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Isolation and Characterization of Halogenated Aniline-Metabolizing Bacteria from Selected Contaminated Sites in Lagos State, Nigeria

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ABSTRACT

Studies have shown that halogenated aniline (HA) contaminants are released into the environments as a result of numerous human activities. This study aimed to unveil the presence of HA bacterial-metabolizing strains from indigenous polluted sites and their degradation potentials on HA contaminant. Soil samples were collected from three contaminated sites (agricultural site ASS, municipal waste MSS and industrial site ISS). Promising bacterial strains capable of utilizing HAs (3-chloroanilines, 4-chloroanilines, 3-chloro-4-fluoroanilines, and 3, 4-dichloroanilines) were isolated by selective enrichment culture technique. The isolates were characterized based on cultural, biochemical characteristics and 16S rRNA gene sequencing methods. In the present study, *Pseudomonas hibiscicola* BT9, *P. hibiscicola* BT7, *Stenotrophomonas maltophilia* BT8, *S. maltoplhilia* BT10, *Bacillus subtilis* WT5 and *Alkalihalobacillus halodurans* BT2 were isolated from contaminated soil sites by enrichment culture containing equivalent amounts of HAs. Growths of strains BT9, BT7 and BT10 were defined in all the incubation system by increased in biomass, residual substrate concentration and stoichiometric chloride released into the culture media during mineralization. However, lower substrate concentration in strain BT9 was obtained when grew on 3,4-dichloroanilines yielding 65% degradation in 120 h, degradation rate of 0.54 mg L^{-1} h^{-1} with stoichiometric chloride release of 1.63 mM. Although strain BT10 appeared to utilize the congener in a similar trend, strain BT9 is more amenable to degradation. This study demonstrated that halogenated anilines utilizing bacteria could be used as potential bioremediation agent for cleaning up this contaminant.

INTRODUCTION

Halogenated anilines (HAs) are aromatic hydrocarbon derivatives that contain an amino group (-NH_2) as well as halogens. The addition of a functional group containing an oxygen, sulfur, or nitrogen heteroatom to benzene, as in the instance of amino (–NH_2) substitution, has been found to reduce benzene toxicity (Kayembe *et al.*, 2013). However, the branching chlorine, bromine, or fluorine atom in HAs makes it more hazardous than aniline (Tratnyek *et al*., 2020). The structural characteristics of the crystals are significantly influenced by the hydrogen bonding between the acceptor and donor (Alfan-Guzman *et al*., 2017).

Hence, they are always resistant to biodegradation due to the presence and positions of the halogens (Prakash *et al*., 2011; Kayembe *et al.*, 2013). HAs are commonly utilized in the manufacturing processes of pharmaceuticals, herbicides, insecticides, dyes, plastics, and cosmetics (Li *et al.*, 2010; Arora *et al.*, 2018). In Nigeria, wastes generated from households, agricultural processes and hospitals are enormous and have always resulted into environmental pollution (Olaniran and Igbinosa, 2011; Raimi *et al*., 2018; Osita and Nnamani, 2021). Various advancements in urbanization, industrialization and civilization generate more pollution, especially if not properly controlled (Arora and Bae, 2014; Kebede, 2022). There are concerns in many countries about the application of pesticides to target only pests, excluding non-target species and humans because of its carcinogenic adverse effect (Marlatt and Martyniuk, 2017). Reports have shown that many herbicides and pesticides degrade naturally into HAs, which are subsequently released as intermediate compounds thereby causing pollution in the environments (Pimviriyaku, 2019). Herbicides such as carbanilates, phenylureas, and acylanilides produce 4-chloroaniline (PCA), 2-chloroaniline, 3-chloroaniline, 3, 4- dichloroaniline, 4-bromoaniline, and 4-methoxyaniline as breakdown products (Rani *et al*., 2017). They adsorb moderately to organic molecules in the soil, especially the root microbiomes, thereafter slowly diffusing as contaminants into the air and surface water microbial communities (Marlatt and Martyniuk, 2017).

Many microorganisms have been isolated and identified based on their capacity to mineralize or convert different halogenated anilines compounds. Several bacteria, including *Moraxella* sp. strain G, *Pseudomonas* sp. strain CA16, *Acinetobacter baylyi* strain GFJ2, and *Alcaligenes* sp., were able to use 4-fluoroaniline, 2-chloroaniline, 3-chloroaniline, 4-chloroaniline, and 4-bromoaniline as sole carbon and nitrogen sources (Zeyer *et al*., 1985; Travkin *et al*., 2003; Vangnai and Petchkroh, 2007; Fuchs *et al*., 2011, Hongsawat and Vangnai, 2011; Megharaj *et al*., 2011). Karishma and Prasad (2016) also reported that *Bacillus amyloliquefaciens* has the potential to mineralize malathion insecticide. The degradation of the textile azo dye (Procion red) was also examined using the bacteria *Pseudomonas stutzeri* SPM-1 collected from textile wastewater dumping sites (Bera and Tank, 2021). Another study investigated that *Alcaligenes denitrificans* and *Cellulomonas* sp. were able to mineralize para-chloroanilie from a contaminated site in textile industry (Fashola *et al.*, 2013). It is noteworthy from all these previous reports that several microorganisms have been documented to degrade HAs.

