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Full Length Research Paper

Studies on aerobic biodegradation activities of 2,4-dichlorophenoxyacetic acid by bacteria species isolated from petroleum polluted site

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Bacteria species were screened and monitored for the efficiency of 2,4–dichlorophenoxyacetic acid (2,4-D) degradation from oil degrading laboratory stock with the view to getting the most efficient 2,4-D degraders, to develop an active indigenous bacterial consortium for the bioremediation of 2, 4-D polluted systems in Nigeria. The 2,4-D was utilized as sole source of carbon attaining maximum cell densities of 10^7 cfu ml^{-1} from an initial 10^5 cfu ml^{-1} in 10 days. The amount of 2,4-D utilized in a batch culture by the isolates varied significantly from an initial inoculum densities of the order of 10^5 cfu ml^{-1} and increased with increasing concentrations of 2,4-D. Growth rates ranged from 0.154 h^{-1} to 0.180 h^{-1} for SERU2 and 0.158 h^{-1} to 0.183 h^{-1} for SERU 11. Dioxygenase specific activity [µg ml^{-1} chloride released/mg protein)$^{-1}$ h^{-1}] in actively growing cell cultures ranged from 0.010 – 0.055 (SERU 2) and 0.009-0.045 (SERU 11). The specific activity of the dioxygenase in the cell-free system ranged between 0.013 – 0.042 (SERU 2) and 0.011-0.046 (SERU 11). The pH optimum for the dioxygenase of the cell-free system was between 7.6 and 8.0 while the temperature optimum was 30°C. In conclusion the results showed that the two bacteria isolates have potential for 2,4-Dichlorophenoxyacetic acid degradation and their cell-free extracts could be used as biological alternatives in the bioremediation of 2,4-D contaminated system.

Key words: 2,4-Dichlorophenoxyacetic acid (2,4-D), petroleum degraders, biodegradation, dioxygenase enzyme.

INTRODUCTION

2, 4-Dichlorophenoxyacetic acid (2,4-D) as a systemic herbicide is used to control many types of broadleaf weed (Young, 2002). 2,4-D mineralization could be because the organism have special nutritional or other cultural requirements which are sensitive to moderate concentrations of 2,4-D, or are members of consortia or because the degradation is by cometabolism (Kamagata et al., 1997). A number of 2,4-D degraders have been isolated and most of the strains have been well characterized (Don and Pemberton, 1981; Fournier, 1980; Fulthorpe et al., 1996; Ka et al., 1994a, b).

Microbial degradation of 2,4-D has been the subject of extensive studies and more recent studies have elucidated the kinetics of degradation of 2,4-D (Igbinosa, 2006). Recognized as one of the most metabolically versatile of bacterial species, *Arthrobacter* sp. and *Pseudomonas* sp. can degrade a wide range of compounds as carbon and energy source including a variety of aromatic compounds pesticides and herbicides (Bhat et al., 1993; Ka et al., 1994a, b; Fulthorpe et al., 1996; Sanni and Ajisebutu, 2003a, b; 1996; Igbinosa, 2006). The wide use of this compound has prompted interest in its biodegradation. However based on available literatures no studies have been carried out on assessing the petroleum degrader on 2,4-dichlorophenoxyacetic acid in the bioremediation of 2,4-D polluted system in Nigeria. This paper reports 2,4-D degradation potential of an axenic culture of a SERU 2 and SERU 11 (*Pseudomonas* and *Arthrobacter* species, respectively) which had previously been studied on crude oil biodegradetion by Sanni and Ajisebutu (2003a,

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b). An attempt was made to investigate the dual purpose of bioremediation of 2,4-D contaminated system.

