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# Isolation and Characterization of a Novel p,p'-DDT-Degrading Bacterium: *Aeromonas* sp. Strain MY1

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***Authors' contributions***

*This work was carried out in collaboration among all authors. Author YM designed and managed the analyses of the study. Authors BCN and LUSE managed the literature searches and wrote the first draft of the manuscript. All authors read and approved the final manuscript.*

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## ABSTRACT

**Background:** Despite the banned on the use of dichlorodiphenyltrichloroethane (DDT) and other Persistent Organic Pollutants (POPs) by the Stockholm Convention for their toxicity, emerging shreds of evidence have indicated that DDT is, however, still in use in developing countries. This might increase the global burden of DDT contamination and its hazardous effects.
**Aim:** This study focused on the isolation and characterization of *p,p'*-DDT-degrading bacterium from a tropical agricultural soil.
**Methodology:** Standard isolation procedure was used for the screening and isolation of the strain. The 16S rRNA and phylogenetic analyses were used to identify the isolate and established protocols were followed to characterize the strain.
**Results:** A new strain belonging to the genus *Aeromonas* was isolated from agricultural soil using minimal salt-*p,p'*-DDT enrichment medium. The 16S rRNA sequencing was used to identify the strain and the partial sequence was deposited in the NCBI GenBank as *Aeromonas* sp. Strain MY1. This mesophilic isolate was capable of utilizing up to 50 $mgL^{-1}$ of *p,p'*-DDT as the sole carbon

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source at an optimum pH of 7.5 and optimum temperature of 35 °C within 120 h under aerobic conditions. $Fe^{2+}$ (0.2 $mgL^{-1}$) demonstrated a stimulatory effect on the *p,p'*-DDT degradation capacity by the strain MY1. However, Zn, Cu, Pb, Hg, Ag and Cr ions have demonstrated various patterns of inhibitory effect on the *p,p'*-DDT degradation capacity of the isolate at 0.2 $mgL^{-1}$. The strain MY1 could be a promising candidate for the bioremediation of *p,p'*-DDT contaminant.

**Conclusion:** *Aeromonas* sp. strain MY1 was capable of utilizing *p,p'*-DDT as a sole carbon source under aerobic conditions. The utilization capacity of the strain was influenced by some heavy metals. Fe was found to enhance the *p,p'*-DDT utilization capacity of the isolate at a lower concentration. While Zn, Cu, Pb, Hg, Ag and Cr showed various patterns of inhibitory effect.

*Keywords: DDT biodegradation; Aeromonas sp.; 16S rRNA; environmental contaminant; heavy metals.*

## 1. INTRODUCTION

Dichlorodiphenyltrichloroethane commonly known as DDT is the most notorious organochlorine pesticide (OCPs) that is still captivating the interest of many researchers due to its degrading impact on ecology and human health. The dangerous ecological impact of DDT is more serious to the environment when there is a co-contamination with other metal pollutants [1]. Despite the banned on the use of DDT and other Persistent Organic Pollutants (POPs) by the Stockholm Convention for their toxicity, DDT is, however, still in use in developing countries of Africa, Asia, and the South Pacific [2,3]. It was reported that in Nigeria over 130,000 tons of pesticides are used annually for agriculture and disease-borne control [4] and consequently DDT was recently detected in water samples [5]. The unique nature of persistence in the environment demonstrated by DDT made it traceable in ambient air, drinking water, vegetables, honey, fish, breast milk and other food varieties [6,7,8]. In many cases, DDT levels in food items were reported to exceed the upper limits of daily intake stated by the U.S. Environmental Protection Agency [9].

DDT is known to accumulate in the environment, potentially dually bio-accumulating in biological systems [6,10]. One of the major health implications of DDT and its metabolites is its link to endocrine disruption [11,12]. Recently, exposure to DDT and its metabolites DDE (dichlorodiphenyldichloroethylene) were reported to be implicated in long-term impairments in muscle health [13], the cognitive decline [14] and some alterations in maternal metabolome [15]. Many studies have also documented the probable involvement of DDT in carcinogenicity [16,17]. It was also demonstrated to pose some risks of inducing apoptosis of skin fibroblasts in some aquatic animals [18].

