

Original Research Article

Isolation, Characterization and Evaluation of *Pseudomonas Aeruginosa* in the Biodegradation of Propanil Contaminated Rice Farms

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Abstract: Propanil are herbicide commonly used on rice farm for the control of weeds and pests. Like other herbicides, tendencies to persists in the soil and impact on the microbial flora of rice farm soils does exists although study finds that propanil is not recalcitrant. This work aimed to isolate, characterize, and evaluate *Pseudomonas aeruginosa* from Enugu propanil contaminated rice farm. A total of nine [9], contaminated soil samples {three each from Nenwe, Ugbawka and Akpuoga-Nike} were collected and analyzed for presence of *P.aeruginosa*. The average total count were 3.8×10^6 [Ugbawka], 3.5×10^6 (Nenwe) and 2.8×10^6 (Akpuoga-Nike). Out of nine samples collected, 7 samples were positive for *P.aeruginosa*. Nine soil samples yielded 13 *Pseudomonas aeruginosa* isolates. Out of 13 *P.aeruginosa* isolated 4(30.8%) from Nenwe, 6(46.1%) from Ugbawka and 3(23.1%) from Akpuoga –Nike soil samples. *P.aeruginosa* were more in Ugbawka followed by Nenwe. The ability to utilize propanil was tested with other isolates based on their growth. It was observed that *Pseudomonas aeruginosa* and mixed culture showed heavy growth in mineral salt propanil media than in the media without propanil. The results of molecular identification revealed that *Pseudomonas aeruginosa* from two different strains utilized propanil heavily than other isolates. The result of the varying concentration of propanil showed that *Pseudomonas aeruginosa* (S1) had increased growth with increase in concentration of propanil from 0.8 at 1ml to 1.2 at 2ml. S2 showed 0.3 at 1ml to 1.0 at 2ml and mixed culture showed 0.6 at 1ml to 0.9 at 2ml. The result showed that *P.aeruginosa* is an efficient propanil degrader. Application of *P.aeruginosa* through fertilizer or soil amendment could be used to manage propanil pollution in the agricultural system.

Keywords: Rice, Propanil, *Pseudomonas Aeruginosa*, Contamination, Soil, Biodegradation, Agriculture.

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INTRODUCTION

Rice farming plays a pivotal role in global food security, yet agricultural practices often involve the use of chemical pesticides to combat pests and weeds which could lead to environmental contamination (Ogah and Nwile 2017). Propanil is widely used herbicide in rice cultivation; it has been identified as a significant environmental pollutant due to its persistence and potential adverse effects on ecosystem (procopio *et al.*, 2012). In propanil contaminated rice farms, microbial communities including bacteria such as *Pseudomonas aeruginosa*, play crucial roles in biodegradation processes (Herrera-gonzalez *et al.*, 2013).

Pseudomonas aeruginosa, a versatile gram negative bacterium known for its metabolic diversity and adaptive capabilities has been implicated in various bioremediation contexts, including the degradation of organic pollutant (Oanh *et al.*, 2020). Understanding its role in propanil contaminated environments is essential for accessing its potential impact on agricultural eco systems and exploring its biotechnological applications (Marin- Bruzos *et al.*, 2021). The biodegradation of toxic compound has been investigated using variety of microbial strains; *Acinetobacter calcoaceticus*, *Rhodococcus* sp, *Pseudomonas* sp, and *Xanthomonas* sp. while *Kokuria* strain grew on 3, 4,-DCA but not on propanil (Herrera-gonzalez *et al.*, 2013). This study

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focuses on the evaluation and characterization of *Pseudomonas aeruginosa* in propanil contaminated rice farms. It discussed the methods employed for isolation, identification and physiological studies of *P. aeruginosa* strains in such environment. Culture and microscopy remain the primary laboratory tools for bacteria detection (Lahlali *et al.*, 2022).

