was designed to assess hydrocarbon degradation by autochthonous species of *Bacillus cereus* and *Pseudomonas aeruginosa.*

Materials and methods

Collection and handling of refinery effluents and crude oil

Effluent samples were collected between October, 2015- March, 2016 from Kaduna Refining and Petrochemical Company (KRPC).The samples were collected in sterile wide mouth bottles. The containers were rinsed with the effluents at the points of collection. The samples were collected by lowering the bottles (with a weighted rope tied to its neck) into the bottom of the well mixed section of the water body, 30 cm deep, and allowed to over flow before withdrawing. After collection, the body of the containers was rinsed thoroughly with water.

Crude oil was collected from fuels laboratory KRPC, Kaduna. To avoid deterioration, the samples were transported in ice chest to the laboratory, Department of Microbiology, Ahmadu Bello University, Zaria for analysis.

Isolation of *Bacillus* and *Pseudomonas species*

Ten (10ml) of the collected refinery effluents was transferred into 90ml of sterile distilled water in a test tube using a sterile pipette to obtain a tenfold dilution. Using sterile 1ml pipette, 0.1ml of the prepared dilutions from 10^{-3}-10^{-6} were aseptically transferred onto the surface of solidified mannitol egg yolk polymyxin agar and centrimide agar for the isolation of *Bacillus* and *Pseudomonas species* respectively and spread evenly using a sterile

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bent glass rod. Plates were prepared in triplicates. The inoculated plates were incubated at 37^0C and observed for bacterial growth after 24hours (Anon, 2007).

Different colonies observed on the incubated plates were purified by repeated streaking of each distinct colony on nutrient agar until pure colonies were obtained. Purified bacterial isolates were transferred on sterile nutrient agar slants and stored at 4^0C for further identification.

Characterization of the bacterial isolates

Characterization of the isolates was achieved using biochemical tests (Cheesebrough, 2001). A 24 hrs pure culture of each of the isolates was used to determine their Gram's reaction (using the primary and secondary dyes, crystal violet and fuchsin). The following biochemical tests were carried out: catalase, oxidase, indole, motility, aerobic growth, anaerobic growth, fluorescent pigmentation, starch hydrolysis, gelatin Liquefaction, o-nitro phenyl beta galactosidase (ONPG), and voges proskauer tests respectively. The isolates identified using conventional biochemical tests were further authenticated using microgen identification system (*Wang et al.*, 2012).

Generation of mutants of *B. cereus* and *P. aeruginosa*

By UV irradiation at 254nm

The wild strains of *Bacillus cereus* and *Pseudomonas aeruginosa* were grown on nutrient broth for 24hours and their cell counts determined using Mac Ferland standard. Ten milliliters of *B. cereus* and *P. aeruginosa* respectively were placed in six sterile petri dishes in a 1mm thick layer and exposed to UV irradiation at 254nm for 5, 10, 15, 20, 25, and 30minutes at a distance of 6cm from the cells. After irradiation, the plates were immediately transferred to the dark to prevent photo reactivation. To avoid dark repair, post irradiation manipulations were carried out. The cells were pretreated with 0.2% (w/v) caffeine for 5 hours and centrifuged at 1500rpm for five minutes to remove the caffeine and the cells re-suspended in 0.85% (w/v) saline and re-centrifuged and supernatant discarded. The treated organisms were serially diluted with sterile 0.85% (w/v) saline and 0.1ml of each of the dilutions was plated on nutrient agar and incubated at 30^0C for 24hours.

By nitrous acid (HNO_2) exposure

A 0.15ml of filter sterilized, freshly prepared, aqueous 2.0M $NaNO_2$ was added to 5ml of a cell suspension in acetate buffer (0.2M, pH4.4). After 20minutes of exposure, the

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reaction was determined by serial dilution in Tris/HCl buffer (0.2M, pH 8.0). Aliquots of 0.1ml of the diluted suspension were plated on nutrient agar and incubated at 30^0C for 24hours.

