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Isolation, Growth and Identification of Chlorpyrifos Degrading Bacteria from Agricultural Soil in Anambra State, Nigeria

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Abstract The extensive use of pesticides is one of the major causes of pollution of soil and water environments. The current method for removing such contaminants from the environment through biodegradation has been shown to be more effective than any other method. Three pesticide degrading bacteria were isolated and identified through cultural and biochemical tests as strains of *Pseudomonas aeruginosa, Serretia marcescens* and *Klebsiella oxytoca*. Their growth in mineral salt medium supplemented with 20mg/l of Chlorpyrifos was monitored at optical density of 600nm. The result showed that *Pseudomonas aeruginosa* had maximum growth in ten days, while *Serretia marcescens* and *Klebsiella oxytoca* recorded highest growth after six days of incubation. HPLC analysis of the residual Chlorpyrifos after 14 days incubation showed that *Pseudomonas aeruginosa* was able to degrade 60% of the pesticide; *Klebsiella oxytoca* degraded 54%, while *Serretia marcescens* had 53% reduction of the pesticide concentration in the mineral salt medium. The results of this research indicated that the isolated bacteria can be used for bioremediation of Chlorpyrifos contaminated soil and water ecosystems.

Keywords Isolation, Bacteria, Biodegradation, Chlorpyrifos, Pollutants, Agricultural Soil

1. Introduction

Pesticide application on agricultural soil is now a common practice and is an important factor of integrated pest management (IPM) strategies[5]. Some of these pesticides persist in the soil to form pollutants which may occasionally lead to surface and groundwater contamination. One of such pesticides is Chlorpyrifos, a widely used organophosphate insecticide[3].

Chlorpyrifos is a broad-spectrum, moderately toxic insecticide that has been widely used in the prevention of both agricultural pests and urban public health pests[15]. It was introduced in 1965 by Dow Chemical Company India[1]. It has large blights on public health and environment resulting from its long residual period in soil and water[7]. The environmental fate of Chlorpyrifos has been studied extensively, and the reported half-life in soil varies from 10 to120 days, with 3,5,6-trichloro-2-pyridinol (TCP) as the major degradation product[11].The manufacture and formulation process of Chlorpyrifos also generate waste that contains the compound, and this has to be treated by physicochemical or biological means[3].

Bioremediation is an environmental clean-up technique involving the use of naturally occurring microorganisms in the decontamination process[17-19]. The bioremediation of organophosphorous pesticides by soil microorganisms has been reported by many workers[13]. Singh et al. (2004) isolated *Enterobacter* B-14, a strain which could degrade Chlorpyrifos. Yang *et al*. (2006) and Li *et al*. (2007) isolated *Stenotrophomonas* species and *Sphingomonas* species respectively, which could utilize Chlorpyrifos as the only source of carbon and phosphorous.

The first step in dealing with pollution caused by Chlorpyrifos is the isolation and screening of microbial species that can degrade the pesticide effectively.

The main objective of this study is to isolate and identify Chlorpyrifos utilizing bacteria from agricultural soil using an enrichment culture technique.

This research was aimed at isolating Chlorpyrifos degrading bacteria from contaminated soil, and determining their growth response in mineral salt medium supplemented with Chlorpyrifos.

2. Materials and Methods

2.1. Pesticide Used

Commercial grade insecticide Chlorpyrifos with 100mg/L concentration was obtained from Agricultural Development Programme (ADP) Awka, Anambra State Nigeria.

2.2. Media

The Mineral Salt Medium (MSM) used contain the following (in gram per litre): 2.4g of KH_2PO_4, 1.2g of K_2HPO_4, 0.5g of NH_4NO_3, 0.1g of $MgSO_4.7H_2O$, 0.02g of $Ca(NO_3)_2$, 0.005g of $Fe(SO_4)_3$. 1ml of trace metal solution[10]. Agar-agar, Urea agar, Nutrient agar, Simon Citrate agar, MacConkey agar, and Nutrient broth were also used during the isolation and identification of Chlorpyrifos degrading bacteria.

2.3. Sample Collection

The rice growing fields in Anaku, Omor, and Igbakwu towns in Ayamelum Local Government Area of Anambra State, Nigeria; which have ten years history of Chlorpyrifos use in pest control were selected for this study. In each of the three farms, soil samples were collected randomly from 12-15cm from four corners in 6-8m apart, and from the centre of the farms. The soil samples were thoroughly mixed; plant debris sorted out, and put into polyethylene bags. The samples were transferred immediately to the laboratory for analysis.