Owing to its inherent environmental recalcitrance, the intensity of ecological and human health effects, still exploring the microbial consortia for novel isolates for the efficient elimination of residual DDT contaminants could be a valuable option. Since microorganisms are known to influence the fate of organochlorine contaminants in the environment. Therefore this study focused on the isolation and characterization of a *p,p*'-DDT-utilizing bacterium inhabiting tropical agricultural soil.

## 2. MATERIALS AND METHODS

### 2.1 Materials

The *p,p'*-DDT and other relevant chemicals used in this research were purchased from the Sigma Aldrich (United Kingdom) unless otherwise specified.

#### 2.1.1 Sampling site

The sampling site is part of Phase I, Kano River Project that is located at the Kadawa Irrigation Scheme, Kura-Garun Malam in Kano State, Nigeria. The site has geographical coordinates of 11°41' 0'' North, 8°22' 0'' East. It has a history of uninterrupted agricultural practice and massive use of agrochemicals including DDT for at least three decades [19].

#### 2.1.2 Soil sample collection

Soil sample was collected from the stated location. The sample was collected at the surface of the soil to the depths of 15 cm. The sampling was focused on these horizons because a large portion of microbial activity occurs in these horizons. The soil sample was mixed evenly and 20 g was carefully put into a sterile container and kept at 4 °C for bacterial isolation.

#### 2.1.3 Nutrient medium

Luria-Bertani (LB) medium was prepared as described by Pant et al. [20] for the isolation of strain MY1 from the soil samples.

#### 2.1.4 Preparation of minimal-salt-*p,p'*-DDT enrichment medium

This medium was prepared following the medium compositions and description by Pant et al. [20] with a slight modification: in a litre of distilled water, 0.1 g $CaCl_2.2H_2O$, 0.08 g $Ca(NO_3)_2$ $4H_2O$, 0.5 g $MgCl_2.6H_2O$, 1.0 g $Na_2SO_4$, and 1.0 g $KH_2PO_4$ were dissolved to form the minimal salt medium. Then 0.05 mg $mL^{-1}$ of *p,p*'-DDT was added to the minimal salt medium for the screening of the *p,p*'-DDT degrading isolate. Thus, inoculation into the final medium ensures that the sole carbon and energy source for the isolate was from the *p'p*-DDT only, and thus, significant formation of biomass in this medium would only occur if the isolate can utilize the *p,p'*-DDT.

### 2.2 Isolation of *p,p'*-DDT-degrading *Aeromonas* sp. strain MY1 from soil Samples

Isolation of *p,p'*-DDT-degrading Aeromonas sp. strain MY1 was carried out using a modified isolation procedure described by Pant et al. [20]. A 25 ml suspension of LB medium containing 0.5 g of air-dried soil was prepared. The suspension was incubated for 48 h at 30 °C on a shaker at 100 rpm. After the incubation, the LB medium was allowed to settle down for 2 h. Then, a 100 µl from the cleared LB supernatant was used to inoculate 4 ml of minimal-salt-*p,p'*-DDT enrichment medium. The enrichment culture was then incubated for 168 h at 30°C on a rotary shaker at 100 rpm. After the incubation, 100 µl of the enrichment culture was transferred into another 4 ml of fresh minimal-salt-*p,p'*-DDT enrichment medium, and the incubation step was repeated. After six sequential sub-culturing, the isolate was inoculated on to minimal-salt-p,p'-DDT enrichment agar plates containing 0.05 mg mL-1 and incubated for 24 h at 30 0C and the isolate formed was preserved. This ensures adequate exposure of the isolate to the *p,p'*-DDT.

### 2.3 DNA Extraction

The bacterial genome extraction was done using the DNA Biospin Bacteria Genomic Extraction Kit, Bioflux Co. (Gapan) following manufacturer's instruction.

