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*Full Length Research Paper*

# Aerobic dehalogenation activities of two petroleum degrading bacteria

[1]*Igbinosa O. Etinosa, [2]Ajisebutu O. Stella and [1]Okoh I. Anthony

[1]Department of Biochemistry and Microbiology University of Fort Hare, Private Bag X1314, Alice, 5700 South Africa.
[2] Department of Microbiology Obafemi Awolowo University Ile-Ife, Osun State, Nigeria.

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**Two petroleum degrading bacteria were screened for 2,4-dichlorophenyacetic acid (2,4-D) degrading abilities and assessed for their dechlorination potentials. The bacterial isolates were previously identified to be *Corynebacterium* sp. (SOGU16) and *Achromobacter* sp. (SOGU11). Axenic cultures of the isolates metabolise 2,4-D as the sole source of carbon and energy. The optimum pH for dioxygenase specific activities was between 7.6 and 8.0 and the optimum temperature was between 30 and 35°C. The cell-free extracts of the cultures in the 2,4- D demonstrated biological degradation of the 2,4-D compound. The observations made in this study are sufficient to conclude that the isolates obtained have reasonable potentials for application in bioremediation of both petroleum and 2, 4-D contaminated sites.**

**Key words:** 2, 4-Dichlorophenoxyacetic, bioremediation, petroleum degraders.

## INTRODUCTION

Pesticides and other xenobiotic compounds are new carbon compounds that have been introduced into the environment during the past several decades. Some of these compounds are good carbon sources for bacteria capable of degrading them. For example 2, 4-dichlorophenoxyacetic acid (2,4-D) is known to be a growth substrate for a number of different soil microorganism (Fournier, 1980; Loos et al., 1979; Miwa and Kuwatsuka, 1990; Ou, 1984). Since such synthetic compounds are usually not present in nature and their supply is under human control, they make good models to study microbial resource competition. 2, 4-dichlorophenoxya-cetic acid possibly the most widely used of the hormone herbicides is moderately persistent in soils.

Bacteria have evolved an extensive range of enzymes and pathways that make them able to degrade a wide array of carbon compounds (Krooneman et al., 2000). Extensive studies have also been carried out to optimize the dehalogenating activities of petroleum degrading bacteria from various settings for bioremediation purpose. Degradation of 2, 4-dichlorophenoxyacetic acid using soil bacteria have been reported (Fournier, 1980; Ou, 1984; Olaniran et al., 2002). However, there is paucity of information on the mechanism of initial uptake by petroleum degrading bacteria. The aim of the current research effort is to report the dehalogenation activities of two petroleum degrading bacteria previously studied by Sanni and Ajisebutu (2003a,b), in an attempt to develop active bacterial strains for use in the bioremediation of systems polluted by 2, 4-ichlorophenoxyacetic acid related compounds in Nigeria.

*Corresponding author. E-mail: ogbomoede@yahoo.co.uk. Tel: +2348028319562.

### Materials and Methods

#### Source of bacteria

Bacteria strains used for the study were obtained from the laboratory stock of already identified petroleum degrading bacteria in Obafemi Awolowo University, Ile-Ife (Sanni and Ajisebutu, 2003a, b).

#### Screening for dioxygenase activities

The identified bacterial isolates were initially screen for dioxygenase activity by streaking on agar plates containing 0.1% 2,4-D containing bromthymol blue indicator (5.0 mg $l^{-1}$) (Fulthorpe et al., 1996). Incubation was carried out at 27 ± 2$^{o}$C for 2 to 7 days and observed for colour change. A change from dark blue green to yellow colouration indicates successful mineralization of 2 4-D in the medium.

