Original Research Article

Biodegradation of Chlorpyrifos Insecticide by *Bacillus cereus ST06 and Chryseobacteriumsp 6024*Isolated from Agricultural Soil, Nigeria.

ABSTRACT

Introduction: Indigenous soil bacteria have the potential to degrade the harmful chlorpyrifos insecticide, this identifies the importance of biodegradation as an eco-friendly method for chemical pollutant cleanup.

Aims: To compare the potential of *Bacillus cereus* ST06 and *Chryseobacteriumsp* 6024 in biodegrading chlorpyrifos insecticide singly or as a consortium in a liquid medium.
Study design: Experimental study design was used toevaluate the bacterial potential in biodegrading chlorpyrifos insecticide.
Place and Duration of Study: Agricultural soil samplecontaining chlorpyrifos degrading bacteria from Ukukwa villageAmansea Nigeria (6^{o}16' 30" N and 7^{o} 07'30"E)soil depths (15cm) was collected and investigated between January and March 2022.
Methodology: In this study,*Bacillus cereus* ST06and*Chryseobacteriumsp* 6024 previously isolated and characterizedusing standard microbiological method based on their phenotypic test, biochemical test, cultural morphology and 16S rRNA sequencing was used for the experiment. Their growth response to 20mg/l and 60mg/l chlorpyrifos in mineral salts medium singly and as a consortium was compared and determined by monitoring the optical density at 600nm and at the optimum condition of pH 6.5 and 30^{o}C for 28days. The residual chlorpyrifos concentration after 28 days was also compared and determined using Gas Chromatography- Electron Cathode Detector (GC-ECD).
Results: The result showed a significant difference (*P< .001)*as*Bacillus cereus* ST06 and *Chryseobacteriumsp* 6024 responded differently to different concentration of chlorpyrifos. *Bacillus cereus* ST06 and *Chryseobacteriumsp* 6024reached maximum growth in medium containing 20mg/l chlorpyrifos with a mean OD of 0.23±0.20 and 0.42±0.02 respectively on day 16 than 60mg/l chlorpyrifos with a mean OD of 0.47±0.02 and 0.81±0.02 respectively on day 20. The bacterial consortium also reached maximum growth on 20mg/l and 60mg/l of chlorpyrifos with mean OD of 0.21±0.31 and 0.29±0.02 on day 20 respectively. The result of residual chlorpyrifos concentration shows that the bacteria consortium degraded 79% and 78% of 20mg/l and 60mg/l chlorpyrifos respectively, while *Bacillus cereus* ST06 and *Chryseobacteriumsp* 6024 degraded 63% and 57% of 20mg/l chlorpyrifos and 61% and 37% of 60mg/l chlorpyrifos.
Conclusion: The study shows that bacteria consortium possessed potential to be used in biodegradation of 20mg/l and 60mg/l Chlorpyrifos than the individual isolates. It is therefore recommended that further studies on RNA profiling of each bacterium and synergistic interaction of the bacterial consortium be studied to better understand regulation of genes and individual bacterial rolesin degradation chlorpyrifos efficiently.

Keywords: Isolation; Characterization; Bacteria; Biodegradation; Chlorpyrifos; Nigeria.