### 2.4 The 16S rRNA Nucleotide Sequencing

For amplification of the target gene from the extracted genomic DNA, 16S rRNA gene primers [16SRNA BAC27F: 5'-AGA GTT TGA TCC TGG CTC AAG-3' and 16SRNA BAC1492R: 5'- GGT TAC CTT GTT ACG ACT T-3'] were used. Polymerase chain reaction (PCR) conditions were set as described by Sangwan et al. [21]. After the final elongation cycle, the size of the DNA fragment was compared with the Hyper Ladder-1K marker Bioline (Lot No: H4-111B). The 3 µl PCR product was mixed with 5XDNA loading buffer blue (1.5 µl) Bioline (Lot No: hLBB-415704) and introduced onto 1% agarose gel electrophoresis that has been subjected to ethidium bromide staining. The electrophoresis was run for 35 minutes under 120V and 300mA current. The product was then visualized with the Syngene Gel Documentation System of Ingenius, England (IG31459). The presence of a product of the expected size was considered to be a positive result. The PCR product obtained was gel-purified and the 16S rDNA sequencing was carried out using the protocols described by Sanger et al. [22]. Then, DNA sequence alignment was done using ClustalW 2.0.12 and the sequence was subjected to the BLAST search program at the NCBI website (http://www.ncbi.nlm.nih.gov/) to identify the isolate.

### 2.5 Phylogenetic Analysis of *Aeromonas* sp. Strain MY1

Phylogeny and evolutionary history of the Aeromonas sp. strain MY1 was constructed using the Neighbor-Joining method. The evolutionary distances were computed using the Maximum Composite Likelihood method [23]. The phylogenetic tree was constructed using MEGA version 6 software program.

### 2.6 Characterization of *Aeromonas* sp. Strain MY1 in *p,p'*-DDT Enrichment Medium by using One Factor at a Time (OFAT)

In the characterization of Aeromonas sp. strain MY1 in the *p'p*-DDT enrichment medium, we have determined the optimum *p,p'*-DDT concentration (as a sole carbon source), pH, temperature, incubation time, and effect of some

heavy metals on the growth of the isolate. The turbidity of the medium is regarded as the index of the biomass and growth of Aeromonas sp. strain MY1 in the *p,p'*-DDT enrichment medium, which was determined spectrophotometrically as optical density (OD) at $600_{nm}$.

Before inoculation, the *Aeromonas* sp. strain MY1 was pre-cultured in LB broth for 48 h and the turbid broth was centrifuged. The supernatant was removed while the cell pellets were introduced into a fresh MSM medium and used as an inoculum source. The *p,p'*-DDT degrading capacity of Aeromonas sp. strain MY1 was determined in vitro by adjusting the cell density (OD600nm) of the inoculum source to 0.5, the cells (100 µl,) were then inoculated into MSM media containing varying concentrations *p,p'*-DDT (10, 20, 30. 40 50, 60 and 70 $mgL^{-1}$) at varying pH ranges (5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5) and incubated at different temperatures (20, 25, 30, 35, 40 and 45°C) and different incubation period (24 h, 48 h, 72 h, 96 h, 120 h, 144 h and 168) under shaking condition (150 rpm). The experiments were conducted one factor at a time in triplicate.

### 2.7 Effects of Heavy Metals on *p,p'*-DDT Utilization Capacity of *Aeromonas* sp. Strain MY1

The effects of heavy metals (Fe, Zn, Cu, Pb, Hg, Ag and Cr) on *p,p'*-DDT degrading capacity of Aeromonas sp. strain MY1 was determined *in vitro* by inoculating into MSM-p,p'-DDT enrichment media containing varying metal concentrations (0.2, 0,4, 0.6, 0.8 and 1.0 $mgL^{-1}$) and incubated on a rotary shaker (140 rpm) at 30°C and pH 7.5 for 168 h. The OD was measured spectrophotometrically at 600nm after blanking with freshly prepared MSM medium containing *p,p'*-DDT for the determination of cell growth as an index of *p,p'*-DDT degradation capacity of Aeromonas sp. strain MY1 [1]. The experiments were set up under aerobic conditions.

## 3. RESULTS AND DISCUSSION

### 3.1 Identification of the Bacterial Strain

The preliminary morphological and biochemical characteristics of Aeromonas sp. strain MY1 on LB agar showed a creamy, shiny colony. Microscopic observation revealed a rod-shaped Gram's negative, motile, non-spore and non-capsule forming bacteria that is positive to catalase, cytochrome oxidase, acetate, and citrate but negative to urease tests (data not presented here).

### 3.2 16S rRNA and Phylogenetic Analyses

For identification of bacteria, although there are many options available, DNA-DNA relatedness using the 16S rRNA sequencing is, however, the most accurate and reliable means of identification [24,25] relative to conventional phenotypic methods that show a low level of accuracy and reliability [26]. The 16S rRNA PCR amplification product of Aeromonas sp. strain MY1 revealed about 1200 bp upon running on 1% agarose electrophoretic gel (Fig. 1). Soltani et al. [27] reported a similar base-pair range for some Aeromonads isolated from Southern and Northern provinces of Iran. The partial sequence of the 16S rRNA from Aeromonas sp. strain MY1 has been determined and deposited in the NCBI Biodata Bank under the accession number MN530936.