Second stage mutation using ultraviolet (UV) light

The crude oil degrading first stage mutant strains of *Bacillus cereus* and *Pseudomonas aeruginosa* obtained after nitrous acid treatment were further exposed to UV irradiation for 30 minutes. The treated organisms were serially diluted with sterile 0.85% (w/v) saline. Aliquots of 0.1ml of the diluted suspensions were plated on nutrient agar and incubated at 30^0C for 24hours and colony counts determined. At the end of the mutagenesis, second stage mutants with higher crude oil degrading potential (UVNABc and UVNAPa) were obtained.

Preparation of Mc Farland turbidity standard

McFarland turbidity standard was prepared by adding 0.5ml of 1.175% (wt/vol) barium chloride dehydrate ($BaCl_2.2H_20$) solution to 99.5ml of 1% (vol/vol) sulfuric acid (H_2S0_4).

The turbidity standard was then poured into test tubes identical to those used to prepare the inoculum suspension and sealed with wax to prevent evaporation.

Assessment of the capacity of mutant strains of *B. cereus* and *P. aeruginosa* to degrade crude oil

Petroleum-hydrocarbon degradation by the isolates obtained was assessed using mineral salt medium (100ml) with the following composition: (KH_2PO_4 2g/l, Na_2HPO_4 3g/l, $NH_4NO_3$1g/l, KCL 1g/l, $MgSO_4$ 4mg/l, $Cacl_2$ 0.2mg/l, $FeSO_4$ 0.2mg/l, $Mncl_2$ 0.2mg/l, Agar 15g, Distilled water 1litre) supplemented with 0.5, 1, 1.5 and 2%, crude oil contained in150ml Erlenmeyer flasks. The pH of the medium was adjusted to 7.0 using dilute hydrochloric acid solution and autoclaved at 121^0C for 15minutes. One (1ml) suspension of each isolate (Mc Farland's standard 7 was used to standardize the inoculum size) was added to the sterile medium. The flasks were continuously aerated during the incubation period in a gyratory shaker incubator (New BrunsWick Scientific Shaker Incubator) programmed at 200r.p.m. Un-inoculated flasks containing the same set of medium were used as control. Seven days after the start of the incubation, each flask was sampled for the determination of optical density (OD_{600nm}) using PG T70 U.V/VIS Spectrophotometer. This was repeated at weekly interval for 8weeks. Increase in level of optical density(bacterial cell density) of the test isolates was used as the evaluation

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criteria for the isolate's adaptation to the medium used and their ability to degrade crude oil being tested (Asmae *et al.*, 2014).

Determination of rate of crude oil degradation by wild and mutant strains of *Bacillus cereus* and *Pseudomonas aeruginosa* using gravimetric analysis

The method used by Latha *et al,* (2012) was employed where one millilitre of standardized inoculum from *Bacillus cereus* and *Pseudomonas aeruginosa* was inoculated in to 100ml of mineral salt medium (KH_2PO_4 2g/l, Na_2HPO_4 3g/l, $NH_4NO_3$1g/l, KCL 1g/l, $MgSO_4$ 4mg/l, $Cacl_2$ 0.2mg/l, $FeSO_4$ 0.2mg/l, $Mncl_2$ 0.2mg/l, Agar 15g, Distilled water 1litre) contained in150ml Erlenmeyer flasks. To each flask was introduced 1ml of crude oil and corked with cotton wool. The experiment was made in replicates. The control flasks contained 100mls of mineral salt medium plus 1ml of crude oil but without organism. The flasks were incubated at 30^0C for 8 weeks in a gyratory shaker (New BrunsWick Scientific Shaker Incubator) programmed at 200rpm. At the start of the experiment (week 1), the weight of 1ml of crude oil was pre -determined using weighing balance (Bench Top Practum 64 – 1s Sartorius) and recorded as initial weight. At the end of week 8, one flask of each representative isolate was removed and used to determine the amount of crude oil left. The amount of residual oil was weighed after extracting the oil from the medium and evaporating it to dryness in rotary evaporator at 40°C under reduced pressure. The weight of extracted oil (final weight) was deducted from the initial weight. Amount of crude oil degraded was calculated as follows:

Initial weight of crude oil – Weight of extracted crude oil (final weight).
The % degradation was calculated as follows:

$$\% \text{ degradation} = \frac{\text{Amount of crude oil degraded}}{\text{Initial weight of crude oil}} \times 100$$

Data analysis

Data obtained from the study was analyzed using statistical package for social sciences (SPSS) version 21. Statistically, significant association between time and increase in bacterial cell numbers was determined and analyzed using the two way analysis of variance (ANOVA). The ANOVA decision criterion was employed using 95% confidence interval with $P < 0.05$ as statistically significant.

