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Full Length Research Paper

Modification of *Bacillus cereus* and *Pseudomonas aeruginosa* isolated from a petroleum refining effluent for increased petroleum product degradation

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***Bacillus cereus* and *Pseudomonas aeruginosa* isolated from Kaduna refining and petrochemical company (KRPC) were subjected to ultra-violet (UV)-irradiation for 30 min followed by nitrous acid treatment and re-irradiated with UV light for 30 min using standard methods and their petroleum degradation abilities were compared with parent strains. Prior to modification, *B. cereus* and *P. aeruginosa* grew on minimal basal medium containing 1.5 and 1% crude oil, respectively, initially but on re-inoculation, both organisms grew on minimal basal medium containing 5% crude oil. 99.83 (0.17% survival) and 96.91% (3.08% survival) death were recorded on UV-irradiation for 30 min; 38.24 (61.76% survival) and 82.02% (10.98% survival) death were recorded after nitrous acid treatment and 29.01 (70.99% survival) and 95.76% (4.24% survival) death were observed on re-irradiation with UV light for 30 min for *B. cereus* and *P. aeruginosa*, respectively. Petroleum product degradation increased from 98.92% for parent *B. cereus* to 99.70% for UV-irradiated nitrous acid treated *B. cereus*, and from 91.34% for parent *P. aeruginosa* to 98.09% for UV-irradiated nitrous acid treated *P. aeruginosa.* However, it decreased from 98.92 to 97.87% and increased from 91.34 to 97.87% for parents and second stage mutants re-irradiated with UV light of *B. cereus* and *P. aeruginosa*, respectively. Higher potential were observed for second stage mutant of *B. cereus* than *P. aeruginosa.* Thus, the modification of the organisms with UV-irradiation for 30 min followed by nitrous acid treatment resulted in their increased petroleum product degradation ability and could therefore be used for bioremediation of environments polluted with petroleum products of 1 - 5% (v/v).**

Key words: UV-irradiation, nitrous acid, petroleum, degradation, modification, refinery.

INTRODUCTION

Petroleum refining produces large amounts of effluents that are toxic and result in environmental pollution of receiving bodies of water and soils. 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). As mechanical cleaning of such polluted environments is nearly impossible, microbial degradation (biodegradation) by natural population of microorganisms represents one of the mechanisms for the elimination of the pollutants from such environments.

Biodegradation could be carried out either by the autochthonous or allouchthonous organisms or both, through seeding (Ajayi et al., 2008). Prior exposure of a microbial community to a hydrocarbon is important in determining how rapidly subsequent hydrocarbon inputs can be degraded, a phenomenon called adaptation which is brought about by either induction or depression of enzymes, genetic changes and/or selective enrichment

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Abbreviations: KRPC, Kaduna refining and petrochemical company; **UV,** ultra-violet; **MBM,** minimal basal medium.

(Prescott et al., 2008). Adapted microbial communities usually have high proportions of hydrocarbon degraders that can respond to the presence of hydrocarbon pollutants.

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 mutants abound and the exposure of organisms to ultra-violet (UV)-light and treatment with nitrous acid has been employed with relative successes. Such mutants, under optional growth conditions, could possess enhanced petroleum degradation potentials than their parents (Anon, 2007a and b).

Bacillus cereus and *Pseudomonas aeruginosa* have been reported by previous studies as petroleum degraders (Nwachukwu et al., 2001; Okerentugba and Ezeronye, 2003; Anon, 2007c and d).This study aimed at improving the petroleum product degradation ability of *B. cereus* and *P. aeruginosa* through mutation using UV-irradiation and nitrous acid treatment.