The partial sequence of 16S rRNA of the Aeromonas sp. strain MY1 contains 678 nucleotides, which upon blasting in the NCBI Biodata Bank (https://blast.ncbi.nlm.nih.gov/Blast.cg) showed that the organism belongs to the genus Aeromonads. More than 60% of the first hundred sequence homology output (blast) of the Aeromonas sp. strain MY1 showed more than 94% sequence similarity with various Aeromonas species, with Aeromonas sanarellii strain A2-67 and Aeromonas enteropelogenes strain CECT having the highest sequence similarity of 98.22% each, whereas Aeromonas cavernicola strain MDC 2508 showed the least similarity of 94.09% (data not presented here). Several kinds of research documented that the genus Aeromonas has a closely related interspecies sequence homology, showing high levels of similarity that is close to 100% [28,29,30].

The phylogenetic tree for the 16S rRNA gene of Aeromonas sp. strain MY1 was derived from the genetic matrices of the first seventeen representatives from Aeromonas species as obtained from the blast analysis and other three DDT degrading species as outliers. The evolutionary tree was constructed using the MEGA 6 program by using the Neighbor-Joining method as stated in the methodology section. Apart from the outliers used in this study, the phylogeny showed minimum divergence (branch lengths) between all the Aeromonas species

used as indicated by higher bootstrap values of 99-100%. However, the tree sandwiches Aeromonas sp. strain MY1 in between Aeromonas enteropelogenes strain CECT 4487 and Aeromonas enteropelogenes strain DMS 6394 in a single cluster (Fig. 2) suggesting stronger closeness with Aeromonas enteropelogenes species. This finding conforms to Martínez-Murcia et al. [28] that the depth of phylogeny of the 16S rRNA gene for the genus Aeromonas is narrow, revealing interspecies identity close to 100%. Also, Thornton et al. [31] observed that some Aeromonads strains showed up to 99.6% identity.

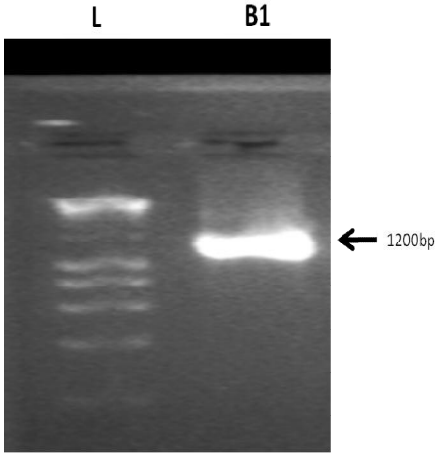


**Fig. 1. Agarose gel electrophoretic image of the 16SrRNA amplicon of Aeromonas sp. strain MY1 isolate coded as B1. L represents Hyper Ladder-1K marker Bioline (Lot No: H4-q 111B)**

### 3.3 Growth Response of *Aeromonas* sp. strain MY1 in *p,p'*-DDT Enrichment Medium

Many researchers have reported the isolation of bacterial species capable of tolerating < 20 mgL-1 of *p,p'*-DDT as a sole carbon source [20]. Though some isolates were documented to tolerate up to 50 mgL-1 when other carbon sources were supplemented [32,33,34]. However, Aeromonas sp. strain MY1 demonstrated higher tolerance and utilization capacity of up to 50 mgL-1 of *p,p'*-DDT as the sole carbon source under aerobic setup. An increase in the concentration beyond 50 $mgL^{-1}$ however, lowers the organism's efficiency to utilize the pesticide contaminant (Fig. 3). A similar scenario was documented by Mwangi et al. [35] that DDT concentration above 50 $mgL^{-1}$ lowers the growth of the DDT degrading isolates. The ability of a microorganism to depend on DDT as sole carbon and energy source for significant growth depends on the organism's capacity to mineralize the DDT. Microbial mineralization of DDT involves multiple biological reactions [34]. The Aeromonas sp. strain MY1 demonstrated a minimal lag phase of < 24 h and precipitated its highest biomass of 0.152 (OD600nm).in 120 h (Fig. 3), efficiently mineralizing the DDT in the medium. This was perhaps, achieved because the strain MYI possesses effective machinery for the efficient utilization of p,p'-DDT as a sole carbon source. Although the majority of literature on Aeromonads has focused on their clinical implications, information on their ability to biotransform DDT is scarcely available. However, Aeromonas hydrophila was reported to biotransform DDT [36,37]. Due to its higher tolerance ability, strain MY1 could therefore be used for efficient bio-cleansing process involving p,p'-DDT decontamination.