Currently, the metabolic capabilities of microorganisms are being tested by immeasurable amounts of halogenated anilines indiscriminately released into the environment (Ilori *et al*., 2008; Das and Dash, 2014). The effectiveness of environmental-friendly and cost-effective techniques for degrading the target pollutant will be dependent on sourcing for competent microorganisms that the target contaminants and microbial communities are established (Adebusoye *et al*., 2008; Bamitele and Ayomikun, 2020; Kebede, 2021). Halogenated anilines are persistent in the environment, therefore there is need to isolate microorganism to degrade the contaminants. Immunotoxicity, mutagenicity, organ damage, and carcinogenicity are all anthropogenic impacts of halogenated anilines that are harmful to living organisms and regarded as hazard to human health, hence their elimination is a major concern (Olaniran and Igbinosa, 2011; Georgiadis *et al*., 2018). The main objective of the present study was to screen for bacteria with

halogenated anilines metabolic capability from three contaminated sources. In this paper we unveil the vast biodegradative potentials of bacterial strains associated with degradation of HA pollutants in Nigeria with a view to significantly reduce the risk pose to human health.

MATERIALS AND METHODS

Chemicals:

Halogenated anilines (HAs) including 3-chloroanilines, 4-chloroanilines, 3, 4-dichloroanilines, and 3-chloro-4-fluoroanilines were acquired from Sigma Aldrich Limited in England and they were of high analytical grade (98-100%). All other chemicals and solvents were of highest purity. The stock solutions of the HAs with low solubility were prepared by dissolving 0.1 g in 100 ml of acetone which equate to 1000 mg L^{-1}. The stock solutions were stored at 4℃ for analysis.

Sample Collection and Preparation:

Soil samples were taken at three distinct places: pharmaceutical industrial site at Ketu (ISS) (Latitude 6.597038', Longitude 3.96932'), agricultural site at Odogunyan (ASS) (Latitude 6.65039', Longitude 3.52262') and a municipal site at Olushosun (MSS) (Latitude 6.57345', Longitude 3.3942'). These sites were selected because HAs are major raw materials used in the industry, continual application of herbicides and pesticides (HAs as active ingredients) on the agricultural farmland and prolonged municipal wastes which are major sources of halogenated anilines. Figure 1, shows the satellite view of selected sites and sampling points. The soils were collected at 5 cm depth with the aid of a sterile spatula into sterile sample bottles, labelled and transported on ice to the laboratory for analysis. The soil samples were air-dried and sieved to remove debris and large particles.

Fig 1: The satellite view of sampling sites.

Physicochemical Analysis of Soil Samples:

The soil physicochemical properties were evaluated using standard analytical protocols (AOAC, 1995; Oyetibo *et al*., 2010, 2019; Salam *et al*., 2014). The methods were used to determine pH, texture, conductivity, and other physico-chemical parameters of the soil samples. The pH of the soil samples was determined using a pH meter (Mettler Toledo SevenMulti 8603, Switzerland). The titrimetric method was used to determine total organic carbon and total organic matter. The total accessible content of the PO_4^{3-} in the soil samples was conducted by

Olsen technique while the soil's cation exchange capacity (CEC) was determined by titrimetry method using ethylene diamine tetra acetic acid. The total heavy metal content of each digest was evaluated using Atomic Absorption Spectrophotometry (Perkin-Elmer Analyst 200, Bridgeport Avenue, Shelton USA). Gravimetry was used to assess the soil's total hydrocarbon content (THC). HPLC analysis with Jasco Analytical Instruments LC-2000 plus Series HPLC Systems (SpectraLab Scientific Incoporation, Markham, Canada) was used to evaluate the concentration of halogenated anilines in soil samples as demonstrated by Mello *et al.* (2013) method.

Enrichment and Isolation of HAs-Degrading Bacteria:

