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STUDIES ON PETROLEUM-DEGRADING POTENTIALS OF WILD AND MUTANT STRAINS OF *BACILLUS* SP ISOLATED FROM AN OIL-CONTAMINATED SOIL IN ABRAKA, DELTA STATE, NIGERIA

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ABSTRACT
Petroleum degrading potentials of Bacillus sp isolated from an oil-contaminated soil was determined in the laboratory through growth in 5% Automated Gas Oil-supplemented minimum basal media at room temperature (30 ± 2^{0}C). Pure cultures of Bacillus sp were then subjected to mutation using 0.01%, 0.1%, 1% and 10% nitrous acid and by exposure to X-rays for 2 and 5 seconds and their petroleum degrading potentials were determined for 7 days. It was observed that both the parent and mutants degraded petroleum with the nitrous acid mutants possessing varying increased potential than the parent stock. F-tests at 95% confidence level showed significant differences in total aerobic counts and pH. There were no significant differences in temperature between parent and mutants in both treatments. Thus, while both nitrous acid and X-rays could initiate mutation in Bacillus sp, only the nitrous acid mutants possessed the desirable greater potentials to degrade the petroleum product and could thus be employed in the bioremediation of petroleum or its products.

Keywords: Petroleum, Degradation, Bacillus sp. Mutants, Nitrous acid, Growth, Bioremediation.

INTRODUCTION

Crude oil is composed of a mixture of aliphatic paraffin, alicyclic and aromatic hydrocarbons which differ in their susceptibility to microbial attack (Osuji and Uwakwe, 2006). Biodegradation of hydrocarbons by natural population of microorganisms represents one of the primary mechanisms of eliminating petroleum from the environment (Fussey and Oudot, 1984; Leahy and Colwell, 1990). The number and types of microorganisms present, availability of oxygen, nutrients and light, and pH and temperature influence this (Okerentugba and Ezeronye, 2003). The ability to degrade and/or utilize hydrocarbon substrates is exhibited by a wide range of bacteria and fungi (Atlas,1981). 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 (Leahy and Colwell, 1990; Okerentugba and Ezeronye, 2003; Asitok and Antal, 2006; Ekpo and Ekpo, 2006; Wuyep *et al.*, 2006).

Since the late 1930s, mutation has been exploited as a method of strain improvement in many industrial processes being an essential and often most direct and least expensive means of improving industrial organisms (Rowlands, 1984). It can result in improved performance of the organism such as improvement of product titer with the production of fewer unwanted properties (Balz, 1986). The induction of mutation by physical and chemical agents has been extensively used in strain improvement programmes featuring both bacteria and fungi (Hopwoods,1970). Mutagenic agents are numerous but selection is usually based on safety of the mutagen, since most are highly toxigenic; simplicity of technique and availability of the mutagen (Okafor, 1987).

This research is thus aimed at isolating *Bacillus* sp from an oil-contaminated soil, initiating mutation using nitrous acid and by exposure to X-rays , determining the mutant(s) with higher petroleum degrading potentials that could be employed in the bioremediation of petroleum pollutants either alone or as part of a consortium of organisms.

MATERIALS AND METHODS

Study site: The soil samples were collected from 'New Discovery' motor mechanic workshop situated along the New Abraka/Sapele Road, Abraka, Delta State, Nigeria.

Sample collection: Composite soil samples were collected in a sterile beaker thrice using a sterile spatula at 3cm from the edge of a motor oil-stained patch in the workshop by scooping to about 3cm. They were immediately transported to the Microbiology laboratory of the Delta State University, Abraka for analyses.

Isolation of *Bacillus* sp: One gram of the oil-stained soil from a motor-mechanic workshop was serially diluted and plated on minimum basal medium (MBM) containing 15g/l agar. The plates were incubated at 37^{o}C for 24 – 48h. Pure cultures were stored in slants at 4^{o}C until needed (Ejechi, 2003).