1. INTRODUCTION

Chlorpyrifos is a wide range organophosphate insecticide known as o, o-diethyl o-3,5,6-trichloro-2-pyridyl phosphorothioate [1]. It is toxic or lethal against worms, moths and hoppers that infest grains, fruits, and vegetables[2]. The use of pesticide has greatly improved crop productivity and reduced the reduction of crop yield. It has also effectively controlled vector borne diseases like malaria [3]. 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 widespread microbial imbalance, environmental pollution, and health hazard [1]. Chlorpyrifos has caused several neurological diseases, water, and soil pollution because of its constant use for agriculture and industrialization[4]. A lot of research have shown that chlorpyrifos is also the leading cause of gene mutation and other negativeeffects on our mental and physical health [5,6]. According to World Health Organization (WHO) and Globally Harmonized System (GHS) pesticides has been classified based on their toxicity or harmful effects, prioritizing public health. [7,8] and chlorpyrifos belongs to class ii pesticides which are moderately toxic. Because of the problems caused by chlorpyrifos, it is important to develop safe methods that can eliminate it from the environment. A lot of research studies towards degradation of chlorpyrifos has been carried out on water and soil, and this include photochemical degradation, nanometal and UV catalytic degradation [9]. Biodegradation, a method which uses microorganisms to breakdown organiccompounds into simple inorganic molecules, has proved more efficient and friendly in decontamination of soil and water polluted by chlorpyrifos [10]. Good number of research has reported the use of biodegradation in cleaning up chlorpyrifos in soil. [11] isolated *Enterobacter* b-14 that was able to breakdown chlorpyrifos. [12,13] also isolated *Bacillus cereus* mcas02 and [14] isolated *Stenotrophomonassp*. and *Sphingomonassp*. respectively, which was able to use chloropyrifos as the source of energy for their growth requirement. Considering that chlorpyrifos is among the insecticides used for insect control , it is therefore pertinent to identify the bacteria with potentials to breakdown chlorpyrifos. Understanding the interplay between bacteria and the insecticide chlorpyrifos is crucial for both environmental assessment and bioremediation strategies. Using different chlorpyrifos concentrations exposesusefulknowledge into bacterial growth under optimal conditions. So,the aim of this study is to evaluate and compare the potentials of *Bacillus cereus* ST06and*Chryseobacteriumsp* 6024 in biodegrading different concentrations of chlorpyrifos insecticidesingly and as a consortium in a liquid medium. The knowledge from this study will giveresearchers and governmentinsight on bioremediation optimizationand helppredicts bacterial behavior in contaminated environments.

2. MATERIALS AND METHODS

2.1 Insecticide

Commercial grade of chlorpyrifos commonly known as Perfect Killer® (containing 20g active ingredient/L, Emulsifiable concentrate 20%) and manufactured by Nantong Jinling Agrochemical co, LTD China was purchased from Eke-Awka Market Anambra State, Nigeria.

2.2 Media

The mineral salts medium (MSM) described by [15] containing(g/l) 1.5g of KH_2PO_4, 0.5g of $NaCl_2$, 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 and biodegradation experiment.

2.3 Sample Collection

Soil sample from UkukwaAmansea, Awka North Local Government Area of Anambra State, Nigeria was collected aseptically by the use of auger.The soil sample (1kg) was collected from the rhizosphere at a depth of 15cm from all corners of the farm. The location of the soil sampling was recorded by GIS Lab, Department of Geography, and meteorology Nnamdi Azikiwe University Awka as latitude $6^o16'$ 30'' N and longitude 7^o 07'30"E.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 Nnamdi Azikiwe University Awka for analysis.

