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Full Length Research Paper

Biodegradation of a refinery effluent treated with organic fertilizer by modified strains of *Bacillus cereus* and *Pseudomonas aeruginosa*

Idise, O. E[1], Ameh, J.B.[2], Yakubu, S.E.[2] and Okuofu, C.A.[3]

[1]Department of Microbiology, Delta State University, Abraka, Nigeria
[2]Department of Microbiology, Ahmadu Bello University, Zaria, Nigeria.
[3]Department of Water Resources, Ahmadu Bello University, Zaria, Nigeria.

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***Bacillus cereus* and *Pseudomonas aeruginosa,* that possess increased potential to degrade petroleum products exposed to UV-irradiation for 30 min followed by nitrous acid treatment, were employed in the biodegradation of effluent of a Refining and Petrochemical Company treated with NPK 15-15-15 organic fertilizer. While the untreated effluent achieved 89.25 and 77.63% degradation of oil and grease, and total petroleum hydrocarbons, respectively, the mutants achieved 69.66 and 49.46%, and 15.91 and 3.49%. The combined mutants achieved 76.8 and 66.24%, respectively, for *B. cereus* and *P. aeruginosa*. On treatment of the effluent with fertilizer prior to addition of the modified organisms, *B. cereus* achieved 95.27 and 80.22% and *P. aeruginosa* achieved 88.82% and 75.24%, while the combined mutants achieved 98.25 and 87.34%, respectively, for oil and grease and total petroleum hydrocarbons. Increased petroleum product degradation was achieved with fertilizer treatment and synergism was observed on combination of the mutants. Concurrent heavy metals assimilation – lead and chromium (VI) – were observed for the modified strains with the petroleum product degradation. Increased assimilation on combination of the modified organisms and application of fertilizer were also observed. Thus, both mutants could be employed in bioremediation of refinery effluents or any environment polluted with up to 5% (v/v) petroleum product, with increased efficiency on application of organic fertilizer, either alone or when combined.**

Key words: Petroleum, fertilizer, biodegradation, effluent, refinery.

INTRODUCTION

Refining of petroleum produces large amounts of effluents that are toxic and about 5-10% of inputs are released as effluent. Accidental discharge of crude petroleum, field oil and grease from the refinery also contribute to effluent (Emoyan et al., 2005). Such contaminated habitats lose their capability to support both plant and animal life and thus constitute public health and socio-economic hazards as well as pose serious aquatic toxicity problems (Okerentugba and Ezeronye, 2003).

The Refining and Petrochemical Company, which refines petroleum products for the domestic markets of the Northeast and west of Nigeria (Bureau of Public Enterprises, 2003–2007) discharges effluent into the Romi stream, a tributary of River Kaduna. Water from the stream is used for both domestic and agricultural purposes. Mechanical cleaning of such polluted environments could be possible but laborious, expensive, ineffective and time consuming. However, microbial degradation (biodegradation) by natural population of microorganisms represents one of the mechanisms for the elimination of the pollutants from such environments.

Improvement in the ability of microorganisms to degrade a pollutant could be achieved through modification of the environment or the organisms. The exposure of organisms to ultraviolet (UV) light and treatment with nitrous acid has been employed with relative successes (Anon., 2007a,b). The application of fertilizer to oil-soaked sites

*Corresponding author. E-mail: emmaidise@yahoo.com. Tel: +2348057592441.

has been reported to stimulate the growth of natural populations of bacteria that metabolize polycyclic aromatic hydrocarbons, organic toxins that are present in spilled oil (Pellerin et al., 2004; Doscher, 2005). *Pseudomonas* sp and *Bacillus* sp have been reported by previous studies as petroleum degraders (Nwachukwu et al., 2001; Okerentugba and Ezeronye, 2003; Anon. 2007c, d).

This research aimed at employing *Bacillus cereus* and *Pseudomonas aeruginosa,* with petroleum product degrading ability, isolated from Refining and Petrochemical Company effluent modified through UV-irradiation and nitrous acid treatment, in the biodegradation of the Refining and Petrochemical Company effluent treated with NPK 15-15-15 fertilizer.