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Results and discussion

The increase in cell population (cfu/ml) with increase in time in a medium containing crude oil as the sole source of carbon and energy was used as the index by which the ability of the tested strains of *B. cereus* and *P. aeruginosa* to biodegrade crude oil was assessed. Thus, the growth of both the wild and mutant strains of these bacteria was monitored at varying concentrations of crude oil over a period of 8 weeks.

Results obtained from the study reveals that both the wild type and the mutant strains can degrade crude oil and grow when concentration of crude oil in the growth environment was 0.5% v/v. In the case of *Bacillus cereus,* the growth of the wild strains remained consistently lower than what were recorded for the mutant strains derived from exposure to UV radiations, Nitrous acid (NA) and combination of both mutagens throughout the 8 weeks period of the study (Figure 1). However, it was noted that, mutants derived from exposure to Nitrous acid exhibited higher growth rates than those obtained from exposure to UV radiations. On the other hand, the growth of mutants obtained following exposure to combination of these mutagens had their growth retarded during the first 3 weeks. However, by the 5th week, their growth rates increased sharply to levels above that of both the UV and Nitrous acid derived mutants (Figure1). Similarly, both the wild and mutant strains of *Pseudomonas aeruginosa* exhibited the ability to degrade crude oil when present in the growth environment at concentration of 0.5%v/v (Fig 2). However, the wild strains of this bacterium grew at slightly higher rates than the UV-radiated and UV radiated-nitrous acid (UVNA) derived mutants during the first 3 weeks of the study. But these same mutants grew at much higher rates than the wild strains between the 5th and the 8th week of the study. On the other hand, the UV derived mutant grew at consistently higher rate than the wild type throughout the study period but at a lower rate compared to the nitrous acid (NA) and UV radiated-nitrous acid (UVNA) derived mutants between the 4th and 8th week period of the study (Fig 2). Increasing the concentration of the crude oil to 1.0% elicited higher growth rates of the NA and UVNA derived mutants of *B. cereus* compared to both the wild type and UV derived mutant strains (Fig 3). However, the growth of the nitrous acid (NA) derived mutants was retarded during the first 2 weeks of the study.

On the other hand, all the three mutant strains of *Pseudomonas aeruginosa* grew at much higher rates than the wild strain at 1.0% crude oil concentration (Fig 4). However, the UV-radiated, nitrous acid (UVNA) derived mutants had the highest growth rates followed

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by the UV derived and the nitrous acid(NA) derived mutants in that order (Fig 4).Further increase in the concentration of crude oil in the growth medium to 1.5% did not alter the growth rates of both the nitrous acid (NA) derived and UV-radiated-nitrous acid (UVNA) derived mutants of *B. cereus* which remained higher than that of the wild strain (Fig. 5). But, the growth of the UV- derived strains of this bacterium was decreased to a level much lower than even that of the wild strain during the first 6 weeks of the study (Fig. 5).

On the other hand, all the three mutant strains of *P. aeruginosa* exhibited much higher growth rates than the wild strain at 1.5% crude oil concentration. The highest growth rate was recorded for the UVNA derived mutants followed by the NA derived and UV derived mutants in that order (Fig 6).

When grown in a medium containing 2.0% of crude oil a similar trend in the growth rates and increase in pH of the wild and mutant strains of *B. cereus* and *P. aeruginosa* was observed (Figs. 7 and 8).

