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Research article

Bacillus velezensis strain MY 83295S: a *p,p'*-DDT-degrader isolated from a tropical irrigation site

Ya'u MURTALA[1,3,*], Bennett Chima NWANGUMA[3], Idris BALA[2], Lawrence Uchenna Sunday EZEANYIKA[3]

[1]Department of Biochemistry, Faculty of Basic Medical Sciences, Bayero University, Kano, Nigeria

[2]Department of Microbiology, Faculty of Life Sciences, Bayero University, Kano, Nigeria

[3]Department of Biochemistry, Faculty of Biological Sciences, University of Nigeria, Nsukka, Nigeria

*Corresponding author: myau.bch@buk.edu.ng

Abstract: Although dichlorodiphenyltrichloroethane (DDT) is prominent in the ban list of the Stockholm Convention on the Persistent Organic Pollutants (POPs), there is recently an emergence of reports indicating the re-surfacing of residual DDT in the domestic waters and some commonly consumed food items. This indicated a progressive increase in the burden of DDT in the ecosystem. In this paper, we report the isolation and characterization of a bacterial strain with the ability to degrade and utilize *p,p'*-DDT as a sole carbon source. The strain was isolated from a tropical irrigation site and tentatively identified as *Bacillus velezensis* strain MY 83295S. This strain demonstrated a mesophilic nature in its aerobic degradation and utilization of the *p,p'*-DDT contaminant. Additionally, a low Fe concentration enhances the strain's ability to degrade and utilize *p,p'*-DDT. While Cu, Pb, Hg, Ag and Cr ions have demonstrated various patterns of an inhibitory effect on the strain's *p,p'*-DDT degradation ability. Strain MY 83295S showed a great potentiality for the bio-decontamination of the *p,p'*-DDT, and its eventual application in the bioremediation process.

Keywords: *Bacillus*, DDT, Bioremediation, Heavy metals, Environment.

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Introduction

Although dichlorodiphenyltrichloroethane (DDT) is prominent in the ban list of the Stockholm Convention on the Persistent Organic Pollutants (POPs), there is recently a strong wave of evidence indicating the re-surfacing of residual DDT in the domestic waters and some commonly consumed food items (Bussolaro et al., 2012; Ogbeide et al., 2015; Mendes et al., 2019; Sheldon et al., 2019). In addition to a long lifespan of DDT due to its persistence in the environment, especially the agricultural sites in some developing countries, some reports have also recently indicated its continual spray for indoor disease vector control (Yadav et al., 2015; UNEP, 2019). This might contribute to the environmental burden of DDT and its metabolites and their eventual bioaccumulation via the food chain (Hickey, 1999; Devi, 2020). Consequently, the detrimental implications of DDT on the ecosystem, particularly on human health have been enormous and recurring, and require a serious concern (Hadara et al., 2016; Yang et al., 2018)

Some abiotic environmental cleanup approaches somehow contributed to the elimination of the recalcitrant DDT contaminants from the polluted environment, mostly without significant alteration in the stability of the inert chemical nature of the DDT (Foght et al., 2001). This simply led to the recycling of this pollutant in the

environment. However, effective bioremediation of the residual DDT can be a savior of the contaminated environment, ensuring a clean and safer ecosystem. Therefore, the addition of DDT-degraders to contaminated sites and the careful manipulation of their degradation conditions will enhance the effective cleansing of the contaminated environment (Aislabie et al., 1997). Therefore, there is a need to explore the microbial consortia for desired strains capable of degrading the residual DDT and ascertain the operational conditions for their application in bioremediation processes.

In this paper, we focused on the isolation and characterization of a *p'p*-DDT degrading bacteria from a tropical pesticide-contaminated irrigation site.

Materials and Methods

Sample collection

The soil sample was collected from an irrigation site located at Phase I, Kadawa Irrigation Site, Hadejia-Jama'are River Basin, Kano State, Nigeria. The site has a history of uninterrupted irrigation activities with the application of a massive pesticide for more than three decades. The sample was collected at the surface of the soil to the depths of 15 cm and taken to the laboratory at 4°C for bacterial isolation.

Preparation of Luria-Bertani (LB) medium for bacterial growth

Sequel to the soil sampling, Luria-Bertani (LB) medium was prepared as described by Pant et al. (2013) for the bacterial growth.

Preparation of p,p'-DDT-minimal salt enrichment medium

The Minimal Salt Medium (MSM) was prepared based on some modifications of the MSM medium described by Pant et al. (2013).

