International Digital Organization for Scientific Research ISSN: 2579-0730
IDOSR JOURNAL OF BIOLOGY, CHEMISTRY AND PHARMACY 8 (2) 102-112, 2023.
https://doi.org/10.59298/IDOSR/JBCP/23/10.128

Isolation, Identification and Characterization of Bacteria Capable of Degrading Chlorpyrifos from Agricultural Soil at Amansea, Anambra State Nigeria.

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ABSTRACT

Chlorpyrifos (O, O-diethyl O-(3,5,6-trichloro-2-pyridyl phosphorothioate) a broad spectrum moderately toxic organophosphorous insecticide is commonly used as pest control on grain, cotton, fruits, and vegetable crops against pest. Although it is important, its use has become one of the major causes of soil and water pollution. The present technique for removing chloryrifos insecticide from the environment which is biodegradation has shown to be more effective than any other physical or chemical methods like chemical hydrolysis. Therefore the aim of this research was to evaluate the biodegradation of chlorpyrifos insecticide by bacteria isolated from agricultural soil in Amansea, Anambra State, Nigeria. In the present study, two bacteria capable of degrading chlorpyrifos were isolated from Agricultural soil using mineral salts medium and characterized based on their physiological, biochemical, morphological and 16S rRNA sequencing as strains of *Bacillus cereus* ST06 and *Chryseobacterium* sp. 6024. Their growth response in mineral salts medium supplemented with 20mg/l concentration of chlorpyrifos was determined by monitoring the optical density at 600nm and at optimium condition of pH 6.5 and 30^0C for 28 days. The residual chlorpyrifos concentration after 28 days was also determined using Gas Chromatography-Electron Cathode Detector (GC-ECD). The result from one way ANOVA indicated that there were significant differences ($P < 0.05$) in the Growth responses to the insecticides by the test organisms. The result showed that *Bacillus cereus* ST06 and *Chryseobacterium* sp. 6024 reached maximum growth on 20mg/l chlorpyrifos with an arithmetic mean difference of 0.23±0.20 and 0.42±0.02 respectively on day 16 .There were also significant (<0.05) difference in concentration of residual chlorpyrifos degraded by the isolates obtained from the GC-ECD curves. *Bacillus cereus* ST06 and *Chryseobacterium* sp. 6024 degraded 63% and 57% of 20mg/l chlorpyrifos respectively. *Bacillus cereus* ST06 showed better results and possess potential to be used in biodegradation of 20mg/l Chlorpyrifos than *Chryseobacterium* sp. 6024. It is therefore recommended that further studies on the effect of varying Chlorpyrifos concentration on the bacteria singly and as a consortium be studied to better understand their potential for degrading chlorpyrifos efficiently.

Keywords: Isolation, Identification, Characterization, Bacteria, Biodegradation, Chlorpyrifos and Insecticides

INTRODUCTION

Chlorpyrifos (O, O-diethyl O-(3,5,6-trichloro-2-pyridyl phosphorothioate) a broad spectrum moderately toxic organophosphorous insecticide [1], used on grain, cotton, fruit, and vegetable crops, as well as, lawns and ornamental plants [2], against common pests like moths, hoppers, midges, worms and