The rate of hydrocarbon degradation by wild and mutant strains of *B. cereus* and *P. aeruginosa* was monitored using gravimetric analysis for the period of 8 weeks. It was observed that the second stage mutant strains of *B. cereus* and *P. aeruginosa* degraded crude oil much higher than the parents and the first stage mutants. This further confirmed the potential of the mutant strains in crude oil degradation as compared to the parent strains. Parent strains of *B. cereus* and *P. aeruginosa* degraded crude oil at 96.59 and 97.58% respectively while the second stage mutant strains of *B. cereus* and *P. aeruginosa* exhibited 99.60 and 99.80% crude oil degradation respectively (Table 1).

The degradation of crude oil by wild and mutant strains of *B. cereus* and *P. aeruginosa* indicate that the mutants were better degraders than their wild or parent strains. It was observed in the second stage mutation that *Pseudomonas aeruginosa* had the highest degradation potential than *Bacillus cereus*. The growth dynamics observed might either be due to their constitutive nature of hydrocarbon assimilating capabilities or adaptation of the strains as a result of previous exposure to exogenous hydrocarbons (Adebusoye *et*

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al., 2007; Wang *et al.*, 2012).This further confirmed that second stage mutants of*Bacillus cereus* and *Pseudomonas aeruginosa* possess highest potential for crude oil degradation. The rapid crude oil degradation exhibited by *Pseudomonas aeruginosa* in the second stage of mutagenesis could be due to the fact that some microorganisms naturally have the potential to utilize crude oil as source of carbon and energy (Rahman *et al.*, 2003; Latha *et al.*, 2012). It was also reported by Latha *et al.*, 2012 that crude oil contains hydrocarbon and does not resist attach by microorganisms. Ferreira *et al.*, 2012 reported that an increase in hydrocarbon degradation corresponds to an increase in cell number during the degradation process demonstrating the microbial ability to utilize hydrocarbon as sole source of carbon and energy. According to Boboye *et al.*, (2010) significant increase in biodegradation of hydrocarbon by *Bacillus cereus* and *Pseudomonas aeruginosa* was found with respect to time i.e., from o hour to 15 days. However, *Pseudomonas aeruginosa* possessed higher potential than *Bacillus cereus*. These results agree with previous reports (Asitok and Antai, 2006; Kim and Crowley 2007; Anon, 2008). Cunliffe and Kertesz, (2006), reported that individual bacteria can metabolize only a limited range of hydrocarbon substrates; hence assemblages of the mixed populations with overall broad enzymatic capacities would be required to achieve considerable biodegradation of petroleum hydrocarbons. Using the analysis of variance (ANOVA) decision criterion, there was statistically significant association between time and the increase in bacterial cell numbers ($P = 0.00 < 0.05$). The % concentrations of crude oil used and the observed increase in bacterial cell numbers were statistically significant ($P = 0.00 < 0.05$). However there was no significant interaction between time and the concentration of crude oil used in this study ($P = 0.00 < 0.05$).

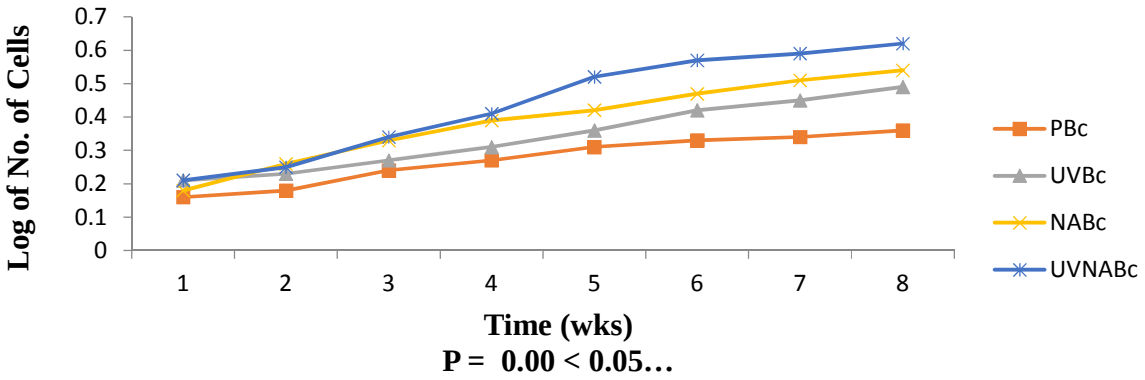

Figure 1: Effects of UV and Nitrous acid mutagenesis on the capacity of *Bacillus cereus* to degrade crude oil at 0.5% V/V