Isolation of p,p'-DDT-degrading bacterium

The isolation of *p'p*-DDT utilization strain was done using a modified isolation protocol described by Pant et al. (2013). Air-dried soil (0.5 g) was suspended in 25 mL of the prepared LB medium. The suspension was kept for 48 h at 30°C on a shaker. Before inoculation, the LB medium was allowed to settle down for 2 h. An aliquot (100 µL) from the cleared LB supernatant was used to inoculate 4 mL of *p,p'*-DDT enrichment MSM. The culture was then incubated for 1 week at 30°C on a rotary shaker at 100 rpm. After incubation, 100 µL of the bacterial suspension was transferred into 5 mL of fresh *p,p'*-DDT enriched MSM and the incubation step was repeated. After six sequential sub-cultivations, the isolate was inoculated on to MSM agar plates enriched with 0.05 mg mL^{-1} of *p,p'*-DDT, and incubated for 72 h at 30°C and the isolate formed was preserved. This ensures adequate exposure of the isolate to the *p,p'*-DDT as a sole carbon source.

Extraction of genomic DNA

The isolate was used to inoculate 8 mL LB medium and incubated overnight at 37 °C and 200 rpm for 24 h. The resulting bacterial suspension (OD_{600} = 0.6) was pelleted at 10,000 rpm for 5 min and the genomic DNA was extracted using the method outlined by Schmidt et al. (1991).

16S ribosomal RNA (16S rRNA) gene amplification

In the amplification of about 1.5 Kb gene from the bacterial genome, 16S rRNA gene primers (BAC27F and BAC1492R) [16SRNA BAC27F: 5'-AGA GTT TGA TCC TGG CTC AAG-3' and 16SRNA BAC1492R: 5'-GGT TAC CTT GTT ACG ACT T-3'] purchased from Sigma-Aldrich, United Kingdom were used according to the protocol described by (Sangwan et al., 2005). The PCR was carried out using TC-E-48FA Gene Touch Thermocycler, Hangzhou Bioer Technology, (China). 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 product was then visualized with the Syngene Gel Documentation System of Ingenius, England (IG31459). The appearance of a product of the expected size has confirmed a positive result.

Agarose gel purification and sequencing of amplified 16S rRNA gene

The gel was purified using the PrepEase gel purification kit (Affymetrix Inc., USA) by following the manufacturer's protocol. The gel-purified product was sequenced using the protocols described by Sanger et al. (1977). Then, DNA alignment was done using ClustalW 2.0.12 version (http://www.clustal.org/) and the sequence was compared to sequences in the public databases with the BLAST search program on the National Center for

Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/) to identify the isolate.

Phylogeny of the isolates

The phylogenetic and evolutionary history of the isolate was constructed using the Neighbor-Joining method. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2013). The phylogenetic tree was constructed using MEGA version 6 software program.

Characterization of the isolate in p,p'-DDT enrichment medium

To characterize the isolate 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 optimum growth of the organism. The turbidity of the medium is an index of growth of the isolate in the *p,p'*-DDT enrichment medium, which was determined spectrophotometrically as optical density (OD) at 600_{nm}. The characterization was performed by modifications of the methods described by Mwangi et al. (2010) and Pant et al. (2013).

Effects of heavy metals on p,p'-DDT utilization capacity of the isolate

The effect of each heavy metals (Fe, Zn, Cu, Pb, Hg, Ag and Cr) on *p,p'*-DDT utilization capacity of the isolate was determined *in vitro* after adjusting the OD_{600nm} of cell density of the inoculum source to 0.6, the cells (100 µL) were then inoculated into MSM-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 (150 rpm) at 30 °C and pH 7.0 for 168 h. This protocol is a modified version of the method described by Sandrin and Maier (2003).

Results

Amplification and sequencing of the amplified 16S rRNA gene

The amplification product for the isolate revealed about 1500 bp upon running on 1.5% agarose gel electrophoresis (Figure 1). Upon gene sequencing, the amplicon revealed 1060 bp as 16S rRNA partial gene sequence for the isolate. A BLAST search was performed on the gene sequence in the NCBI nucleotide archive and revealed *Bacillus* as the genus of the isolate. The 16S rRNA gene partial sequence was deposited in the public databases of the NCBI GenBank as *Bacillus velezensis* strain MY 83295S under the universal accession number MN812291.