Furthermore it explored the metabolic activities involved in propanil degradation by *P. aeruginosa*, shedding light on the biochemical mechanisms underlying its environmental adaptation (Sharma *et al.*, 2014).

This study aims to contribute to a comprehensive understanding of *Pseudomonas aeruginosa* ecological role in propanil contaminated rice farms from Enugu, Nigeria. It's potential as a biocontrol agent or bioremediation tool for developing sustainable agricultural practices that mitigate pesticide pollution while preserving soil health and crop productivity.

MATERIALS AND METHODS

Sample Collection

Soil samples were collected from three experimental farms (Ugbawka, Nenwe and Akpoga-Nike) contaminated with propanil. Each selected rice farm was divided into five portions to ensure representative sampling from various areas within the farm. Triplicate soil cores were obtained from each portion from 0-15cm depth which were pooled, sieved, homogenized and transferred to microbiology laboratory, Enugu State University of Science and Technology using polyethylene bags.

Media used are nutrient Agar, Macconkey Agar and Minarel Salt Media.

Isolation of Propanil Degrading Organisms

One gram (1g) of each sample was weighed into an Erlenmeyer flask and 9ml of distilled water was mixed with the sample. This was placed on a laboratory shaker (S 150) for 3 hours to homogenize the solution and used as the stock solution. Ten fold serial dilutions were performed. Six test tubes containing 9ml of the distilled water were used for serial dilution. Inoculums from 10^{-4} fold dilution was inoculated on macConkey Agar medium by streak plate technique and incubated at 35°C for 48 hours.

After incubation, different colonies were subcultured on fresh macConkey agar media to get a pure culture. The number of colonies of bacteria grown for each sample were counted using colony counter (LT-37 Labtronics, India).

Calculation of Bacteria Plate Count

Colony forming unit (CFU/ml) = Number of colonies x reciprocal of dilution.

Gram Staining of the Isolates

The bacteria isolate were gram stained to know the Gram reaction.

Gram staining reagent: Crystal violet solution, lugol's iodine solution, ethanol and safranin.

A glass slide was prepared and a smear of microbial growth was made. The smear was air dried and heat fixed to the slide. Crystal violet solution was applied to the smear and allowed to stand for at least one minute. The slide was rinsed with water and iodine solution was poured over the slide and allowed to stand for 30 seconds. The slide was differentiated with ethanol solution and counter stained with safranin. The slide was gently rinsed with water, blotted dried and observed under a microscope using oil immersion objective lenses.

Biochemical Tests for Identification

These biochemical tests were conducted to identify the isolated microorganisms based on their metabolic characteristics.

Catalase Tests

A small amount of the microbial colony was placed onto a clean glass slide. A drop of hydrogen peroxide was added to the sample. Bubbles were observed for the production of catalase activity. Emission of bubbles indicated a positive reaction while an absence of bubble indicated a negative reaction.

Oxidase Test

A small amount of the microbial colony was placed onto a filter paper. A drop of oxidase reagent was added to the colony. The development of a color change, dark- blue or purple was observed within some time.

Citrate Utilization

One loopful of the isolate was inoculated into simmon's citrate agar containing two drops of bromothymol blue indicator and incubated at 37°C for 24 hours. Growth with blue colour indicated positive test while negative result has no change in colour. Positive result is to show that the organism utilizes citrate as carbon source.

Gas Production

One (1) loopful of the isolate was inoculated into each 50ml culture containing sterile peptone water broth with 10% D-glucose. Two drops of phenol red indicator solution were added into the broth and an inverted Durham tube was inserted in the culture tube. The broth was incubated for 24 hours at 37°C .

Acid production was indicated by the change of yellow colour to red while the presence of gas was shown by appearance of gas bubble in the Durham tube. Negative result showed no change in colour or appearance of gas.

Indole Test

Two percent weight per volume (2% w/v) peptone water (2.0g of peptone powder in 100ml of distilled water) was prepared and 10ml each were measured into sterilized bijou bottles that were incubated with test isolates. Unincubated bottle served as a control.