Key: PBc = Parent *Bacillus cereus*, UVBc = UV radiated *Bacillus cereus*, NABc = Nitrous acid modified *Bacillus cereus*,UVNABc = UV-radiated nitrous acid modified *Bacillus cereus*

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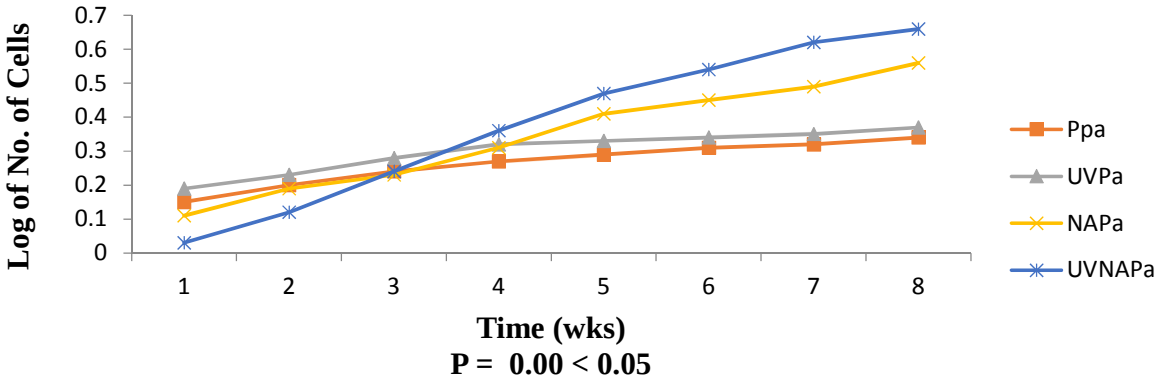

Figure 2: Effects of UV and Nitrous acid mutagenesis on the capacity of *Pseudomonas aeruginosa* to degrade crude oil at 0.5% V/V

Key: PPa = Parent *Pseudomonas aeruginosa,* UVPa = UV modified *Pseudomonas aeruginosa,* NAPa = Nitrous acid modified *Pseudomonas aeruginosa,* UVNAPa = UV-radiated Nitrous acid modified *Pseudomonas aeruginosa*

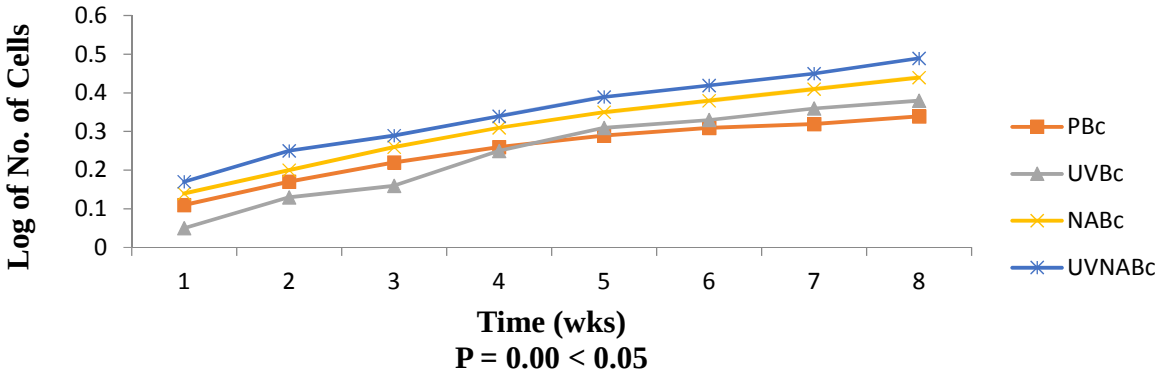

Figure 3: Effects of UV and Nitrous acid mutagenesis on the capacity of *Bacillus cereus* to degrade crude oil at 1% V/V

Key: PBc = Parent *Bacillus cereus*, UVBc = UV- radiated *Bacillus cereus* NABc = Nitrous acid modified *Bacillus cereus,* UVNABc = UV- radiated nitrous acid modified *Bacillus cereus*