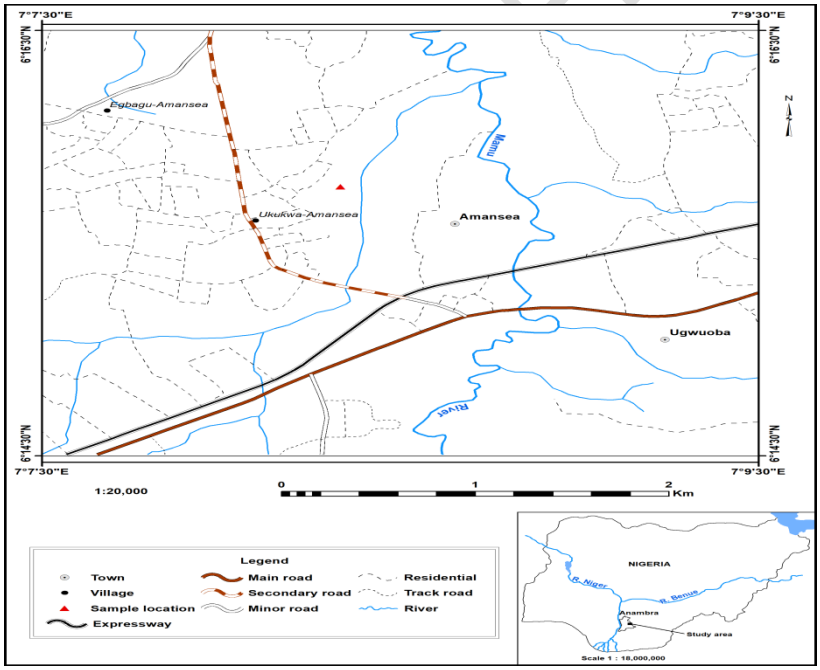

Figure 1: Geographic map Anambra state showing Amansea
Source: GIS Lab, Department of Geography and meterology Nnamdi Azikiwe University Awka Anambra State, Nigeria.

2.4 Culture and Isolation of Bacteria

Bacillus cereus ST06 and *Chryseobacteriumsp* 6024 with potential of degrading chlorpyrifos which was previously isolated and characterized by [16] based on modified methods of [17,18,19] was used for the study.

2.5 Biodegradation experiment

2.5.1 Inoculum standard Preparation

The inoculum used for all the experiments was prepared using viable count method according to [20].1ml of the 24hours culture containing $1.3X10^4$ CFU/ml (determined by viable count method) was used as inoculum for the biodegradation proper.

2.5.2 Assessing the growth response of the isolated bacteria to varying the initial concentration of chlorpyrifos in mineral salts medium singly and as a consortium during biodegradation process by monitoring the optical density (OD) at optimum condition of 600nm, pH 6.5 and 30O C for 28 days.

One milliliter of the 24 hours culture ($1.3 X 10^4$) individually and as a consortium were used to inoculate 250ml flasks containing 100ml MSM and Chlorpyrifos in the concentration of 20mg/l and 60mg/l respectively in triplicates. The chlorpyrifos was added as a source of carbon. There were un-inoculated flasks which served as controls. The flasks were incubated on a rotary shaker at 150rpm at 30℃ for 28 days. The optical density of the isolates were determined at 4days intervals for 28days using a Spectrophotometer (OD 600nm) as described by [20].

2.5.3 Determination of residual Chlorpyrifos after biodegradation and Percentage degradation.

After 28 days incubation the method of [21] 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.

2.6 Data analysis

Statistical Package for Social Sciences (SPSS) version 23.0 was used to perform data calculation and statistical analysis of the data generated in order to show mean significant differences between the two treatments on isolated bacteria. Two-ways Analysis of variance (ANOVA) was used. The rate of degradation was calculated from the following equation:

Rate of degradation was determined = $(ConI_0 – ConI)/(ConI_0) X 100$ (1)

$ConI_0$= Initial Concentration
ConI = Final Concentration

3.0 RESULTS

3.1 IsolationandIdentificationofBacteria

Bacilluscereus ST06 and *Chryseobacteriumsp* 6024 were identified by 16s rRNA gene amplification using thermocycler and were sequenced in previous research by [16]. The partial 16s rRNA gene sequences were compared with that of referred strains gene sequences in the GenBank. The Accession No of the isolated bacteria represented in (table 1)shows that the two isolates are identical to *Bacillus cereus* ST06 and *Chryseobacteriumsp*6024.

Table 1: Bacterial strain identification by I6S rRNA.

Isolates	Accession No	Identity	Confirmed Name
A1	MH475925.1	97%	*Bacillus cereus* ST06
A2	KY056237.1	99%	*Chryseobacterium sp.* 6024

3.2 Growth response of the isolated bacteria to 20mg/l of chlorpyrifos at optimum condition of 600nm, pH 6.5 and 30^{0}C for 28 days.