MATERIALS AND METHODS

Mutation with UV irradiation at 254 nm

This was carried out by using a modification of the procedure reported by Ado (2004). The organisms were grown on nutrient broth for 18-24 h and their microbial counts were determined. Ten milliliters (10 ml) of 5.9 x 10^{14}cfu/ml and 8.44 x 10^{14}cfu/ml of *B. cereus* and *P. aeruginosa,* respectively, were aseptically transferred into separate sterile Petri dishes and placed at 6 cm from the source of UV light for 5, 10, 15, 20 25 and 30 min in a dark room. The UV irradiated organisms were then transferred into a sterile 20 ml test tube in a dark room and treated with 0.2% (w/v) caffeine and allowed to stand at room temperature (30 ± 2°C) in the dark for 5 h. The irradiated cells were then centrifuged at 1500 rpm for 5 min, re-suspended in normal saline and re-centrifuged and discarded the supernatant. The treated organisms were then incubated at 18°C for 16 h and their microbial counts were determined.

Mutation with nitrous acid

The organisms were grown on nutrient broth for 18-24 h and their microbial counts were determined. Acetate buffer (0.2 M, pH 4.4) was prepared in accordance with the procedure in Ado (2004). To 50 ml of 50:50 organism: acetate buffer suspension in a 150 ml flask, was added 1.5 ml of membrane filter (0.2 µm pore size) sterilized aqueous 2.0 M sodium nitrate. This was allowed to stand at room temperature (30 ± 2°C) for 20 min. The reaction was terminated by serial dilution with Tris HCl prepared by dissolving 121 g of Tris base in 800 ml of distilled water. The pH value was adjusted to 7.4 by adding 84 ml of 0.1 M HCl to 100 ml of 0.1 M Tris base. The mixture was made up to 1 L with distilled water. The treated organisms were inoculated, using pour plate technique, on nutrient agar and incubated at 37°C for 24 h.

Petroleum product-degradation by the modified strains: The organisms were inoculated in nutrient broth and incubated at 37°C for 18 - 24 h and their microbial counts determined. Fifty milliliters of each organism was transferred into two hundred milliliters of minimal basal medium containing 1% crude oil in a 250 ml flask. The flasks were placed in a mechanical shaker (Made in China) preset at 120 rpm. After 1 h of agitation and thereafter daily, pH, temperature, $OD_{560\ nm}$ and Total viable counts (TVC) were determined in accordance with the method of Okerentugba and Ezeronye (2003).

Refining and Petrochemical Company effluent degradation by modified organisms

This was carried out by using a modification of the procedure reported by Okerentugba and Ezeronye (2003). The modified organisms were grown in nutrient broth for 18-24 h and their microbial counts determined. 500 ml of each organism was added to 2000 ml of Refining and Petrochemical Company effluent in a bioreactor (Plate I) that was slowly aerated continuously. The pH, temperature and $OD_{560\ nm}$ were determined after 1 h and daily until the organisms attenuated. To the bioreactors containing the combined organisms, 250 ml each of the organisms were added to 2000 ml of Refining and Petrochemical Company effluent.

Degradation of Refining and Petrochemical Company effluent treated with fertilizer by modified organisms

This was carried out by using a modification of the procedure reported by Okerentugba and Ezeronye (2003). The modified organisms were grown in nutrient broth for 24 h and their microbial counts determined. 500 ml of each organism was added to 2000 ml of Refining and Petrochemical Company effluent in a bioreactor that was slowly aerated continuously. 0.023 g of the fertilizer (NPK 15-15-15) was added to the total volume of 2.5 L (Pellerin et al., 2004). The pH, temperature and $OD_{560\ nm}$ were determined after one hour and daily until the organisms attenuated. To the bioreactors containing the combined organisms, 250 ml each of the organisms were added to 2000 ml of Refining and Petrochemical Company effluent.

Analyses

Temperature was measured using a thermometer with the range of 0 – 100°C. pH was determined using a Horiba M – 13 pH meter. Optical density (OD) was measured using a Spectrophotometer 20A (Made in China) at 560 nm. Lead (Pb) was determined with Unicam 929 Atomic Absorption Spectrophotometer (AAS) using Test method A of ASTM (2002). Chromium (VI) was determined with the colorimetric method of ASTM (2002).