Preparation of 5%-supplemented Minimum Basal Medium *MBM): This was prepared in accordance with the method reported by Ejechi (2003) thus: 2g/l KH_2PO_4, 3g/l Na_2HPO_4, 1g/l NH_4NO_3, 1g/l KCL, 4mg/l $MgSO_4$, 0.2mg/l $CaCl_2$, 0.2mg/l $FeSO_4$, 0.2mg/l $MnCL_2$ and 1 l of water. After sterilizing the MBM, 50ml Automated Gas Oil (AGO), dissolved in 250ml Carbon tetrachloride (CCl_4) in a flask, was added. The mixture was allowed to stand at 45°C for 24h for volatization of the CCl_4 before use. For the preparation of MBM (A), 15g/l agar was added to above MBM prior to sterilization.

Characterization and identification of *Bacillus* sp: This was carried out using methods reported by Cowan and Steel (2004).

Growth of organisms in 5% AGO-Supplemented MBM (A): Pure cultures of the isolates were grown in nutrient broth and 1 ml (10^5cfu/ml) of each isolate was inoculated on 5% AGO-Supplemented MBM (A) plates and incubated at 37°C for 24 – 48h. Growth after incubation was recorded as positive result (Ejechi , 2003).

Growth of organisms in 5% AGO-Supplemented MBM: Pure cultures of the isolates were grown in nutrient broth and 1 ml (10^5 cfu/ml) of each isolate was added to 9 ml of 5% AGO-Supplemented MBM and incubated at 37°C. Plate counts, using pour plate technique, were determined daily for 7 days (Ejechi , 2003).

Determination of pH and Temperature of samples: These were carried out using a pH meter (Metler Telodo 320) in accordance with the manufacturer's directions and a thermometer of a range 0 – 100°C (Cowan and Steel (2004).

Mutagenesis of isolates using nitrous acid:. Initiated mutation using the method reported by Shonukan and Nwafor (1989) by adding 0.8ml of each nitrous acid concentration (0.01%, 0.1%, 1% and 10%) to 2.0ml of washed exponential phase nutrient broth cultures of the organisms at 1.5 x 10^8 cells/ml. The mixture of the nitrous acid and cells were incubated at 37°C for 60mins. Cells were then washed twice with normal saline solution and diluted 1:10 in a sterilized MBM. The mixture of cells and mutants were incubated at 37°C overnight to allow for segregation of the mutants.

Mutagenesis of isolates with X-rays: Using a modified method of Lederberg and Lederberg (1952), plates of pure colonies of *Bacillus* sp were exposed to X-rays for 2secs and 5secs. The plates were then wrapped in Aluminium foil to avoid photo reactivation. The X-ray treated colonies were then subjected to replica plating by aseptically pressing sterile velvet cloth on the plates and imprinting the velvet on freshly prepared nutrient agar plates. These secondary plates were then incubated at 37°C for 24 – 48h. Growth after incubation was recorded as positive mutation result.

Statistical method of analyzing the data: The Microsoft excel (1997-2003) program was used for the statistical analyses at 95% confidence level.

RESULTS

A total of four bacteria were isolated from the oil-contaminated soil– *Pseudomonas* sp,*Bacillus* sp, *Acinetobacter* sp and *Enterococcus* sp. All the isolates possessed the ability to degrade the petroleum product It is noteworthy that exposure for ≥ 2 sec is highly undesirable as its mutants grew rather poorly for 2 days. (AGO) as they grew on the 5% AGO-supplemented Minimum Basal Medium (Table 1). The Total aerobic counts for the parent and the mutants is presented in Table 2. The parent, aside from the initial decrease from 3.2 x 10^3cfu/g in day 1 to 2.7 x 10^3 cfu/g in day 2, increased steadily from 3.5 x 10^3cfu/g in day 3 to 10.1 x 10^4cu/g in day 7. All the nitrous acid mutants, except the 10% mutants which increased from 1.31 x 10 ^{3}cfu/g in day 1 to 7.3 x 10^4cfu/g in day 3 and thereafter had no growth, grew throughout the period of study with the 0.1% and 1% mutants having 2 and 3 days of lag phase before achieving a constant growth of 7.3 x 10^4cfu/g while the 0.1% mutants had a constant growth of 7.3 x 104cfu/g throughout the period of study. The X-ray mutants however had growth far lower than the parent stock. While the 2-sec mutants grew poorly throughout the period of study, the 5-sec mutants grew for only 2 days albeit poorly.

