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RESEARCH ARTICLE

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The aerobic dechlorination activities of two bacterial species isolated from a refuse dumpsite in Nigeria

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Abstract Two bacterial species isolated using enrichment culture techniques from the topsoil of a main refuse dumpsite in Nigeria were assessed for their dehalogenation potentials. The bacterial isolates were identified as belonging to the *Bacillus* and *Pseudomonas* genera. Axenic cultures of the isolates utilized monochloroacetic acid (MCA), trichloroacetic acid (TCA), trichloromethane ($CHCl_3$) and tetrachloromethane (CCl_4) as the sole source of carbon for growth up to a final substrate concentration of 0.1% (w/v). The mean generation times of the isolates in all the growth media ranged significantly ($P < 0.05$) from 2.41 to 10.04 h and were generally higher than that observed in glucose medium (1.46–1.51 h). The numbers of the chloride atoms in the different organochlorides were negatively correlated with the ability of the organisms to degrade the compounds. Dehalogenase specific activities of the cell-mediated cultures ranged from 0.1 to 0.96 μg ml^{-1} chloride release (mg protein)$^{-1}$ h^{-1} and were significantly ($P < 0.05$) higher than that of the cell-free extract [0.09–0.8 μg ml^{-1} chloride release (mg protein)$^{-1}$ h^{-1}]. The optimal pH of the dehalogenase activity was found to be 8.0, and the optimal temperature was between 30 and 35 °C.

Keywords Dechlorination · Bioremediation · Dehalogenation · Dumpsite

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Introduction

Rapid developments in industry and agriculture have led to the production and use of enormous quantities of organochlorine compounds, which have been introduced into the environment through their use as pesticides [4], or from effluents from pulp and paper industries, as well as from bleaching plants. Many of these compounds later become widely dispersed into the biosphere either deliberately or accidentally and can be detected in water, air and also in humans, thus bringing about undesirable effects on both the biotic and abiotic components of the ecosystem [10].

Organochlorides persist in the environment because they are not readily susceptible to biological transformation. However, microorganisms have evolved an extensive range of enzymes and pathways that make them able to degrade a wide array of these compounds [9]. Extensive studies have been carried out to optimize the dehalogenating activities of indigenous microbiota from various settings for bioremediation purposes.

Little work has been done on dehalogenation of organochlorides using bacteria that are indigenous to the Nigerian environment. Yet, the Nigerian Niger delta has long suffered very serious problems of pollution, especially from crude oil exploration, without appropriate remediation efforts [11]. Here, we describe the dehalogenating activities of two bacterial species isolated in Nigeria in an attempt to develop active bacterial consortia for use in the bioremediation of systems polluted by organochlorine-containing compounds in Nigeria.

Materials and methods

Isolation of bacterial strains

The two bacterial species were isolated from the topsoil of the main refuse dumpsite of the Obafemi Awolowo University, located in Ile-Ife, Nigeria. Ten g of the soil samples, these being subsamples from composite sets obtained from at least four points within the

sampling area, were added separately to 100 ml sterile Bushnell-Hass medium (BHM) [3], pH 7.0, in 250-ml conical flasks containing 0.1% (w/v) of the desired organochloride – either monochloroacetic (MCA), trichloroacetic (TCA), $CHCl_3$ or CCl_4– as the carbon and energy source. The composition of BHM includes (per liter): $MgSO_4{\cdot}7H_2O$ (0.2 g), $CaCl_2$ (0.02 g), KH_2PO_4 (1.0 g), K_2HPO_4 (1.0 g), NH_4NO_3 (1.0 g) and $FeCl_3$ (two drops of 60% solution). The cultures were incubated at room temperature (26 ± 1 °C) on an orbital shaker at 100 rpm. After 3 days incubation, samples were serially diluted in 0.1 M KH_2P0_4/K_2HPO_4 buffer, pH 7.0, and 100-µl portions were plated on solidified defined growth medium containing 1.5% (w/v) agar and the same concentration of chlorinated compound [9]. Discrete colonies of axenic cultures of the isolates were thereafter transferred to agar slants as working stock cultures for further use and strain characterization. The pure strains of the isolates were preserved in 80% (v/v) glycerol at –20 °C, while the nutrient agar slant cultures were stored at 4 °C.

Screening for dehalogenase activities and identification of bacterial isolates

The pure bacterial isolates were initially screened for dehalogenase activity as described elsewhere [12]. Isolates having dehalogenase activities were further confirmed by streaking on agar plates containing the organochloride enrichment and bromocresol purple indicator (5 mg/l). The plates were incubated at 30 °C for 5–7 days and observed for color change from purple to yellow, which would indicate the release of hydrochloric acid after dechlorination of the organochloride [17]. The bacterial isolates were tentatively identified using standard cultural, morphological, biochemical and microbiological techniques [7,15].