The HAs degrading bacteria were isolated using mineral salt medium (MSM) previously described by Travkin *et al.* (2003). The medium contained per litre 0.2g $MgSO_4$, 0.73g Na_2HPO, 0.5g, KH_2PO_4, 0.25g $NaHCO_3$, 0.001g $MnSO_4$, 0.75g NH_4NO_3, 0.02g $FeCl_3$ and 0.1 g $Na_2SeO_3.5H_2O$. The medium was fortified with nystatin (50 μg mL^{-1}) to suppress fungal growth and the pH was adjusted to 7.0. Trace element (1 ml) described by Shah (2015) was sterilized separately and added aseptically to the medium and ammonium chloride was excluded. The medium was supplemented with equal proportion 0.1% (v/v) each of 3-chloroanilines, 4-chloroanilines, 3,4 dichloroaniline, 3-chloro-4-fluoroanilines and inoculated with 5 g of soil sample. The conical flask was sealed with cotton plug and incubated at 27 ± 3°C on a rotary shaker at 150 rpm for 30 days until there was turbidity. Enrichment cultures were transferred to fresh medium using 10% inoculums and cultivated under the same condition. Subsequent transfer was carried out by adding one percent inoculums of the enrichment culture to a freshly prepared MSM and incubated under the same conditions. The transfer was repeated four successive times, after which HAs degraders were obtained by plating out on Petri plates containing MSM, substrates and agar. Colonies of HAs degraders were observed as those forming halo zones on the MS agar by venting off dissolve acetone sprayed on the agar.

Cultural and Biochemical Characterization of Halogenated Aniline Bacteria:

The samples used for microscopy were 24 h young cultures. The subculturing was done by plating out aliquots of the cultures onto MSM plate containing 200 mg L^{-1} of HAs. Typical bacteria colonies that were able to form zones of clearance (halo zones) after 10 days were recorded and classified as HA degrading bacteria. They were screened for utilization of the HA in MSM broth. For further testing, cultures were kept on basal slants maintained in glycerol (50:50) at -18°C. They were examined for Gram reaction and cellular characteristics using a Hitachi S-3500N model compound microscope (ThermoNaran, Hitachi Technologies, America Inc.). The biochemical characterization was carried out by testing catalase reaction, oxidase test, urease test, indole test, citrate utilization, nitrate reduction test, methyl-red-Vogues Proskaeur reaction and sugar fermentation were studied as described by Lanyi (1987). Pure cultures of bacterial isolates were identified according to the identification scheme of Bergey's Manual of Systemic Bacteriology, 9th edition (Holt, 1994).

Characterization of Halogenated Anilines Degrading Bacteria Based on 16S rRNA:

Genomic DNA was extracted and purified according to standard protocols for bacterial genomic DNA preparation using Jena Bioscience DNA preparation kits (Germany). The 16S rDNA was amplified using Polymerase Chain Reaction (PCR). The primers for the forward 27F (16S rDNA) (5'-AGA GTT TGA TCM TGG CTC AG-3') and reverse 1492R (5'-GGT TAC CTT GTT GTT ACG ACT T-3') were used to amplify 16S rDNA gene. The PCR

was prepared in 10 µl reaction mixtures containing 4 µl of PCR grade H_20 (The BigDye Terminator), 2 µl of terminator mix (9600 emulation mode), 1 µl of BigDye sequencing buffer (0.1 ng μl^{-1}) (The BigDye Terminator), 0.5µ l of 3.3 pmol of each primer and 2 µl of DNA template. The PCR conditions used consisted of an activation time for the master mix for 15mins at 96 ℃, 50 cycles at 96 ℃ for 50 s, 55 ℃ for 10s 72 ℃ for 4 min and 10 min of primer extension at 72 ℃. The amplicon was purified using the BigDye Terminator v3:1 PCR purification kit. The DNA sequence of the PCR amplified product was determined with an automated sequencing apparatus (ABI PRISM 377, PE Biosystems Inc.). Homology searches of the 16S rDNA sequences of the strains were performed using the BLAST program (http://www.ncbi.nlm.nih.gov/) to identify closely related bacterial 16S rDNA genes. Nucleotide sequence data were deposited in the NCBI nucleotide sequence database under accession number OK605024-9. MEGA version 6 (Tamura *et al*., 20018) was used for phylogenetic and molecular evolutionary analyses while the phylogenetic tree was constructed using neighbor-joining method.

Determination of Degradation Potentials of 3, 4-DCA Degrader:

The six bacterial isolates were cultured in MSM with 3, 4-DCA as carbon and nitrogen sources at 200 mg L^{-1} while growth was monitored by turbidity. About 1.0 ml inoculum from 24 h grown pure isolate was inoculated in a 250 mL Erlenmeyer flask containing mineral salts medium (MSM 100 ml, pH 7.0, HA 200 mg) and incubated at 27±3°C under 150 rpm. Cell growths of the bacterial isolates were assessed by visual observation for turbidity. For each strain, two sets of flasks were set up: the controls (MSM + substrate) and (the bacterial strain + MSM + 3, 4-DCA) and contamination was avoided. The experiment was set up in triplicate to determine cell biomass (λ_{600nm}), chloride concentration and residual concentration at each time point as biodegradation indices while the control flasks were inoculated with heat-killed bacterial isolates. The bacterial growth was determined by recording the turbidity of the growth medium against the controls in UV-visible spectrophotometer (Jenway 6270, UK) at 600 nm. The HAs utilization was determined by sampling cultures at 24 h intervals for 6 days and determining the residual concentration of HAs congeners using GC-MS. A Hewlett Packard (HP) 5890 series II (Agilent Technologies, San Francisco, California, USA) chromatograph with a flame ionization detector (FID) was used for determination of the levels of halogenated anilines in the sample. The stationary phase of separation of the compounds was HP-5 capillary column coated with 5% Phenyl Methyl Siloxane (30 m length x 0.32 mm diameter x 0.25 µm film thickness) (Agilent Technologies). The carrier gas was Helium used at constant flow of 1.1 ml min^{-1} at an initial nominal pressure of 5.5467 psi and average velocity of 20.036 cm/sec at an initial temp of 40°C and holdup time of 2.4955 min. One microliter (1µL) of the samples were injected in a pulsed split less mode at 250 °C and calibrated. After calibration, the samples were analyzed and corresponding halogenated anilines concentration obtained to determine growth kinetics and biodegradation rates of isolates.