The growth of the mutants in 5% AGO-supplemented minimum basal medium is shown in Table3. While all the nitrous acid mutants grew luxuriantly, the X-ray mutants grew sparingly.

The values of the pH and temperature are presented in Tables 4 and 5 respectively. With the exception of the 10% nitrous acid mutants with pH Standard Error (SE) of 0.68, all the other mutants had pH SE which ranged from 0.03 – 0.06 which is lower than the pH SE of the parent hence the null hypothesis was rejected. The SE for Temperature for both the parent and the mutants were within the range of ± 0.1 SE for the parent hence the null hypothesis was accepted.

DISCUSSION

The ability to degrade and/or utilize hydrocarbon substrates is exhibited by a wide range of bacteria and fungi (Atlas,1981; Leahy and Colwell, 1990; Okerentugba and Ezeronye,2003). Thus, the ability of *Bacillus* sp from the oil-contaminated soil to utilize the 5% AGO for growth agrees with reports by previous workers (Atlas, 1981; Leahy and Colwell, 1990). The organisms in Table 1 have been reported isolated from soil samples, being petroleum degraders (Atlas, 1981; Fusey and Oudot, 1984; Leahy and Colwell, 1990; Okerentugba and Ezeronye, 2003; Asitok and Antai, 2006).

The increase in total aerobic counts of nitrous acid mutants over the parent in Table 2 indicates that mutation with the beneficial effect of increased ability to degrade the petroleum product had occurred, while the 0.01%, 0.1% and 1% nitrous acid mutants possessed this quality, the 0.1% mutants is preferred since it lacked any period of lag phase. The attainment of a constant growth rate of 7.3 x 10^4cfu/g by all the nitrous acid mutants indicated the tolerable level permissible by the hydrocarbon concentration in the medium. The inability of the 10% nitrous acid mutants to grow beyond Day 3 could be adduced to a lethal mutation. Its pH range of 4.35 (1.96 - 6.31) attested to this. Whereas there was mutation in the X-ray mutants as shown in Table 2, the mutants lacked the desired beneficial effects of increased ability to degrade the petroleum product than the parent as their Total aerobic counts were lower than those for the parent.

X-rays, being an ionizing irradiation, cause ionization in the molecule of DNA thereby producing reactive radicals

which cause changes in the DNA (Okafor, 1987) or out rightly kill the cells.

The statistical analyses at 95% confidence level in Tables 4 and 5 indicate that while there were significant differences in the total aerobic counts and pH for the parent and the mutants of both treatments, there was none for the temperature values. Thus, while nitrous acid treatment and X-rays could initiate mutation in *Bacillus* sp, only the mutants of nitrous acid possessed the desirable beneficial effect of increased ability to degrade the tested petroleum product with the 0.1% nitrous acid mutants being the most preferred as it lacked any period of lag phase prior to attainment of the permissible level of 7.3 x 10^4cfu/g. Exposure of the organism for ≥ 2 sec produced a lethal mutation. The 0.01%, 0.1% and 1% nitrous acid mutants could therefore be employed either singly or as part of a consortium of organisms in the bioremediation of environments polluted with about 5% petroleum or its products.

Table 1: **Cultural, morphological and biochemical characterization of the bacterial isolates from soil.**

Tests	**A**	**B**	**C**	**D**
Cultural characteristics	Bluish small rough colonies with smooth edges	Round circular colonies with smooth edges	Cream circular colonies with entire edges	Cream circular colonies with entire edges
Odor	Fruity	-	-	-
Shape	Rods	Rods in clusters	Rods	Short rods
Gram stain	-	-	-	-
Growth on 5% AGO	+	+	+	+
Aerobic growth	+	+	+	+
Anaerobic growth	-	+	-	-
Growth at 45°C	+	NA	NA	NA
Fluorescence under UV light	+	NA	NA	NA
Motility test	+	+	-	+
Catalase test	+	+	+	-
Endospores test	-	+	-	-
Urea test	-	±	-	-
Oxidase test	+	-	-	-
Glucose fermentation	-	-	±	+
Lactose fermentation	-	-	-	+
Identity	*Pseudomonas aeruginosa*	*Bacillus* sp	*Acinetobacter* sp	*Enterococcus* sp

Key: + = positive. - = negative. ± = positive/negative. NA = not applicable. AGO = automated gas oil.