MATERIALS AND METHODS

Screening for the ability to utilize petroleum

The isolates were inoculated on minimal basal medium (MBM), glucose 25 g, $NaNO_3$ 15 g, $MgSO_4.7H_2O$ 1.3 g, KCl 1.3 g, KH_2PO_4 3.8 g, $FeSO_4$. $7H_2O$ 10 mg, $ZnSO_4$.$7H_2O$ 1.0 mg, agar 37.5 g, distilled water 2.5 L and pH 6.8 - 7.2 (Pontecorvo, 1949) containing 0.5, 1, 1.5, 2. 2.5 and 5% crude oil, and incubated at 37°C for 24 – 48 h. Growth of organisms indicated positive result. From positive plates, the organisms were re-inoculated on another set of MBM plates above, incubated at 37°C and observed for growth to determine enhanced petroleum utilization with prior exposure.

Mutagenic treatments of the *B. cereus* and *P. aeruginosa*

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 of 5.9 x 10^{14} cfu/ml and 8.44 x 10^{14} cfu/ml of B and P, 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 twenty milliliter 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 three to five minutes, re-suspended in normal saline and re-centrifuged and the supernatant was discarded. The treated organisms were then incubated at 18°C for 12 – 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 fifty milliliters 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 twenty minutes. The reaction was terminated by serial dilution with Tris HCl prepared in accordance with the procedure in Ado (2004): 121 g of Tris base was dissolved 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.

RESULTS AND DISCUSSION

The characterization of the isolates from the Kaduna refining and petrochemical company (KRPC) effluent presented in Table 1 identified the isolates to be *B. cereus* and *P. aeruginosa*. Both organisms have been reported to be petroleum degraders (Okerentugba and Ezeronye, 2003; Israel et al., 2007).

The growth of *B. cereus* and *P/ aeruginosa* in MBM containing crude oil presented in Tables 2a and b indicate the occurrence of enzyme induction which enabled growth in all the tested concentrations of the crude oil (0.5 – 5%) after prior exposure in Table 2b. These results are in agreement with previous reports (Nwachukwu et al., 2001; Okerentugba and Ezeronye, 2003; VanHamme et al., 2003).

The effects of mutational treatments on *B. cereus* and *P. aeruginosa* presented in Tables 3 - 5 revealed 0.17, 61.76, 70.99 and 3.08, 10.98, 4.24% survival for *B. cereus* and *P. aeruginosa*, respectively, for 30 min UV-irradiation, nitrous acid treatment and 30 min UV-irradiation of the organisms. There was progressive increase in percentage survival of *B. cereus* with each mutational treatment while *P. aeruginosa* increased with second mutational treatment and thereafter decreased with the third mutational treatment. The increase in percentage survival with mutational treatment could be due to the presence of nucleotide excision repair and base excision repair mechanisms. Nucleotide excision repair is a system which works on DNA damage which is 'bulky' by creating a block to DNA replication and translation. It involves cleavage of the damaged DNA by endonuclease followed by removal of short segments of the damaged DNA by exonuclease, while DNA polymerase then fills the resulting gap. With nitrous acid treatment, base excision repair could be employed in the removal of the damaged or inappropriate base from its sugar with glycosylase enzymes while a new base is incorporated by DNA polymerase using the other strand as a template. Both phenomena have been reported by previous workers (VanHamme et al., 2003; Anon., 2007a).

The degradation of crude oil by parents and mutants of *B. cereus* and *P. aeruginosa* presented in Figures 1 and 2, respectively, indicate that the mutants were better degraders than their parents. The petroleum degrading potential of the parents and mutants of *B. cereus* and *P.*

Table 1. Characterization of isolates.

Test	Isolate a	Isolate b
Cultural characteristics		
Growth on nutrient agar	Gray dry opaque colonies	Green colored colonies whose color intensity increased on incubation at room temperature for 24 h
Growth on MacConkey agar	ND	Pale colored colonies
Growth on Blood agar	ND	Greenish
Gram reaction	+	-
Motility	+	+
Catalase	+	+
Aerobic growth	+	+
Anaerobic growth	+	-
Fluorescence under UV light at 254nm	ND	+
Citrate utilization	+	ND
Growth in 7% NaCl	+	ND
Growth at 42^{o}C	ND	+
Growth at 4^{o}C	ND	+
Growth at 50^{o}C	-	ND
Glucose fermentation	+	+
Acid from glucose fermentation	+	-
Nitrate utilization	+	ND
Maltose fermentation	ND	-
Acid from Mannitol	ND	+
Spore formation	♦+	ND
Identity	*B. cereus*	*P. aeruginosa*

♦ = Single intracellular spore; + = positive; - = negative; ND = Not determined.