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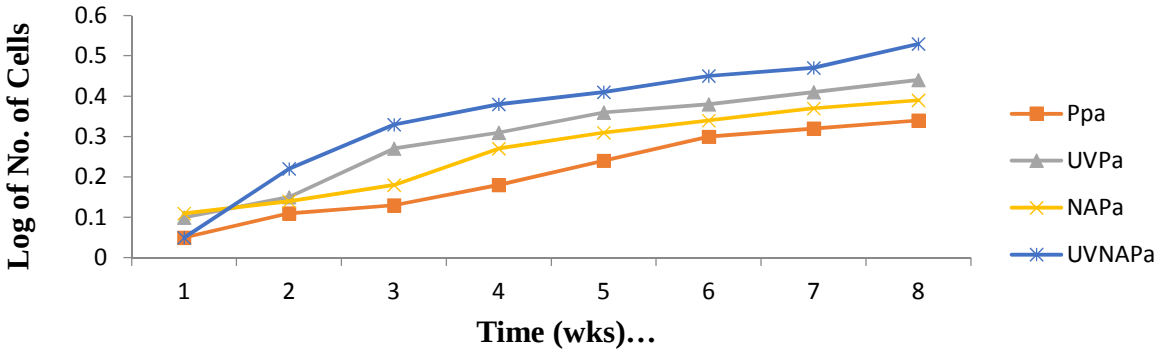

Figure 4: Effects of UV and Nitrous acid mutagenesis on the capacity of *Pseudomonas aeruginosa* to degrade crude oil at 1% (v/v)

Key: PPa = Parent *Pseudomonas aeruginosa*, UVPa = UV - radiated *Pseudomonas aeruginosa,* NAPa = Nitrous acid modified *Pseudomonas aeruginosa,* UVNAPa = UV- radiated+ Nitrous acid modified *Pseudomonas aeruginosa*

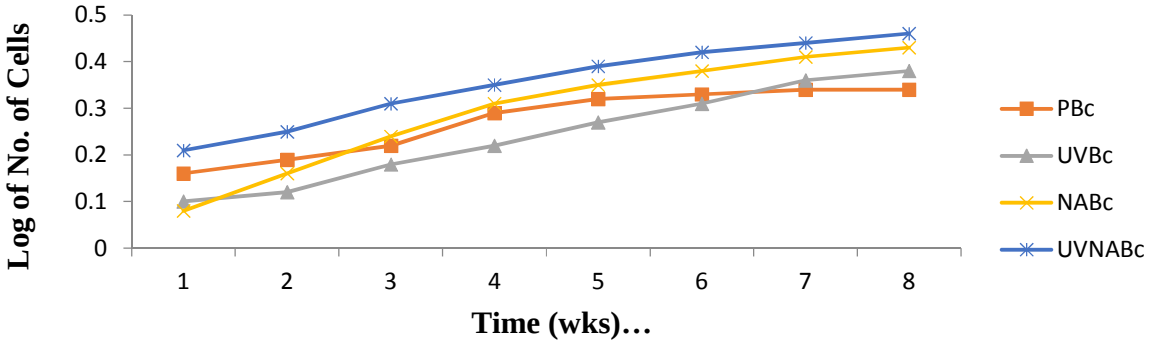

Figure 5: Effects of UV and Nitrous acid mutagenesis on the capacity of *Bacillus cereus* to degrade crude oil at 1.5% V/V

Key: PBc = Parent *Bacillus cereus*, UVBc = UV - radiated *Bacillus cereus* NABc = Nitrous acid modified *Bacillus cereus,* UVNABc = UV- radiated nitrous acid modified *Bacillus cereus*