spiders [3]. The use of Pesticide has greatly improved crop productivity and reduced reduction of crop yield. It has also effectively controlled vector borne diseases like Malaria [4]. Although, pesticides have an important role in agriculture to solve the problem of feeding the world's over growing population, the extensive use has led to the wide spread microbial imbalance, environmental pollution and health hazard [5]. The frequent application of Chlorpyrifos has caused several toxicological, environmental pollution and residue problems, which threatens human health and the environment [6]. Some researchers in previous studies were able to show that Chlorpyrifos cause acute toxicity, chronic toxicity, neurotoxicity and genotoxicity on mammals, aquatic organisms and other non-target organisms [7]. According to WHO and Globally Harmonized System (GHS) pesticides has been classified based on their toxicity or harmful effects, prioritizing public health. [8] chlorpyrifos belongs to class II pesticides with moderate toxicity. Due to these problems, development of technologies that guarantee their elimination in a safe, efficient and economical way is very important. A lot of significant studies related to the decontamination of soil pollution by Chlorpyrifos has been reported, this includes photochemical degradation and the use of nanometal materials or UV/H2O2 catalytic degradation [9]. Compared with these methods, biodegradation is an efficient and environmental friendly method to remove chlorpyrifos from water and soil [10]. The biodegradation of organophosphate pesticide by soil microorganisms has been reported by many researchers and its biodegradation has helped to solve the problem of recalcitrance in water and soil. [11] isolated *Enterobacter* B-14, a strain which could degrade chloropyrifos. [12,13] isolated *Bacillus cereus* MCAS02 and [14] isolated *Stenotrophomonas* sp *and Sphingomonas* sp respectively, which could utilize Chloropyrifos as the source of carbon and phosphorous for their growth. Considering that Chlorpyrifos is one of the most commonly applied insecticides for control of pests and insects, it is therefore of great significance to identify strain that can efficiently degrade Chlorpyrifos. So the aim of this research was to isolate and characterize Bacteria with potential of degrading Chlorpyrifos efficiently.

MATERIALS AND METHODS

Study Area

The study was carried out in Amansea, Awka North LGA of Anambra State, Nigeria. Awka is in the tropical rainforest region and is located between latitude 6°16' 18.7'' N and longitude 7° 07'23.8''E. Awka North has an estimated population of 112,192 in 2006 and 159,900 projected by Nigeria statistic of bureau 2022. The land mass is 460.2/km^2. The people are mainly farmers and itinerant traders. Agricultural crops include yam, cocoyam, cassava, maize, fruits and vegetables.