RESULTS AND DISCUSSION

The potential of the modified organisms, UV-irradiated nitrous acid treated *B. cereus* (B_{UVNA}) and *P. aeruginosa* (P_{UVNA}), to degrade petroleum oil in Refining and Petrochemical Company effluent presented in Figure 1 indicates better performance of the modified organisms than the control. There was reduction in pH with degradation of the oil with the presence of diauxic growth phenomenon. The use of modified organisms for bioremediation reportedly results in high substrate affinity which permits the removal of the pollutant to reduced concentrations and/or the recycling of the pollutant to natural compounds. They also employ low energy for the removal of the pollutant. These results are in agreement with the reports of Van Hamme et al. (2003).

The potential of the modified organisms – B_{UVNA} and P_{UVNA}–to degrade petroleum oil in Refining and Petrochemical Company effluent that had been treated with fertilizer presented in Figure 2 indicates higher petroleum product degradation by the modified organisms with the fertilizer addition. Oil and greased degradation increased from 49.46 to 95.27, 15.91 to 88.82 and 66.24 to 98.28% while total petroleum product degradation increased from 69.61 to 80.22, 3.49 to 73.24 and 76.8 to 87.34% (Figure

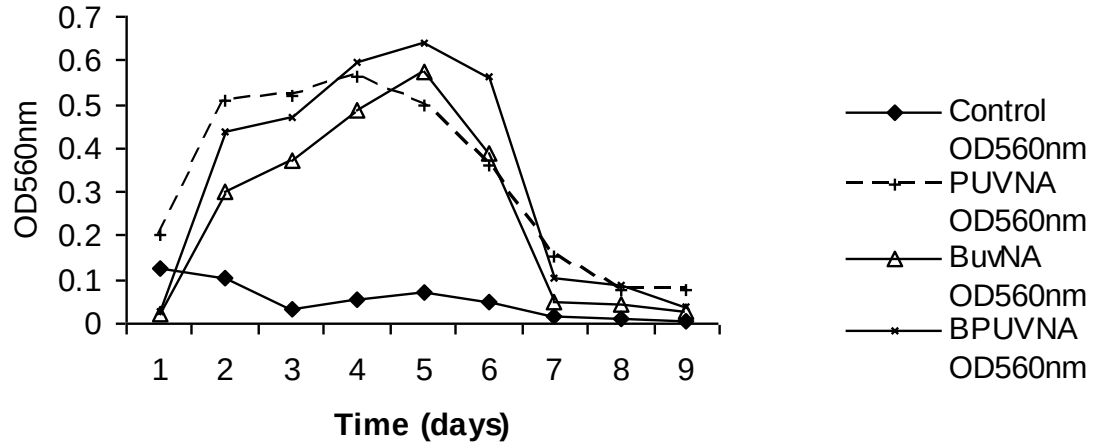

Figure 1. degradation of the KRPC effluent by the modified strains. B = *Bacillus cereus*; P = *Pseudomonas aeruginosa*; UVNA = ultraviolet-irradiation followed by nitrous acid treatment; OD = optical density.

