Degradation of Anthracene by Bacteria Isolated from Oil Polluted Tropical Soils

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Four bacteria, identified as *Pseudomonas aeruginosa, Alcaligenes eutrophus, Bacillus subtilis* and *Micrococcus luteus* were isolated from crude oil polluted soils using anthracene as the sole carbon and energy source. All the organisms utilized n-hexadecane, n-tetradecane, diesel oil, engine oil and naphthalene as sole carbon sources. None could utilize hexane, cycloheptane, xylene, benzene, toluene, phenol, fluoranthene,and kerosene as carbon sources. Highest cell density obtained with 0.1% (w/v) anthracene were 4.5×10^7 (cfu/ml), 8.6×10^6 (cfu/ml), 5.4×10^6 and 2.4×10^6 (cfu/ml) respectively, for *P. aeruginosa, A. eutrophus, B. subtilis* and *M. luteus* after 30 days incubation. Growth of the organisms on a Nigerian crude oil resulted in a residual oil concentration of 22.2%, 33.3%, 39.3% ,44% and 91.7% respectively, for *P. aeruginosa, A. eutrophus, B. subtilis, M. luteus* and the noninoculated control on the 14 th day. Ring fission enzymes of the *meta* pathway were detected in induced cells of *P. aeruginosa* and *A. eutrophus* while *ortho* pathway enzymes were detected in *B. subtilis* and *M. luteus. P. aeruginosa* and *A. eutrophus* had specific catechol-2,3-dioxygenase activities of 3.8 ± 0.183 and 0.64 ± 0.032 μmol / min × mg protein respectively while catechol-1,2-dioxygenase activities of 1.95 ± 0.029 and 1.89 ± 0.026 μmol / min × mg protein were detected in *B. subtilis* and *M. luteus* respectively. This work , highlights the capability of these unreported tropical strains of *A. eutrophus, B. subtilis* and *M. luteus* as anthracene degraders.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are organic molecules that consist of two or more fused benzene rings in linear, angular or cluster arrangement. They are components of coal, crude and refined oils and are also formed during pyrrolytic processes. Consequently, they have been detected in numerous aquatic and terrestrial ecosystems especially in industrial sites, at concentrations high enough to warrant concern about bioaccumulation. For example, PAH ranges from a low concentration of 5ng/g of soil in an undeveloped area to 1.79×10^6 ng/g at an oil refinery (Cerniglia, 1992). The most frequently occuring PAHs include naphthalene, phenanthrene, anthracene, pyrene, benz(a)pyrene and benz(a)anthracene. There is concern about the presence of these compounds in the environment as they have been shown to exhibit toxic, mutagenic and carcinogenic effects. Many PAHs are known to function as precarcinogens that require metabolic activation before they are able to bind to DNA, RNA or proteins (Sutherland *et al.*, 1995).

Anthracene is one of the low molecular weight PAHs with unaided or natural water solubility of 0.07–0.08 mg/l at 25–30 °C (Cerniglia, 1992; Willumsen and Karlson, 1997). There is, therefore, interest in the metabolic fate of the compound, firstly because of its recalcitrance which is due to poor aqueous solubility and secondly the fact that the anthracene structure is found in carcinogenic PAHs such as benz(a)pyrene, benz(a)anthracene and 3-methyl-cholanthrene (Cerniglia, 1984). Aqueous solubility of anthracene is even poorer than some higher molecular weight PAHs such as fluoranthene (0.26 mg/l) and pyrene (0.14 mg/l). This, therefore, gives anthracene a higher environmental persistence over some of the high molecular weight PAHs because of its poorer bioavailability.