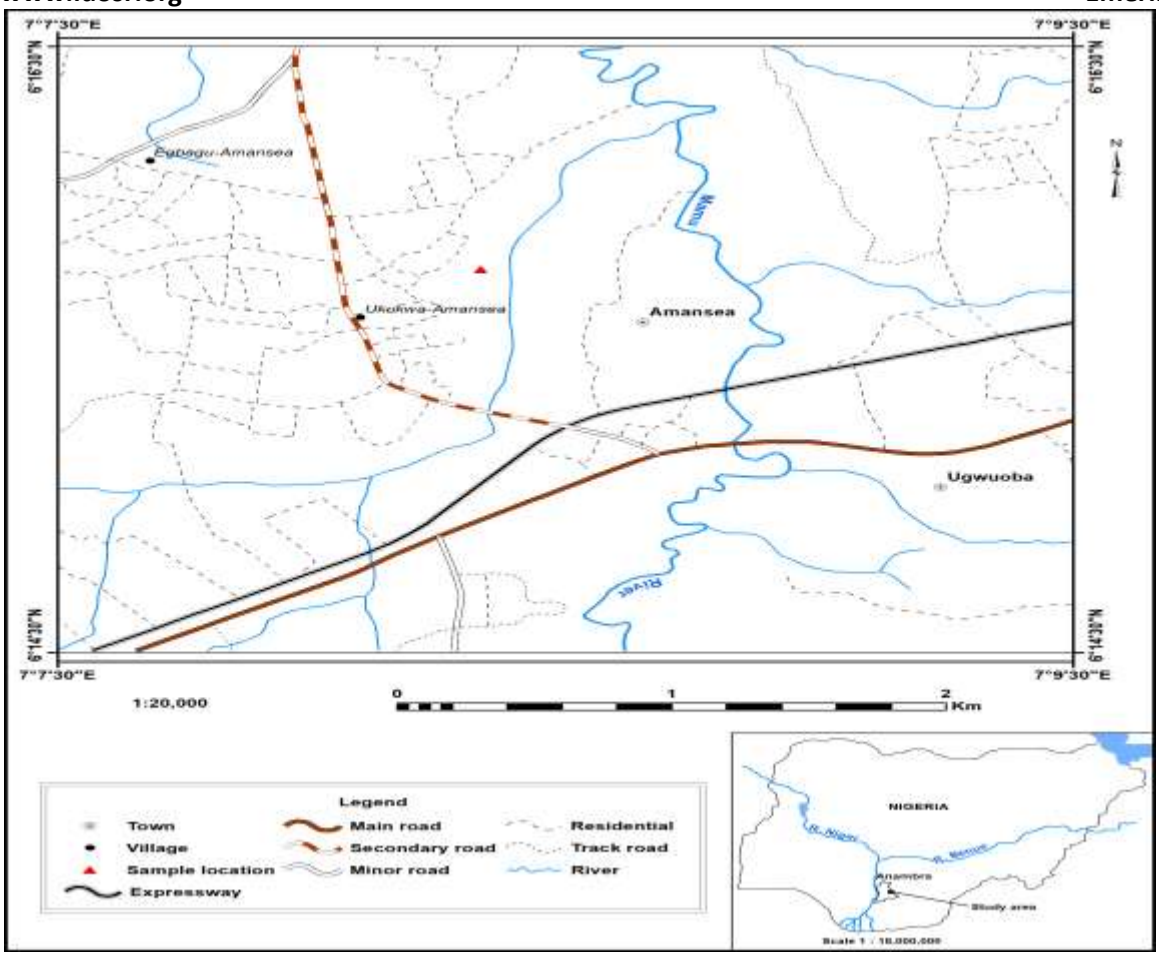

Figure 1: geographic map of study area showing ukukwa Amansea.
Source: GIS Lab, Department of Geography and meterology Nnamdi Azikiwe University Awka Anambra State, Nigeria (2023).

Insecticide and media

Commercial grade of Chlorpyrifos commonly known as PERFECT KILLER® (containing 20g active ingredient/L, Emulsifiable concentrate 20%) was purchased from Eke-Awka market Anambra State, Nigeria. The mineral salts medium (MSM) described by Benslama and Brulahruf (2013) containing(g/l) 1.5g of K_2PO_4, 0.5g of NaCl, 0.6g of Na_2HPO_4, 2g NH_4SO_4, 0.2g $MgSO_47H_2O$, 0.01 $Cacl_2$ and 0.001g $FeSO_4.7H_2O$ was used for the isolation of bacteria. Agar agar, peptone water, nutrient agar and Simmon's citrate agar were also used.

Sample collection

Chlorpyrifos-contaminated agricultural soil was collected from a farm within Amansea. The soil sample (1kg) was collected from the Rhizosphere at a depth of 0-15cm from four corners of the farm. The soil was sorted, mixed and put into a sterile polyethylene bag and then conveyed immediately to the Laboratory of the Department of Applied Microbiology and Brewing laboratory Nnamdi Azikiwe University Awka for analysis.

Isolation and identification of Chlorpyrifos degrading bacteria

The method described by [12] with little modification was adopted to isolate chlorpyrifos-degrading bacteria. The soil sample was air-dried and sieved using a 2mm mesh. 5g of the soil sample was added into a 250ml Erlenmeyer flask containing an autoclaved mixture of 100ml of mineral salts medium and 1ml of Perfect killer ® (20mg/l of Chlorpyrifos) at 121^oC for 15mins. This set up was done

in triplicate. The flasks were incubated on a rotary shaker at 150 rpm for 7 days at 30^{o}C. Isolation was done by sub-culturing 0.1ml of the broth culture on mineral salts medium agar supplemented with 20mg/l Chlorpyrifos using the pour plate method. The plates were incubated at 30^{o}C for 72 hours. Distinct colonies that appeared after 72 hours incubation were sub-cultured in nutrient agar to obtain pure culture. Pure cultures of the isolates inoculated on nutrient agar were maintained on slant and observed morphologically after 24hours incubation. To identify the bacterial isolates biochemical tests like gram staining test, motility test, catalase test, oxidase test, methyl red test, indole test, citrate utilization test, sugar fermentation test, urease production test, Voges proskaeur test, gelatin hydrolysis test, spore test and nitrate reduction test were conducted as described by [12].