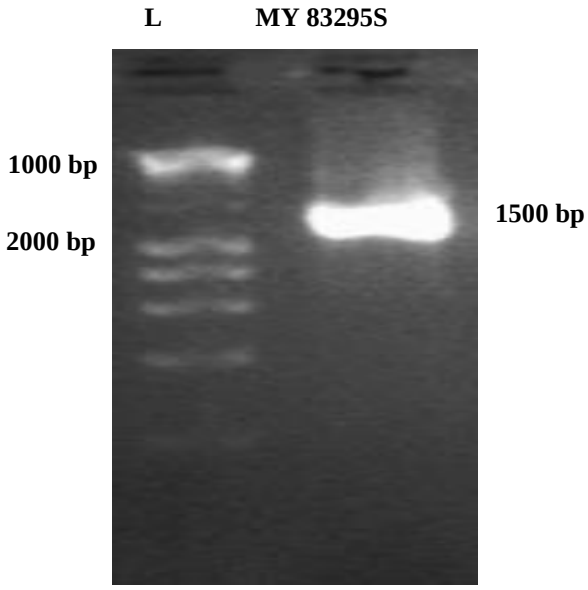

Figure 1. Gel electrophoretic image of the 16S rRNA gene from strain MY 83295S. L represents Hyper Ladder-1K marker Bioline (Lot No: H4-q111B)

Phylogenetic analysis of strain MY 83295S

The phylogenetic analysis of strain MY 83295S (Figure 2) revealed a cluster of members of the genus *Bacillus*. The strain specifically formed a small cluster with *Bacillus velezensis* strain CBMB205. This cluster was supported by a bootstrap value of 64%. The first nineteen members of the genus *Bacillus* of the BLAST search and *Aeromonas* sp. strain MY1 (as out-group) were used for the construction of this phylogenetic tree using the Neighbour-Joining method.

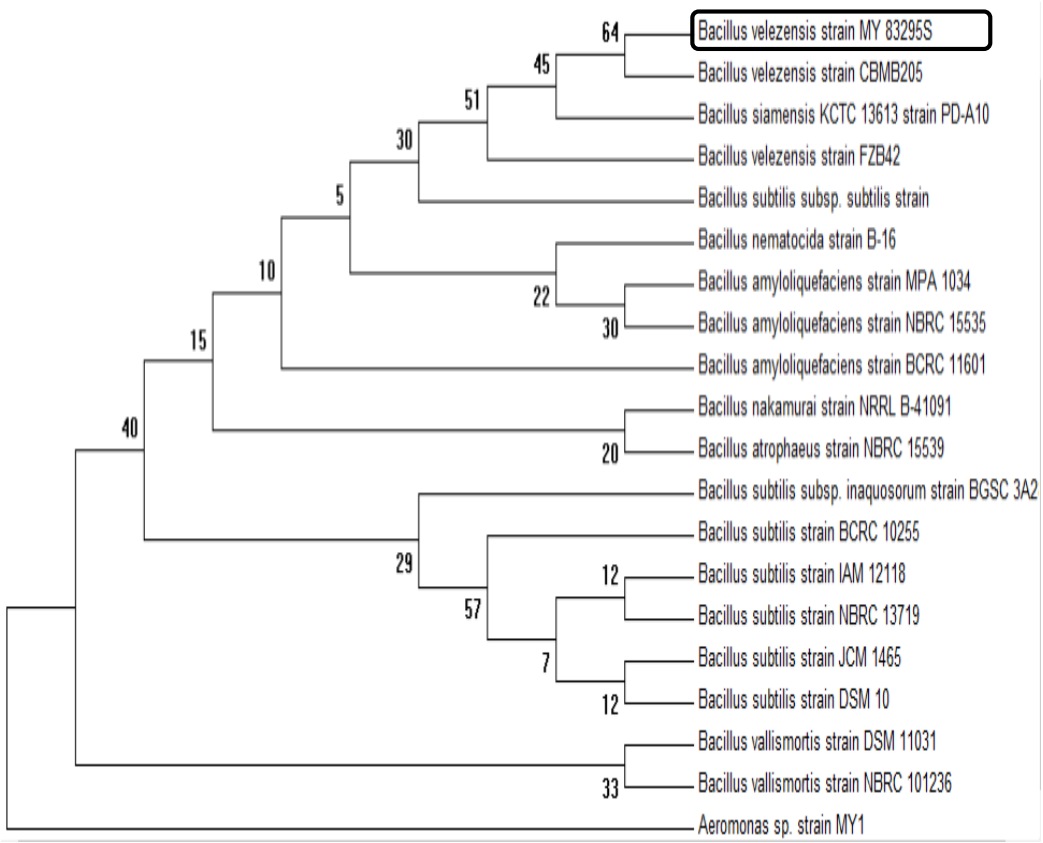

Figure 2. Phylogenetic and evolutionary relationships of taxa of strain MY 83295S

Growth capacity of strain MY 83295S in p,p'-DDT enrichment medium

The strain MY 83295S showed a moderate *p,p'*-DDT tolerance, precipitating its highest turbidity at 30 mgL^{-1}. The relative growth of this strain in *p,p'*-DDT enrichment medium was observed to be concentration-dependent (Figure 3).