Studies have shown that microbiological degradation of PAHs is the major process that results in

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the decontamination of sediment and surface soils (Cerniglia, 1992). Some bacteria and fungi have been reported to have the ability to grow on anthracene. They include *Beijerinckia* sp., *Mycobacterium* sp., *Pseudomonas* sp., *Arthrobacter* sp., *Cunninghamella* sp. and *Trametes* sp. (Cerniglia, 1992). Aerobic biodegradation mechanism requires the presence of molecular oxygen to initiate enzymatic attack on the rings. The initial reaction in the bacterial degradation of anthracene involves oxygenation at the 1,2 positions with the formation of *cis* 1,2-dihydroxy-1,2-dihydroanthracene which is then oxidized to 1,2-dihydroxyanthracene prior to ring fission (Menn *et al.*, 1993). The 1,2-dihydroxyanthracene is further metabolized to 2-hydroxy-3-naphthoic acid, 2,3 dihydroxynaphthalene, salicylic acid and then to catechol. The resultant catechol is degraded in what is referred to as the lower pathway through *meta* or *ortho* cleavage depending on the bacterial strain and other factors while the degradative steps up to salicylate is referred to as the upper pathway. Catechol metabolism through the *meta* and *ortho* pathways are mediated by intracellular dioxygenases which include catechol 2,3 dioxygenase and catechol 1,2 dioxygenase respectively. Organisms utilizing the two pathways form products which are capable of entering the tricarboxylic acid cycle (Cerniglia, 1984).

Microbial seeding of polluted soils and sediment is gaining worldwide acceptance as one of the environmentally acceptable alternatives to physical remediation such as chemical washing and incineration. The search for suitable PAH degrading bacteria with bioremediation potential is an important project in every environmental contigency plan. In this paper, we report the degradation of anthracene up to the lower pathway by four genera of tropical bacteria, three of which to our knowledge has not been reported previously.

Materials and Methods

Chemicals and media

All chemicals were of analytical grade. PAHs such as anthracene and naphthalene were purchased from Sigma (U.K). Media such as nutrient agar and broth were purchased from Oxoid (U.K). Crude oil (Escravos light) was obtained from Chevron Nigeria Limited.

Soil samples

Samples were taken from 2 experimental soil sites within the University of Lagos polluted for over 1 year with crude oil. Composite samples were placed into sterile bottles and transported immediately to the laboratory for further work.

Isolation and identification of PAH degrading bacteria

Bacteria capable of degrading anthracene as sole carbon and energy source were isolated from the soil samples using minimal salts agar medium as described by Kästner *et al.* (1994). The medium contained per liter: Na_2HPO_4, 2.13 g; KH_2PO_4, 1.3 g; NH_4Cl, 0.5 g; $MgSO_4$. $7H_2O$, 0.2 g. Sterilization was carried out by autoclaving at 121 °C for 15 min. Trace elements solution (1 ml per liter) as described by Bauchop and Elsden (1960) was sterilized separately and then added aseptically to the medium. The plates were poured and allowed to dry overnight after which 0.2 ml of a filtered ethanol solution (containing 0.5 g anthracene per 100 ml of ethanol) was aseptically pipetted and uniformly spread on the agar surface as described by West *et al.* (1984). The ethanol was allowed to dry under sterile condition before inoculation. Plates prepared in this manner had an opaque film of anthracene on the agar surface. Diluted soil samples were inoculated onto the agar surface ,the plates were kept in clean polythene bags to conserve moisture and incubated in the dark for 12 to 15 days. Inoculated anthracene free minimal salts agar medium were included in all cases as control for comparison with test plates. Colonies on the control plates were counted and taken as oligothrophs able to grow on medium components without any further carbon source. The colonies that appeared on the anthracene plates with crystal cleared zones were replicated onto fresh anthracene coated agar plates and incubated for another 15 days. Isolates which fail to grow were excluded from further tests. The anthracene degraders were identified as described by Cowan (1974) and Holt *et al.* (1994). Diagnostic properties used include Gram reaction, motility, colonial morphology, production of oxidase, catalase, indole, gelatin liquefaction, starch hydrolysis and sugar utilization.