Molecular identification of isolates

Molecular identification of the Bacterial isolates was done for proper confirmation of the Bacteria isolates in three stages DNA Extraction, PCR and 16s rRNA sequencing. Bacterial DNA was extracted by the method of Quick-DNATM Miniprep plus Kit [Edwards, 1998). The 16S rRNA genes of the strains were amplified PCR using two universal primers 27F:5'-AGAGTTTGATCGTGGCTCAG-3' and 5'-1492R TACGGTTACCTTGTTACGACTT -3'. The PCR was performed with Eppendorf nexus gradient Mastercycler (Germany). The genes were sequenced by the Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit. The genes were compared with most similar sequences available in the Genbank nucleotide database using the NCBI BLAST Program.

Growth response of the isolated bacteria in Mineral salts medium to 20mg/l Chlorpyrifos at OD 600nm, pH 6.5 and 30^{o}C

1ml of the 24 hours of the bacteria culture were used to inoculate 250ml flasks containing 100ml MSM and Chlorpyrifos in the concentration of 20mg/l in Triplicates. There were un-inoculated flasks which served as controls. The flasks were incubated on a rotary shaker at 150rpm at 30^{o}C for 28 days. The optical density of the isolates was determined at intervals of 4 days for 28 days using Spectrophotometer (OD 600nm) as described by [2].

Determination of residual Chlorpyrifos

After 28 days incubation the method of [5] was used to determine residual chlorpyrifos. 5ml of each of the culture were taken from each flask and placed in centrifuge tubes. These portions of the culture were extracted with equal volume of ethyl acetate as the extracting reagent by centrifuging at 150rpm for 20minutes. The ethyl acetate with residual Chlorpyrifos was filtered through Whatman No 1 filter paper. The final extracts were analyzed by Gas Chromatograph-Buck M910 scientific gas chromatography equipped with Electron capture detector (GC-ECD) that allowed the detection of contaminants even at trace level concentrations (in the lower µg/g and µg/kg range) from the matrix to which other detectors do not respond. The GC conditions used for the analysis were capillary column HP 88 capillary column (100m x 0.25µm film thickness,) CA, USA. The injector and detector temperature were set at 250ºC and 290ºC respectively. The oven temperature was programmed as follows: 110ºC held for 10 min, ramp at 10 ºC/ min to 200ºC, held for 5min, and finally ramp at 10ºC/ min to 320ºC. Helium was used as carrier gas at a flow rate of 1.0 mL/ min and detector make-up gas of 29 Ml min-1. The injection volume of the GC was 8.0 µL. The total run time for a sample was 48 min. The residue levels of PCB were quantitatively determined by the external standard method using peak area. Measurement was carried out within the linear range of the detector. The peak areas whose retention times coincided with the standards were extrapolated on their corresponding calibration curves to obtain the concentration.

Data analysis

Statistical analysis of the data generated was carried out to indicate mean significant differences between the treatments using Statistical Package for Social Sciences (SPSS) version 16.0. one ways ANOVA, Graphs and tables were used for data presentation.

RESULTS

Identification of bacterial isolates

Two morphologically distinct bacterial colonies were observed on the mineral salt agar supplemented with Chlorpyrifos. The result of the morphological, cultural and biochemical test are shown in Table 1. The two bacterial isolates were identified by 16S rRNA gene amplification using thermocycler and were sequenced. The Partial 16Sr RNA gene sequences were compared with that of referred strains gene sequences in the Genbank and they were related to *Bacillus cereus strain ST06* and *Chryseobacterium* sp *strain 6024.* The FASTA Sequence of the 16S rRNA sequences of the bacterial strains and related bacterial are shown in Figure 2 and 3