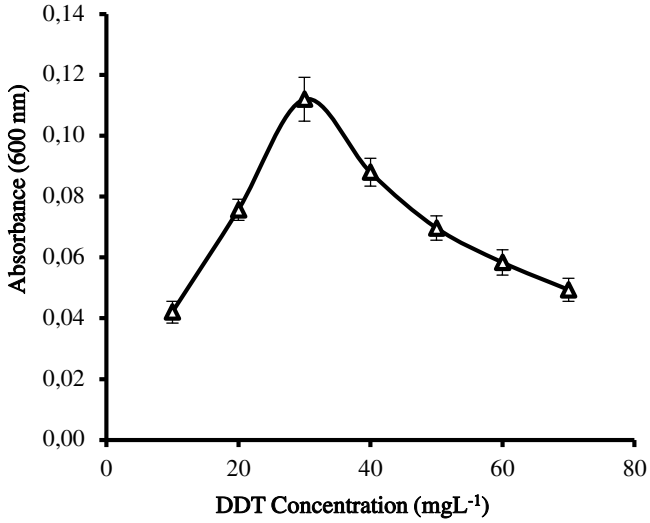

Figure 3. Effect of *p,p'*-DDT concentration on the growth of strain MY 83295S in *p,p'*-DDT enrichment medium

Effect of incubation time on p,p'-DDT utilization capacity of strain MY 83295S

The strain MY 83295S demonstrated a lag phase of 72 h for the initial *p,p'*-DDT utilization. However, the strain was observed to reach its optimum turbidity in 168 h (Figure 4).

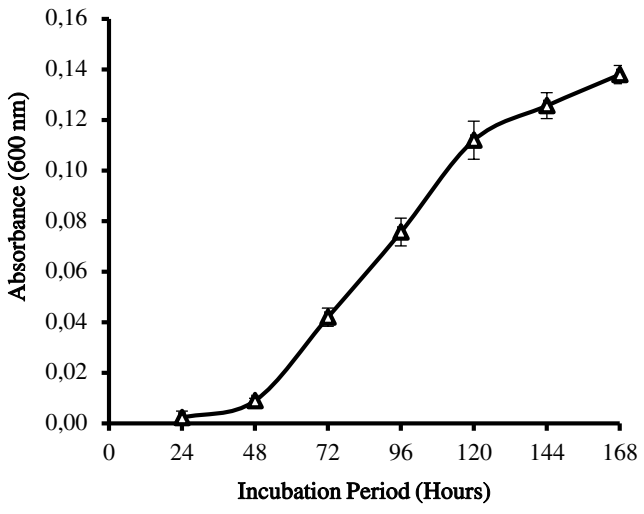

Figure 4. Effect of incubation time on the growth of strain MY 83295S in *p,p'*-DDT enrichment medium

Effect of temperature on p,p'-DDT utilization capacity of strain MY 83295S

Strain MY 83295S demonstrated a mesophilic behaviour by exhibiting *p,p'*-DDT degradation and utilization within a temperature range of 20-45°C. However, the strain had an optimum temperature of 30°C (Figure 5).

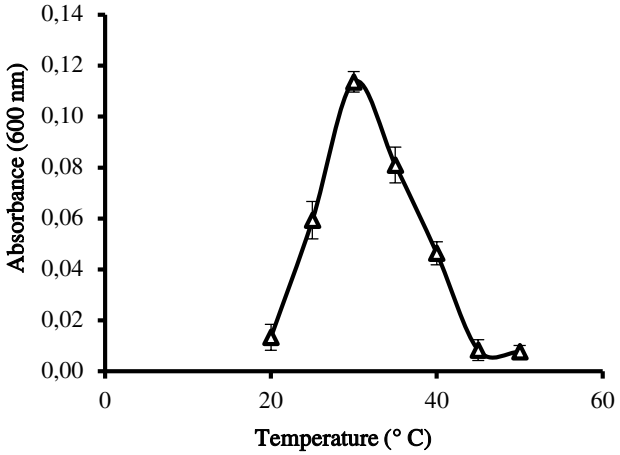

Figure 5. Effect of temperature on the growth of strain MY 83295S in *p,p'*-DDT enrichment medium

Effect of pH on p,p'-DDT utilization capacity of strain MY 83295S

The strain MY 83295S showed utilization capacity of *p,p'*-DDT within both slightly acidic and alkaline scales, ranging from a pH of 6.0 to 8.0 and an optimum pH of 7.0 (Figure 6).

