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Full Length Research Paper

Biodegradation of Paraeforce Using Yeast Cells Isolated From Arable Farmland in Obio/Akpor Local Government Area of Rivers State.

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The aim of the research is explore the biodegradation potential of yeast on paraeforce impacted soil and the analysis of the associated enzymes and metabolites. Soil sample was collected from an agrarian soil previously exposed to paraeforce herbicide. Physicochemical analysis was done on the soil sample. Fungal isolation was done using traditional plate culture and molecular techniques. The biodegradation potential of the isolates was determined using *titrimetric* method. The physicochemical analysis of the treated soil sample recorded a pH of 6.96, temperature (33^{0}C), moisture content (71.8%), nitrate (25.6mg/kg), total organic carbon (42mg/kg), biochemical oxygen demand (5.68mg/kg), and conductivity (4.236µscm^{-1}). Yeasts isolated from the soil sample were *Pichia kudriavzevii MT366877, Hanseniaspora opuntiae* MT366875, Candida. These yeasts were able to degrade the paraeforce. There was a synergy in the degradative activity of the mixed culture. At the end of 90 days, paraeforce degradation was significant in *Pichia kudriavzevii MT366877 (27.5mg/kg), Hanseniaspora opuntiae MT366875 (22.4mg/kg)* and *Pichia cecembensis MT366876 (23.1mg/kg).* Natural attenuation recorded a degradation rate of 41.37mg/kg in 90 days. When the sample was optimized using poultry wastes, the degradation rate improved. The mixed culture achieved 100% (50mg/kg) degradation of paraeforce in 56 days. Natural attenuation achieved 100% degradation in 70 days. *Pichia kudriavzevii MT366877, Hanseniaspora opuntiae MT366875 and Pichia cecembensis MT366876* recorded 36.7mg/kg, 29.5mg/kg and 26.08mg/kg respectively at the end of 90 days. The growth of fungi was influenced by pH, temperature, nitrate, total organic carbon and the biochemical oxygen demand of the growth medium. The research showed that living cells of the yeasts have great potential for the degradation of paraeforce in an impacted soil and may be used bioremediation of impacted soil.

Keywords: Biodegradation, Bioremediation, Contamination, Paraeforce, Soil And Yeasts

INTRODUCTION

Paraeforce is used to eliminating unwanted weeds in the farm which compete with food crop for limited growth nutrient in the soil. The wrong application of this herbicide has resulted in the contamination of soils, streams, rivers and ground water which are important natural resources

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(Baran *et al.,* 2007). These contaminations do not pose danger to only the non-target organisms and the environment but exposes human beings to many health implications.

Some physicochemical methods of paraeforce degradation are quite cumbersome and expensive, and sometimes leave behind toxic metabolic intermediate products that further contaminate the soil. According to Belal *et al* (2008), most microorganisms can detoxify this compound or use it for microbial growth. Biodegradation is achieved through microbial complex enzyme systems and their ability to withstand adverse environmental conditions (Castillo *et al*., 2011). Fungi feature among the nature's most vigorous agent of wastes' decomposition and are essential component of the soil food web (Rhodes, 2012). Baldwin *et al* (1977) found that the most effective organisms for decomposing herbicides are fungi, isolated mainly from several soils.

The aim of this study is to investigate the potential of some yeast in the remediation of paraeforce impacted soil. It is expected to contribute immensely towards enriching the available new technology employed in the remediation of pesticides impacted soils by using fungal living cells.

MATERIALS AND METHODS

Sample site and collection

The site sampled is situated on Gbei road in Nkpolu Rumuigbo, Obio/Akpor local government area of River State. It is a sandy loamy soil used for agriculture with previous known history of exposure to herbicide treatment. Soil samples from the site were collected from different locations at a depth of 20cm in a sterile container and pooled together for use. The physicochemical analysis was done immediately after sample collection. The microbiological analyses were done in Rexall diagnostic and research laboratory, Port Harcourt.

MATERIALS

Culture media: Potato dextrose agar (PDA), nutrient agar and broth from Oxoid Company, UK and were used and prepared according to manufacturer's instruction (Oxoid Manual, 2007).

Herbicide: Paraeforce used is nitrogen based herbicide and served as nitrogen and carbon source. It prepared according to manufacturers' instruction

METHODS

Physicochemical properties of the soil sample.

The following parameters were determined using the methods of American public health association (APHA, 2005); biochemical oxygen demand (BOD), nitrate concentration, phosphate concentration, pH, temperature, conductivity and exchangeable cations. The total organic carbon content will be determined by modified Walkley-Black titration method described by Busse *et al*. (2001). Also, soil particle size and water content were measured.