Table 2: **Total Aerobic counts (TAC) of parents and mutants in 5% AGO-supplemented MBM. Total aerobic counts (x 10^3 cfu/g) in days**

Organism	**1**	**2**	**3**	**4**	**5**	**6**	**7**
B	3.2	2.7	3.5	4.8	5.1	9.0	10.1
N1	0.30	0.30	73.0	73.0	73.0	73.0	73.0
N2	73.0	73.0	73.0	73.0	73.0	73.0	73.0
N3	1.0	1.5	6.6	73.0	73.0	73.0	73.0
N4	1.31	1.80	73.0	0	0	0	0
X2	0.014	0.0022	0.0049	0.0064	0.0028	0.0030	0.0045
X5	0.42	0.15	0	0	0	0	0

Keys: B = Parent *Bacillus* sp. N1 = 0.01% nitrous acid mutants, N2 = 0.1% nitrous acid mutants.. N3 = 1% nitrous acid mutants, N4 = 10% nitrous acid mutants. X2 = 2 sec X-ray mutants, X5 = 5 sec X-ray mutants .

Table 3: Growth of mutants in 5% Ago-supplemented minimum basal medium containing 15g/l agar.

Organism	Growth in 5% AGO-MBM containing agar
N1	++
N2	++
N3	++
N4	++
X2	+
X5	+

Keys: N1 = 0.01% nitrous acid mutants, N2 = 0.1% nitrous acid mutants, N3 = 1% nitrous acid mutants, N4 = 10% nitrous acid mutants, X2 = 2 sec X-ray mutants, X5 = 5 sec X-ray mutants, ++ = Good growth, + = Poor growth

Table 4: **pH values on growth in 5% AGO-supplemented Minimum basal medium with time.**

Organism	N	Range	Minimum	Maximum	X	SE	X ± SE
B	7	0.27	6.21	6.48	6.32	0.36	5.96 – 6.58
N1	7	0.4	6.35	6.75	6.55	0.06	6.49 – 6.51
N2	7	0.11	6.54	6.65	6.60	0.02	6.30 – 6.34
N3	7	0.16	6.23	6.38	6.32	0.02	6.30 – 6.34
N4	7	4.35	1.96	6.31	4.49	0.68	3.81 – 5.17
X2	7	0.39	6.33	6.72	6.47	0.05	6.42 – 6.52
X5	7	0.17	6.32	6.49	6.41	0.03	6.38 – 6.

Keys: B = Parent *Bacillus* sp. N1 = 0.01% nitrous acid mutants, N2 = 0.1% nitrous acid mutants. N3 = 1% nitrous acid mutants, N4 = 10% nitrous acid mutants. X2 = 2 sec X-ray mutants, X5 = 5 sec X-ray mutants .

Table 5: **Temperature values on growth in 5% AGO-supplemented Minimum basal medium with time**

Organism	N	Range	Minimum	Maximum	X	SE	X ± SE
B	7	3	26.2	29.2	27.74	0.37	27.37 – 28.11
N1	7	2.6	27.2	29.8	28.5	0.33	28.17 – 28.83
N2	7	3.4	27.3	30.7	28.61	0.47	28.14 – 29.08
N3	7	2.8	27.4	30.2	28.53	0.39	28.14 – 28.92
N4	7	2.7	27.4	30.1	28.34	0.38	27.96 – 28.72
X2	7	3.3	26.9	30.2	28.13	0.41	27.72 – 28.54
X5	7	3	26.9	29.9	27.91	0.43	27.48 – 28.34

Keys: B = Parent *Bacillus* sp. N1 = 0.01% nitrous acid mutants, N2 = 0.1% nitrous acid mutants. N3 = 1% nitrous acid mutants, N4 = 10% nitrous acid mutants. X2 = 2 sec X-ray mutants, X5 = 5 sec X-ray mutants. AGO = automated gas oil.

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