Preparation of starter cultures

Cells grown for 12 days, were harvested from the surface of anthracene agar and resuspended in 10 ml sodium phosphate buffer (50 mM, pH 7.2) in a test tube. Centrifugation was carried out at 10,000×*g* for 10 min at 4 °C. The supernatant was discarded while the pelleted cells were resuspended in the same buffer and the washing repeated for 2 more times at the same condition. The pelleted cells were finally resuspended in sterile minimal salts medium (10 ml) to a final population of about 10^4 per ml of the medium and used for the anthracene growth experiment.

Growth of the organisms on different carbon sources

The ability of the isolates to utilize different carbon sources were tested by growing each organism in sterile 500 ml Erlenmeyer flasks containing 100 ml of filter sterilized minimal medium with trace elements. Sterile substrates including octane, hexadecane, dodecane, decane, tetradecane, undecane, cycloheptane, cyclohexanol, xylene, phenol, toluene, phenanthrene, anthracene, fluoranthene, pyrene, crude oil, kerosene, diesel oil, engine oil at 0.1% v/v or w/v were added.The solid aromatic hydrocarbons were dissolved in ethyl acetate and added aseptically to the medium. The ethyl acetate was allowed to evaporate under sterile condition. Each flask was inoculated with a loopful of the organism. Incubation, carried out at 28 °C on a shaker (150 rpm), was for 21 days in flask containing aromatic hydrocarbons and for 5 days in flasks with other hydrocarbons. Noninoculated but incubated flasks containing each substrate served as control. Growth was measured by daily optical density (OD) at 600 nm. Turbidity measurement started on the day of incubation until the end of the experiment. Cultures without any increase in turbidity over the initial optical density and noninoculated control were scored as no growth (−) while cultures with increase in turbidity that were significantly greater than the control were scored as growth (+). They had OD reading above 0.2.

Growth test of the isolates on anthracene

The minimal salts medium (90 ml) were put into 250 ml Erlenmeyer flasks. Finely crushed anthracene crystals (0.1%, w/v) was added. The medium was sterilized and inoculated with 10 ml of a starter culture of each organism. Incubation was carried out in the dark at 28 °C on a shaker set at 150 rpm. Growth of the organisms were assayed by plating in triplicate aliquots of the cultures on to sterile nutrient agar. The plates were incubated at 28 °C for 48 h after which the colonies were counted. Assays for growth was carried out on the day the medium was inoculated and subsequently at 3 days interval for 30 days.

Growth test on crude oil (Escravos light)

Minimal salts medium (90 ml) was dispensed into 5 sets of 16 Erlenmeyer flasks (250 ml). To each of the flasks was added 500 µl of crude oil as the sole carbon source. The mixture was autoclaved at 121 °C for 15 min and allowed to cool. The bacteria were grown in nutrient broth overnight and then washed trice in sterile sodium phosphate buffer (10 ml, 50 mM, pH 7.2). The cells were resuspended im 10 ml of sterile minimal medium (to about 10^5). Each of the organisms treated in this way was used as starter culture to inoculate 4 sets of the flasks in duplicate. Noninoculated medium (5th set) kept under the same condition served as control The flasks were incubated in the dark at room temperature (28 °C) with shaking (100 rpm) for 14 days. Growth of the organisms on oil was estimated by plating aliquots of the cultures in triplicate on nutrient agar on the day the incubation was carried out and subsequently at a 2 day interval. Residual oil in the culture medium and the control was extracted with carbon tetrachloride. This was carried out by pouring the content of the flasks into a separating funnel after which 20 ml of carbon tetrachloride was added. After shaking, the aqueous phase was re-extracted with half volume of carbon tetrachloride. The organic phases were pooled, allowed to evaporate to a constant weight which was thereafter measured gravimetrically.