The inoculated bottles were incubated at 37°C for 48 hours after which 0.5ml of Kovacs' reagent and concentrated hydrochloric acid was added to the content of each bottle. The bottles were shaken and examined for a red colouration in the surface layer within 10 minutes to allow the reagent to rise to the top. Presence of indole was indicated by a deep red colour at the reagent layer and the negative result was indicated by no visible reaction.

Preliminary Test for Propanil Utilization by the Isolates

A 99ml of mineral salt media was prepared with the addition of 1ml of propanil. The mineral salt media contained the following nutrients: NaNO₃ -0.1g, KH₂PO₄ 0.05, CaCl₂·2H₂O-0.001g and MgSO₄·7H₂O-0.02g.

A control medium, mineral salt media was prepared without propanil. The isolates were inoculated singly and as mixed culture in the mineral salt propanil media and in the control media. The isolates were incubated at 37°C for 24-48 hours. The utilization of propanil was observed based on the growth of the isolate on the culture media plates.

Effect of Varying Concentration of Propanil on the Growth of Monocultures and Mixed Cultures

Each bacteria isolates were incubated into different test tubes containing 10ml of nutrient broth and incubated for 24 hours. A 0.1ml of aliquot 10⁻⁴ dilution factor was collected from each culture tube and placed into different 250ml Erlene Meyer flasks containing the following mixtures: 99ml of MSM with 1ml of propanil, 98.5ml of MSM with 1.5ml of propanil and 98ml of MSM with 2mls of propanil individually (monoculture). For mixed culture, all the bacteria isolates were inoculated into one test tube containing 10ml of nutrient broth and incubation for 24 hours. A 0.1ml aliquot of 10⁻⁴ dilution factor was collected from the culture tube and placed into a 250ml Erlene Meyer flask containing 99ml of MSM with 1ml of propanil, 98.5ml of MSM with 1.5ml of propanil and 98ml of MSM with 2mls of propanil.

Both the monocultures and mixed cultured flasks were incubated at 150rpm in a rotary shaker for 14 days at 30°C. The temperature and pH values were taken

then the optical densities of the medium were checked at 660nm.

Molecular Identification of Propanil Degrading Bacteria Isolates

The molecular identification was done in three stages DNA Extraction, PCR and sequencing. The isolates were confirmed at bioinformatics services Ibadan, Nigeria. DNA extraction was done using ZR bacterial DNA miniprep (manufactured by Zymo Research). After the DNA extraction, the electrophoresis for DNA and PCR were done to visualize and solidify DNA under UV light.

16S rRNA gene amplification of the bacterial isolates were carried out. Sequencing of the implied fragments were done with a genetic analyzer 3130x1 sequencer from Applied Biosystems using manufacturer's manual. The sequencing kit used was that of Big Dye Terminator V3.1 cycle sequencing kit.

RESULT

A total of nine propanil contaminated soil samples (three each from Nenwe, Ugbawka, and Akpoga-Nike) were collected.

Table one showed that *Bacillus sp*, *Pseudomonas sp* and *E. coli* were isolated. The average total of *P.aeruginosa* count in the tested soil samples were 3.5x10⁶cfu/ml, 3.8x10⁶ cfu/ml and 2.8x10⁶ in Nenwe, Ugbawka and Akpoga-Nike soil samples as shown in (Table 2). Table 3 showed the total number of samples and the number of occurrence of *P.aeruginosa* in the contaminated soil. Out of the total 9 samples, 7 samples were positive for *P.aeruginosa*. Table 4 showed total of 13 of *P.aeruginosa* obtained from 9 soil samples and their percentage occurrence indicating 4(30.8) from Nenwe, 6(46.1) from Ugbawka and 3(23) from Akpoga-Nike.