Determination of Chloride Ion Concentration:

The MSM (5 ml) was supplemented with 200 mg L^{-1} (1.234 mM) of 3,4-DCA containing 5% of cells of isolates at exponential growth phase. The Mohr method was used to determine the concentration of inorganic chloride eliminated into the culture medium by titrating with $AgNO_3$ standard solution using chromate ions as an indicator. In this procedure, the chloride is precipitated during the titration and the silver nitrate standardized against standard chloride solution prepared from NaCl.

Chloride ion concentration (mg/L) = (V_{SN} × N_{SN} × 35.45) × 1000 / V_w Where: V_{SN} = volume of $AgNO_3$ used, N_{SN} is normality of $AgNO_3$ (0.02N) and V_w = volume of sample used (ml). In all the trials, tubes inoculated with heat-killed cells were used as the control. The tests were performed in triplicate concurrently.

RESULTS

Physicochemical Properties of Soil Samples and Existence of Pollutant in the Soil:

The physiochemical properties result of the contaminated soil samples as summarized in Table 1 indicated that ASS, MSS and ISS were weakly acidic, neutral and slightly alkaline soil respectively. They were specified with low organic matter content, organic carbon and high halogenated anilines contaminants (10.56 to 44.16 mg kg^{-1}) (Table 1). Generally, the concentrations of HAs obtained were higher than the tolerable limit for safe environment as prescribed by National Environmental Standards and Regulations Enforcement Agency (NESREA) and World Health Organization (WHO) (Table 1).

Table 1: Physicochemical properties of the contaminated soil samples.

Parameters	ASS	MSS	ISS	WHO Limit	NESREA Limit
pH	5.72	7.35	8.32	6.5-9.0	5.5-9.5
Halogenated anilines conc. (mg kg^{-1})	10.56	44.16	24.64	0.04	-
Conductivity (S m^{-1})	180	421	127	1200	-
Total Organic Carbon (%)	3.35	3.75	1.52	-	-
Total Organic Matter (%)	5.79	6.49	2.63	-	-
NO_3^- (mg kg^{-1})	27.1	24.05	11.2	50	50
PO_4^{3-} (mg kg^{-1})	102.6	63.4	39.2	-	-
SO_4^{2-} (mg kg^{-1}) Cl^- (mg kg^{-1})	33.2 1835	15.3 1168	10.1 749	500 250	620 250
TPH (mg kg^{-1})	BDL	0.007	BDL	-	-
CEC (mg eq.100 g^{-1})	2.05	1.82	0.96	-	-
Cd (mg kg^{-1})	0.10	BDL	0.05	0.03	0.003
Cr^{3+} (mg kg^{-1})	11.0	0.65	0.45	2.0	2.0
Cu^{2+} (mg kg^{-1})	5.10	5.35	5.70	1.0	1.0
Pb^{2+} (mg kg^{-1})	0.15	19.2	80.6	0.03	0.01
Mn^{2+} (mg kg^{-1})	1.00	2.05	0.35	0.3	0.4
Mg^{2+} (mg kg^{-1})	3.02	4.03	6.41	2	-
Ni^{2+} (mg kg^{-1})	0.10	0.11	BDL	0.03	0.01
Fe^{3+} (mg kg^{-1})	60.1	75.5	26.1	50	50
Zn^{2+} (mg kg^{-1})	35.2	33.4	52.1	3.0	3.0

ASS: Agricultural Soil Site, MSS- Municipal Soil Site, ISS- Industrial Soil Site. TOC-Total Organic Carbon, CEC- Cation Exchange Capacity, TPH-Total Hydrocarbon Content, WHO-World Health Organization (2011), NESREA- National Environmental Standards and Regulations Enforcement Agency (2011).