Detection of ring fission enzymes

The method of Stanier *et al.*, (1966) was employed. The bacterial strains were grown in anthracene minimal salts medium (0.1, w/v) for 6 days. The cells were harvested from 30 ml broth by centrifugation (10,000×*g*, 10 min, 4 °C). The cells

were resuspended in 4 ml of Tris-(hydroxymethyl)-aminomethane-HCl buffer (0.02 M, pH 8.0) in a test tube. Catechol solution (4 ml, pH 8.0, 0.01 M) was added to the mixture after which it was shaken for 5 min. The appearance of a yellow colour within a few minutes indicate *meta* fission. When no yellow colour appeared, the mixture was further shaken for an additional 2 h at 30 °C and tested for β-ketoadipic acid by the method of Rothera (1908). Control experiment was set up with noninduced cells grown in minimal salts medium (200 ml) supplemented with yeast-extract (0.05%, w/v) and glucose (0.01%, w/v). The control cells were similarly treated as reported for the test strains.

Preparation of crude cell extracts

Cells of the organisms grown on anthracene minimal salts medium (200 ml , 0.1%, w/v) were harvested, washed twice in 50 mM potassium phosphate buffer (pH 7.2) and resuspended in 2 ml of the buffer.The cell suspension was then distrupted by ultasonication in an MSE soniprep 150 ultrasonic disintegrator (MSE Scientific instument, U. K.) at an amplitude of 10–12 μm. Sonication was for 5×30 s with cooling on ice between cycles. The cell debris were removed by centrifugation at 27,000×*g* for 20 min at 4 °C. The supernatants were decanted, stored on ice and used within 5 min of preparation as sources of crude dioxygenases.The experiment was repeated with noninduced cells grown in glucose medium.

Assays of catechol-2,3-dioxygenase and catechol-1,2-dioxygenase activities in crude cell extracts

Catechol-2,3-dioxygenase and catechol-1,2-dioxygenase activities were determined in the extracts as described by Kataeva and Golovleva (1990) and Ka-Leung *et al.* (1990) respectively. Protein concentration of cell extract was determined as described by Bradford (1976).

Results and Discussion

Four bacteria strains were isolated on the basis of their ability to form clearing zones and grow after purification on anthracene as the sole carbon source of energy. The bacteria strains were identified as *Pseudomonas aeruginosa, Alcaligenes eutrophus, Bacillus subtilis* and *Micrococcus luteus.* The *P. aeruginosa* was a gram negative rod, catalase positive, oxidase positive, motile and did not produce indole. It, however, produced pyocyanin and pyoverdin pigments.The organism grew at 42 °C and utilized glucose, raffinose, sucrose, mannitol and maltose but did not utilize trehalose and sorbitol as sources of carbon. The organism was subsequently identified as a strain of *P. aeruginosa.* The *A. eutrophus* was gram negative and coccobacillus in shape. It was motile, urease and catalase positive. Citrate, oxidase, methyl red and Voges-Proskauer tests were all negative, the organism did not utilize xylose, raffinose, sucrose, mannitol, maltose and salicin. The *B. subtilis* colonies were creamy,dull surfaced and spreading on agar. The cells were short rods with spores, gram positive, motile and oxidase positive. Indole, citrate, urease and methyl red tests were all negative. The organism, however, utilized glucose, sucrose, lactose, mannitol and salicin. The colonies of *M. luteus* were golden yellow in pigmentation. The cells were coccoid and gram positive. Indole, citrate, urease, methyl red and Voges-Proskauer tests were negative. The organism did not utilize raffinose, lactose, mannose, maltose and salicin but glucose, xylose and sucrose were utilized.

The substrate specificity determination for the organisms revealed that the four organisms could utilize some hydrocarbons as shown in Table I. None of the organisms could utilize some substrate which included hexane and cycloheptane. The abilty to utilize some of these carbon sources indicate the possession of the necessary enzymes (Finnerty, 1977). Inability of any of the organisms to utilize hexane may be due to the possibility of membrane distruption by the hydrocarbon. The ability to utilize both anthracene and naphthalene by all the organisms was an indication of the possession of the ring fission enzymes and similarity in the anthracene and naphthalene catabolic pathways as previously reported by Evans *et al.* (1965) and Sanseverino *et al.* (1993). Ability of the *P. aeruginosa* to utilize pyrene, however, indicated that its dioxygenase system is active towards more than one type of PAH and this points to its usefulness as a potential seed for bioaugmentation.