The result of different bacteria isolates response to 20mg/l chlorpyrifos at the interval of 4 days for 28 days shown in (figure 2) shows that *Bacillus cereus* ST06 and *Chryseobacteriumsp* 6024 chlorpyrifos had a mean OD of 0.55 and 0.67 for the period of 28 days respectively. The bacterial consortium also had mean OD of 0.47 for the same period. Also, from the graph *Bacillus cereus*ST06and*chryseobacteriumsp*6024 had maximum growth on day 16 with mean OD of 0.23 and 0.42 respectively. The bacteria consortium had maximum growth on day 20 with mean OD of 0.21. This means each bacterial responded to 20mg/l of chlorpyrifos differently. The ANOVA result revealed that *P*<.001 meaning that there is a significant difference in the mean. This confirms that the three isolates responded differently to 20 mg/L of chlorpyrifos.The bacterial consortium tolerated 20mg/l of CP most and *chryseobacteriumsp* 6024 tolerated the least. This suggests that the bacterial consortium may be more effective at bioremediation of chlorpyrifos-contaminated environments than *Bacillus cereus* ST06 or *Chryseobacteriumsp*6024 individually. The control also showed variable and nonlinear pattern in the decrease and increase in the OD value. This can be attributed to some biotic and abiotic factors that contribute to reaction in the control.

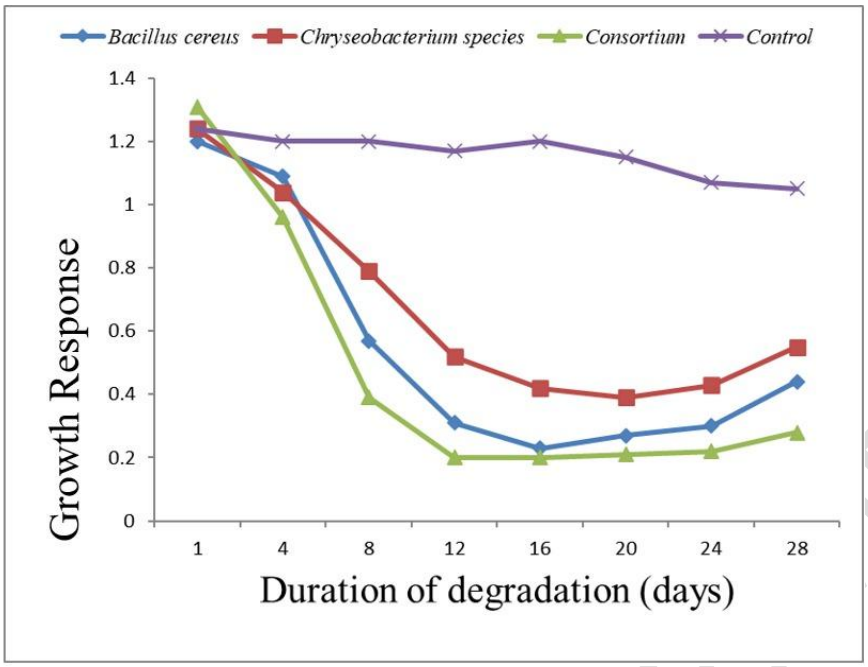

Figure 2: Growth Response of bacteria isolate on 20mg/l Chlorpyrifos.

3.3 Growth response of the isolated bacteria to 60mg/l of chloropyrifos at optimium condition of 600nm, pH 6.5 and 30^{0}C for 28 days.