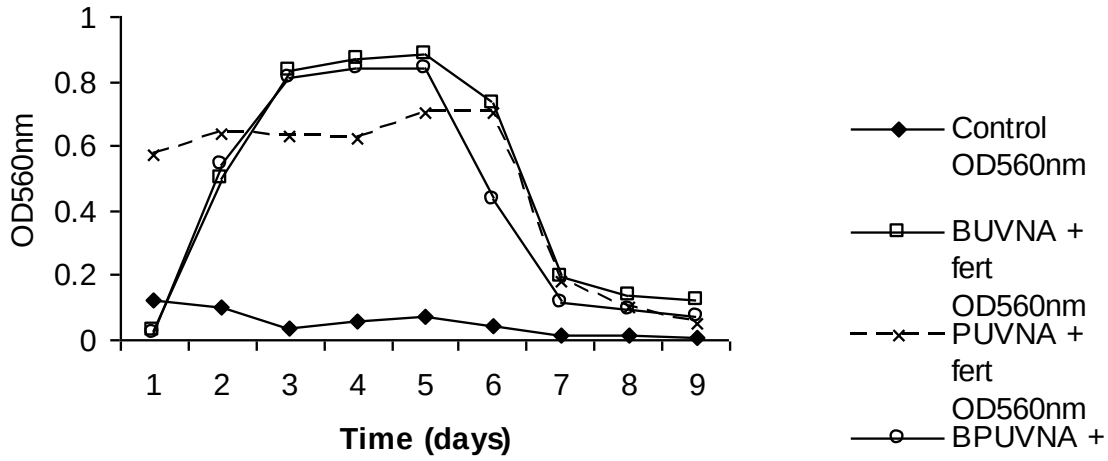

Figure 2. Degradation of the KRPC effluent by the modified strains after prior fertilizer treatment. B = *Bacillus cereus*; P = *Pseudomonas aeruginosa*; UVNA = ultraviolet-irradiation followed by nitrous acid treatment; fert = NPK 15-15-15 fertilizer; OD = optical density.

3), respectively, for *B. cereus* and *P. aeruginosa* modified by UV-irradiation for 30 mins followed by nitrous acid treatment. These results are in agreement with the reports of Pellerin et al. (2004).

There was reduced petroleum product degradation ability of the modified organisms from 99.7 and 98.089% to 49.46 and 15.91% (Figure 3), respectively, for *B. cereus* and *P. aeruginosa* modified by UV-irradiation for 30 min followed by nitrous acid treatment on introduction into the Refining and Petrochemical Company effluent. Modified organisms are often confronted with the problems of survival and the competition for nutrients with inherent microbes in the environmental sample. Usually, an open system arrives at population equilibrium irrespective of whether it was inoculated or not. Thus, possessing the degradation ability *in vitro* does not confer same ability on application to the environment. These results are in agreement with reports of previous studies (Okerentugba and Ezeronye, 2003; VanHamme et al., 2003).

Synergistic effect of increased petroleum product degradation on combination of the selected strains observed for *B. cereus* and *P. aeruginosa* modified by UV-irradiation for 30 min followed by nitrous acid treatment in Figure 3 agrees with the reports of VanHamme et al. (2003) and Adoki (2007).

The application of chemical fertilizer was observed to have greatly enhanced the performance of the modified strains in the degradation of petroleum pollutants from the Refining and Petrochemical Company effluent. There was an increase in performance by 15.24 and 1998.57% for O/G and 92.62 and 458.27% for TPH on application of fertilizer with B_{UVNA} and P_{UVNA}, respectively. This represented an increase of 8.88 and 19.25% for O/G and

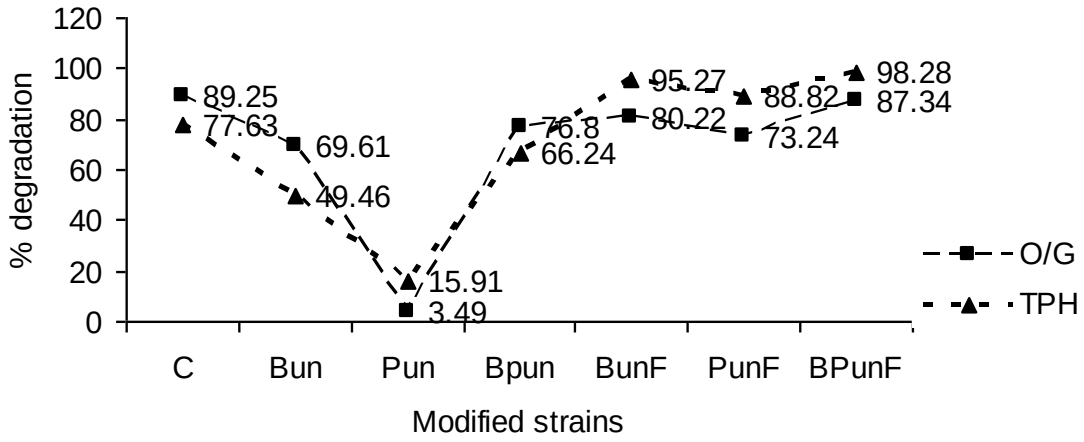

Figure 3. Percentage amounts degraded by the modified strains. O/G = Oil and grease; TPH = total petroleum hydrocarbons; Bun = UV-irradiated nitrous acid treated *Bacillus cereus*; Pun = UV-irradiated nitrous acid treated *Pseudomonas aeruginosa*; BPun = combined UV-irradiated nitrous acid treated *Bacillus cereus* and *Pseudomonas aeruginosa*; F = NPK 15-15-15 fertilizer.

