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

Ifediegwu, M.C.1, Agu, K.C.1*, Awah, N.S.1, Mbachu, A.E.1, Okeke, C.B.1, Anaukwu, C.G.1, Uba, P.O.1, Ngenegbo, U.C.2, Nwankwo, C.M.3

1Department of Applied Microbiology and Brewing, Faculty of Biosciences, Nnamdi Azikiwe University, Nigeria
2Department of Parasitology and Entomology, Faculty of Biosciences, Nnamdi Azikiwe University, Nigeria
3Department of Science Laboratory Technology, School of Applied Science and Technology, Federal Polytechnic Oko, Nigeria

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) strategies5. 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 insecticide3.
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$_2$PO$_4$, 1.2g of K$_2$HPO$_4$, 0.5g of NH$_4$NO$_3$, 0.1g of MgSO$_4$7H$_2$O, 0.02g of Ca(NO$_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$^\circ$C 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$^\circ$C for 24 hours. 1ml of the 24 hour culture containing approximately 1.1x10$^6$ 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$^\circ$C 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.

2.5. 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-Proskauer 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$^\circ$C on a rotary shaker for 24 hours. 1ml of the 24 hour culture containing approximately 1.1x10$^6$ 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$^\circ$C for 14 days.

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, Slightly raised, Opaque looking colonies |
| Gram’s Reaction                | – /short rods                                                             | – /short rods                                                             | – /short rods                                                             |
| Methyl red                     | –                                                                         | –                                                                         | –                                                                         |
| Voges-Proskauer                | –                                                                         | +                                                                         | –                                                                         |
| Indole                         | –                                                                         | +                                                                         | +                                                                         |
| Motility                       | +                                                                         | –                                                                         | +                                                                         |
| Citrate                        | +                                                                         | +                                                                         | +                                                                         |
| Oxidase                        | +                                                                         | –                                                                         | –                                                                         |
| Nitrate                        | +                                                                         | +                                                                         | –                                                                         |
| Starch                         | –                                                                         | –                                                                         | –                                                                         |
| Urease                         | –                                                                         | +                                                                         | –                                                                         |
| H₂S                            | –                                                                         | –                                                                         | –                                                                         |
| 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 14 days 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. 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. 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 found to have the potential to degrade Chlorpyrifos. Rani et al., (2008) isolated Serratia sp, Klebsiella sp, Providencia sp found to have the potential to degrade Chlorpyrifos. 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 to 19.80mg/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. 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 Serratia 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.

REFERENCES

[1] Eiser, R (2000). Handbook of Chemical Risk Assessment, Health Hazards to Humans, Plantand Animals 2: Organics Lewis Publishers, Washington D.C. p 883-902.
[2] Fulekar, M.H. (2008). UGC Major Research Project “Development of Bioremediation Technology for Pesticide Industrial Wastes using Novel Cow Dung Microorganisms in Sequence Biological Reactor and Two Phase Partitioning Bioreactor”
[3] Fulekar, M.H. and Geetha, M. (2008).Bioremediation of Chlorpyrifos by Pseudomonas aeruginosa using scale up technique. Journal of Applied Bioscience, 12: 657-660.
[4] Furhan, M., Khan, A.U.; Wahid, A.; Ali, A.S. and Ahmed, F.(2013). Potential of Indigenous Klebsiella sp for Chlorpyrifos biodegradation. Pakistan Journal Science 65(1) 133-138.
[5] Gilani, S.T.S.; Ageen, M.; Shah. H. and Raza, S. (2010). Chlorpyrifos degradation in soil and its effect on soil microorganisms. Journal of Animal and Plant Sciences, 20(2) 99-102.
[6] Li, X.; He, J.; and Li, S. (2007). Isolation of Chlorpyrifos Degrading Bacterium, Shengomonas sp, strain DSP-2 and Cloning of the MPD Gene. Research Microbiology, 158: 143-149.
[7] Mohan, S.V.; Sirisha, K. and Rao, N.C. (2004). Degradation of Chlorpyrifos contaminated soil by Bioshurry Reactor operated in Sequencing Batch Mode. Bioprocess Monitoring. Journal of Hazard Matter, 116:39-48.
[8] Munazza, A.; Nusrat, J.; Shahida, A. and Sheikh, A.R. (2005). Chlorpyrifos resistant bacteria from Pakistani soils: Isolation, Identification, Resistance profile and Growth Kinetics. Pak. J. Bot. 37(2): 382-388.
[9] Racke, K.D. (1993). Environmental fate of Chlorpyrifos. Review in Environmental Contamination and Toxicology. 131:1-150.
[10] Rani, S.M.; Lakshm, K.V.; Devi, S.P.; Madiian, F. and Aruna, K. (2008). Isolation and Characterization of Chlorpyrifos Degrading Bacteria from Agricultural Soil and its Growth Response. African Journal of Microbiology 2:2-31.
[11] Singh, B.K.; Walker, A.; Morgan, A.J. and Wright, D.J. (2003). Effects of soil pH on the Biodegrading of Bacterium. Applied Environmental Microbiology, 9: 5198-5206.
[12] Singh, B.K.; Walker, A.; Morgan, A.J. and Wright, D.J. (2004). Microbial Degradation of Chlorpyrifos by Enterobacter strain B-14 and its use in Bioremediation of Contaminated soil. Applied Environmental Microbiology, 70: 4855-4863.
[13] Sumit, K. (2004).Bioremediation of Chlorpyrifos by Bacteria Isolated from the Cultivated Soils. International Journal of Pharms and Biosciences, 2: 359-366.
[14] Topp, E.; Zhu, H.; Nour, s.m.; Houost, S.; Lewis,M. and Cuppels, D.(2000). Characterisation of an Atrazine Degrading Pseudaminobacter sp. isolated from Canadian and French soil. Applied Environ. Microbial, 66(7): 2773-2782
[15] Yang,C.; Liu, N.; Guo, X. and Qiao, C. (2006). Cloning of mpd gene from a Chlorpyrifos degrading bacterium and use of this strain in bioremediation of contaminated soil. FEMS Microbiology Letter 265: 118-125.
[16] Zhu, J.; Zheo, Y. and Qui, J. (2010). Isolation and Application of a Chlorpyrifos degrading Bacillus licheniformis ZHU-1. African Journal of Microbiology Research, 4(24): 2716-2719.
[17] Mbachu, A.E., Onochie, C.C. Agu, K.C. Okafor O.I. and Awah N.S. (2014). Hydrocarbon Degrading Potentials Of Indigenous Bacteria Isolated From Auto-Mechanic Workshops at Mgbuka-Nkpor, Nigeria. *Journal of Global Biosciences*, 3(1): 321-326

[18] Agu, K.C., Ogbue, M.O. Abuchi, H.U. Onunkwo, A.U. Chidi-Onuorah, L.C. and Awah, N.S. (2013). Lipase Production by Fungal Isolates from Palm Oil-Contaminated Soil in Awka Anambra State, Nigeria. *International Journal of Agriculture and Biosciences*, 2(6): 386-390.

[19] Agu, K.C., Orji, M.U., Onuorah, S.C., Egurefa, S.O., Anaukwu, C.G., Okafor, U.C., Awah, N.S., Okafor, O.I., Mbachu, A.E. and Anyaegbunam, B.C. (2014). Influence of Solid Waste Dumps Leachate on Bacteriological and Heavy Metals Contamination of Ground Water in Awka. *American Journal of Life Science Researches*, 2 (4): 450-457.