MATERIALS AND METHODS

Source of bacteria

The bacteria species used in this study were isolated and identify by Sanni and Ajisebutu (2003a, b) and was preserved in an agar slants at 4°C. After resuscitation by growing it in 2,4-D bromothymol blue (2,4-D-BTB) supplemented with 0.1% (v/v) sterile, 2,4-D. The composition of 2,4-D-BTB included (per liter): Mg $S0_4$. $7H_20$ (0.02 g), $CaCl_2$ (0.02 g), Na_2HP0_4 (0.141 g), K_2HP0_4 (0.136 g), (NH_4) $S0_4$ (0.12 g), casamino acid (0.025 g), yeast extract (0.025 g), $CaCl_2.H_2O$ (0.0023 g), bromothymol blue (BTB) (0.05 g), agar (20 g) and $FeCl_3$ (two drops of 60% solution), pH 7.0-7.2. The plates were incubated at 37°C for 2 – 7 days and observed for a colour change. A change from dark blue green to yellow indicated the successful mineralization of 2,4-D in the medium.

Screening for biodegradation potential

The bacterial isolate was initially selected by enrichment, using 0.1% (v/v) sterile 2,4-D in Minerals Salts Medium (MSM). Turbidity and/or emulsification (after 10 days) were used as index of biodegradation potential. The purity of the culture was confirmed by streaking on solidified 2% (v/v) glucose in minimal medium (GMM) followed by incubation at 37°C for 24 h. A total of 10 ml of the pure enrichment culture was then centrifuged to pellet out the cells, washed twice with sterile physiological saline solution and the suspension was adjusted to an optical density at 540 nm (OD_{540}) of 0.1 which is equivalent to a cell population of between 2.5 – 3.0 x 10^6 cells/ml on the McFarland standard. From this 1.0 ml was inoculated into 0.1% (w/v) sterile 2,4-D in 50 ml of MSM in 125 ml Erlenmeyer flasks. Incubation was at room temperature (27±2°C) on a horizontal shaker fixed at 150 rpm for 10 days. Turbidity and/or emulsification were used as index of degradation activity.

Kinetic Studies

A 1.0 ml of each sample of the bacterial suspension prepared as described above was inoculated into separate 125 ml Erlenmeyer flasks containing 50 ml of MSM to yield an initial bacteria count of approximately 10^5 cfu ml^{-1}. The final concentrations of 2,4-D (weathered by autoclaving) in the flasks were 0.1, 0.2, 0.4, 0.6 and 0.8% (w/v). Uninoculated controls were also setup to determine the extent of 2,4-D loss by evaporation, incubation conditions were as explained earlier. Samples were taken at 0, 2, 4, 6, 8 and 10 days.

The content of each flask was emptied into separate sterile separation funnels and allowed to stand for 1 - 2 h, during which almost all the 2,4-D fraction separated at the top. The aqueous fraction was then drained and analyzed for pH and cell densities and was returned into the separation funnels. Cell densities were determined by estimating the viable count as previously described (Seeley and Van Denmark, 1981). .

Total residue 2,4-D was estimated by gravimetric method as described elsewhere (Ajisebutu, 1988) with modifications, using dichloromethane as extraction solvent. Chloride ion released was used as index of biodegradation (Fulthorpe et al., 1996). The quantity of the chloride ion released was measured at appropriate intervals using the argentometric method (American Public Health Association, 1971). Specific growth rate constants were estimated as described elsewhere (Alexander and Snow, 1989).

Measurement of dioxygenase activity

Mineral Salts Medium (MSM) supplement with sterile 2,4-D at 0.1% (w/v) was inoculated with 24 h cultures of the each isolates grown on the same medium and incubated at 30°C on a horizontal shaker at 100 rpm. Cultures were harvested by centrifugation at 3,500 x g for 10 min and the dioxygenase activity in the crude cell free extracts were determined as previously described (Igbinosa, 2006). The dioxygenase enzyme activity, which is an index of 2,4-D degradation in the cell-free extract of the two bacteria were measured by the amount of chloride ion released after ascertaining that the system is cell-free by using nutrient agar plate. Chloride ion released was measured as mentioned earlier, and the protein concentration of the extracts was estimated by the Biuret method (Gornall et al., 1949) using bovine serum albumin as the standard protein. Dioxygenase specific activity was expressed as µg ml^{-1} chloride ion released (mg protein)$^{-1}$ h^{-1}.