For the result bacterial response to 60mg/l chlorpyrifos shown in (figure 3). *Bacillus cereus* ST06 and *Chryseobacteriumsp* 6024 reached maximum growth on 60mg/l chlorpyrifos with a mean OD of 0.47±0.02 and 0.81±0.02 respectively on day 20. The bacterial consortium also reached maximum growth on 60mg/l of chlorpyrifos with a mean OD of 0.29±0.02 on day 20 too. This means that as the concentration of chlorpyrifos increases the growth response of the bacteria to chlorpyrifos reduces. Generally, for both graphs there is a significant difference (*P*<.001) in the mean OD after 28 days implying that at different concentration of chlorpyrifos bacterial isolates behaviors differently.
The growth response to chlorpyrifos by *Bacillus cereus*ST06 decreased slowly with increase in chlorpyrifos concentration and metabolite accumulation.
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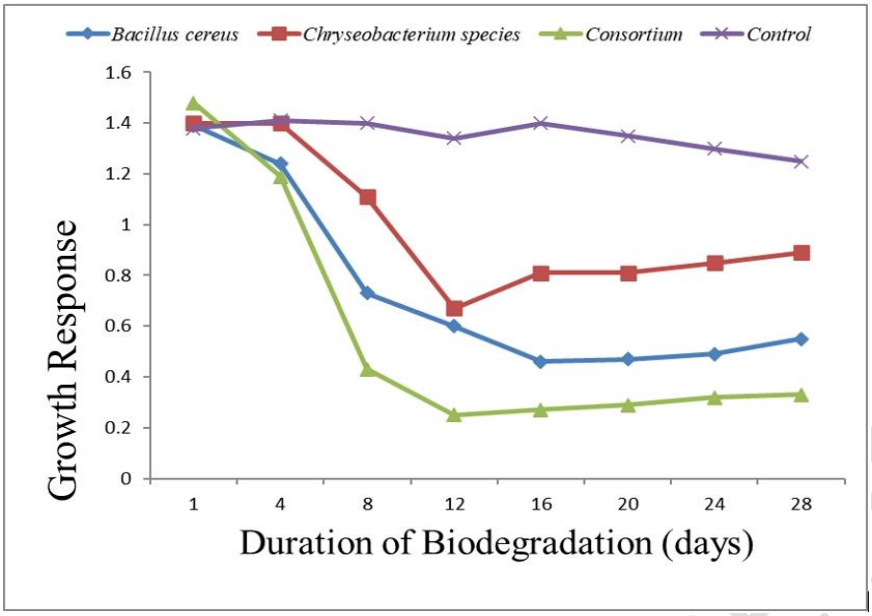

Figure 3: Growth Response of bacteria isolate on 60mg/L Chlorpyrifos

3.4 Determination of residual Chlorpyrifos after biodegradation and Percentage degradation

The result of residual Chlorpyrifos determination (table 2) shows that all treatments, including the control, resulted in a decrease in chlorpyrifos concentration. The chlorpyrifos-degrading bacteria isolated singly and in consortium has the ability to catabolize 20mg/L and 60mg/L of chlorpyrifos in mineral salts medium, in vitro. The two organisms showed variation in their ability to degrade 1ml volume of 20g/L and 3ml vol of 60mg/L Chlorpyrifos after 28 days. *B. cereus* ST06 reduced 20mg/L Chlorpyrifos to 7.41mg/L while *Chryseobacteriumsp*6024 reduced 20mg/L chlorpyrifos to 8.80mg/L. B. cereus reduced 60mg/L Chlorpyrifos to 23.40mg/L and *Chryseobacteriumsp* 6024 reduced 60mg/L chlorpyrifos to 37.82mg/L. The consortium showed highest reduction of both 20mg/L and 60mg/L at 4.20mg/L and 13.20mg/L respectively. This indicates that degradation occurred under the experimental conditions. The isolates *Bacillus cereus* ST06, *Chryseobacteriumsp* 6024, and the consortium showed higher degradation rates than the control. This suggests that these bacterial isolates have the potential to enhance chlorpyrifos degradation. The degradation rates were generally higher at the lower initial concentration of 20 mg/l compared to the higher initial concentration of 60 mg/l showing a significant different of (P<.001). The Control showed some degradation, likely due to abiotic factors or the activity of naturally occurring microorganisms. The rate of degradation as shown in (figure 4 and 5) shows that *Bacillus cereus*ST06and*chryseobacteriumsp*6024 showed 63% and 56% Chlorpyrifos degrading capacity at 20mg/l respectively, and 61% and 37% at 60mg/l respectively within a time period of 28days. The consortium showed 79% and 78% degradation of chlorpyrifos at 20mg/l and 60mg/l respectively. This means that chlorpyrifos degradation is concentration dependent. The Two-way ANOVA result shows it significantly different (P < .001) meaning that as chlorpyrifos concentration in liquid medium increases, the bacteria response or growth to it decreases.