The results of the growth test of the organisms on anthracene is presented in Fig. 1A. *P. aeruginosa* was the best anthracene degrader, it had the

Table I. Growth test on different carbon sources.

Substrate	*P. aeruginosa*	*A. eutrophus*	*B. subtilis*	*M. luteus*
Octane	+	+	−	+
Hexadecane	+	+	+	+
Dodecane	+	−	+	+
Undecane	+	−	+	−
Decane	+	−	+	−
Hexane	−	−	−	−
Tetradecane	+	+	+	+
Cycloheptane	−	−	−	−
Cyclohexanol	+	−	−	−
Xylene	−	−	−	−
Phenol	−	−	−	−
Benzene	−	−	−	−
Toluene	−	−	−	−
Naphthalene	+	+	+	+
Phenanthrene	−	−	−	−
Fluoranthene	−	−	−	−
Anthracene	+	+	+	+
Pyrene	+	−	−	−
Crude oil	+	+	+	+
Kerosene	−	−	−	−
Diesel oil	+	+	+	+
Engine oil	+	+	+	+

highest cell density of 4.5×10^7 cfu/ml after 30 days incubation. *A. eutrophus*, *B. subtilis* and *M. luteus* had cell densities of 8.6×10^6, 5.4×10^6 and 2.4×10^6 cfu/ml respectively. There was appearance of a yellow colouration in the culture broths of *P. aeruginosa* and *A. eutrophus* within the 1 st week of cultivation, the yellow colour then changed to brown which persisted till the end of the experiment. Many Pseudomonads have been reported to possess the ability to grow on anthracene. They include *P. putida, P. cepacia, P. paucimobilis* and *P. fluorescens* (Dagher *et al.*, 1997; Cerniglia, 1992; Mueller *et al.*, 1990; Foght and Westlake, 1988). *Alcaligenes denitrificans* has been reported to have degraded naphthalene, phenanthrene, fluoranthene, pyrene and benz(a)anthracene (Cerniglia, 1992) while *A. faecalis* also grew on phenanthrene (Kiyohara *et al.*, 1982, 1990). *Bacillus cereus* was reported to have transformed naphthalene to 1-naphthol (Cerniglia *et al.*, 1984) Furthermore, *Micrococcus* sp. with ability to utilize phenanthrene as sole carbon source has been isolated (Cerniglia, 1992). In this work, we confirmed the ability of tropical soil isolates of *P. aeruginosa*, *A. eutrophus, B. subtilis* and *M. luteus* as anthracene degraders. Other known anthracene degraders include *Beijerinckia* sp. (Akhtar *et al.*, 1975; Jerina *et al.*, 1976), *Flavobacterium* sp. (Colla *et al.*, 1959), *Mycobacterium* sp. and *Rhodococcus* sp. (Cerniglia, 1992), *Arthrobacter* sp. (Savino and Lollini, 1997) and *Sphingomonas* sp. (Dagher *et al.*, 1997). It has been well established that the first step in bacterial aerobic degradation of PAH requires a dihydroxylation reaction and subsequent formation of *cis*-dihydrodiol which is carried out by membrane bound enzyme systems. Further oxidation leads to the formation of catechol which becomes substrate for other dioxygenases (Cerniglia, 1992).