### 3.4 Effects of pH, Temperature and Inoculum Size on the Growth of *Aeromonas* sp. strain MY1 in *p,p'*-DDT Enrichment Medium

As a complex process, bacterial DDT mineralization is largely influenced by some environmental determinants such as pH, temperature and concentration of the DDT [38,39]. Concerning this, the strain MY1 demonstrated some mesophilic characteristics by exhibiting maximum growth at pH 7.5 and 35 °C, whereas fluctuations in the above parameters lower the biomass and decrease the OD of the isolate (Fig. 4). This is not surprising by considering the environmental conditions of the tropical region where the organism was isolated. Data from a pilot study on the physicochemical characteristics of the soil from the sampling site fall within a similar range of pH and temperature values upon which the organism had been inherently adapted to (data not presented here). Pant et al. [20], Fang et al. [34] and Mwangi et al. [35] also reported isolates that degrade DDT close to the above pH and temperature ranges.

### 3.5 Effects of Heavy Metals on *p,p'*-DDT Utilization Capacity of *Aeromonas* sp. strain MY1

Virtually all agricultural sites are co-contaminated with organic and metal contaminants. The

presence of these pollutants must have some effects on the microbial consortia and the way these microbes biotransform the accumulated contaminants. Thus, assessing the effects of Fe, Zn, Cu, Pb, Hg, Ag and Cr on the *p,p'*-DDT utilization capacity of strain MY1 is important. The growth of A. sp. strain MY1 in *p,p*'-DDT medium was enhanced by a presence of 0.2 mgL-1 of $Fe^{2+}$ relative to the absence of this divalent ion. However, a further increase in the concentration to 0.4 $mgL^{-1}$ decreased the growth of the organism (Fig. 5). Depending on the concentration, $Fe^{2+}$ showed both stimulatory and inhibitory effects. The stimulatory effect of $Fe^{2+}$ could be the result of an increase in biogenic $Fe^{2+}$ formation as reported by Glass, [40] and Cao et al. [37] with a possible increase in the reductive biotransformation and utilization of p,p'-DDT observed in the strain MY1. Indeed, iron is required for various metabolic processes in bacteria. Thus, it is not surprising when iron enhanced the growth of strain MY1 in p,p'-DDT medium. The enhancement of the reductive transformation of DDT by Aeromonas hydrophila HS01 in the presence of zerovalent iron was also reported by Cao et al. [36].