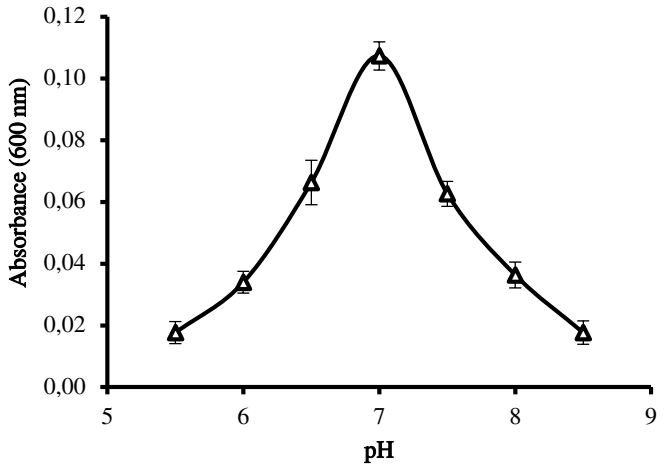

Figure 6. Effect of pH on the growth of strain MY 83295S in *p,p'*-DDT enrichment medium

Effect of Fe^{2+} on p,p'-DDT utilization capacity of strain MY 83295S

Fe^{2+} was found to enhance the *p,p'*-DDT degradation and utilization capacity of strain MY 83295S at a concentration of 0.2 mgL^{-1} within 168 h. Increasing the Fe^{2+} concentration to 0.4 mgL^{-1}, decreased the *p,p'*-DDT utilization capacity of this strain (Figure 7).

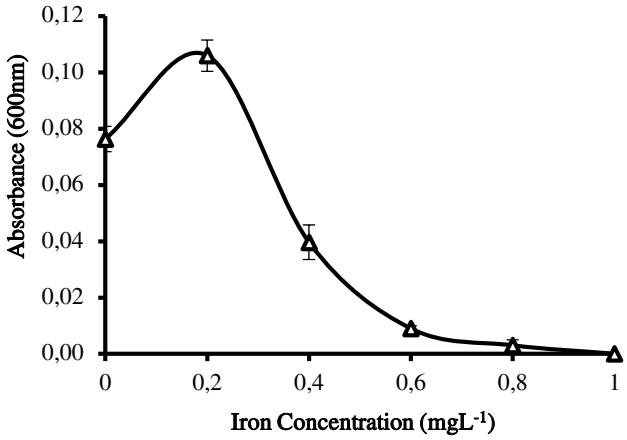

Figure 7. Effect of Fe^{2+} concentration on the growth of strain MY 83295S in *p,p'*-DDT enrichment medium

Effect of Cu^{2+} on p,p'-DDT utilization capacity of strain MY 83295S

A pattern of inhibition demonstrated by Cu^{2+} was favourable to the growth of strain MY 83295S relative to that of other heavy metals assessed. The intensity of the inhibition was observed at 0.6 mgL^{-1} where the growth was completely inhibited (Figure 8).

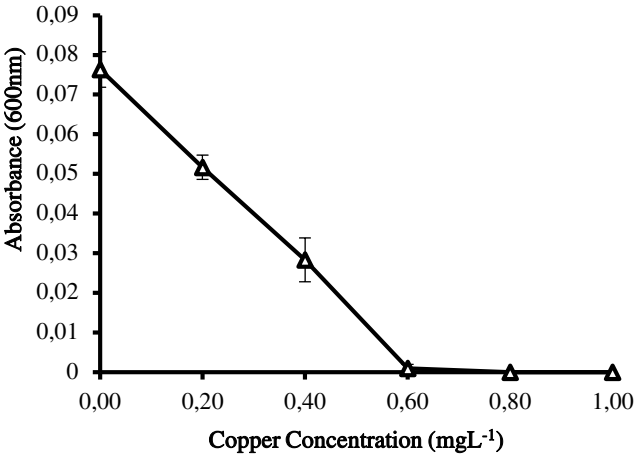

Figure 8. Effect of Cu^{2+} concentration on the growth of strain MY 83295S in *p,p'*-DDT enrichment medium

Effect of Pb^{2+}, Hg^{2+}, Ag^{2+} and Cr^{2+} on p,p'-DDT utilization capacity of strain MY 83295S

Pb^{2+}, Hg^{2+}, Ag^{2+} and Cr^{2+} (Figures 9-12) showed virtually similar pattern of growth inhibition of strain MY 83295S in the *p,p'*-DDT enrichment medium at a concentration of 0.2 mgL^{-1}. Except for Cr^{2+} that showed relative turbidity

of 0.02 (OD_{600nm}), the other metals precipitated a little turbidity of near 0.01 (OD_{600nm}) at a metal concentration of 0.2 mgL^{-1}.