Table 1. Statistical analyses of Refining and Petrochemical Company effluent degradation by the modified organisms

Organisms	f-cal	f-crit	Ho
C/B/P/BP	3.905476	2.90112	Rejected
C/P+ fert/B+ fert/BP+fert	4.52603	2.90112	Rejected
C/B/B+fert/BP+fert	4.362206	2.90112	Rejected
C/P/P+fert/BP+fert	5.171421	2.90112	Rejected

C = Control; B = UV-irradiated nitrous acid-treated B*acillus cereus*; P = UV-irradiated nitrous acid-treated *Pseudomonas aeruginosa*; BP = UV-irradiated nitrous acid-treated B*acillus cereus* and *Pseudomonas aeruginosa*; fert = NPK 15-15-15 fertilizer.

3.16 and 11.45% increment when the combined strains received fertilizer treatment. These results agree with the reports of previous researchers (Prince et al., 2003; VanHamme et al., 2003; Adoki, 2007).

Statistical analyses in Table 1 showed statistically significant differences at 95% confidence level in the petroleum product degradation by the control (that received no modified organisms and fertilizer treatment), parents alone and when combined and the control, modified organisms alone and when combined on fertilizer treated effluent.

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. Alkanes are usually degraded before the rings in the aromatic chains. This usually results in increased removal of the polycyclic aromatic hydrocarbons (PAHs) as the transformation products from one PAH may affect the removal of other PAHs (VanHamme et al., 2003). This phenomenon could have accounted for the increased ability of the selected strains to degrade the pollutant both *in vitro* and in the environmental sample (Kanaly et al., 2000; VanHamme et al., 2003).

The biodegradation of heavy metals from the Refining and Petrochemical Company effluent by the modified strains presented in Table 2 shows concurrent assimilation of lead (Pb) and chromium (VI) with the petroleum products with *B. cereus* being more efficient than *P. aeruginosa*. There was synergistic effect of increased efficiency on combination of the two modified organisms. Increased heavy metals assimilation efficiencies were observed with the prior addition of NPK15-15-15. Synergistic effect of the combined strains on application of the fertilizer was equally observed. These results agree with the reports of previous researches (VanHamme et al., 2003; Adoki, 2007).

Thus, the two modified strains could be employed in the bioremediation of refinery effluent to acceptable levels prior to discharge into the receiving environment. The introduction of these strains in the process waste water treatment plant could be a plausible solution to the environmental pollution problem therein in the discharge of petroleum refining effluent. Aside this, the modified strains could be employed in the biodegradation of environments polluted with 1-5% (v/v) petroleum products

Table 2. Bioassimilation of heavy metals from the Refining and Petrochemical Company effluent by the modified strains

Sample		Initial amount (ppm)	Final amount (ppm)	Amount degraded (ppm)	% Assimilation
Control					
	Pb	0.08	0.005	0.075	93.75
	Cr	0.010	0.002	0.008	80.00
B_{UVNA}					
	Pb	0.08	0.006	0.074	92.50
	Cr	0.010	0.00	0.010	100.00
P_{UVNA}					
	Pb	0.08	0.007	0.073	91.25
	Cr	0.010	0.003	0.007	70.00
BP_{UVNA}					
	Pb	0.08	0.007	0.073	97.25
	Cr	0.010	0.00	0.010	100.00
B_{UVNA} + Fert.					
	Pb	0.08	0.007	0.073	91.25
	Cr	0.010	0.00	0.010	100.00
P_{UVNA} + Fert.					
	Pb	0.08	0.007	0.073	91.25
	Cr	0.010	0.00	0.010	100.00
BP_{UVNA} + Fert.					
	Pb	0.08	0.007	0.073	91.25
	Cr	0.010	0.00	0.010	100.00

B_{UNVA} = UV-irradiated nitrous acid treated *Bacillus cereus*; P_{UVNA} = UV-irradiated nitrous acid treated *Pseudomonas aeruginosa*; BP = combined *Bacillus cereus* and *Pseudomonas aeruginosa*; Fert. = NPK 15-15-15; Pb = lead; Cr = chromium (VI).

with increased efficiency on prior introduction of organic fertilizer. The other environmental effects of introducing bacterial mutants in the environment for effluent treatment also need to be studied in detail.

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