The effects of pH on the specific dioxygenase activity of the cell-free culture extract of the isolates against 2,4-D were determined by incubating the enzyme solution at different pH conditions using phosphate buffer (pH 6-8) and glycine-NaOH buffer (pH 8-9). The effect of temperature on dioxygenase activity was also evaluated by incubating the enzyme solution at 25, 30, 35, 40 and 45°C. All data were statistically analysis using the SPSS 11.0 software package. Means were compared using the one-way ANOVA and student's t-test, while relationships were tested for using the Pearson correlation index.

RESULTS

The preliminary screening for the ability of *Pseudomonas* sp. (SERU 2) and *Arthorbacter* sp. (SERU 11) suggested appreciable biodegradation potential, which was the basis for further investigation. Kinetic studies revealed a varying response of the bacteria to different concentrations of 2,4-D. Biomass increased with time in all cases reaching maximal densities that ranged from 1.0 x 10^6 to 2.2 x 10^6 cfu ml^{-1} for SERU 2 and 1.0 x 10^6 to 2.4 x 10^6 cfu ml^{-1} for SERU11 in 10 days for the various concentrations of 2,4-D (Table 1). The specific growth rate was lowest at 0.154 and 0.158 h^{-1} for SERU 2 and SERU 11 respectively at 0.1% 2, 4-D concentration. The pH declined slightly but significantly in all cases from an initial 6.89 to values of 6.50 – 6.89 for SERU 2 and 6.52 – 6.85 for SERU 11 in 10 days (Table 2).

The growth profile at 0.2% concentration of the 2,4-D was in the order of 1.08 x 10^5 – 1.40 x 10^8 and 1.2 x 10^5 – 1.3 x 10^8 cfu ml^{-1} for SERU 2 and SERU 11, respectively, while at 0.4% concentration of the 2,4-D was in the range of 9.11 x 10^5 – 3.84 x 10^7 and 1.68 x 10^5 – 1.18 x 10^8 cfu ml^{-1} for SERU 2 and SREU 11, respectively (Figure 1). Although there were differences in the cell densities value of the two organisms most of the differences were not observed to be statistically significant. The amount of 2,4-D mineralized by the bacterial during the 10 day incubation increased significantly ($p< 0.05$) (Table 3). The quantity of chloride ion released in the 2,4-D cultures was observed to be 0.064 µg ml^{-1} for SERU 2 and 0.053 µg ml^{-1} for SERU 11. The dioxygenase specific activity of the cell-mediated isolates at different growing time showed a range of 0.01 – 0.0576 µg ml^{-1} Cl^{-1} (mg protein)$^{-1}$ h^{-1} and

Table 1. Maximum cell densities attained during 10 days of incubation of *Pseudomonas* sp. SERU 2 and *Arthrobacter* sp. SERU 11 in various concentrations of 2,4-D.

2,4-D concentration (%, w/v)	SERU 2		SERU 11	
	Starting cell density ($x10^5$ cfu ml^{-1})	Maximum cell density ($x10^5$ cfu ml^{-1})	Starting cell density ($x10^5$ cfu ml^{-1})	Maximum cell density ($x10^5$ cfu ml^{-1})
0.1	0.2±0.04	1.0±0.5	0.1±0.05	10±0.7
0.2	0.1±0.06	12±0.7	0.1±0.08	15±0.6
0.4	0.1±0.04	15±0.9	0.2±0.06	18±0.5
0.6	0.1±0.05	20±1.2	0.2±0.04	21±1.5
0.8	0.1±0.06	22±1.8	0.1±0.06	24±2.0