The metal inhibitory concentrations that reduce *p,p'*-DDT degradation capacity of the strain appeared to be within the range 0.6 mgL^{-1} for Fe^{2+} and Cu^{2+}. While for Pb^{2+}, Hg^{2+}, Ag^{+} and Cr^{2+}, the inhibitory concentration observed was 0.2 mgL^{-1}.

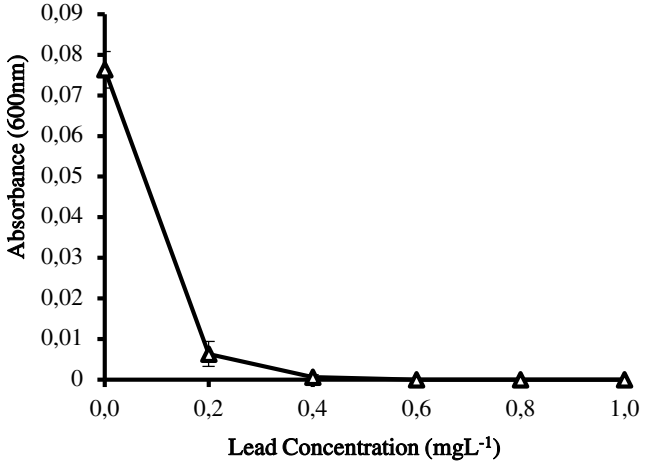

Figure 9. Effect of Pb^{2+} concentration on the growth of strain MY 83295S in *p,p'*-DDT enrichment medium

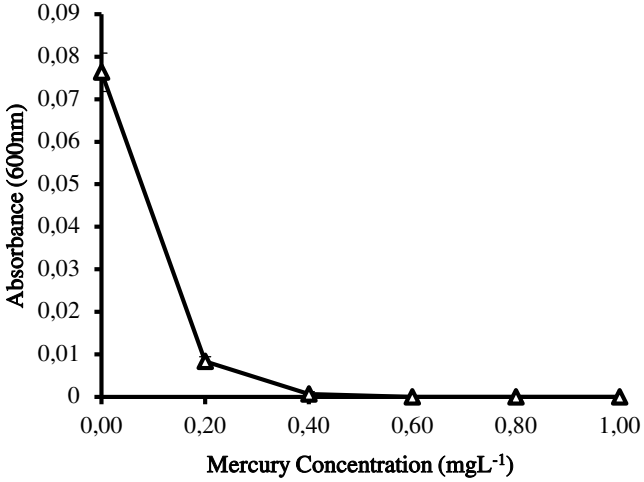

Figure 10. Effect of Hg^{2+} concentration on the growth of strain MY 83295S in *p,p'*-DDT enrichment medium

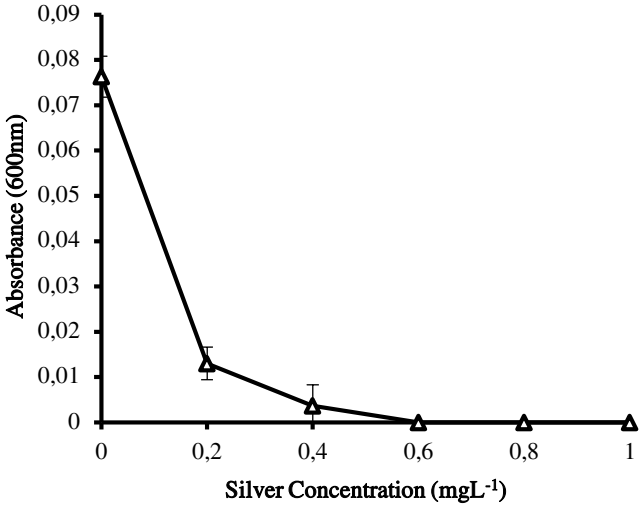

Figure 11. Effect of Ag^{2+} concentration on the growth of strain MY 83295S in *p,p'*-DDT enrichment medium

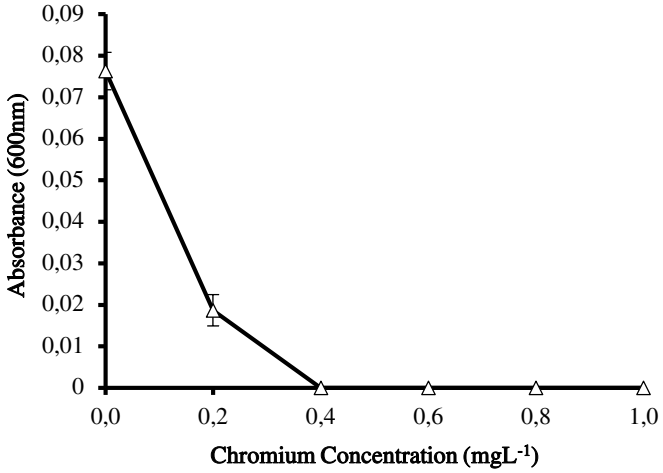

Figure 12. Effect of Cr^{2+} concentration on the growth of strain MY 83295S in *p,p'*-DDT enrichment medium