Isolation and Identification of Halogenated Aniline-Degrading Strains:

A total number of 10 bacterial colonies based on those that formed typical colonies surrounded by zones of clearance were selected and isolated from the enrichment media containing the MSM and the HAs. Subsequent screening established 6 isolates that had the capability to utilize 3, 4-DCA as source of carbon and energy. They were identified as *Pseudomonas hibiscicola* BT9, *P. hibiscicola* BT7,

Stenotrophomonas maltophilia BT10, *S. maltoplhilia* BT8, *Bacillus subtilis* WT5 and *Alkalihalobacillus halodurans* BT2 (Table 2). They were identified on the basis of the colony pigmentation where only strains BT2 and WT5 revealed whitish pigment while other strains were yellowish in colour. A rod-shaped motile was observed in all strains. The morphological characteristics under the light microscopy showed strains BT2 and WT5 as gram-negative while other strains were gram-positive. They were all catalase positive while only BT7 and BT9 were oxidase positive and others negative. The conventional biochemical characterization result showed that they were all sugar-fermenting strains.

The 16S rRNA gene sequence analysis was used to establish a positive genotypic identification of the six isolates. (Table 3). The sequence (1,185 bp) of 16S rRNA gene of strain BT9 had similarity of 99% to *Stenotrophomonas maltophilia* (Table 2). The dendrogram showed two major distinct clusters, the *Pseudomonas* and *Stenotrophomonas* group, that were closely related and likely to have evolved from same ancestors, and the *Bacillus* group that was also closely related but distant from the *Pseudomonas* and *Stenotrophomonas* group. The 16S rRNA gene sequence of *Microbacterium* sp., an unrelated organism, was used as an out-group (Fig 2).

Table 2: Genomic identities of 16S rRNA fragments of bacterial strains isolated from different HAs-degrading consortia

Tentative identity	Source	Nucleotide length	GenBank accession no.	Related strain/Accession no	Identity %
Alkalihalobacillus halodurans BT2	MSS	1059	OK605024	*Alkalihalobacillus halodurans* strain DSM 497 NR_025446	97
Bacillus subtilis WT5	ASS	1058	OK605025	*Bacillus subtilis* strain IAM 12118NR_112116	98
Pseudomonas hibiscicola BT7	MSS	1066	OK605026	*Stenotrophomonas maltophilia* strain IAM 12423NR_041577	98
Stenotrophomonas maltophilia BT8	1SS	1000	OK605027	*Stenotrophomonas maltophilia* strain IAM 12423NR_041577	99
Pseudomonas hibiscicola BT9	1SS	1185	OK605028	*Stenotrophomonas maltophilia* strain IAM 12423 NR_041577	99
Stenotrophomonas maltophilia BT10	ISS	1207	OK605029	*Stenotrophomonas maltophilia* strain IAM 12423 NR_041577	98

ASS: Agricultural Soil Site, MSS- Municipal Soil Site, ISS- Industrial Soil Site.

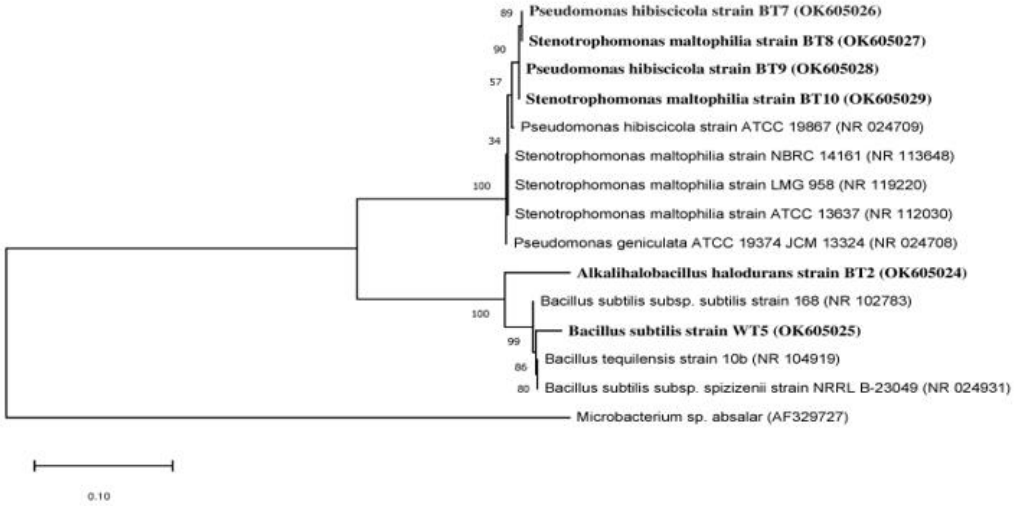

Fig. 2: Phylogenetic tree based on 16S rRNA gene sequences of 3,4- dichloroaniline-degrading bacteria strains using the Neighbor-Joining method (Saitou and Nei, 1987) Bootstrap test = 1000 replicates. The evolutionary distances were computed using the Kimura 41 parameter method. Analysis involving 20 nucleotide sequences was computed using Mega 6 software. *Microbacterium* sp. partial sequence was used as an out-group.