2.4. Isolation of Chlorpyrifos Degrading Bacteria

Chlorpyrifos degrading bacteria were isolated from the soil samples by the enrichment culture technique on mineral salt medium, using Chlorpyrifos as the sole source of carbon as described by Zhu *et al*., (2010). The enrichment preparation comprised of 10 mg/L Chlorpyrifos in 100 ml of the mineral salt medium in 250ml Erlenmeyer's flask. This was autoclaved at 121℃ for 15 mins before adding 5g of the soil sample. The flasks were incubated in a rotary shaker at 120 revolutions per minute (rpm) and 30℃ for 7 days. After incubation, 1ml of the culture was inoculated on Chlorpyrifos mineral salt agar, and the plates incubated at 30℃ for 48hours. The Chlorpyrifos mineral salt agar comprised of mineral salt medium, 1.5 % agar-agar, 1% Sodium Citrate, 0.5 % methanol (solvent carrier for pesticide), and 10mg/L Chlorpyrifos[14]. Three morphologically distinct bacterial growths (A, B and C) on the agar were selected and sub-cultured repeatedly until pure cultures were obtained. The pure cultures were stored in Chlorpyrifos mineral salt agar slant at 4℃ for further studies.

2.5. Identification of the Isolates

The three bacterial isolates grown on Chlorpyrifos agar were subjected to physiological and biochemical tests. The tests carried out include: Gram staining, catalase test, citrate utilization, oxidase test, indole production, motility, sugar fermentation, methyl-red test, nitrate reduction, starch hydrolysis, Voges-Proskeur test and hydrogen sulphide production. Identification was based on recommendations of Gerhard *et al*., (1981), and then using Bergey's Manual of Determinative Bacteriology for confirmation.

2.6. Growth of the Isolates and Biodegradation of Chlorpyrifos in Liquid Culture

The inoculum used for all the experiments was prepared by growing the bacterial isolates in separate 250ml Erlenmeyer flask containing 50ml of mineral salt medium (MSM) at 120 revolutions per minute (rpm) and 37℃ on a rotary shaker for 24 hours.1ml of the 24 hour culture containing approximately $1.1x10^4$ CFU/ml (determined by viable count method) was used as inoculum. This was used to inoculate 250ml flasks containing 100ml MSM and 20ml/L of Chlorpyrifos in triplicate. The un-inoculated flask was used as a control. The flasks were incubated in a rotary shaker at 120rpm and 30℃ for 14 days.

The growth (optical density) of the isolates were determined at intervals of 0, 2, 4, 6, 8, 10, 12, and 14 days; using Spectrophotometer (model PD 303 UV-VIS) at 600nm.

2.7. Chlorpyrifos Extraction and HPLC Analysis

After 14 days incubation, 5ml of the culture was taken from each flask and was placed in centrifuge tubes. This portion of the culture was extracted twice 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.

An Agilent FS720 High Performance Liquid Chromatography (HPLC), model SPD-10A, UV-VIS DETECTOR, equipped with tandem reverse phase cartridge column, (C-18) was used to analyse the residual Chlorpyrifos in the culture. The tanden reverse phase cartridge column was fitted at a temperature program of 170℃ for 30seconds at a flow rate of 0.8ml/min at 25℃. The retention time was 15seconds, while the detection limit was 20µg/L in aqueous phase for Chlorpyrifos.

The concentration of Chlorpyrifos pesticide was determined by removing 1ml of the filtrate and transferring into 15ml high performance liquid chromatography vial containing 60% methanol and 40% water (3:2) and analysed by HPLC. Calibration curves from 0 to 100mg/L were made for the Chlorpyrifos. The extract peaks appearing in the chromatogram were identified with the help of already standardized curve and the concentration pesticide residue determined.

3. Results

3.1. Identification of the Isolates

Three morphologically distinguishable bacterial colonies were observed on the mineral salt agar containing Chlorpyrifos pesticide. The results of morphological, cultural and biochemical tests carried out are shown in Table 1. The three isolates were identified as *Pseudomonas aeruginosa, Serretia marcescens* and *Klebsiella oxytoca*.