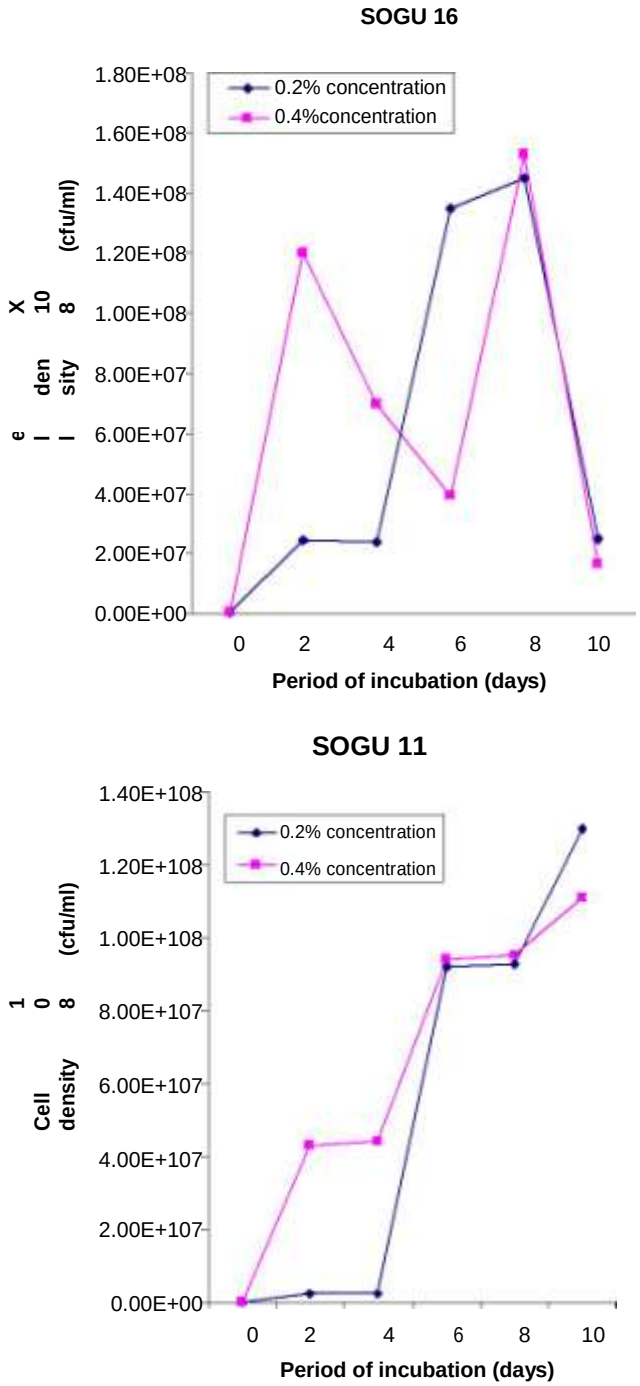


**Figure 1.** Growth profiles of the SOGU 16 and SOGU 11 isolates in 0.4% and 0.2% concentrations of the 2, 4-D medium.

**Growth profile and chloride release**

The growth patterns of the axenic cultures of the bacterial isolates were determined by cultivating 1.0 ml of standardized ($OD_{540}$ nm) culture of each isolates in 10 ml mineral salt medium pH 7.2 in 250 ml. Erlenmeyer flask containing 0.1% of 2, 4-D as described previously. Control was set up containing only 2, 4-D in the medium. Incubation was as at room temperature (27 ± 2$^{o}$C) for 2 – 7 days. At each sampling time, viable bacterial counts were carried out using standard pour plate techniques (Seeley and Van Denmark, 1981). The quantity of free chloride ion released was measured at the appropriate intervals using the Argentometric method (American Public Health Association, 1971). Specific growth rate constant were estimated as described else where (Alexander and Snow, 1989).

**Estimation of dioxygenase activity**

Defined medium containing the desired 2,4-D at the final substrate concentration of 0.1% (w/v) was inoculated with 24 h cultures of the isolates grown on the same medium and incubated at 37$^{o}$C on an orbital shaker at 100 rpm. Late exponential phase cultures were harvested by centrifugations at 3,500 x g for 10 min and the dioxygenase activity in the crude cell-free extract was determined as previously described (Igbinosa, 2006). Free chloride ion relea-sed was measured as earlier described and the protein concentra-tion of the extracts was estimated by the Biuret method (Gornall, 1948) using bovine serum albumin as the reference protein. Dioxygenase specific activity was expressed as g/ml chloride released (mg protein)$^{-1}$ h$^{-1}$. The effects of pH on the specific dioxygenase activities of the cell- free culture extract of the isolates against 2,4-D were determined by incubating the enzyme solution at different pH using $KH_2P0_4$ and $Na_2$ $HP0_4$ buffer (pH 6.0 – 8.0) and glycine (NaOH) buffer (pH 8.0 - 90). The effect of temperature on dioxygenase activities was also evaluated by incubating the enzyme solution at various temperatures of 25, 30, 35, 40 and 45$^{o}$ C. All data were statistical analysed using the SPSS 11.0 software package. Means were compared using the one-way ANOVA and the student's t-test while relationships were tested for using the Pear-son correlation index.