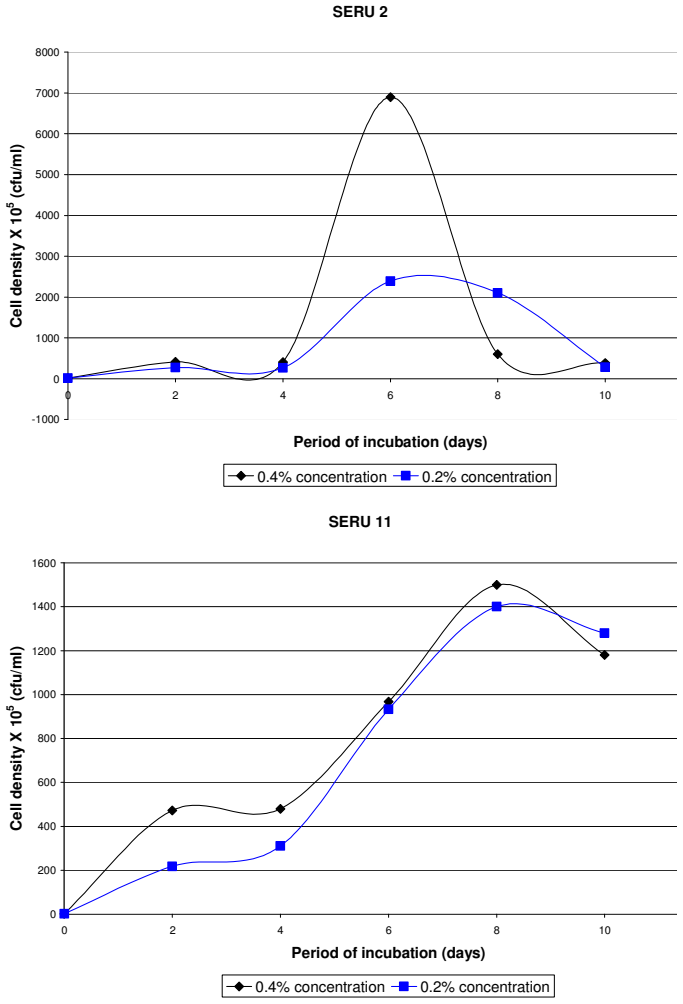

Figure 1. Growth profiles of the SERU 2 and SERU 11 isolates in 0.4 % and 0.2% concentrations of the 2, 4- D medium.

0.009 – 0.048 µg ml^{-1} Cl^{-1} (mg protein)$^{-1}$ h^{-1} for SERU 2 and SERU 11 respectively (Table 4). The highest value was obtained at the 120 h for SERU 2 and 96 h for SERU 11. The values of the cell-mediated dioxygenase specific activity varied significantly among the isolates at the different time ($p< 0.05$). Dioxygenase specific activity of the cell-free extract, values ranged from 0.0125 – 0.0456 µg ml^{-1} Cl^{-1} (mg protein)$^{-1}$ $h^{-1}$and 0.0112 – 0.0487 µg ml^{-1} Cl^{-1} (mg protein)$^{-1}$ h^{-1} for SERU 2 and SERU 11 respectively (Table 5). As was observed in the cell-mediated highest values were observed at 120 h for SERU 2 and at 96 h for SERU 11. The value of the dioxygenase activity varied significantly ($p< 0.05$) between the different hours. The optimum pH for dioxygenase specific activity for the two isolates was 8.0 and 7.6 of the SERU 2 and SERU 11 respectively (Table 6). The optimum temperature for enzyme activity was 30°C for the two isolates (Table 7).

DISCUSSION

The results obtained from this study revealed that some petroleum degraders have the potential to degrade 2,4-dichlorophenoxyacetic acid (2,4-D). These bacteria had previously been identified by Sanni and Ajisebutu (2003a, b). They were screened for 2,4-D degrading ability of various concentrations (0.1 – 0.8% w/v) as sole carbon and energy source. Preliminary screening for the ability to utilize 2,4-D for growth revealed a 10 to 10^3-fold increase in biomass with 10 days of the isolates SERU 2 and SERU 11 (*Pseudomonas* sp. and *Arthrobacter* sp.), respectively, yielded the highest 2,4-D reduction and were thus selected for further studies.

Growth of axenic cultures of SERU 2 and SERU 11 isolates on various concentrations of 2,4-D in shake flask exemplified typical bacterial growth pattern in utilizable complex substrate medium with irregular specific growth rate which peaked variously at different concentrations of 2,4-D (Table 2). This findings agrees with the works of Ka et al. (1994a, b) and Fulthrope et al. (1996) who reported that the varying growth peaks observed is a phenomenon characteristics of microbial growth pattern in a multi-component media that 2,4-D is, and it is suggestive of differences in metabolic responses of the isolates.