Table 1: Cultural and biochemical Characteristics of isolated organisms

Test	Isolate A1	Isolate A2
Colony morphology	Circular, creamy, entire, opaque and rough edge	Rhizoid,light yellow, smooth colonies
Grams reaction	Gram +ve rod	Gram –ve rod
Methyl red	-ve	-ve
Voges proskauer	+ve	-ve
Indole	-ve	+ve
Motility	+ve	-ve
Citrate	+ve	+ve
Oxidase	-ve	+ve
Gelatin hydrolysis	+ve	-ve
Spore test	+ve	-ve
Nitrate reduction	+ve	-ve
Catalase	+ve	+ve
Lactose	-ve	-ve
Glucose	+ve	-ve
Sucrose	+ve	-ve
Maltose	+ve	-ve
Galactose	-ve	-ve
Fructose	+ve	-ve
Mannitol	-ve	-ve
Identity	*Bacillus cereus* ST06	*Chryseobacterium* sp. *6024*

Key: NLF= Non Lactose fermenter

TGGCGGAGTGCTTAATGCGTTAACTTCAGCACTAAAGGGCGGAAACCCTC
TAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATC
CTGTTTGCTCCCCACGCTTTCGCGCCTCAGTGTCAGTTACAGACCAGAAA
GTCGCCTTCGCCACTGGTGTTCCTCCATATCTCTACGCATTTCACCGCTA
CACATGGAATTCCACTTTCCTCTTCTGCACTCAAGTCTCCCAGTTTCCAA
TGACCCTCCACGGTTGAGCCGTGGGCTTTCACATCAGACTTAAGAAACCA
CCTGCGCGCGCTTTACGCCCAATTATTCCGGATAACGCTTGCCACCTACG
TATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTA
CCGTCAAGGTGCCAGCTTATTCAACTAGCACTTGTTCTTCCCTAACAACA
GAGTTTTACGACCCGAAAGCCTTCATCACTCACGCGGCGTTGCTCCGTCA
GACTTTCGTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTC
TGGGCCGTGTCTCACCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTAC
GGATCGTTGCCTTGGTGAGCCGTTACCTCACCAACTAGCTAATGCGACGC
GGCTCCATCCATAAGTGACAGCCGAAGCCGCCTTTCAATTTCGAACCATG
CGCCTCAAAACGTTATCCGGTATTAGCCCCGGCTTCCCGGAGTTATCCCC
ACTCTTATGGGGCAGGTTACCCACGTGTTACTCACCCGTCCGCCGTCACT
TCATAAGAGCAACTCTTAATCCATTCCCTCGACTTGATGTATCAGGACSC
CGCCAGCGCTCATCTTGAACCATGATCAAACTTTAGGC
Figure 2: Partial sequence of *Bacillus cereus strain ST06*

TAACTTATCACTTTCGCTTAGTCTCTGAATCCGAAAACCCAAAAACGAGT
TAGCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCC
CCACGCTTTCGTCCATCAGCGTCAGTTGTTGCTTAGTAACCTGCCTTCGC
AATTGGTGTTCTAASTAATATCTATGCATTTCACCGCTACACTACTTATT
CCAGCTACTTCAACAACACTCAAGACCTGCAGTATCAATGGCAGTTTCAC
AGTTGAGCTGTGAGATTTCACCACTGACTTACAGATCCGCCTACGGACCC
TTTAAACCCAATAAATCCGGATAACGCTTGCACCCTCCGTATTACCGCGG
CTGCTGGCACGGAGTTAGCCGGTGCTTATTCTTATAGTACCTTCAGCTAC
CCTCACGAGAGTAGGTTTATCCCTATACAAAAGAAGTTTACAACCCATAG
GGCCGTCGTCCTTCACGCGGGATGGCTGGATCAGGCTCTCACCCATTGTC
CAATATTCCTCACTGCTGCCTCC
Figure 3: Partial sequence *Chryseobacterium* sp *Strain 6024*

Growth response of the isolated bacteria in Mineral salts medium to 20mg/l Chlorpyrifos at OD 600nm, pH 6.5 and 30^{0}C