Table 2: Residual chlorpyrifos concentration after degradation

S/n	Isolates	Residual Chlorpyrifos concentration			
		Initial conc.(mg/l)	Final conc.(mg/l)	Initial conc.(mg/l)	Final conc.(mg/l)
1	Bacillus cereus ST06	20.00	7.41[b]	60.00	23.40[b]
2	Chryseobacteriumspp 6024	20.00	8.80[c]	60.00	37.82[c]
3	Consortium	20.00	4.20[a]	60.00	13.20[a]
4	Control	20.00	13.46[d]	60.00	53.40[d]

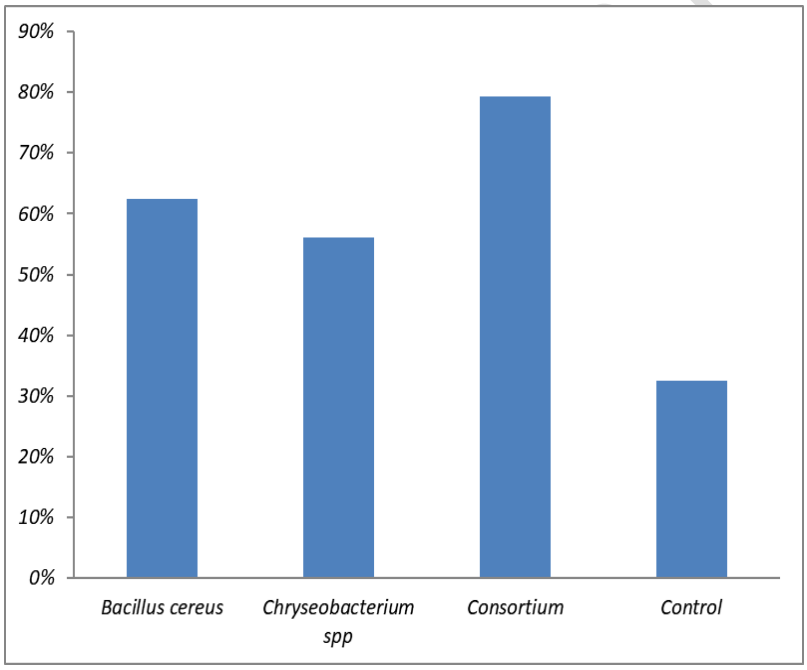

Figure 4: Percentage degradation ability of the isolate at 20mg/L Chlorpyrifos

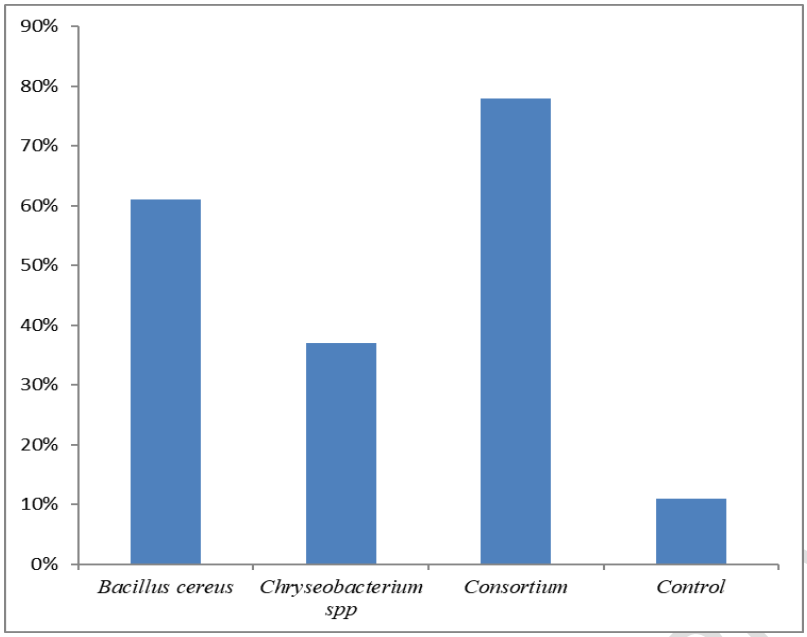

Figure 5:Percentage degradation ability of isolates at 60mg/L Chlorpyrifos

4 DISCUSSION

This study investigated the potential of two indigenous soil chlorpyrifos-degrading strains *Bacillus cereus* ST06 and *Chryseobacteriumsp* 6024, and their consortium in biodegrading of moderately harmful chlorpyrifos insecticide. In our previous research variety of microorganisms capable of degrading chlorpyrifos have been isolated from the environment and identified [16]. However, only *Bacillus cereus* ST06 and *Chryseobacteriumsp* 6024 was found to possess potential to degrade chlorpyrifos insecticide at certain range of chlorpyrifos concentration. Several studies investigated bacterial strains capable of degrading chlorpyrifos as shown in (table 3) and have isolated and characterized variety of bacteria from local environments for biodegradation applications. The research evaluates bacterial growth responses to chlorpyrifos in liquid medium at different concentrations, both singly and in a consortium. The research shows that *Bacillus cereus*ST06 slightly tolerated 20mg/l and 60mg/l chlorpyrifos and degraded about 63% and 61% chlorpyrifos in 28days at 30^{o}C and 6.5 pH as against previous research of [22] that isolated *Bacillus cereus* Ct3 that resisted up to 125 mg/l of chlorpyrifos and successfully degraded 88% of chlorpyrifos in 8 days at pH 8 and temperature of 30–40^{o}C. [23], successfully isolated another *Bacillus sp* that degraded 12 mg/l up to 79.5% in 35 days. [24] also reported *Bacilluspumilus* C2A1 resistant strain that tolerated 50mg/l chlorpyrifos and degraded up to 97% in 45 days. [25]isolated *B. cereus* CP6 and *Klebsiella pneumoniae* CP19 which was able to degrade above 70% chlorpyrifos at 200-300 mg/L initial concentrations. The ability of *Bacillus cereus* to degrade chlorpyrifos was also investigated under different culture conditions, such as pH, temperature, and chlorpyrifos concentration by [26]. The results shows that the optimum temperature, pH, concentration of chlorpyrifos degradation were 30^{o}C, 7.0, less than 100 mg/l respectively. Based on these results Different strains of *Bacillus sp* tolerated wide range of chlorpyrifos concentration. The possible reason for the differences in the level of tolerance can be attributed to the accumulation of intermediate metabolites in the medium inhibiting bacterial response to Chlorpyrifos, effect on lag phase and inoculum size [27,28,29].

Our research also showed that *Chryseobacteriumsp*6024 susceptible to Chlorpyrifos attack. There has been little or no involvement of *Chryseobacteriumsp*6024 in biodegradation of chlorpyrifos in the soil[30]. It was slightly susceptible to 20mg/l and 60mg/l concentration of chlorpyrifos. At initial concentration of 20mg/l chlorpyrifos, *Chryseobacteriumsp*6024 degrade 57% in 28days indicating slight potential to degrade chlorpyrifos. However, with increase in the concentration of chlorpyrifos to 60mg/l, the growth response reduced drastically to degrading 37%. This shows that the ability of *Chryseobacteriumsp*6024 to degrade chlorpyrifos is concentration dependent as revealed by the Two-way ANOVA result. The result shows that there is a significant difference in the growth response of bacterium to varying concentrations of chlorpyrifos. Research about *Chryseobacterium* participation in biodegradation insecticide was discovered by [31,32,33]. However, *chryseobacterium* was identified as organochlorine pesticides, poly lactic acid and glyphosate degraders respectively.*Chryseobacteriumsp.*Y16C degraded up to 400mg/l of glyphosate in 4days indicating potentials in degrading glyphosate[32]. All the results shows that *Chryseobacteriumsp*isindeednovel for biodegradation of chlorpyrifos implying the importance of further research.