Growth profile and chloride release

The growth patterns of the axenic cultures of the bacterial isolates were determined by cultivating 1 ml of standardized ($OD_{540nm} = 0.1$) cultures of the isolate in 100 ml BHM, pH 7.0, in 250-ml Erlenmeyer flasks containing the desired organochloride. The cultures were incubated as described earlier (30 °C) and, at each sampling time, total bacterial counts were carried out using standard pour-plate technique. The amount of free chloride ion release was also measured at appropriate intervals using the argentometric method [2]. Specific growth rate constants were estimated as described elsewhere [1].

Estimation of dehalogenase activity

Defined medium containing the desired organochloride at a final substrate concentration of 0.1% (w/v) was inoculated with overnight cultures of the isolates grown on the same medium and incubated at 30 °C on an orbital shaker at 100 rpm. Late-exponential-phase cultures were harvested by centrifugation at 3,500 rpm for 10 min and the dehalogenase activity in the crude cell-free extracts was determined, as previously described [12]. Free chloride ion released was measured as mentioned earlier, and the protein concentration of the extracts was estimated by the Biuret method [8] using bovine serum albumin as the reference protein. Dehalogenase specific activity was expressed as µg ml^{-1} chloride release (mg protein)$^{-1}$ h^{-1}.

The effects of pH on the specific dehalogenase activities of the cell-free culture extract of the isolates against TCA were determined by incubating the enzyme solution at different pH conditions using KH_2PO_4/Na_2HPO_4 buffer (pH 6–8) and glycine/NaOH buffer (pH 8.6–9.0). The effect of temperature on dehalogenase activities was also evaluated by incubating the enzyme solution at 25, 30, 35, 40 and 45 °C. All data were analyzed statistically by using one-way analysis of variance (ANOVA).

Results and discussion

Twelve bacterial species were isolated based on their ability to utilize at least one of the four organochlorides as sole source of carbon and energy. Of these, only two (GBB416 and GBB417) were able to utilize all four compounds for growth, and also tolerate high concentrations of the compounds [up to 0.1% (w/v)] which were toxic to the others. Hence, GBB416 and GBB417 were selected for further work. The cultural and biochemical characteristics of the two bacterial isolates revealed that they belong to the *Bacillus* (GBB416) and *Pseudomonas* (GBB417) genera. The versatility of these groups of bacteria in organochloride dehalogenation has been reported [13].

Both isolates were also observed to grow steadily in all the compounds, attaining peak growths in about 12 h (Fig. 1), with mean generation times ranging significantly ($P < 0.05$) from 2.41 to 10.04 h for the organochloride substrates (Table 1). The amount of chloride released in the organochloride cultures ranged from 0.038 to 0.058 µg colony-forming units $(cfu)^{-1}$ for GBB416, and from 0.047 to 0.063 µg cfu^{-1} for GBB417. TCA was the compound least utilized for growth by the two isolates, which suggests that the number of chloride

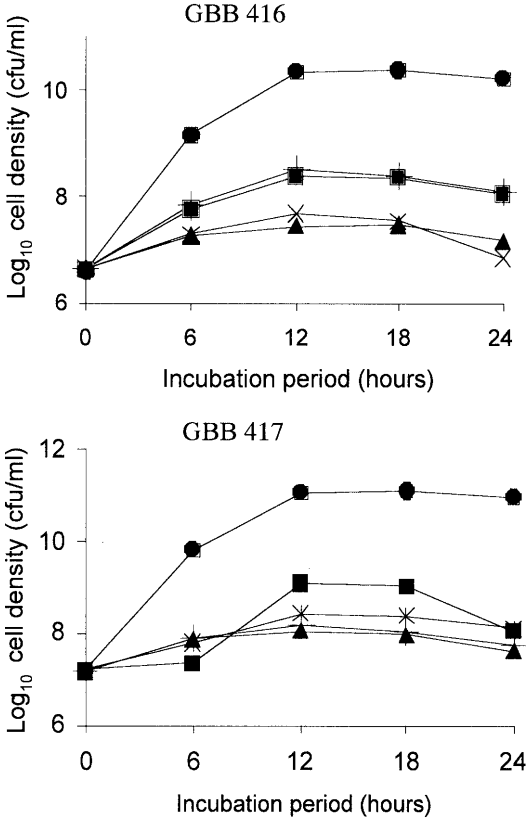

Fig. 1. Growth profiles of isolates GBB416 and GBB417 in the different organochloride medium. ■ represents monochloroacetic acid (MCA). × Tetrachloromethane (CCl_4), ▲ trichloroacetic acid (TCA), + chloroform ($CHCl_3$), ● glucose

Table 1. Mean generation time of the bacterial isolates during growth in the different organochloride media

Isolates code	Mean generation time (h)				
	MCA	TCA	$CHCl_3$	CCl_4	Glucose
GBB416	2.91 ± 0.05	9.51 ± 0.6	4.63 ± 0.51	2.87 ± 0.06	1.51 ± 0.02
GBB417	2.51 ± 0.21	10.04 ± 0.42	6.69 ± 0.31	2.41 ± 0.04	1.46 ± 0.02

atoms is negatively correlated with the ability of the organisms to degrade the compounds. Similar observations have been reported previously [12, 16]. Our results suggest that biomass is required only to a particular threshold, enough to produce the appropriate enzyme system to carry out dehalogenation even when biomass production ceases. This observation is in line with a previous report indicating that the toxic effect of organochlorides increases with the number of chlorine atoms attached, and also confirms our earlier report [12], although some exceptions have been observed for some anaerobes including *Dehalococcoides ethenogenes* strain 195.