Figure 4 presented the growth response of the isolated bacteria to 20mg/l Chlorpyrifos contained in mineral salts medium while monitoring their optical density at 600nm at 4 days interval for 28 days. There was gradual increase in the proliferation of *Bacillus cereus* ST06 from day 8 with a mean concentration of 0.57±0.12 as indicated by decrease in absorbance value and its growth started decreasing from day 24 with a mean concentration of 0.30±0.02 as indicated by increase in absorbance value. Similar result was obtained from *Chryseobacterium* sp. 6024 as increase in proliferation took place from day 4 with a mean concentration of 1.04±0.03 and started decline from day 20 with a mean concentration of 0.39±0.15.

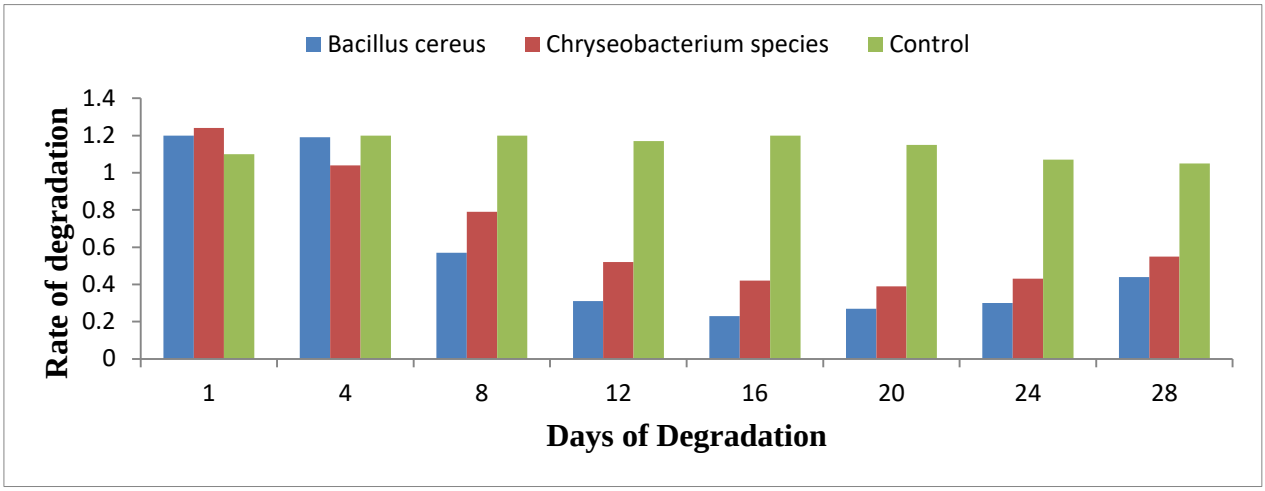

Figure 4: Growth response of bacteria isolates on 20mg/l Chlorpyrifos

Determination of residual Chlorpyrifos

Table 2 showed the ability of these chlorpyrifos-degrading bacteria to catabolize 20mg/l of chlorpyrifos in mineral salts medium, in vitro. The two organisms showed variation in their ability to degrade 1ml volume of 20g/l chlorpyrifos after 28 days. *B. cereus* reduced 20mg/l Chlorpyrifos to a mean concentration of 7.41±0.01 beter than *Chryseobacterium* sp. which reduced 20mg/l chlorpyrifos to a mean concentration of 8.80±0.01. Figure 5 presented the percentage degradation of 20mg/l chlorpyrifos by the two isolates. *Bacillus cereus* ST06 degraded 63% of 20mg/l chlorpyrifos in mineral salts medium after 28days while *Chryseobacterium spp* 6024 degraded 57% of 20mg/l chlorpyrifos in mineral salts medium after 28days.

Table 2: Residual Chlorpyrifos concentration

s/n	Isolates	CP initial conc.(mg/l)	CP final conc. (mg/l)
1	*Bacillus cereus*	20.00	7.41±0.01[a]
2	*Chryseobacterium* sp	20.00	8.80±0.01[b]
3	Control	20.00	13.46±0.01[c]

Mean values along the same column with different affixes are different significantly (P<0.05)
*Chlorpyrifos concentration in Perfect killer = 20g/l equivalent to 20000mg/l.