3.2. Growth of the Isolates and Biodegradation of Chlorpyrifos in Liquid Culture

The result of the growth response of the isolates in the presence of Chlorpyrifos showed that all the isolates utilized the insecticide as the only carbon and energy source. *Pseudomonas aeruginosa* showed maximum growth in 10 days, while *Klebsiella oxytoca* and *Serretia marcescens* recorded highest growth in 6 days incubation (Figure 1a-c).

Table 1. Morphological, Cultural and Biochemical Characteristics of the Isolates.

Tests	Isolate 1	Isolate 2	Isolate 3
Colony Morphology	Circular, Smooth Whitish, Entire, Convex, Translucent looking colonies	Circular, Dry, Cream Undulate, Flat, Opaque looking colonies	Circular, Moist, Cream, Swarming, Slighlty raised, Opaque looking colonies
Gram's Reaction	– /short rods	– /short rods	– /short rods
Methyl red	–	–	–
Voges-Proskauer	–	+	–
Indole	–	+	+
Motility	+	–	+
Citrate	+	+	+
Oxidase	+	–	–
Nitrate	+	+	–
Starch	–	–	–
Urease	–	+	–
H_2S	–	–	–
Growth on MacConkey	+ /NLF	+/LF	+/NLF
Glucose Fermentation	–	A/G	A/G
Sucrose	–	A/G	A/G
Maltose	–	A	A
Mannitol	–	A	A
Lactose	–	+	–
Fructose	+	–	–
NaCl (2-5%)	+	+	+
Identity	***Pseudomonas aeruginosa***	***Serretia marcescens***	***Klebsiella oxytoca.***

Key: A/G= Acid/Gas,
NLF= Non lactose fermenter,
LF= Lactose fermenter

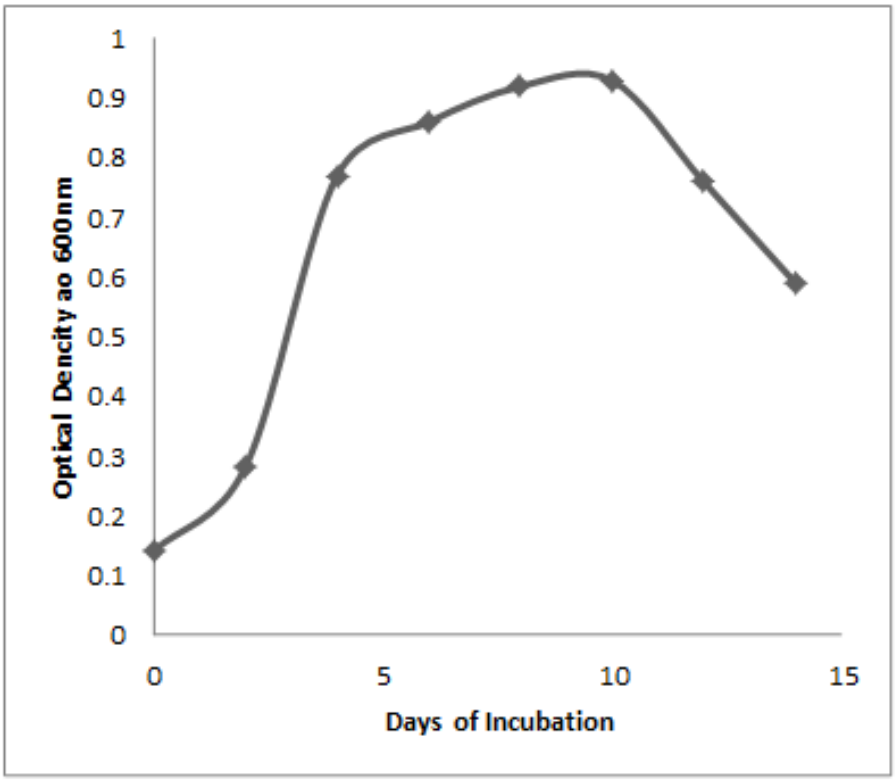

Figure 1a. Growth response of *Pseudomonas aeruginosa* in the presence of Chlorpyrifos pesticide.