Degradation Capability of Bacterial Strains on 3, 4-DCA:

All the six bacterial strains grew exponentially without displaying lag phase (Fig. 3), as the cell biomass increased within 24 h. The metabolism of the substrate was measured as a significant reduction of 3, 4-DCA, increase in cell biomass indicated by turbidity and chloride elimination in each of the bioaugmented culture system. The entire bacterial cell maintained constant increase at all the time points in the utilization of the 2.468 mM substrate supplemented whereas the control flask showed no reduction in the concentration of the substrate due to no microbial action. The isolates showed significant growth at 120 h of incubation ranging from an OD of 0.15 ± 0.007 to 0.35 ± 0.004, indicating significant differences ($p < 0.001$) in their ability to degrade the HA. The data obtained suggest that BT9, BT10 and BT7 could utilize the substrate as the only source of C and N because only 3, 4-DCA was introduced into the culture medium as sole source of C and N. The result showed that >51% of the 3, 4-DCA was utilized by the three bacterial strains except in WT5, BT2 and BT8 where poor utilization of the substrate was observed during 120 h incubation period. The incubation of the HA congener with both BT9 and BT10 demonstrated similar trend; however, the former showed higher growth and utilized the substrate more than the latter. The growth of strain BT9 produced significant biomass at three-orders-of–magnitude with specific with corresponding decrease in substrate concentration and stoichiometric elimination of chloride into the culture fluid (Fig 3). Overall, strain BT9 degraded nearly 65% of the substrate in 120 h at a degradation rate of 0.54 mg L^{-1} h^{-1}, whereas, approximately 61% was obtained for BT10 at a degradation rate of 0.51 mg L^{-1} h^{-1} in the broth cultures.

Although all the tested organisms demonstrated similar trend of dechlorination from the aromatic ring near stoichiometric amount during mineralization, the rate evaluated for strain BT9 during the first 48 h nearly doubled strain BT2 and BT8. The 1.63 mM chloride eliminated after 120 h in BT9 implied mineralization of nearly 70% of the initial substrate concentration with an approximate dechlorination rate (ADR) of 0.007mMh^{-1} (Table 3). The amount of Cl^- recovered throughout the incubation period was stoichiometric to the residual concentration of 3, 4-DCA in the culture medium. There was biomass increase concomitant with increase in chloride in the culture fluid, suggesting progressive metabolism. The remaining isolates WT5, BT2 and BT8 could not utilize the substrate extensively due to low biomass with degradation rate of <33% from the initial concentration of the isolate. Interestingly, degradation was relatively rapid in BT9, especially at the onset (24-48 h) of incubation but subsequently decreased. Decrease in growth was observed from 72 h which suggests depletion of substrate and toxic metabolites accumulation in the culture fluid. The kinetic data summarized in Table 3 show that strain BT9 and BT10 are more efficient in detoxification of the halogenated anilines congener.

Table 3: Degradation kinetics of 3, 4-DCA-degrading bacterial strains cultured in aerobic condition

Isolate + 3, 4-DCA	% degradation	degradation rate mg $L^{-1}h^{-1}$	Cl^- release mM	% MZT	ADR mMh^{-1}
BT9	65.26	0.54	1.63	33.95	0.007
BT10	61.31	0.51	1.45	41.25	0.008
BT7	51.21	0.43	1.24	49.8	0.010
WT5	33.93	0.28	0.87	64.7	0.013
BT2	28.64	0.24	0.73	70.4	0.014
BT8	23.09	0.19	0.58	76.5	0.016

Note:3, 4-Dichloroanline (3,4-DCA) was supplied at a concentration of 2.468 mM. %MZT: Percent mineralization; ADR: Approximate dechlorination rate.