The amount of high concentration of 2,4-D metabolized at the end of the reaction period increased with increasing concentrations of starter 2,4-D, while degradation appeared to be more pronounced between the 0.2 and

Table 2: Effect of 2,4-D concentration on the specific growth rate of SERU 2 and SERU 11 and pH decline of the cultures (after 10 days of degradation reaction) Specific growth rate was calculated from mean values of cell densities.

2,4-D concentration (%, w/v)	SERU 2		SERU 11	
	Specific growth rate (h^{-1})	Decline in pH	Specific growth rate (h^{-1})	Decline in pH
0.1	0.154	6.89 ± 0.05	0.158	6.85 ± 0.06
0.2	0.167	6.74 ± 0.04	0.162	6.78 ± 0.03
0.4	0.171	6.70 ± 0.03	0.160	6.60 ± 0.05
0.6	0.179	6.65 ± 0.03	0.172	6.55 ± 0.02
0.8	0.180	6.50 ± 0.02	0.183	6.52 ± 0.04

Table 3. Percentage degradation of 2,4-D by two bacterial isolate.

Isolates	Quantity of 2,4-D (g)		% Degradation
	2,4-D Recovered	2,4-D Degraded	
Control	0.2447	-	%
SERU 2	0.0509	0.1938	79.2%
SERU 11	0.0434	0.2013	82.3%

Table 4. Dioxygenase specific activities of cell-mediated cultures of the isolates against 2,4-D at different growing time.

Isolate code	Dioxygenase specific activity [µg ml^{-1} Cl^{-1} (mg protein)$^{-1}$ h^{-1}] at different time (h)							
	O h	24 h	48 h	72 h	96 h	120 h	144 h	168 h
SERU 2	0.010±0.03	0.046±0.23	0.056±0.26	0.056±0.26	0.0573±0.27	0.058±0.27	0.057±0.27	0.055±0.25
SERU 11	0.009±0.05	0.041±0.22	0.046±0.23	0.047±0.24	0.048±0.24	0.047±0.24	0.047±0.24	0.045±0.23

Table 5. Dioxygenase specific activities of cell-free extract culture of the isolates against 2,4-D at different growing time.

Isolate code	Dioxygenase specific activity [µg ml^{-1} Cl^{-1} (mg protein)$^{-1}$ h^{-1}] at different time (h)							
	O h	24 h	48 h	72 h	96 h	120 h	144 h	168 h
SERU 2	0.010±0.04	0.010±0.04	0.010±0.04	0.010±0.04	0.016±0.010	0.016±0.010	0.016±0.010	0.016±0.010
SERU 11	0.0112±0.004	0.0112±0.004	0.0112±0.004	0.0137±0.006	0.0175±0.013	0.0175±0.013	0.0175±0.013	0.0175±0.013

Table 6. Effect of pH on the dioxygenase activity of cell free culture extract of the isolates against 2,4-D. Vaules in parentheses represent the percentage of activity at optimal pH.

Isolate code	Dioxygenase specific activity [µg ml^{-1} Cl^{-1} (mg protein)$^{-1}$ h^{-1}] at different pH						
	6.0	6.6	7.0	7.6	8.0	8.6	9.0
SERU 2	0.019±0.01 (32.8)	0.025±0.05 (43.1)	0.033±0.06 (56.9)	0.043±0.01 (74.1)	0.058*±0.01 (100)	0.042±0.02 (72.4)	0.036±0.01 (62.1)
SERU 11	0.023±0.02 (46.9)	0.029±0.01 (59.2)	0.038±0.01 (77.6)	0.049*±0.01 (100)	0.036±0.01 (73.5)	0.022±0.02 (449)	0.025±0.01 (51.0)

*Optimal value.

0.4% (w/v) of 2,4-D. This finding is in line with those of earlier workers (Ka et al., 1994a, b; Kamagata et al., 1997), who reported that biomass is required only to a particular threshold enough to produce the appropriate enzyme system that carry through the degradation process even when biomass seizes.