As shown in Fig. 1B, *P.aeruginosa* grew on the crude oil and increased in cell density to a peak of 9.8×10^8 (cfu/ml) on the 10th day. *A. eutrophus* and *M. luteus* had their peaks on the 12 th day with populations of 7.2×10^8 and 5.9×10^8 (cfu/ml) respectively. Continuous increase was however noticed with *B. subtilis* with a maximum cell density of 5.5×10^8 on the 14 th day. As the organisms were growing, the amount of oil in the flasks were decreasing. The percentages of the oil remaining in the flasks on the 14 th day ranged from 22.2–91.7 (Fig. 1C). *P. aeruginosa* was found to have utilized the oil best. Many bacteria possess the enzymatic capability to degrade crude petroluem. Hydrocarbon utilizing bacteria are ubiquitously distributed in the soil environment (Atlas, 1995). Some bacteria degrade aliphatic, others aromatic, while some degrade both aliphatic and aromatic hydrocarbons (Leahy and Colwell, 1990; Atlas and Bartha, 1992). The organisms used in this study belonged to the later class. Crude oils are never completely degraded and always leave some complex residues (Atlas, 1995) it is plausible that these organisms participate in the degradation of first, the alkane component and at a latter stage, some of the PAH residues. Decrease in the concentration of the oil in the control flasks was mainly due to evaporation since chances of photooxidation was excluded.

There was appearance of a bright yellow colour within a few seconds of the addition of catechol solution to the test tubes containing induced cell suspensions of *P. aeruginosa* and *A. eutrophus* showing the presence of the enzymes of *meta* pathway. The cell suspensions of *B. subtilis* and *M. luteus* were further tested for the presence of β-ketoadipic acid. The appearance of purple colouration in the test tubes with induced cells of *B. subtilis* and *M. luteus* confirmed the presence of the enzymes of *ortho* pathway in the 2 organisms. Assays of the cell free extracts of the organisms

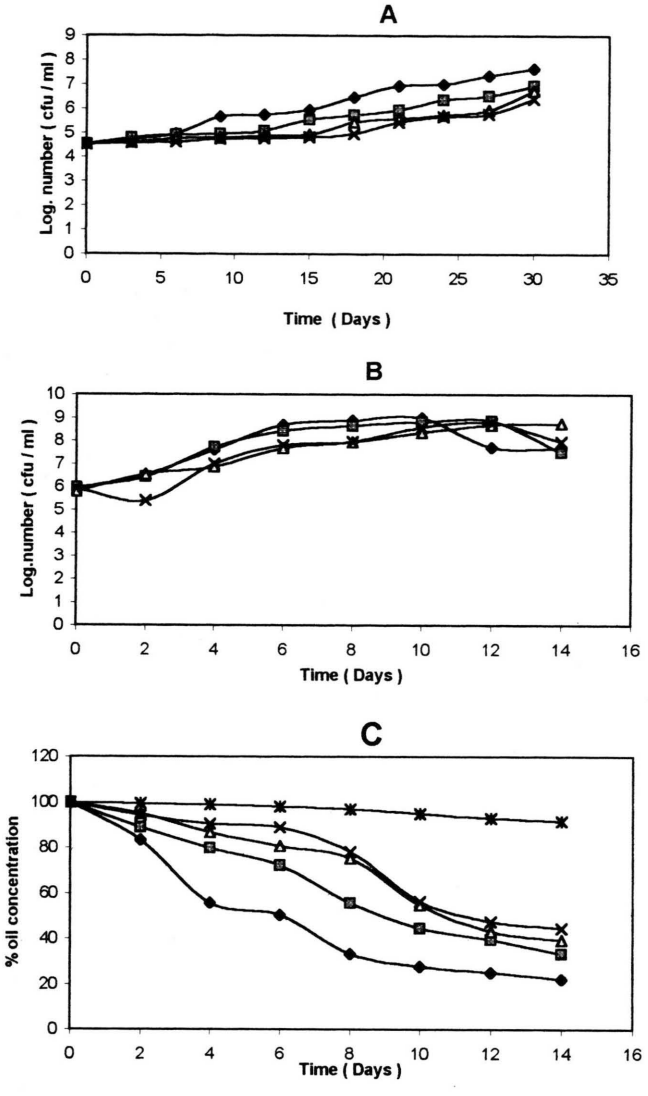

Fig. 1A. Growth of organisms on anthracene:
◆, *P. aeruginosa*;
■, *A. eutrophus* ;
△, *B. subtilis*;
x, *M. luteus*.