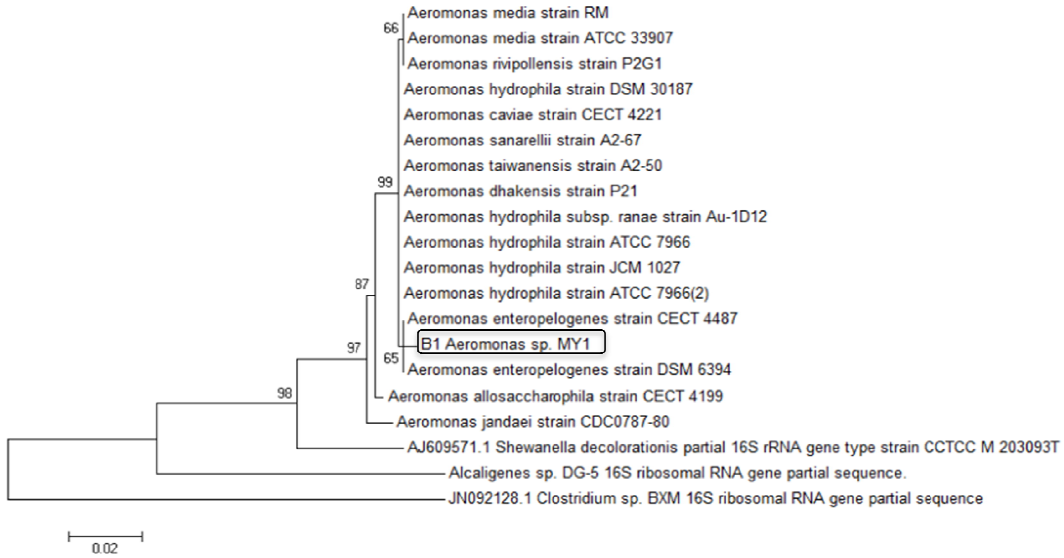


**Fig. 2. Evolutionary relationships of taxa of *Aeromonas* sp. Strain MY1 isolate. The strain was shown in a box coded as B1 *Aeromonas* sp. MY1**

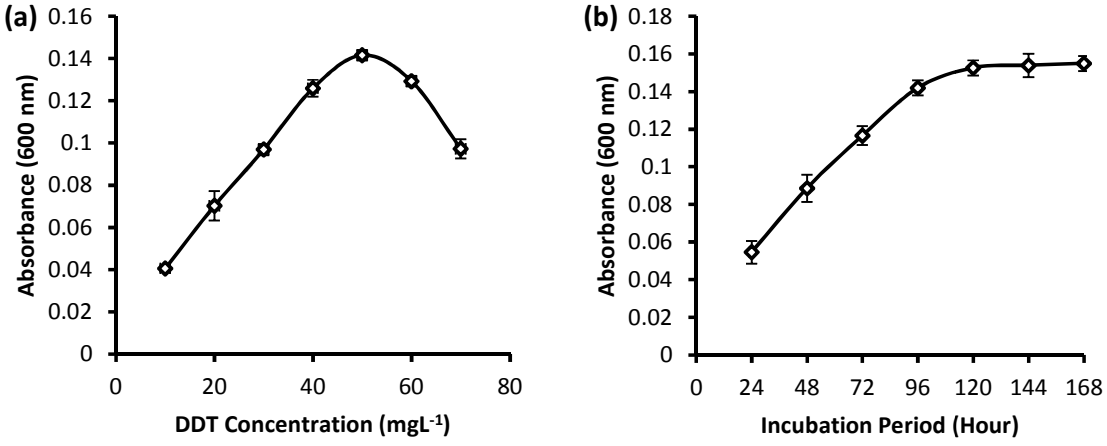


**Fig. 3. Effects of *p,p'*-DDT concentration (a) and incubation time (b) on the growth of *Aeromonas* sp. strain MY1 in *p,p'*-DDT enrichment medium**

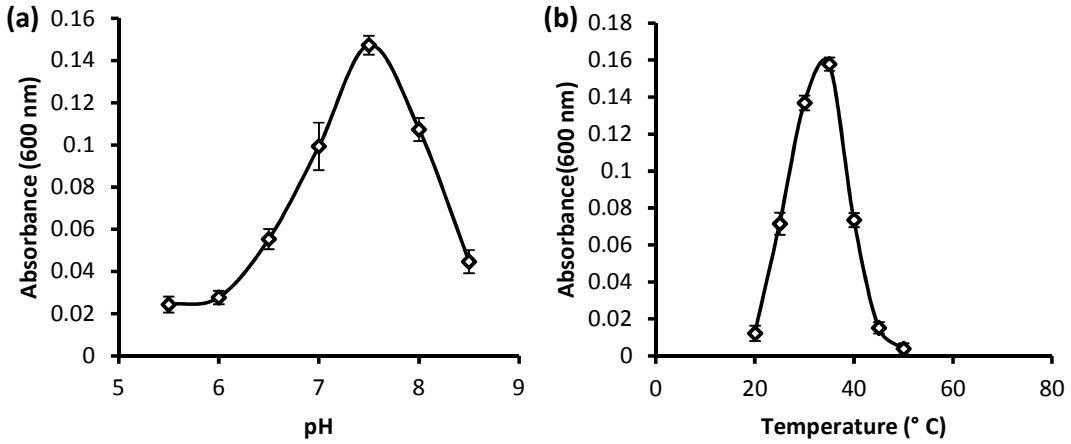


**Fig. 4. Effect of pH (a) and temperature (b) on the growth of *Aeromonas* sp. strain MY1 in *p,p'*-DDT enrichment medium**