Biodegradation of 2,4-D was appreciable as shown by the result of this research (Table 3), which is in agree-

Table 7. Effect of temperature on the dioxygenase activity of cell-free culture extract of the isolate against 2,4-D. Values in parentheses represent the percentage of activity at optimal temperature.

Isolate code	Dioxygenase specific activity [µg ml^{-1} Cl^{-1} (mg protein)$^{-1}$ h^{-1}] at different temperatures (^{o} C)				
	25	30	35	40	45
SERU 2	0.027±0.02 (46.6)	0.058*±0.04 (100)	0.030±0.01 (51.7)	0.024±0.01 (41.4)	0.016±0.01 (27.6)
SERU 11	0.032±0.02 (69.6)	0.046*±0.05 (100)	0.033±0.03 (71.7)	0.026±0.04 (56.5)	0.016±0.01 (34.8)

*Optimal value.

ment with the work of Ka et al. (1994a, b) who reported that 70% of the extractable 2,4-D was cleared by micro-organisms. As reported earlier, isolates used in this study were those that had been used by Sanni and Ajisebutu (2003a, b), in a study carried out to ascertain the crude oil degrading ability of some selected bacteria. In this study the prominent crude oil degraders were not observed to be prominent in the degradation of 2,4-D. This is in line with the finding of Alexander (1994) who reported that biodegradation was a major process that determined the early fate of 2,4-D with as much as 1000 ppm being biodegraded within 3 to 5 day. It is suggested that strains of the members of the group to which these isolates belong were very efficient degraders of crude oil and chlorinated aromatic compounds. The ability of the isolates to tolerate high concentration of 2,4-D up to 0.8% w/v is an added advantage. The potentials of the cell-free extracts from the cultures of the isolate in the degradation of 2,4-D was ascertained using chloride ion released as index of biodegradation process. A lag period of about 24 h was observed, probably a period of acclimation to the new environment. An appreciable amount of chloride concentration was detected. Considering the specific activity of the cell-free extracts of the isolates it is projected that 37.5 x 10^{-4} mol of 2,4-D will degrade per milliliter per hour per milligram of protein content of the crude cell-free extracts from the cultures of the isolates of growth. This is in agreement with the work of Ventullo and Larson (1986) who observed acclimation period in the mineralization of docecyltrimethyl ammonium chloride. Fulthorpe et al. (1996) reported acclimation period in the mineralization of 2, 4-Dichlorophenoxyacetic acid (2,4-D) and 3-chloropbenzoate (3CBA). Olaniran et al. (2002) also reported acclimation period in oranochloride. The rate of chloride released in the cell-free system compared appreciably well with the values obtained for the corresponding actively growing culture system (Tables 4 and 5). The enzymes responsible for the dechlorination process were inducible. This observation is relevant especially in the light of public clamour for the use of cell-free system in bioremediation work against the skepticism that has befallen the application of live organisms, especially Genetically Modified Organisms (GMOs) in bioremediation of polluted environment.

The optimum pH value of 2,4-D dioxygenase activity was between 7.6 and 8.0. This pH value agrees with previous works of Slater et al. (1979) and Olaniran et al. (2002) who reported a pH optimal range of 7.9 – 8.1 for organochloride. It would appear that chlorination process is favoured in an alkaline medium. It is suggestive that a change in pH alters the state of ionization of charge amino acids that may play crucial roles in substrate binding. The optimum temperature of 2,4-D dioxygenase activity was 30^{o}C, which fell within the range reported by Hausinger and Fakumori (1995). Microorganisms found effective in bioremediation have been shown to perform well in the temperature range of 10 – 40^{o}C (Cookson, 1995).

The findings in this study show that *Pseudomonas* sp. SERU 2 and *Arthrobacter* sp. SERU 11 could effectively use their cell-free extract to bioremediate 2,4-dichlorophenoxyacetic acid and petroleum hydrocarbon contaminated systems instead of the live organisms which are themselves hazardous to the environment.

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