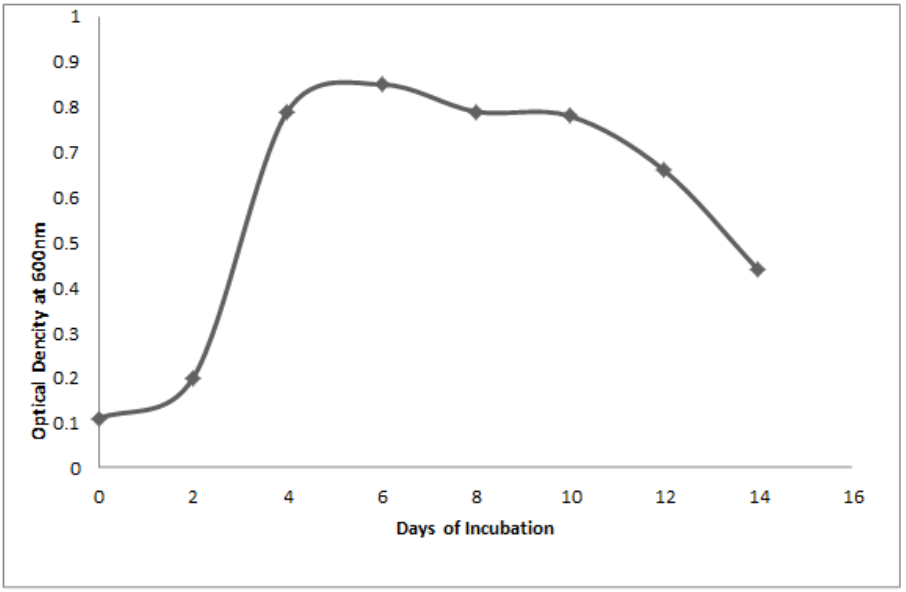

Figure 1b. Growth response of *Klebsiella oxytoca* in the Presence of Chlorpyrifos Pesticide

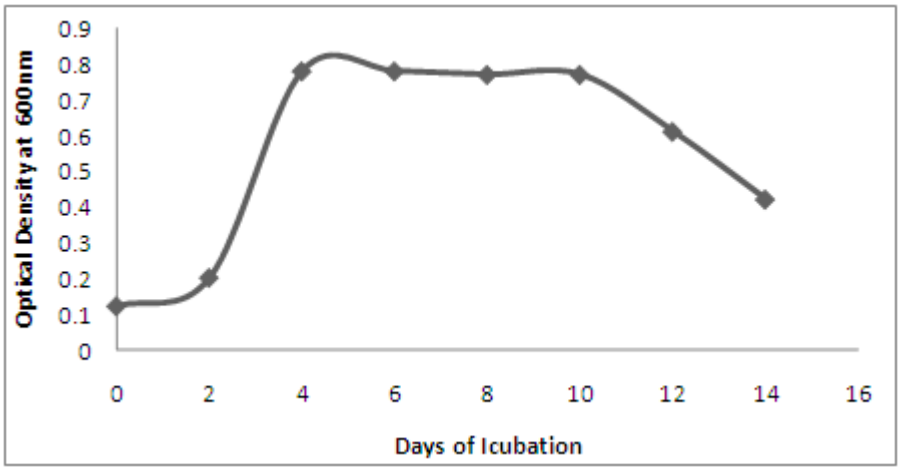

Figure 1c. Growth response of *Serretia marcescens* in the Presence of Chlorpyrifos Pesticide