Table 3: Degradation of other chlorpyrifod degraders

Microorganisms	Initial concentration (mg/l)	Rate of degradation(%)	Time (days)	references
Cupriavidusnantongensis X1T	200	100	2	[34]
Bacillus cereus ST06	20	63	28	This study
B. cereus ST06	60	61	28	This study
*Chryseobacteriumsp*6024	20	56	28	This study
C. sp 6024	60	37	28	This study
*Consortia (B.cerus*ST06 *and C. sp*6024*)*	20	79	28	This study
*Consortia (B.cerus*ST06 *and C. sp*6024*)*	60	78	28	This study
Pseudomonas putida T7, Pseudomonas aeruginosa M2, Klebsiella pneumoniae M6 and *Aspergillussp*	500	100	30	[35]
Alcaligenes faecalis	100	98.6	20	[36]
Pseudomonas aeruginosa DKC2	100	71	2	[37]
Bacillus cereus Ct3	125	88	8	[22]
Staphylococcus aureus	50	82.06	14	[38]
Kocuriakristinae	50	30.78	14	[38]

A corresponding decrease in utilization of chlorpyrifos by the isolates individually and in consortium was noticed from 16 days (figure 2 and 3). This could be as a result of some environmental factors which is the release and accumulation of 3,5,6-trichloro-2-pyridinol (TCP) an intermediate of chlorpyrifos degradation into the liquid medium, therefore making it resistant to microbial attack. This agrees with the work of [39] that TCP demonstrated antimicrobial effect againstthe bacteria. Also, the work of [40] confirmed that TCP limited its biodegradation and chlorpyrifos biodegradation by microorganisms. This study discovered that the consortium of the two bacterial strains enhanced chlorpyrifos degradation compared to individual isolates.The medium containing the bacterial consortium at 20 mg/l and 60mg/l Chlorpyrifos were significantly (P<.001) higher than the medium with individual isolates with the maximum growth of 0.29 (OD at 600nm) observed at day 20. The percentage utilization

by the bacterial consortium in the medium containing 20 mg/l and 60mg/l Chlorpyrifos were significantly (*P*<.001) higher than the medium with individual isolates with the maximum percentage utilization of 78% and 79% respectivelyafter 28 days of biodegradation.In a previous study by [35], bacterial consortia of *Pseudomonas. putida* T7, *Pseudomonas aeruginosa* M2, and *Klebsiella pneumoniae* M6, and *Aspergillus. terreus* TF1 showed the greatest potential in degrading chlorpyrifos in mineral salt medium, natural soil, and sterile soil up to100%. This means that developing bacterial consortia are better for insecticide degradation than individual isolates. The result of the study also showed that the concentration of uninoculated control was reduced from 20mg/l to13.40mg/l (33% reduction) and from 60mg/l to 53.40mg/l (11% reduction). This reduction could be traced to the fact that once Chlorpyrifos is in a reaction, it may be facedwithvolatilization and photodegradative conditions either directly or indirectly according to [41].

5. CONCLUSION

In the present study, two Chlorpyrifos- degrading bacteria were identified. They were *Bacillus cereus*ST06 and *Chryseobacteriumsp*6024.The biodegradation study of the bacterial isolates was done singly and as a consortium to determine the response to chlorpyrifos. The result showed that *Chryseobacteriumsp* 6024 is a novel bacterium in biodegradation studies with minimal potential to degrade Chlorpyrifos. *Bacillus cereus* ST06 can remove up to 63% and 61% of 20mg/l and 60mg/l chlorpyrifos from the liquid medium, indicating it can be employed to degrade chlorpyrifos. Result of this study also showed that the consortium of the isolates (*Bacillus cereus*ST06and*Chryseobacteriumsp*6024) can remove up to 79% and 78% of 20mg/l and 60mg/l Chlorpyrifos after 28 days better than the individual isolates; hence they can be used for the degradation of Chlorpyrifos for sustainable agriculture.

I recommend that further studiesshould focus on sequencing of chlorpyrifos degrading genes from*Bacillus cereus* ST06 and *Chryseobacteriumsp* 6024, gene profiling of both bacteria to know the downregulated and upregulated genes during biodegradation, in order to harness the use of the two bacterial isolates to properly degrade Chlorpyrifos and its intermediate TCP. It is also important to study the mechanism of action of the bacterial consortia to understand their roles in degrading chlorpyrifos.

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