Following Table 5 it was observed that *Pseudomonas sp* grew heavily on mineral salt propanil media but do not grow on mineral salt media without propanil. There was also heavy growth on mixed culture while *Bacillus sp* and *E. coli* had slight growth. Those isolates that had heavy growth utilized propanil more than any other organisms. Molecular identification using 16srDNA typing revealed that *Pseudomonas aeruginosa* from two different strains were the only bacteria that utilized propanil heavily. Table 6 showed that *P.aeruginosa* (S1) had increased growth with increase in concentration of propanil from 0.8 at 1ml to 1.2 at 2ml. *P.aeruginosa* (S2) also showed increased growth more significantly from 0.3 at 1ml to 1.0 at 2ml while mixed culture showed moderate growth from 0.6 at 1ml to 0.9 at 2ml.

Table 1: Biochemical Characteristics of Bacteria Isolated from Contaminated Soil Samples

	Cultural Characteristics	Gram Stain	Citrate	Catalase	Oxidase	Indole	Suspected Organisms
1.	Large gray white colonies	+ ve in chains	+	+	+	-	<i>Bacillus</i> spp
2.	Green colonies	- ve rods	+	+	+	+	<i>Pseudomonas</i> spp
3.	Mucoid slightly raised colony	- ve rods	-	+	-	+	<i>E.coli</i>

Keys + Positive
- Negative

Table 2: Mean Total of Bacteria Counts in Propanil Contaminated Soil

Rice Soil Sample	Mean Total (cfu/ml)
Nenwe	3.5 x 10 ⁶ cfu/ml
Ugbawka	3.8 x 10 ⁶ cfu/ml
Akpoga-Nike	2.8 x 10 ⁶ cfu/ml

Table 3: Incidence of Bacterial Propanil Contaminated Soil

Sample	No. of Samples	No. of Occurrence	% of Occurrence
Nenwe	3	2	67
Ugbawka	3	3	100
Akpoga-Nike	3	2	67
Total	9	7	77.8

Table 4: Frequency of *P.aeruginosa* from Propanil Contaminated Soil

Farm	Occurrence	% Occurrence
Nenwe	4	30.8
Ugbawka	6	46.1
Akpoga-Nike	3	23.1
Total	13	100

Table 5: Preliminary Test of Isolates of Propanil Utilization

Mineral Salt Media Without Propanil	Mineral Salt Medium with Propanil	Level of Utilization	Suspected Organisms
++	+	Slight	<i>Bacillus</i> spp
-	+++	Heavy	<i>Pseudomonas</i> spp
-	+++	Heavy	<i>Pseudomonas</i> spp
+++	+	Slight	<i>E.coli</i>
+	+++	Heavy	Mixed culture

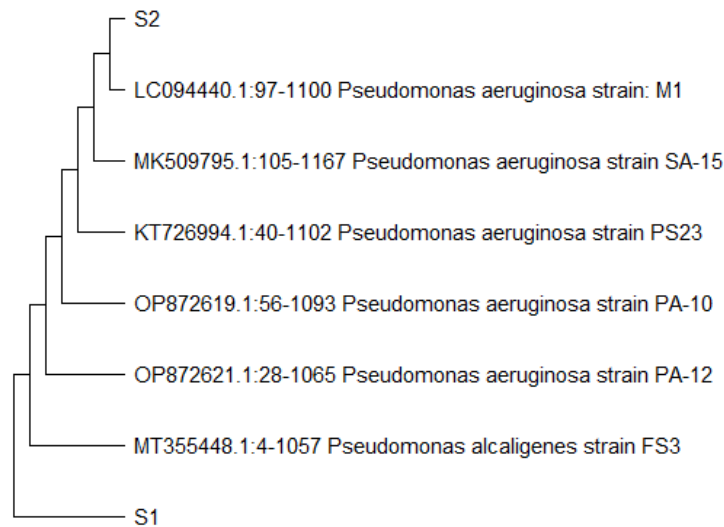
Key - No growth, no utilization
+ Slight growth
++ Moderate growth
+++ Heavy growth