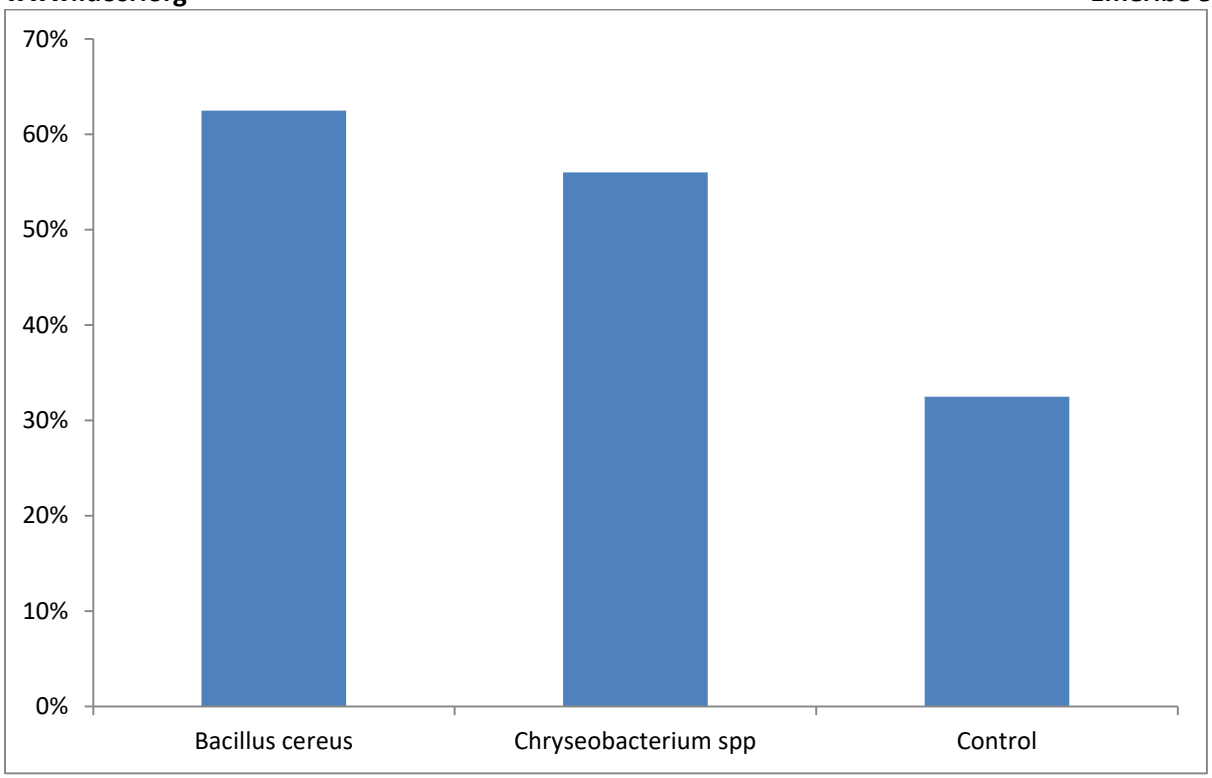

Figure 5: Percentage degradation ability of the isolates.

DISCUSSION

In this study, two distinct bacteria were isolated from agricultural soil and their actions were shown to be significant in Chlorpyrifos insecticide degradation. The Partial 16S rRNA gene sequences were compared with that of referred strains gene sequences in the Genbank and they were related to *Bacillus cereus strain ST06* and *Chryseobacterium* sp *strain 6024* (Figure 2 and 3). The result obtained in this study (Table 1) is in agreement with earlier reports that indicated the involvement of different species of Gram positive bacteria, especially the members of *Bacillaceae* in the degradation of organophosphorous insecticides like Chlorpyrifos [15]. There is still no experimental evidence for the involvement of members of *Weksellaceae* in the biodegradation of chlorpyrifos, indicating that *Chryseobacterium* sp 6024 in this group is novel for this purpose.