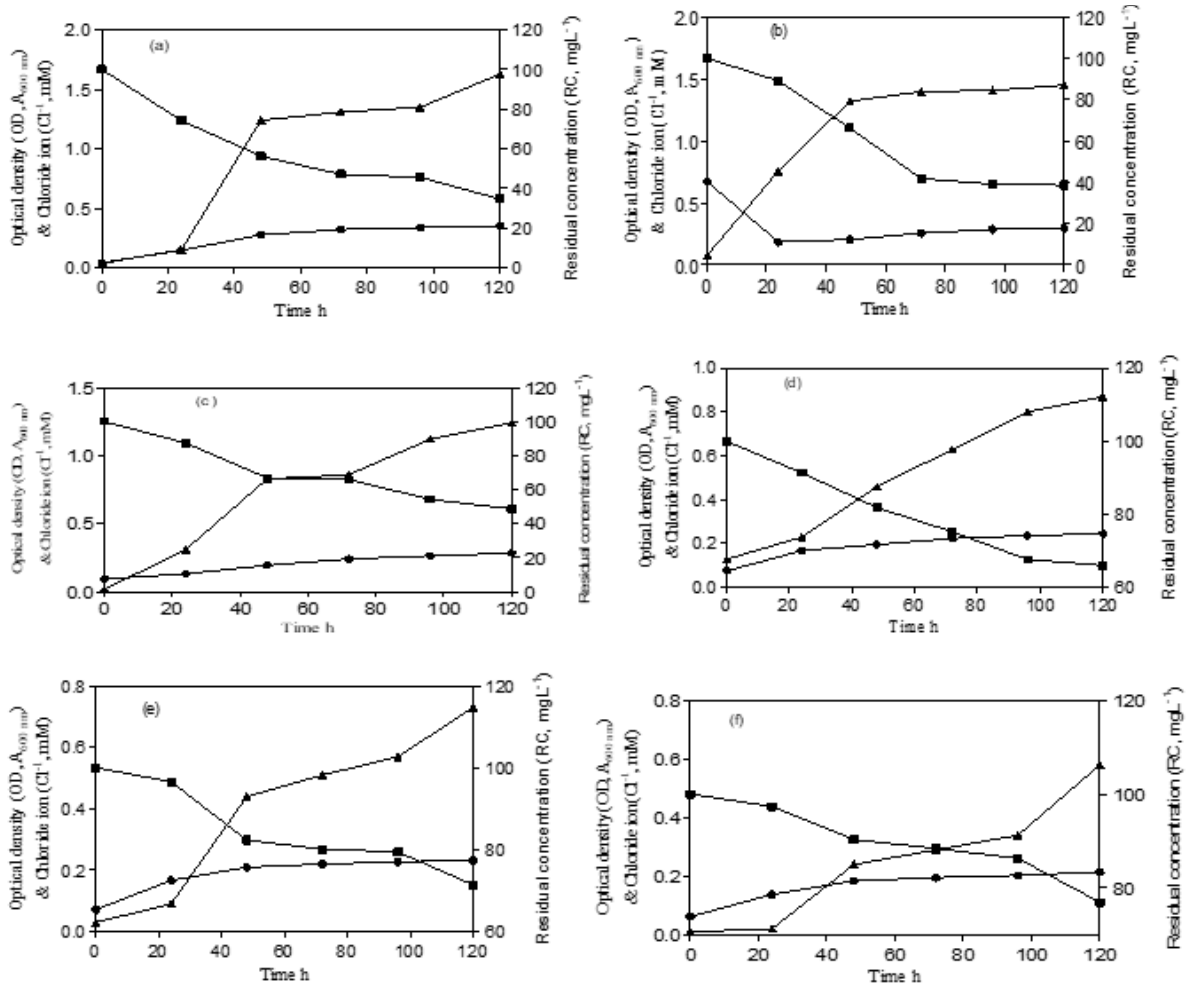

Fig.3: Growth profiles of 3,4 DCA-degrading bacterial by *Pseudomonas hibisciola* BT9 (a); *Stenotrophomonas maltophilia* BT10 (b); *Pseudomonas hibisciola* BT7 (c) and *Bacillus subtilis* strain WT5 (d); *Alkalihalobacillus halodurans* strain BT2 (e) and *Stenotrophomonas maltophilia* strain BT8 (f) in mineral salt medium at pH 7.0 supplemented with 2.468 mM of 3,4 DCA. ■, residual concentration of 3,4 DCA; ●, optical density at 600nm (OD_{600nm}); ▲, chloride eliminated into culture medium. Data presented are means ± SD of three replicate flasks. In most cases, error bars are too small to be observed.

DISCUSSION

Halogenated anilines are utilized in numerous industrial processes to manufacture a wide range of goods. The research into halogenated anilines has drawn the interest of numerous scholars because to this wide range of applications (Alfan-Guzman *et al*., 2017). They are crucial synthetic intermediates employed in large quantities during the manufacture of many different chemical compounds, including insecticides, herbicides, medicines, and plant dyes. An important disposal issue has been caused by the production, distribution, and usage of the chemical components. This is aggravated by improper discharge of industrial wastewater, urban sewage, and agricultural runoff, leading to inadvertent release of HAs into the environment and consequent pollution of the ecosystem (Megharaj *et al*., 2011). HAs have been demonstrated as recalcitrant to microbial degradation especially due to the presence of the chlorine atom which makes them less vulnerable to microbial attack during mineralization of the compound. It is noteworthy that indigenous bacteria capable of degrading halogenated anilines appear to be widely distributed in this extreme environment (Travkin, *et al*., 2003).

The results obtained in this study readily suggest that the sites had microorganisms with metabolic capabilities for halogenated anilines compounds. However, the high HAs concentration is attributable to the constant application of herbicides, pesticides, industrial effluents and incineration processes on the sites, which are examples of sources of HAs pollution of the environment (Pimviriyakul, 2019). The low conductivity obtained in the three soil samples could be due to high concentration of the pollutants which led to low water holding capacity and consequently reduce the soil conductivity. The proportion of inorganic nutrient sources (N, P and S) needed for growth and development of the microorganisms are reduced due to adsorption of the contaminant onto the organic matter in soil (Saibu *et al*., 2020). The presence of heavy metals in the soil samples shows the contamination of the environments. The high concentration of these heavy metals in the environments might be from discharge of industrial effluents, burning of fossil fuels, corrosion of metals and natural degradation of wastes.