## RESULTS AND DISCUSSION

The results obtained from this study revealed that petroleum degraders have the potential to mineralize 2-4-dichlorophenoxyacetic acid (2, 4- D). The results agreed with those of Senior et al. (1976) about the versatility of these groups of bacteria as 2, 4-dichlorophenoxyacetic acid degraders. These bacteria had previously been studied and identified (Sanni and Ajisebutu, 2003a,b) as *Corynebacterium* sp. (SOGU16) and *Achromobacter* sp. (SOGU11). The growth profiles of SOGU16 and SOGU11 at 0.2% and 0.4% concentrations of 2, 4- D is shown in Figure 1. This observation suggested that the test isolates had difficulty in biodegrading 2, 4- D at high concentration than at low concentration. The result implies that high concentration could be important for inducing increased selection pressure for the isolation of 2, 4-D degrader with enhanced efficiency.

The difference between the cell densities of the two bacteria was found not to be significant ($p < 0.05$). The mean generation time for each of the bacterial isolates cultured with the different concentrations of 2,4-D is shown in Table 1. Cell growth in the inoculated flask was found to exemplify typical bacterial growth curve patterns in an utilizable substrate medium. Specific growth rates ranging from 0.145 to 1.860 h$^{-1}$ were recorded for different 2, 4-D concentrations compared to 1.500 and

**Table 1.** Mean generation time of bacteria isolate in various concentration of 2,4-D and glucose.

| 2,4-D Concentration (% w/v) | Specific growth rate ($h^{-1}$) | |
|---|---|---|
| | SOGU16 | SOGU11 |
| 0.1 | 0.152 | 0.145 |
| 0.2 | 0.167 | 0.156 |
| 0.4 | 0.177 | 0.160 |
| 0.6 | 0.179 | 0.175 |
| 0.8 | 0.186 | 0.172 |
| Control glucose | 1.690 | 1.500 |

*All values are means determined by using two independent broth cultures.

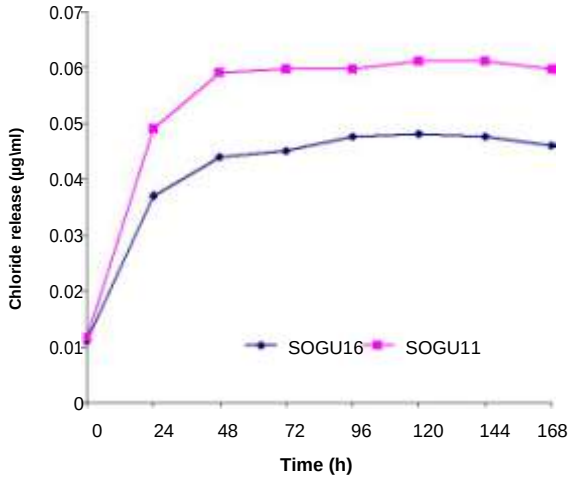


**Figure 2.** Quantity of chloride ion released by SOGU16 and SOGU11 when grown in 2, 4-D over a period of time.

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

| Isolate | Dioxygenase specific activity [ug/ml$^{-1}$ $Cl^-$ (mg protein) $^{-1}$ $h^{-1}$] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 h | 24 h | 48 h | 72 h | 96 h | 120 h | 144 h | 168 h |
| SOGU16 | 0.011±0.04 | 0.037±0.16 | 0.044±0.22 | 0.045±0.23 | 0.048±0.24 | 0.048±0.24 | 0.048±0.23 | 0.046±0.23 |
| SOGU11 | 0.012±0.04 | 0.049± 0.24 | 0.057± 0.26 | 0.059±0.27 | 0.059±0.27 | 0.061±0.28 | 0.061±0.28 | 0.059±0.27 |

1.690 $h^{-1}$ for SOGU16 and SOGU11, respectively, on glucose.

The quantity of chloride released in the batch cultures of the isolates ( g/ml) measured at various incubation times for the 2, 4- D is shown in Figure 2. The chloride ion ($Cl^-$) moiety of the 2, 4-D molecule in particular have been shown to be very bactericidal (Parker and Doxtander, 1982). Fulthorpe et al. (1996) reported that chloride ions are released in the dechlorination of chlorinated aromatic compounds. It would therefore appear that the concen-tration of chloride ion has a negative effect on the ability of the microorganisms to degrade 2, 4-D compounds. Such toxic effects of chlorinated aromatic compound have been reported especially for compounds with more $Cl^-$ (Kaufmar, 1966). It is therefore possible that the popu-lation of the bacteria that degraded the 2, 4-D were the survivors of the initial toxic effect.