The result of the growth response of the isolated bacteria during biodegradation process of chlorpyrifos by monitoring optical density at 4 days interval for 28 days (Figure 4) indicated that *Bacillus cereus* reached maximium growth on day 16, while *Chryseobacterium* sp reached maximium growth on day 20 at 20mg/l concentration. There was a decrease in the absorbance value of both isolates which brought about a corresponding increase in the growth of the bacterial isolates for the period of from day 8 and 4 respectively. The degradation of chlorpyrifos by *Bacillus cereus* decreased slowly with increase in chlorpyrifos concentration and metabolite accumulation. This is in agreement with the research of [16] on bacterial degradation of chlorpyrifos by *Bacillus cereus.* Their report demonstrated that the degradation rate of chlorpyrifos was decreased slowly with the concentration of chlorpyrifos being increased. A corresponding decrease in utilization of chlorpyrifos by the isolates was noticed from 16th day. This could be as a result of some environmental factors which is the release and accumulation of TCP (3,5,6-trichloro-2-pyridinol) an intermediate of chlorpyrifos degradation into the liquid medium, therefore making it resistant to

microbial attack. This is in agreement with the work of [17] that TCP shows relatively high antimicrobial effect on microorganisms. Also the work of [18] confirmed that TCP prevents its own biodegradation by microorganisms and also limits chlorpyrifos degradation. The medium containing *Bacillus cereus* was significantly ($P< 0.05$) higher than the medium containing *Chryseobacterium sp* with the maximum growth of 0.25 (optical density at 600nm) observed at day 20.

From the result of residual Chlorpyrifos determination (Table 2 and Figure 5), *Bacillus cereus* and *chryseobacterium* sp showed 63% and 56% Chlorpyrifos degrading capacity of 20mg/l to 7.41mg/l and 8.80mg/l respectively after 28 days. This report is in agreement with [19] on *Bacillus sp.* and *Micrococcus* sp. and reported that both possess potential to degrade chlorpyrifos. Also in a previously published reports by [20] *Bacillus cereus* strain isolated from the municipal soil sediment have been studied for their role in pesticide biodegradation. The result of the study also showed that the concentration of uninoculated control was reduced from 20mg/l to13.40mg/l 33% reduction (Table 2). This reduction could be traced to the fact that once Chlorpyrifos is applied to the soil, it may be exposed to photodegradative conditions either directly or indirectly according to [21]. The decrease might also be as a result of volatilization exhibited by pesticides.

CONCLUSION

In the present study, two isolates capable of utilizing Chlorpyrifos as the only source of carbon and energy were identified. They are *Bacillus cereus ST06 and Chryseobacterium* sp*. 6024*. The biodegradation study of the strains where done to know optimal degradation. The result showed that *Chryseobacterium* sp .is a novel bacterium in the biodegradation studies with 57% rate of 20mg/l Chlorpyrifos degradation. The Result of this study also showed that *Bacillus cereus ST06* can remove up to 63% of 20mg/l Chlorpyrifos concentration better than *chryseobacterium sp. 6024*. Hence they may be used for bioremediation of Chlorpyrifos contaminated soil.

ACKNOWLEDGEMENT

The authors wish to thank the Managing Director Fountain of success medical laboratory, Amansea, Anambra State for her assistance in supplying some reagents for the research.

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CITE AS: Emeribe, Chiemeka Elochi, Onuorah, Samuel Chinedu and Chukwukelo, Chinonyelum Dilys (2023). Isolation, Identification and Characterization of Bacteria Capable of Degrading Chlorpyrifos from Agricultural Soil at Amansea, Anambra State Nigeria. IDOSR JOURNAL OF BIOLOGY,CHEMISTRY AND PHARMACY8(2)102-112. https://doi.org/10.59298/IDOSR/JBCP/23/10.128