Isolation of fungal species

Plate culture method described by Cheesbourgh (2001) was used. The pure colonies on culture plates were characterized based on cell and colonial morphology (Holt *et al*., 1994 and Aneja, 2005). Cell morphology was based on lactophenol cotton blue wet mount and gram stain reaction (Holt *et al*., (1994). Observed characteristics were compared with the established identification key by Barnett and Hunter (1972).

Molecular method for characterization of fungal isolates

This involved DNA extraction using a ZR fungal/bacterial DNA mini prep extraction kit, sequencing using the BigDye Terminator kit on a 3510 ABI sequencer from Inqaba Biotechnological, Pretoria, South Africa. Sequencing obtained will be edited using the bioinformatics algorithm. Trace edit similar sequences will be downloaded from the National Centre for Biotechnology Information (NCBI) data base using BLASTIN. The evolutionary history will be inferred using the Neighbor-Joining method in MEGA 6.0 (Saitou and Nei, 1987).The bootstrap consensus tree will be inferred 500 replicates (Felsenstein, 1985) taken to represent the evolutionary history of the taxa analyzed.

Screening of fungal isolates for growth

The different fungi isolated were inoculated into PDA containing 50mg/L of 1/80 concentration of the test paraeforce (herbicides). The inoculated plates were incubated at 28^0C for 48 to 72hours and growth on plates observed (Okpokwasili and Nwosu, 1990).

Biodegradation of Paraeforce

Experimental design
A = 50g of Sterilized soil sample only (negative control)
B = 50g of unsterilized soil sample with 50ml of 50mg/l paraeforce (natural attenuation-positive control)

C = 10ml broth culture of *Pichia kudriavzevii MT366877* was seeded into 50g of sterilized soil sample containing 50ml of 50mg/l paraeforce
D =10ml broth culture of *Hanseniaspora opuntiae MT366875*was seeded into 50g of sterilized soil sample containing 50ml of 50mg/l paraeforce
E = 10ml broth culture of *Pichia cecembensis MT566876 sp* seeded into 50g of sterilized soil sample containing 50ml of 50mg/l paraeforce
F = 10ml broth culture of mixed culture of *Pichia kudriavzevii*, *Hanseniaspora opuntiae* and *Pichia cecembensis MT566876* seeded into 50g of sterilized soil sample containing 50ml of 50mg/l paraeforce.

Soil extraction on the treated soil was done using the method of Polese (2002) and the rate of paraeforce degradation determined using the method described by Okpokwasili and Nwosu (1990).

Optimization of the fungal degradation process using poultry wastes

Poultry waste was added to the soil sample in the ratio of 1:5 and 50ml of 1/80 dilution of the paraeforce added to the mixture. A 50ml broth culture of the different fungal isolates was added into 50g of treated samples, mixed properly and incubated at 28^0C for ninety (90) days. The rate of paraeforce degradation by the fungal species was determined using the method described by Okpokwasili and Nwosu (1990).

Assessment of the effect of different paraeforce concentration on fungal growth.

A mineral salt medium without paraeforce served as control while medium containing different concentration of paraeforce (10, 20, 40, and 80 mg/L) served as test. To each tube was inoculated 1ml of each of fungal isolates and incubated at 28^0C for eight (8) days. Growth and tolerance of the fungi to the paraeforce was measured spectrophotometrically and compared with that of the control cultures (Abd El-Ghany and Masmali, 2016).

Statistical analysis

Analysis of variance and t-test were used to compare the data generated.

RESULTS

Physicochemical parameters of the soil sample

The physicochemical paraments of the untreated sample was measured immediately after sample collection and the results were as follows:- the pH of the sample was 6.05, temperature was 32.5^0C, moisture content was 69.5%, total organic carbon was 34.6mg/kg, nitrate was 1.6mg/kg, available phosphate was 37.4mg/kg and residual paraeforce concentration was 0.56mg/kg. the calcium was 1.444mg/kg, potassium level was 0.607mg/kg, sodium level was 0.580mg/kg, magnesium was 0.507mg/kg and electrical conductivity of 0.190µscm^{-1}. The soil particle composition were clay (27.2%), sand (46.8%) and silt (26.0%). After treatment and incubation for ten days, the physicochemical parameters were measured and the results were as follows:- the pH of the sample was 6.96, temperature was 33^0C, the moisture content of the treated sample was 71.8% while the total organic carbon was 42mg/kg. Nitrate concentration was 25.6mg/kg and available phosphate was 13.86mg/kg. the paraeforce concentration at the time of analysis was 18.8mg/kg), calcium concentration was 1.066mg/kg, potassium level was 0.508mg/kg, sodium was 0.279mg/kg, magnesium was 0.265mg/kg with an electrical conductivity of 0.136µscm^{-1}.. These results are recorded in Table 1.