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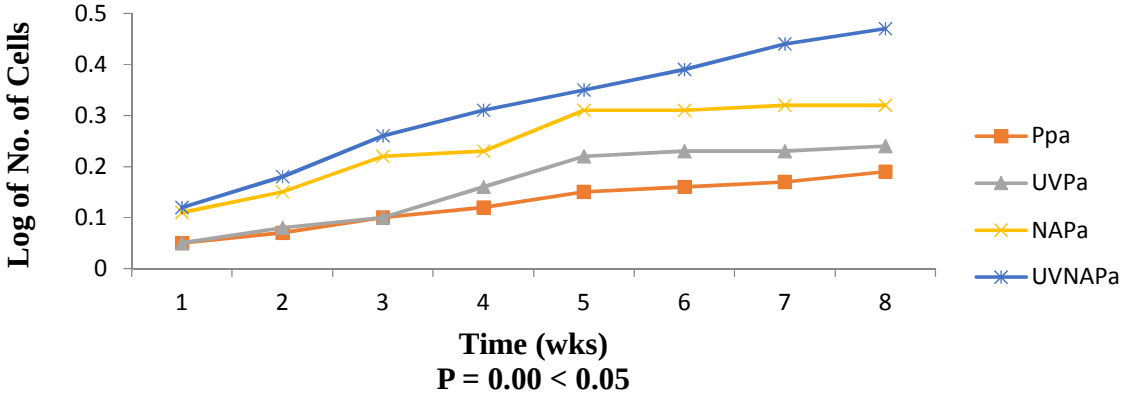

Figure 6: Effects of UV and Nitrous acid mutagenesis on the capacity of *Pseudomonas aeruginosa* to degrade crude oil at 1.5% V/V

Key: PPa = Parent *Pseudomonas aeruginosa*, UVPa = UV – radiated *Pseudomonas aeruginosa* NAPa = Nitrous acid modified *Pseudomonas aeruginosa,* UVNAPa = UV-radiated nitrous acid modified *Pseudomonas aeruginosa*

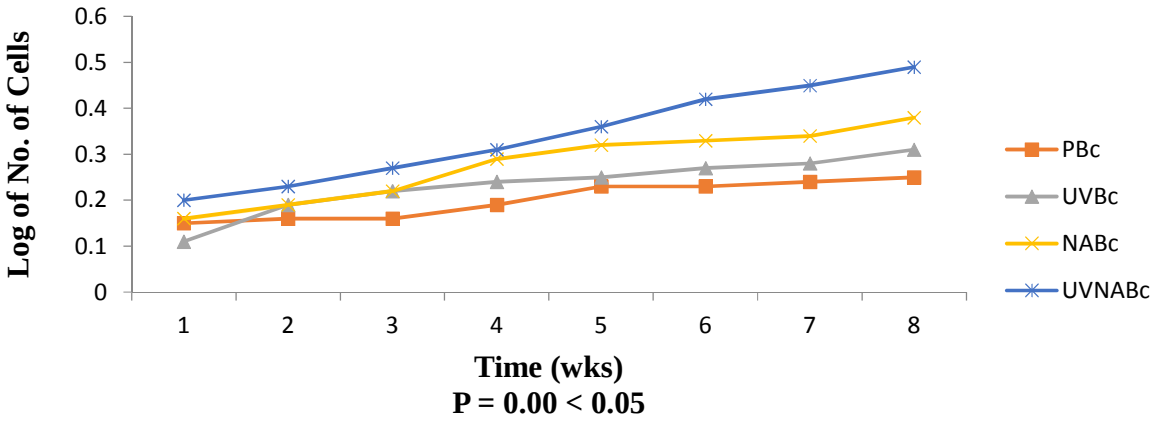

Figure 7: Effects of UV and Nitrous acid mutagenesis on the capacity of *Bacillus cereus* to degrade crude oil at 2% V/V

Key: PBc = Parent *Bacillus cereus*, UVBc = UV - radiated *Bacillus cereus* NABc = nitrous acid modified *Bacillus cereus,* UVNABc = UV- radiated nitrous acid modified *Bacillus cereus.*