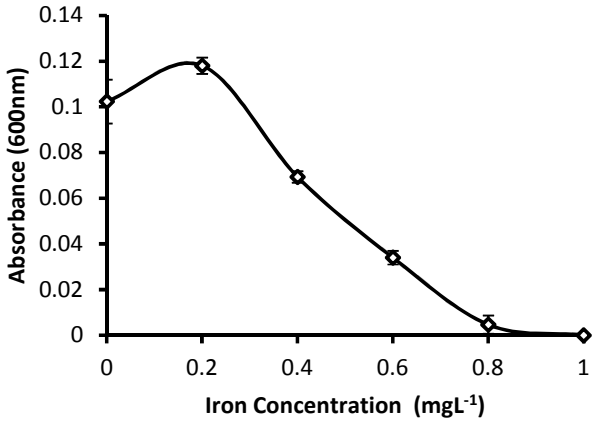


**Fig. 5. Effect of iron (II) ions on the growth of *Aeromonas* sp. strain MY1in *p,p'*-DDT enrichment medium**

The other metals tested, Zn, Cu, Pb, Hg, Ag, and Cr showed different extremes of inhibitory effect on the p,p'-DDT utilization capacity of strain MY1 with Pb and Cr (Fig. 6) exhibiting the maximum inhibitory effect at the minimum p,p'-DDT concentration of 0.2 $mgL^{-1}$.

However, the pattern of inhibition demonstrated by Zn and Cu (Figs. 7a and b) was more favourable to the growth of strain MY1 relative to that of Hg and Ag (Figs. 7c and d). This could be linked to the essentiality of Zn and Cu for some metabolic processes in bacteria. However, it has been observed that the presence of both organic and metal pollutants resulted in metal toxicity in bacteria, mostly by interacting and inhibiting the bacterial enzymes and thus, inhibiting organic pollutant biodegradation [1,41]. Also, metal oxyanions, such as chromate, mimic the structure of essential non-metal oxyanions, such as sulfate [1]. Furthermore, mercuric and silver cations form harmful complexes that may disrupt some physiological functions, in addition to their inhibitory binding to the SH group of the variety of bacterial proteins [42]. These metals reduced the strain MY1 ability to degrade and utilize the *p,p'*-DDT contaminant and therefore decrease its capacity to efficiently bio-eliminate the p,p'-DDT from the environment. However, a controlled environment must be created where the heavy metals with the inhibitory effect are chelated to favour the *p,p'*-DDT bio-cleansing capacity of the strain. Indeed, addition of low concentrations of Fe at the site of decontamination process could help the *p,p'*-DDT bio-cleansing process.

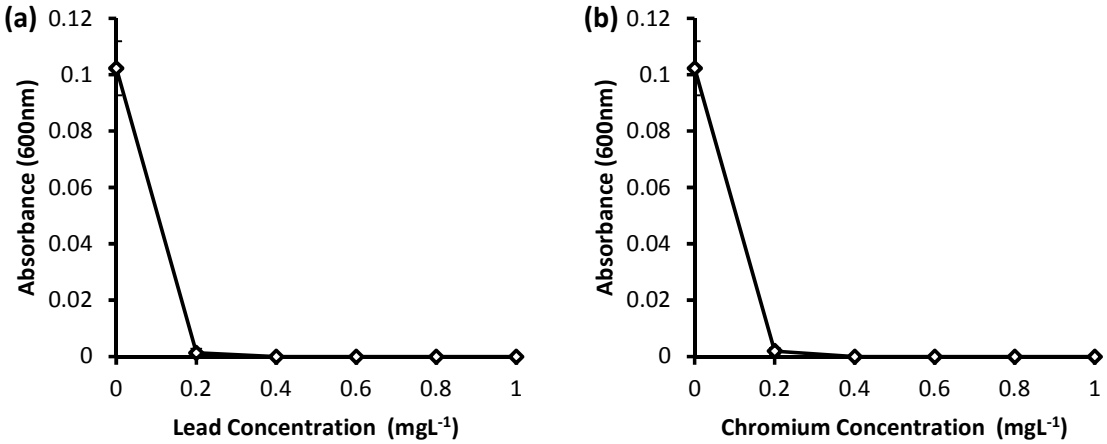


**Fig. 6. Effects of lead (a) and chromium (b) ions on the growth of *Aeromonas sp.* strain MY1 in *p,p'*-DDT enrichment medium**