The dehalogenase specific activity profile (Table 2) varied, ranging from 0.1 to 0.96 μg ml^{-1} chloride released (mg protein)$^{-1}$ h^{-1} for cell-mediated dehalogenation, and from 0.09 to 0.8 μg ml^{-1} chloride released (mg protein)$^{-1}$ h^{-1} for cell-free extracts. Dehalogenase specific activity for TCA was significantly ($P < 0.05$) higher than that for other organochlorides. The insignificant variation in enzyme activities observed between cell-mediated and cell-free dehalogenation suggests that cell-free extracts could be a preferred option for use in the bioremediation of organochloride polluted systems as previously proposed [5] – more so, in the light of public anxieties on the release of live organisms into the environment.

The optimum pH for dehalogenase specific activities for the two isolates was 8.0 (Table 3), with optimal enzyme activities being 1.15 and 0.47 μg ml^{-1} Cl^{-1} (mg protein)$^{-1}$ h^{-1} for GBB416 and GBB417, respectively. This suggests that the dehalogenation process would be favored in an alkaline medium, as reported elsewhere [14]. However, the optimum temperature for enzyme

Table 2. Dehalogenase specific activities of cell-mediated cultures and cell-free extracts of the bacterial isolates against the different organochloride substrates

	Dehalogenase specific activity [μg ml^{-1} Cl^{-1} (mg protein)$^{-1}$ h^{-1}]			
	Cell-mediated culture		Cell-free culture	
Substrate	GBB416	GBB417	GBB416	GBB417
MCA	0.18 ± 02 (50.7)	0.20 ± 03 (56.3)	0.16 ± 01 (45.1)	0.16 ± 008 (45.1)
TCA	0.96 ± 04 (90.1)	0.31 ± 07 (29.1)	0.80 ± 08 (75.1)	0.30 ± 02 (28.2)
$CHCl_3$	0.11 ± 006 (10.3)	0.10 ± 03 (9.4)	0.09 ± 012 (8.5)	0.09 ± 01 (8.5)
CCl_4	0.47 ± 05 (33.1)	0.21 ± 02 (14.8)	0.45 ± 04 (33.1)	0.18 ± 04 (12.7)

Table 3. Effect of pH on the dehalogenase activity of the cell-free culture extract of the isolates against trichloroacetic acid (TCA). Values in *parentheses* represent the percentage of activity at optimal pH. *Optimal value

Isolate	Dehalogenase specific activity [μg ml^{-1} Cl^{-1} (mg protein)$^{-1}$ h^{-1}]					
	pH					
	6.0	6.6	7.0	7.6	8.0	9.0
GBB416	0.72 ± 04 (62.6)	0.80 ± 11 (69.6)	0.96 ± 14 (83.5)	1.1 ± 21 (95.7)	1.15 ± 26 * (100)	0.85 ± 13 (73.9)
GBB417	0.17 ± 03 (36.2)	0.23 ± 01 (48.9)	0.31 ± 07 (66.0)	0.38 ± 10 (80.9)	0.47 ± 09 * (100)	0.27 ± 06 (57.5)

Table 4. Effect of temperature on the dehalogenase activity of the cell-free culture extract of the isolates against trichloroacetic acid (TCA). Values in *parentheses* represent the percentage of activity at optimal temperature. *Optimal value

Isolate	Dehalogenase specific activity [μg ml^{-1} Cl^{-1} (mg protein)$^{-1}$ h^{-1}]				
	Temperature (°C)				
	25	30	35	40	45
GBB416	0.88 ± 13 (91.7)	0.96 ± 13 * (100)	0.92 ± 15 (95.8)	0.77 ± 17 (80.2)	0.66 ± 11 (68.8)
GBB417	0.24 ± 08 (70.6)	0.31 ± 07 (91.2)	0.34 ± 11 * (100)	0.28 ± 04 (82.4)	0.22 ± 03 (64)

activities was found to vary for the two isolates, being 30 °C for GBB416 and 35 °C for GBB417. These optimal temperature ranges fall within the established range for dehalogenase enzyme activities [6] (Table 4).

This study corroborates our previous report that cell-free extracts of organochloride-degrading bacterial species could be as efficient in the degradation of complex compounds as the corresponding live cultures. From the point of view of public anxieties on the effect of releasing live organisms (especially genetically modified microorganisms) into the environment, cell-free systems are more likely to be useful in the treatment of effluents containing these organochlorides, especially in contained systems. Optimization of the process conditions for the use of these cell-free extracts in the bioremediation of polluted systems in Nigeria is the subject of an on-going collaboration.

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