This study yielded six promising halogenated-degrading bacterial strains from polluted sites in Nigeria, and the two most versatile of them, strains BT10 and BT9, were established as members of the genus *Stenotrophomonas* and *Pseudomonas* respectively. Many aerobic bacterial cultures have previously been reported to be able to grow solely on halogenated anilines as carbon and nitrogen sources with their capacity to degrade halogenated aniline congeners (Alfan-Guzman *et al*., 2017; Payne *et al*., 2015; Wang *et al*., 2018). Findings by some authors have shown that bacterial isolates *Delftia acidovorans* CA28, *Delftia tsuruhatensis* H1, *Comamonas testosteroni* 12, *Acinetobacter baumannii* CA2, *Pseudomonas* sp. JL2, and *Pseudomonas* mineralized 4-chloroaniline, 3-chloroanilineand and 2-chloroaniline (Latorre *et al*., 1984; Loidl *et al*., 1990; Surovtseva *et al*., 1992; Boon *et al*., 2001; Vangnai and Petchkroh, 2007; Zhang *et al*., 2010; Hongsawat and Vangnai, 2011). This could be a function of diverse metabolic networks and ability to secrete biosurfactants to make hydrophobic substrates more bioavailable, thereby facilitating degradation. In this study, all the bacterial species isolated from the three polluted soils were able to use halogenated anilines as a single source of carbon, nitrogen and energy. The population of the 3,4-DCA degrader increased concomitantly with decreasing HAs residual concentration showing direct linkage between observed growth and utilization. *Pseudomonas*

hibiscicola BT9 grew exponentially on the substrate and was shown to be the top degrader in this investigation, capable of degrading halogenated anilines by 65 percent, with halogenated anilines disappearing from the medium after 120 hours and a minimum chloride content of 387 mg/L after 120 h. Under aerobic conditions, *Pseudomonas* sp. JL2, *P. putida* C, *P. stutzeri* SPM-1 and other *Pseudomonas* strains have been shown to break down different halogenated anilines compounds (Loidl *et al*., 1990; Ascon-Cabrera and Lebeault, 1993; Bera and Tank, 2021). The outstanding degradative ability of P. *hibiscicola* could be because it has a distinct adaptive potential to survive in extreme conditions, including environments that harbor substantial concentrations of recalcitrant chemical sources such as halogenated anilines (Pimviriyakul, 2019).

In this study, *Bacillus subtilis* WT5 was also shown to be able to use halogenated anilines as the sole source of carbon and energy. *Bacillus megaterium* IMT21 and *Rhodococcus* sp. T1-1 have been studied for their ability to use five isomers of dichloroaniline as their only source of carbon and energy, including 3,4-dichloroaniline, 3,5-dichloroanilines, 2,3-dichloroaniline, 2,4-dichloroaniline, and 2,5-dichloroaniline (Lee *et al*., 2008; Yao *et al*., 2011). *Stenotrophomonas maltophilia* strain BT10 was the study's second-best degrader. Previous research demonstrated that the *Pseudomonas* genus, which are allied to the *Stenotrophomonas* genus, are capable of utilizing a wide range of chlorinated and fluorinated pesticides such as flubendiamide, tetraclorophenol, or DDT as a source of nitrogen and energy (Deng *et al*., 2015; Pan *et al*., 2016).

In the present study, the bacterial strains capable of utilizing halogenated anilines were isolated, characterized, and identified as *Pseudomonas hibiscicola* strain BT9 and *Stenotrophomonas maltophilia* strain BT10. These strains were able to grow successfully in tested substrate as determined by their growth kinetics. Therefore, these two strains may be adjudged as the promising tool for the remediation of sites contaminated with halogenated anilines. Further research focused on deciphering the metabolic pathways, determining the degradative enzymes involved and their metabolic products is on-going.

CONCLUSION

Bioremediation is one of the current approaches that can be applied for the reduction and/or removal of halogenated anilines pollutants. The present study provides an investigation on halogenated anilines-degrading bacteria obtained from different soil environments based on culture-dependent techniques in Nigeria. It was demonstrated that potential bacterial degraders of halogenated anilines could be isolated from indigenous contaminated soil samples and used for bioremediation strategy. This study confirms that bacterial species inhabiting different ecosystems are potential biological agents for the efficient biodegradation of halogenated anilines. It also adds to the existing body of knowledge towards reclaiming polluted environments contaminated with halogenated anilines.

Declarations:

Ethical Approval: Not applicable.

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Availability of Data and Materials: All datasets analyzed and described during the present study are available from the corresponding author upon reasonable request.

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