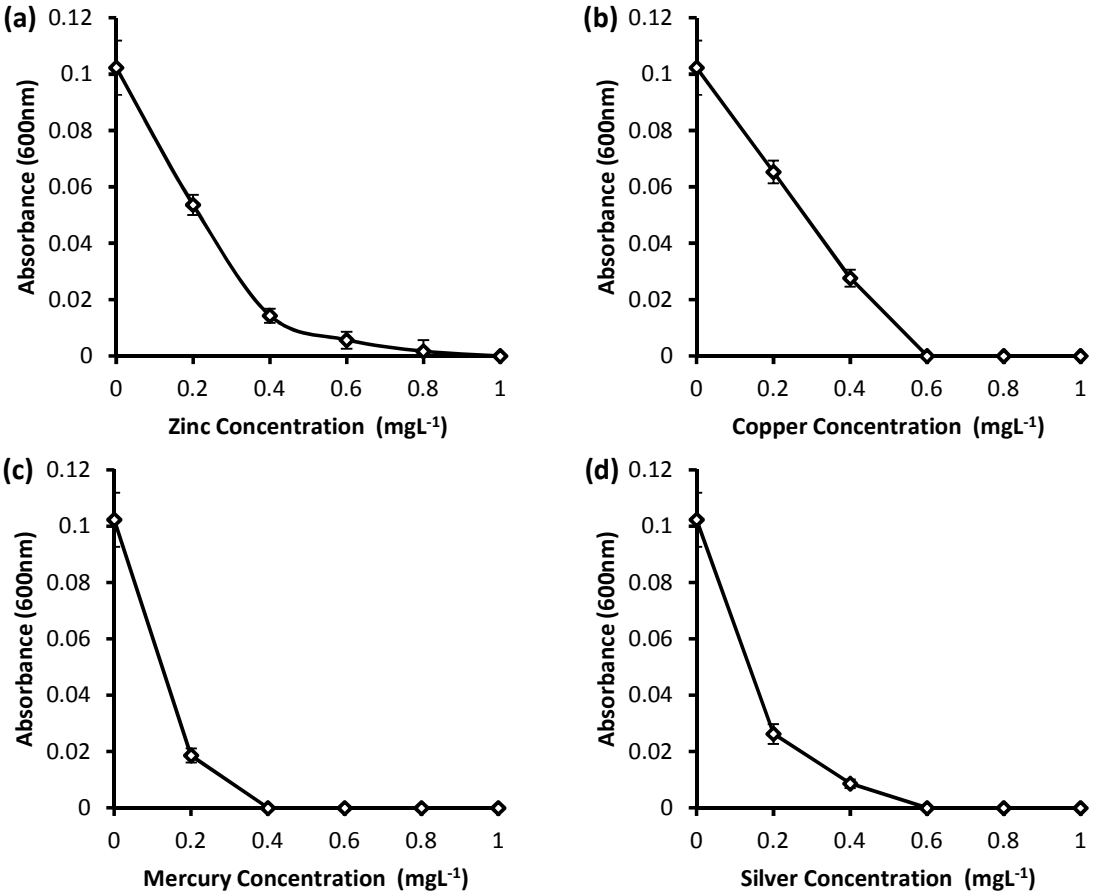


**Fig. 7. Effects of zinc (a), copper (b), mercury (c) and silver (d) ions on the growth of *Aeromonas sp.* strain MY1 in *p,p'*-DDT enrichment medium**

## 4. CONCLUSION

A new strain of the genus Aeromonas has been isolated from agricultural soil. Genomic 16S rRNA sequencing was used to identify the strain and the partial sequence was deposited in the NCBI Biodata Bank as Aeromonas sp. MY1 with accession number MN530936. The isolate was capable of utilizing up to 50 mg $L^{-1}$ of *p,p'*-DDT as only carbon and energy source at an optimum pH of 7.5 and 35 °C within 120 h. The *p,p'*-DDT utilization capacity of the strain MY1 was influenced by some heavy metals. Fe was found to enhance the *p,p'*-DDT utilization capacity of the isolate at a lower concentration. While Zn, Cu, Pb, Hg, Ag and Cr showed various patterns of inhibitory effect. This isolate could serve a potential role of a bio-cleaner for the removal of p,p'-DDT contaminant.

## DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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