The dioxygenase specific activities of the cell mediated culture at different growing time are show in Table 2. The highest values were obtained at 96 h for isolate SOGU16 and at 120 h for SOGU11. The values obtained for the cell mediated dioxygenase specific activities was found to

**Table 3.** The dioxygenase specific activities at different growing time as cell-free extracts of the cultures grown in the presence of the substrate (0.2% 2, 4-D).

| Isolate | Time/h | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 h | 24 h | 48 h | 72 h | 96 h | 120 h | 144 h | 168 h |
| SOGU16 | 0.011 ± 0.05 | 0.012 ±0.06 | 0.017 ± 0.12 | 0.039 ±0.24 | 0.041 ± 0.25 | 0.043 ± 0.26 | 0.036 ± 0.23 | 0.031 ±0.21 |
| SOGU11 | 0.012 ± 0.07 | 0.018 ± 0.11 | 0.018 ± 0.13 | 0.039 ±0.21 | 0.036 ± 0.23 | 0.038 ± 0.24 | 0.035 ± 0.22 | 0.030 ± 0.21 |

**Table 4.** Effect of pH on the dioxygenase specific activity of the cell-free culture extract of the isolate against 2,4-D.

| Isolate | Dioxygenase specific activity [ug/ml$^{-1}$ Cl$^{-}$ (mg protein) $^{-1}$ h$^{-1}$] | | | | | | |
|---|---|---|---|---|---|---|---|
| | pH | | | | | | |
| | 6.0 | 6.6 | 7.0 | 7.6 | 8.0 | 8.6 | 9.0 |
| SOGU 16 | 0.0113 | 0.017 | 0.033 | 0.046* | 0.039 | 0.018 | 0.017 |
| | ±0.001 | ±0.007 | ±0.01 | ±0.01 | ±0.01 | ±0.005 | ±0.002 |
| | (24.6) | (36.9) | (71.7) | (100) | (84.7) | (39.1) | (36.9) |
| SOGU 11 | 0.022 | 0.033 | 0.043 | 0.057 | 0.067* | 0.036 | 0.031 |
| | 0.01 | 0.03 | 0.04 | 0.02 | 0.03 | 0.01 | 0.01 |
| | (32.8) | (49.3) | (64.2) | (85.1) | (100) | (53.7) | (46.3) |

Values in parentheses represent the percentage of activity at optimal pH. *Optimal value.

**Table 5.** Effect of temperature specific activity of the cell-free culture extract of the isolates against 2,4-D.

| Isolate | Dioxygenase specific activity [ g/ml$^{-1}$ Cl$^{-1}$ (mg protein)$^{-1}$ h$^{-1}$] | | | | |
|---|---|---|---|---|---|
| | Temperature ($^{o}$C) | | | | |
| | 25 | 30 | 35 | 40 | 45 |
| SOGU 16 | 0.027 | 0.032 | 0.036* | 0.019 | 0.014 |
| | ±0.02 | ±0.01 | ±0.03 | ±0.01 | ±0.01 |
| | (75.0) | (88.9) | (100) | (52.8) | (38.9) |
| SOGU 11 | 0.029 | 0.078* | 0.043 | 0.026 | 0.017 |
| | ±0.02 | ±0.01 | ±0.03 | ±0.01 | ±0.003 |
| | (37.2) | (100) | (55.1) | (33.3) | (21.8) |

Values in parentheses represent the percentage of activity at optimal temperature. *optimal value.

vary significantly ($p<0.05$) among the isolates at the different times ($p < 0.05$). Results obtained for the dioxygenase specific activities of the cell-free extracts are presented in Table 3. Highest values were observed at the 120 h for SOGU16 and 72 h for SOGU11. The values obtain-ed for the dioxygenase activities varied significantly ($p < 0.05$) between the different hours except in SOGU16 isolates. The cell-free extracts of the cultures in 2, 4-D mediated media demonstrated biological degradation of the 2, 4-D compound.

The optimum pH for dioxygenase specific activities was between 7.6 and 8.0 in SOGU16 and SOGU11 (Table 4). The highest activity obtained was 0.046 and 0.067 g/ml/Cl$^{-}$ released per mg protein per hour in the two isolate. This was probably because the dechlorination process is favoured in an alkaline medium (Igbinosa, 2006; Hausinger and Fukumori, 1995; Olaniran et al., 2002). However, the optimum temperature for enzyme activities was observed to be 30 and 35$^{o}$C for isolate SOGU11 and SOGU16 (Table 5). This finding agreed

with the result of Hausinger and Fukumori, (1995) and Olaniran et al. (2002).

The study showed that even the cell- free extracts of the isolates were almost as efficient in the degradation of the complex compounds as the corresponding live cultures. Thus cell-free enzyme system may subsequently provide a way out of the rejection of the use of live organisms. However, further studies on the subject are on going.

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