Table 2a. Growth of isolates on mineral basal medium containing crude oil.

Organism	Mineral basal medium with 0.5% (v/v) crude oil	Mineral basal medium with 1% (v/v) crude oil	Mineral basal medium with 1.5% (v/v) crude oil	Mineral basal medium with 2% (v/v) crude oil	Mineral basal medium with 2.5% (v/v) crude oil	Mineral basal medium with 5 % (v/v) crude oil
B	+	+	+	-	-	-
P	+	+	-	-	-	-

B = *B. cereus;* P =*P. aeruginosa;* + = growth; - = no growth.

Table 2b. Growth of isolates previously exposed to crude oil on mineral basal medium containing crude oil.

Organism	Mineral basal medium with 0.5% (v/v) crude oil	Mineral basal medium with 1% (v/v) crude oil	Mineral basal medium with 1.5% (v/v) crude oil	Mineral basal medium with 2% (v/v) crude oil	Mineral basal medium with 2.5% (v/v) crude oil	Mineral basal medium with 5% (v/v) crude oil
Bc	+	+	+	+	+	+
Pc	+	+	+	+	+	+

Bc = *B. cereus* previously exposed to crude oil; Pc = *P. aeruginosa* previously exposed to crude oil; + = growth; - = no growth.

aeruginosa presented in Table 6 shows the second stage mutants of both organisms as possessing highest potential of 99.70 and 98.09%, respectively. However, *B. cereus* possessed higher potential than *P. aeruginosa.* These results agree with previous reports (Asitok and Antai, 2006; Anon., 2007c, 2008).

In conclusion, the mutational treatments of 30 min UV-irradiation followed by nitrous acid treatment produced

Table 3. Effects of irradiation of isolates with UV light.

Organism	Initial TVC (cfu/ml)	TVC with 30 min UV irradiation	Reduction	% kill	% Survival
B	5.90×10^{14}	1.38×10^{11}	5.89×10^{14}	99.83	0.17
P	8.44×10^{14}	2.56×10^{14}	8.18×10^{14}	96.92	3.08

B = *B. cereus*; P = *P. aeruginosa*; TVC = total viable counts.

Table 4. Treatment of UV-irradiated isolates with nitrous acid.

Organism	Initial TVC (cfu/ml)	TVC with 20 min treatment	Reduction	% kill	% Survival
B_{UV}	6.8×10^{12}	4.2×10^{12}	2.6×10^{12}	38.24	61.76
P_{UV}	4.92×10^{13}	5.4×10^{12}	4.38×10^{13}	89.02	10.98

B_{UV} = UV-irradiated *B. cereus*; P_{UV} = UV-irradiated *P. aeruginosa*; TVC = total viable counts.

Table 5. UV-irradiation of nitrous acid-treated UV-irradiated isolates.

Organism	Initial TVC (cfu/ml)	TVC with 30 min UV irradiation	Reduction	% kill	% Survival
B_{UVNA}	1.31×10^{15}	9.3×10^{14}	3.8×10^{14}	29.01	70.99
P_{UVNA}	2.0×10^{16}	8.48×10^{14}	1.92×10^{16}	95.76	4.24

B_{UVNA} = UV-irradiated nitrous acid treated UV-irradiated *B. cereus*; P_{UVNA} = UV-irradiated nitrous acid treated UV- irradiated *P. aeruginosa;* TVC = total viable counts.