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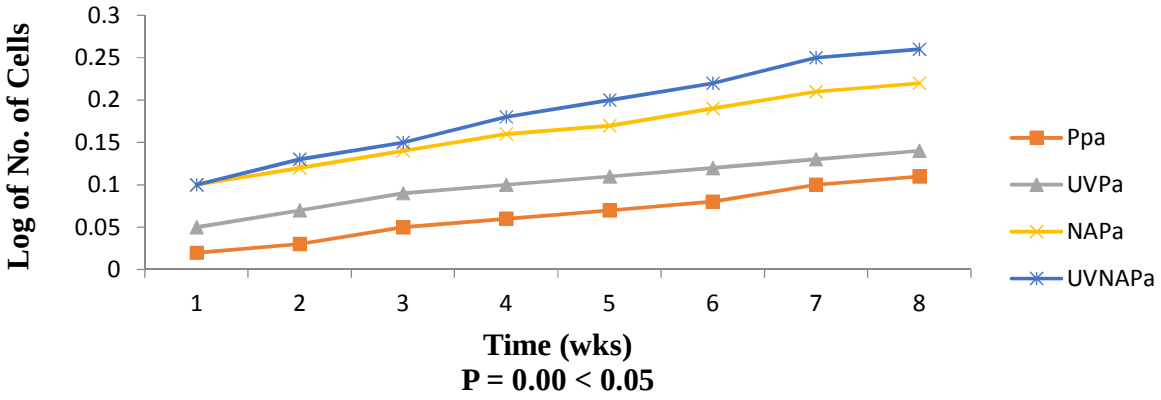

Figure 8: Effects of UV and Nitrous acid mutagenesis on the capacity of *Pseudomonas aeruginosa* to degrade crude oil at 2% V/V

Key: PPa = Parent *Pseudomonas aeruginosa*, UVPa = UV - radiated *Pseudomonas aeruginosa* NAPa = Nitrous acid modified *Pseudomonas aeruginosa,* UVNAPa = UV- radiated nitrous acid modified *Pseudomonas aeruginosa*

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Table 1 Rate of hydrocarbon degradation by wild and mutant strains of *B. cereus* and *P. aeruginoas*a using Gravimetric analysis

Organism	Start TPH (ppm)	End TPH (ppm)	Amount and (%) degraded (ppm)	Total and (%) hydrocarbon dissipated
PBc	8910	303.83	8606.17 (96.59)	7354.17 (82.54)
UVBc	8910	50.87	8859.13 (99.43)	7607.13 (85.38)
NABc	8910	31.84	8872.25 (99.58)	7620.25 (85.53)
UVNABc	8910	31.84	8878.16 (99.60)	7626.16 (85.59)
Ppa	8910	215.62	8694.38 (97.58)	7442.38 (83.53)
UVPa	8910	39.20	8870.79 (99.56)	7618.79 (85.51)
NAPa	8910	33.48	8876.52 (99.62)	7624.52 (85.57)
UVNAPa	8910	17.82	8892.18 (99.80)	7640.18(85.75)
CONTROL	8910	7658	1252 (14.05)	

Key: TPH = Total Petroleum Hydrocarbon, ppm = Parts per million, PBc = Parent *Bacillus cereus*, UVBc = UV- radiated *Bacillus cereus* NABc = Nitrous acid modified *Bacillus cereus,* UVNABc = UV- radiated nitrous acid modified *Bacillus cereus,* PPa = Parent *Pseudomonas aeruginosa*, UVPa = UV - radiated *Pseudomonas aeruginosa,* NAPa = Nitrous acid modified *Pseudomonas aeruginosa,* UVNAPa = UV- radiated Nitrous acid modified *Pseudomonas aeruginosa*

Conclusion

Biodegradation is one of the most rapidly growing areas of biotechnology, which has been used for the clean-up of pollutants. This is because of its low costs and its public acceptability. Crude oil degradation by environmental microflorae involves microorganisms having specialized metabolic capacities. In polluted environments, specialized microorganisms are abundant because of the adaptation of the microflorae to pollutants. It is evident from this study that crude oil degrading bacteria are ubiquitous in refinery effluents and they can be isolated from hydrocarbon polluted sites and waste water. The degradation process was observed to gradually increase with the peak value at week 8. The crude oil concentrations at 0.5, 1and 1.5% were found to be most effective for biodegradation as compared to 2% concentration. In this study, *Pseudomonas*

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aeruginosa exposed to UV - radiation and nitrous acid (second stage mutants) demonstrated significantly higher potential to degrade crude oil.

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