Isolation and characterization of fungal species

The characterization of fungal isolates was based on culture characteristics and cell morphology.
Candida sp had tiny to moderate colonies, whitish and smooth entire edge with single, paired and short chain cells. *Pichia sp* had cream white large colonies with entire edge, spherical budding and single cells which were occasionally paired and elongated. *Hanseniaspora sp* had whitish, convex, smooth and entire edged colonies with spherical sometimes oval and budding yeast cells in pairs or single while Generally, yeast cells are gram positive Molecular characterization of the screened isolates identified them as listed in Table 2 and Figure 1.

Screening test

Screening test was done on the isolated fungal species using 1/80 dilution of the stock paraeforce (50mg/L) in potato dextrose agar. *Pichia kudriavzevii MT366877, Haneniaspora opuntiae MT366875,* and *Pichia cecembensis MT566876* showed tolerance and were able to grow on the paraeforce medium.

Growth of *Pichia kudriavzevii MT366877*on paraeforce medium

Growth of individual fungal species on paraeforce medium was measured over a period of eight days and changes on the physicochemical parameters of medium due to fungal growth determined. These parameters were used as indices for growth measurement. Every two days, cell count was done using the pour plate technique and expressed in $\log_{10}$. Also, measured alongside the cell count were BOD, TOC, Nitrate, pH and Temperature. Figure 2 illustrated the growth of *Pichia kudriavzevii MT366877* and

Table 1: Physicochemical parameters of soil before and after treatment

Parameters	Untreated	Treated	** Standard Values
pH	6.05± 0.5	6.96±0.04	6.5-8.5
Temperature	31.5±0.5	33 ±1.0	21.8-33.4^{0}C
Moisture content (%)	69.49±2.2	71.8±1.2	ND
Biochemical oxygen demand	4.26mg/kg	5.68mg/kg	
Total organic carbon (mg/kg)	34.6 ± 0.8	42±0.6	
Nitrate (mg/kg)	1.6±0.15	25.6±0.1	50
Phosphate (mg/kg)	37.4±1.2	13.86±0.3	20
*Paraeforce (mg/kg)	0..56	50.0±0.1	ND
Exchangeable cations (mmol/kg)			
Calcium	1.444±.0.054	1.466±0.2	60-120
Potassium	0.607±0.02	0.508±0.1	30
Magnesium	0.507±0.1	0.765±0.21	150
Sodium	0.580±0.013	0.479±0.04	70-120
Electrical Conductivity (uscm^{-1})	0.190± 0.05	4.236±1.0	13.70-860.0
Particle size of soil			
Clay (%)	27.20±1.25		
Sand (%)	46.80±1.0		
Silt (%)	26.00±0.85		

***Paraeforce**
****APHA = American Public Health Association (20th Edition 1998)**

Table 2: Growth and tolerance to Paraeforce

Isolates	Identity of Isolates
F1	*Hanseniaspora opuntiae MT366875*MT366875 *(Sacharomycetes)*
F2	*Pichia cecembensis MT566876* MT566876
F4	*Pichia kudriavzevii MT366877MT366877*MT366877 *(formerly Candida cruzei)*

the effect on some physicochemical parameters of the paraeforce medium. In day two, the cell count was 0.27 in $\log_{10,}$ the BOD was 1.05mg/l and TOC (32.8mg/l), Nitrate (26.2mg/l), pH and temperature were 6.0 and 31^{0}C respectively. On the fourth day, the cell count was 0.86, BOD was 2.33mg/l and TOC was 30.91. Nitrate was 26.2mg/l. pH and temperature were 6.1 and 32^{0}C respectively. In day six, the cell count was 2.18, BOD (3.9mg/l), TOC (27.34mg/l), Nitrate (17.3mg/l), pH (6.0) and temperature was 32.8^{0}C. On the eight day, the data generated were; cell count (4.48), BOD (8.2mg/l), TOC (24.26mg/l), Nitrate (13.8mg/l), pH (5.8) and temperature was 33^{0}C.