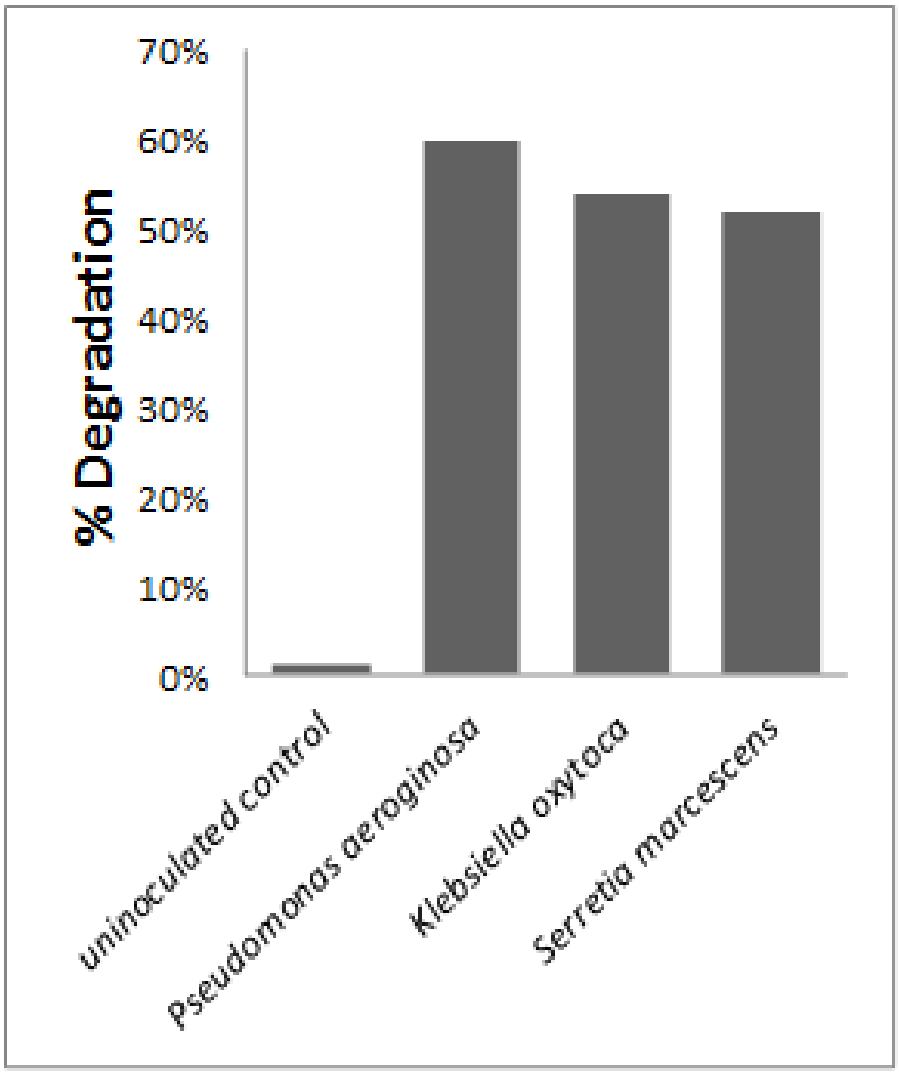

Figure 2. Percentage degradation of Chlorpyrifos by the isolates after 14days incubation period

4. Discussion

In this study, three different bacteria isolated from agricultural soil, are species whose actions were reflected in significant pesticide depletion. All of the isolates are Gram negative bacteria, two of them belong to the family *Enterobacteriaceae*. The result obtained in this study were in agreement with earlier reports that indicated the involvement of different species of Gram negative bacteria, especially the members of *Enterobacteriaceae* in the degradation of organophosphorous insecticides like Chlorpyrifos[12]. Many pure and mixed Chlorpyrifos transforming cultures have been isolated from a variety of sources, and majority of them are cultures of Gram negative organisms[14]. Bioremediation of Chlorpyrifos by *Pseudomonas aeruginosa* using scale up technique was earlier reported by Fulekar and Geetha (2008). It was previously reported by Fulekar (2008) that *Pseudomonas aeruginosa* was the most common Gram negative bacterium found in soil and this bacterium has been found to have the potential to degrade Chlorpyrifos. Rani et al., (2008) isolated *Serratia sp, Klebsiella sp, Providencia sp* and *Bacillus sp* implicated in the degradation of Chlorpyrifos. Munazza *et al*., (2005) and Furham *et al* (2013) also isolated *Klebsiella sp* capable of degrading Chlorpyrifos from soil.

Among the three bacteria isolates, *Pseudomonas aeruginosa* showed the highest Chlorpyrifos degrading capacity (60% reduction). This further affirms the claim made by Fulekar (2008); that *Pseudomonas aeruginosa* is the most Gram negative bacteria in the soil with Chlorpyrifos degrading potential. The result of the study showed that the concentration of uninoculated control was reduced from 20mg/l to19.80mg/l (1% reduction). 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[9]. The decrease might also be as a result of volatilization exhibited by pesticides.

5. Conclusions

In the present study, three isolates capable of utilizing Chlorpyrifos as the only source of carbon and energy were identified. Biodegradation of pesticides by microorganisms is an effective means of preventing environmental pollution. Result of this study showed that the isolates (*Pseudomonas aeruginosa, Klebsiella oxytoca* and *Serretia marcescens*) can remove up to 50% Chlorpyrifos from the medium; hence they may be used for bioremediation of Chlorpyrifos contaminated soil, water or industrial effluents.

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