Fig. 1B. Growth of organisms on crude oil:
◆, *P. aeruginosa*;
■, *A. eutrophus*;
△, *B. subtilis*;
x, *M. luteus*.

Fig. 1C. Residual oil during growth of organisms:
◆, *P. aeruginosa*;
■, *A. eutrophus*;
△, *B. subtilis*;
x, *M. luteus*;
*, Control.

grown on anthracene revealed the presence of either of these enzymes. Neither enzyme was detected in noninduced cells. Catechol-2,3-dioxygenase activity was found in cell free extracts of *P. aeruginosa* and *A. eutrophus* (Table II) while extracts of *B. subtilis* and *M. luteus* had activities of catechol 1,2 dioxygenase. Anthracene ring fission, therefore, takes place through *meta* pathway in *P. aeruginosa* and *A. eutrophus* and *ortho* pathway in *B. subtilis* and *M. luteus*. Further metabolism of the intermediate of *meta* pathway was inferred when the yellow colour of the assay mixture for *P. aeruginosa* and *A. eutrophus* faded with overnight incubation. The high level of activity found in the extract of *P. aeruginosa* was probably due to the presence of high concentration of the active com-

Table II. Activities of dioxygenases in induced cells.

Organisms	Specific enzyme activity (μmol/min × mg protein)	
	Catechol-2,3-dioxygenase	Catechol-1,2-dioxygenase
P. aeruginosa	3.8 ± 0.183	0.0
A. eutrophus	0.64 ± 0.032	0.0
B. subtilis	0.0	1.95 ± 0.029
M. luteus	0.0	1.89 ± 0.026

The expt. was repeated four times.

ponents of the dioxygenase since the extracts were used within 5 min of preparation. Catechol-2,3-dioxygenase is extremely sensitive to oxygen and is easily inactivated in the presence of air (Gibson, 1968). Growth of *Pseudomonas* sp. on naphthalene has been reported to induce two other enzymes that are involved in further metabolism of 2-hydroxymuconic semialdehyde. These are the NAD^+ dependent 2-hydroxymuconic semialdehyde dehydrogenase and the non NAD^+ dependent 2-hydroxymuconic semialdehyde hydrolase. The activity of 2-hydroxymuconic semialdehyde dehydrogenase is generally higher than that of 2-hydroxymuconic semialdehyde hydrolase (Sutherland *et al.*, 1995).The *ortho* pathway enzymes are produced in most strains when the *meta* pathway enzymes have not been induced and catechol has accumulated. In the *ortho* pathway, catechol-1,2 dioxygenase metabolizes catechol to *cis cis* muconate (Williams *et al.*, 1975).

Other bacteria known to have metabolized PAHs through *meta* pathway include *P. fluorescens* 5RL (Menn *et al.*, 1993), *P. putida* NCIMB11767 (Heald and Jenkins, 1996), *P. testosteroni* and *P. stutzeri* (Garcia-Valdes *et al.*, 1988). *P. putida* PpG7 utilized *ortho* pathway to metabolized naphthalene (Singer and Finnerty, 1984) while *Pseudomonas* sp. strain PG also metabolized salicylate through the *ortho* pathway (Sutherland *et al.*, 1995). Bacteria dioxygenases have great potential in environmental biotechnology since they participate in biodegradation of many natural and xenobiotic compounds (Heald and Jenkins, 1996). The organisms reported in this work have metabolic activities against some aliphatic and aromatic hydrocarbons, this points to their usefulness as potential seeds for tropical soil bioremediation. The substrate range of degradative organisms with potential commercial value is an important consideration for bioaugmentation of soils and sediments contaminated with a mixture of compounds such as found in industrial wastes and in petroleum spills.

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