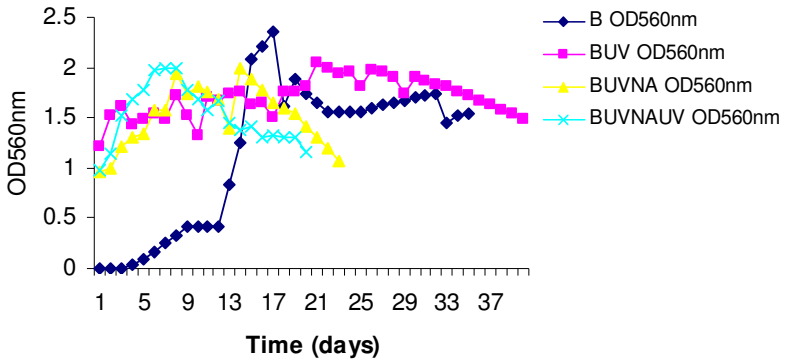

Figure 1. Crude oil degradation by parent and mutants of *B. cereus.* OD, optical density; B, parent *B. cereus*; B_{UV}, UV-irradiated *B. cereus;* B_{UVNA}, UV-irradiated nitrous acid treated *Bacillus cereus;* B_{UVNAUV}, re-UV-irradiated UV-irradiated nitrous acid treated *B. cereus.*

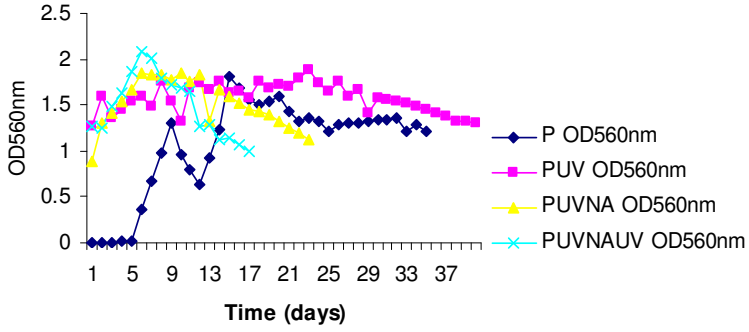

Figure 2. Crude oil degradation by parent and mutants of *P. aeruginosa*. OD, optical density; P, parent *P. aeruginosa*; P_{UV}, UV-irradiated *P. aeruginosa;* P_{UVNA}, UV-irradiated nitrous acid treated *P. aeruginosa;* P_{UVNAUV}, re-UV-irradiated UV-irradiated nitrous acid treated *P. aeruginosa.*

Table 6. Petroleum degrading potential of the tested strains.

Organism	Start Total Petroleum Hydrocarbons (ppm)	End Total Petroleum Hydrocarbons (ppm)	Amount Degraded (ppm)	% Degradation
B	6,000	64.87	5935.13	98.92
B_{UV}	6,000	115.31	5884.69	98.08
B_{UVNA}	6,000	17.74	55982.26	99.70
B_{UVNAUV}	6,000	120.35	5879.65	97.99
P	6,000	519.65	5480.35	91.34
P_{UV}	6,000	188.87	5811.13	96.85
P_{UVNA}	6,000	114.78	5885.22	98.09
P_{UVNAUV}	6,000	127.83	5872.17	97.87

B = *B. cereus*; B_{UV} = UV-irradiated *B. cereus;* B_{UVNA} = UV-irradiated nitrous acid treated *B. cereus;* B_{UVNAUV} = UV-irradiated nitrous acid treated UV-irradiated *B. cereus;* P = *P. aeruginosa;* P_{UV} = UV-irradiated *P. aeruginosa;* P_{UVNA} = UV-irradiated nitrous acid treated *P. aeruginosa;* P_{UVNAUV} = UV-irradiated nitrous acid treated UV- irradiated *P. aeruginosa*.

mutants that possessed higher petroleum product degrading potential than their parents. Both mutants could thus be employed, either alone or in combination in the bioremediation of refinery effluents as well as environments polluted with 5% (v/v) petroleum products.

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