Discussion

The recalcitrant nature of DDT and its metabolites made them be among the high ranking environmental contaminants since the 1970s. In this study, we used minimal salt-*p,p'*-DDT enrichment medium for the screening and isolation of *p,p'*-DDT degrader that used *p,p'*-DDT as a sole carbon and energy source. The isolate, *Bacillus velezensis* strain MY 83295S was found to be a Gram-positive, motile, endospore-forming, catalase-positive, urease and cytochrome oxidase negative *bacilli*. It was also found to metabolize citrate, D-glucose, D-mannitol and starch (data not presented here). These characteristics conform to those reported earlier for the genus *Bacillus* (Berkeley et al., 1984; Parvathi et al., 2009; Lee et al., 2017).

To obtain a valid molecular identification of the isolate, the 16S rRNA analysis was performed due to its accuracy and reliability in the bacterial identification (Roy et al., 2013; Hong & Farrence, 2015). The amplified 16S rRNA gene product for the *Bacillus velezensis* strain MY 83295S revealed about 1500 bp upon running on 1.5% agarose gel electrophoresis (Figure 1). Some researchers have previously reported a similar range of 16S rRNA gene amplification products for the genus *Bacillus,* which depends on the segment amplified or the type of primers used (Jill & Clarridge, 2004; Mitra & Roy, 2010).

A BLAST search for the strain MY 83295S in the NCBI nucleotide archive revealed a sequence similarity of 98.87% with *Bacillus amyloliquefaciens* strain MPA 1034 and *Bacillus amyloliquefaciens* strain NBRC 15535, indicating their genus relatedness, and perhaps, members of the *amyloliquefaciens* group. *Bacillus velezensis* strain FZB42 was the next in closeness to strain MY 83295S with a percentage sequence similarity of 98.78%. All the hundred representatives from the search result were found to belong to the genus *Bacillus*, with the last member (*Bacillus crescens* strain JC247) in the sequence similarity ranking, showing similarity of 94.06%.

The phylogenetic analysis of strain MY 83295S revealed that the strain was positioned in the same cluster with *Bacillus velezensis* strain CBM8205 with a bootstrapping of 64%. This cluster was contained within a clade containing *Bacillus siamensis* KCTC 13613 strain PD-A10 with a low bootstrap value of 45% (Figure 2). Going by the phylogenetic analysis of strain MY 83295S, though, the strain was designated as *Bacillus velezensis* strain MY 83295S, it is difficult to ascertain the actual species of this organism, considering the low bootstrap value obtained. However, both the BLAST search and phylogeny confirmed the genus of strain MY 83295S to be *Bacillus*. Many researchers have reported accurate identification of the genus *Bacillus* using 16S rRNA gene sequencing and phylogenetic analysis (Goto et al., 2000; Wang & Sun, 2009; Mwangi et al., 2010; Pant et al., 2013; Yoon et al., 2017).

Microorganisms demonstrated the capacity for the removal of various toxic environmental pollutants such as DDT (Reineke et al., 2011). Several researchers have reported the isolation of DDT-degraders (Mwangi et al., 2010; Pant et al., 2013, 2016, 2017). Strain MY 83295S was found to biodegrade and utilize *p,p'*-DDT as a sole carbon and energy source. Though some bacterial isolates were reported to utilize < 20 mgL^{-1} of *p,p'*-DDT as sole carbon and energy (Pant et al., 2013, 2016), strains MY 83295S however, demonstrated an ability to utilize up to 30 mgL^{-1} of *p,p'*-DDT as the sole carbon source under aerobic condition (Figure 3). Substantial literature, however, showed that many isolates were capable of degrading up to 50 mgL^{-1} of DDT when additional carbon sources were supplemented into the DDT-containing medium (Barragan-Huerta et al., 2007; Fang et al., 2010; Bajaj et al., 2014).

The bacterial ability to depend on the DDT's carbon skeleton largely depends on the organism's capacity to remove the chlorine substituent and utilize the carbon atoms to harness energy from the process. This process involved mineralization of DDT which was reported to be a complex process involving several stepwise reactions (Fang et al., 2010; Pan et al., 2016).