Table 6: Effect of Varying Concentration of Propanil on the Growth of Monoculture and Mixed Culture

Propanil Concentration (ml)	S1 <i>P.aeruginosa</i>	S2 <i>P.aeruginosa</i>	S C mixed culture
1	0.8	0.3	0.6
1.5	1.0	0.6	0.7
2	1.2	1.0	0.9



Gel image of high molecular weight DNA extracted from the bacteria isolates



DISCUSSION

This study showed that *Pseudomonas aeruginosa* was present in soil samples from rice farm. Samples contaminated with *Pseudomonas aeruginosa* (77.8%) which is relatively the same with other studies (procopio *et al.*, 2012). According to procopio *et al.*, (2012) *Pseudomonas* species had more capability of growing in medium contaminated with propanil when compared with *serratia* and *Acinetobacter* specie. Onwona-kwakyee *et al.*, (2020) reported *Pseudomonas aeruginosa* as the most abundant genus with a series of catabolic pathways and enzymes in pesticide degradation. *Pseudomonas* species are group of bacteria present in large amount in the soil that have major role in mineralization of organic matter. This study falls in line

with Table 2 and percentage of occurrence in Table 3 within the study area. Nenwe had 2(67%), Ugbawka 3(100%) and Akpoga-Nike had 2(67%) of occurrence. Among the different types of soil samples, *Pseudomonas aeruginosa* strain were more prevalent in Ugbawka and Nenwe soil sample than the Akpoga-Nike (Table 4) due to contamination caused by use of herbicide in the soil (Sharma *et al.*, 2014). As shown in Table 5 *Pseudomonas aeruginosa* and mixed culture showed heavy utilization by their growth when compared with growth in *Bacillus* species and *E.coli*. According to Procopio *et al.*, (2012) *Pseudomonas* strains had the highest degradation activity of propanil with other strains. Most of the *Pseudomonas* strain improved growth of rice, produce inoleactic acid, soluble mineral phosphate and fixed nitrogen. Sinong *et*

al., (2021) performed a high throughput amplicon analysis demonstrating a higher abundance and greater diversity of root microbiome under unfertilized propanil natural farming conditions. Herrera-gonzalez *et al.*, (2013) examined the ability of microbial consortia *Pseudomonas aeruginosa* included to degrade propanil completely.

This study showed that microbial growth is concentration-dependent and varies significantly among different isolates. *Pseudomonas aeruginosa* (S1) showed increase in growth with increase in concentration of propanil from 0.8 at 1ml to 1.2 at 2ml. Another strain of *Pseudomonas aeruginosa* (S2) also increased from 0.3 at 1ml to 1.0 at 2ml. The mixed culture increased from 0.6 at 1ml to 0.9ml at 2 ml as shown in table 6. The increased in growth of *Pseudomonas aeruginosa* indicated that they are capable of utilizing propanil as carbon source. Herrera-gonzalez *et al.*, (2013) reported how *Pseudomonas fluorescens* degraded propanil. Similarly Sinong *et al.*, (2021) investigated the biodegradation potential of *Pseudomonas aeruginosa* in contaminated soil.

CONCLUSION

Pseudomonas aeruginosa plays a significant role in degrading propanil by using enzyme such as amidases which hydrolyze the amide bonds in propanil leading to the formation of 3, 4-dichloroaniline. Further degradation is facilitated by dioxygenase that break down aromatic compounds into less harmful substances. Bioremediation potential of bacteria has demonstrated its application in managing propanil pollution in agricultural settings.

Recommendation

Toxicity of the contaminant depended on its physical, chemical composition, concentration of the contaminants, the plant or animal species and the time of application as well as other environmental condition. *Pseudomonas* species known to have strong resistance to toxic substances with high susceptibility to genetic manipulation should be isolated from more larger sources to know the specific strain of high efficiency. From the previous studies different enzymes are responsible for this biodegradation as such, the enzymes need to be characterized and the genes identified for more efficient degradation.

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