The strain MY 83295S demonstrated a longer lag phase of nearly 72 h for the initial DDT degradation (Figure 4). The delay observed in the initial rate of DDT degradation could be linked to the delay in the initial synthesis of molecular machinery for the degradation process. However, the strain MY 83295S effectively mineralized and utilized *p,p'*-DDT, precipitating total biomass of 0.14 (OD_{600nm}) in 168 h (Figure 4).

As a bio-molecular process, bacterial DDT mineralization is greatly modulated by some environmental determinants such as temperature, pH and DDT levels in the contaminated site (Aislabie et al., 1997; Bidlam & Manonmani, 2002). Optimization of these determinants is thus, critical for the application of a bacterium for the effective decontamination process. Though the strain MY 83295S demonstrated a mesophilic behaviour in the *p,p'*-DDT mineralization, it, however, displayed an optimum temperature of 30°C (Figure 5). This was perhaps emanated from the strain's adaptational strategy to the conditions of the tropical region where the strain was isolated. Temperature is a key to microbial processes, especially metabolic activities that involve enzymes of detoxification pathways and other functional proteins that are susceptible to denaturation and consequent inactivation when the optimum temperature is altered.

The pH range of strain MY 83295S in the *p,p'*-DDT mineralization process was both slightly acidic and alkaline with a neutral as the optimum pH value. The pH plays a significant role in changing the ionic character of the constituent amino acids in enzymes and other intracellular and membrane proteins. This perhaps,

influenced the DDT mineralization of the strain MY 83295S, either by affecting the DDT membrane transport system or the degradation enzymes. A stream of literature had reported bacterial DDT degradation close to the above pH and temperature ranges (Mwangi et al., 2010; Fang et al., 2010; Wang et al., 2010; Pant et al., 2013, 2016).

A typical contamination site is often loaded with both organic contaminants like DDT and heavy metals, and more or less, the later might influence the degradation rate of the former. The effect of metals on the mineralization of DDT by strain MY 83295S showed both stimulatory and inhibitory effects. Fe^{2+} was found to enhance the mineralization of DDT in strain MY 83295S (figure 7). This stimulatory effect might be linked to an increase in biogenic Fe^{2+} formation as observed in some DDT degrading strains as reported by Glass, (1972) and Cao et al. (2012) with a possible increase in the reductive biotransformation and utilization of *p,p'*-DDT observed in strain My 83295S. Additionally, some bacterial species were reported to express a protein called ferric-uptake-regulator (RUF) that contributes to the modulation of intracellular iron pool in bacteria. Iron is required for various metabolic processes in some bacteria (Troxell & Hassan, 2013; Kaushik et al., 2019).

An inhibitory pattern demonstrated by Cu^{2+} (Figure 8) was more favourable to the growth of strain MY 83295S relative to that of Pb^{2+} (Figure 9), Hg^{2+} (Figure 10), Ag^{2+} (Figure 11) and Cr^{2+} (Figure 12). This could be linked to the essentiality of copper to some bacterial enzymes and proteins, though elevated levels are associated with toxicity (Zevenhuizen et al., 1979; Trevors & Cotter, 1990; Fowler et al., 2019). It has been documented that the co-existence of both organic and metal pollutants led to the metal toxicity in bacteria, mostly by interacting and inhibiting the vital proteins and consequently affecting the biodegradation of organic pollutants (Angle & Chaney, 1989; Sandrin & Maier**,** 2003; Murata et al*.,* 2005). For instance, metal oxyanions such as chromate, mimic the molecular structure of essential non-metal oxyanions such as sulfate and interfere with their molecular functions (Sandrin & Maier, 2003). Furthermore, cationic complexes of silver and mercury may disrupt many physiological functions, in addition to their inhibitory binding to the thiol group of the essential biomolecules in bacteria (Nies, 1999). Metal ions generally affect organic pollutants biodegradation by altering both the physiology and ecology of the bio-degraders (Sandrin & Maier**,** 2003).

Conclusion

A bacterial strain with a *p,p'*-DDT degradation and utilization ability was isolated from a tropical irrigation site and tentatively identified as *Bacillus velezensis* strain MY 83295S. The strain was able to degrade up to 30 mgL^{-1} of *p,p'*-DDT as a sole carbon source. Low Fe concentration enhances the strain's ability to grow on *p,p'*-DDT enrichment medium while Cu, Pb, Hg, Ag and Cr ions demonstrated inhibitory effects on the *p,p'*-DDT degradation.

Ethical Approval

The authors don't declare ethical approval.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Funding Statement

The authors don't declare any fund.

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