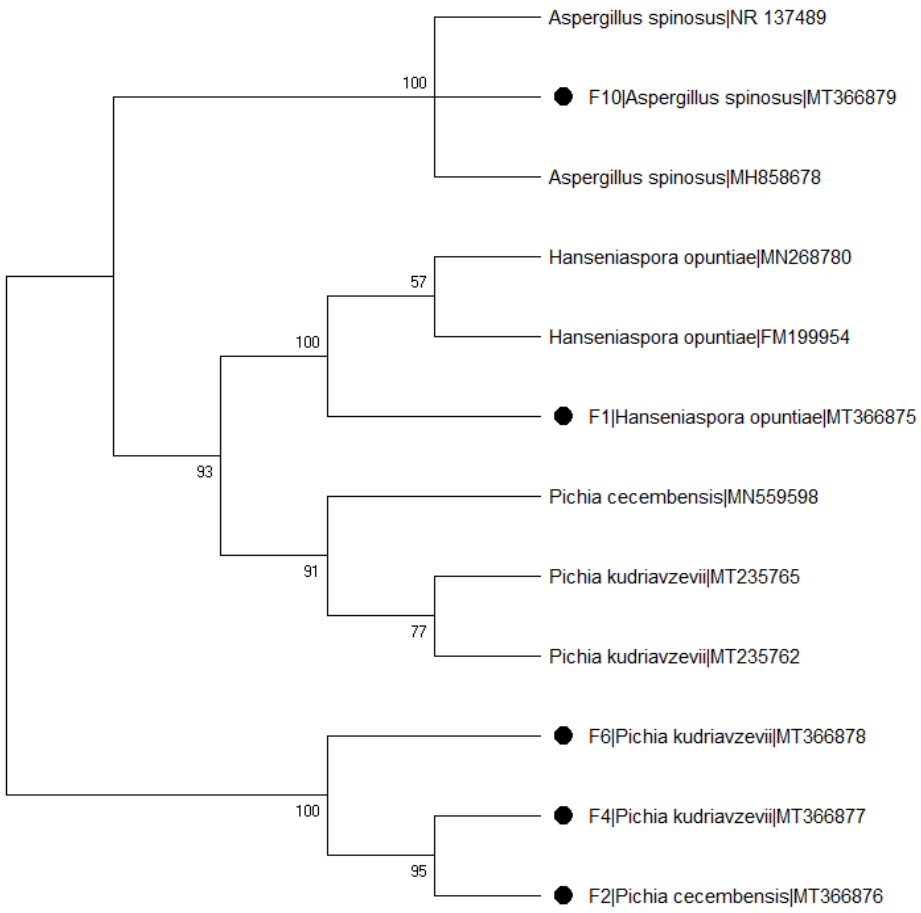

Figure 1: Neighbour joining tree showing evolutionary relationship between fungal isolates and close relatives

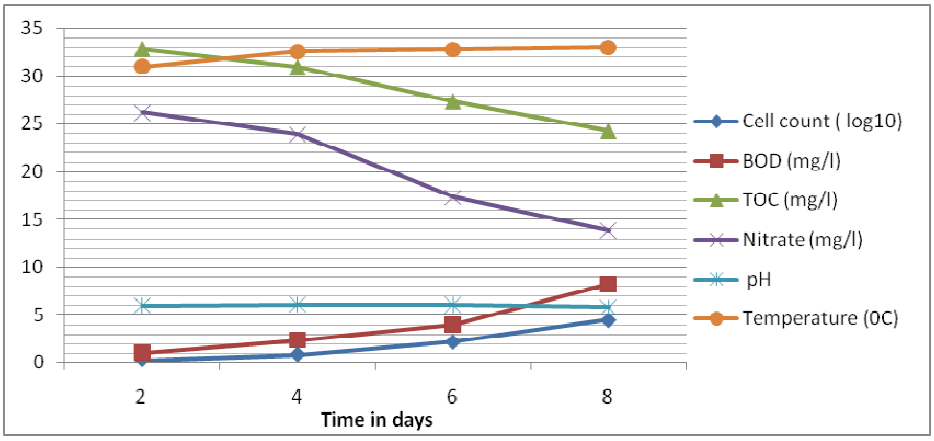

Figure 2: Measurement of growth of *Pichiakudriavzevii MT366877*on paraeforce medium

Figure 3 below illustrated the data generated using fungus *Hanseniaspora opuntiae MT366875.* In day two, the cell count was 0.22, BOD was 1.02mg/l, TOC was 32.9mg/l, Nitrate concentration was 27.2, pH was 6.6 and

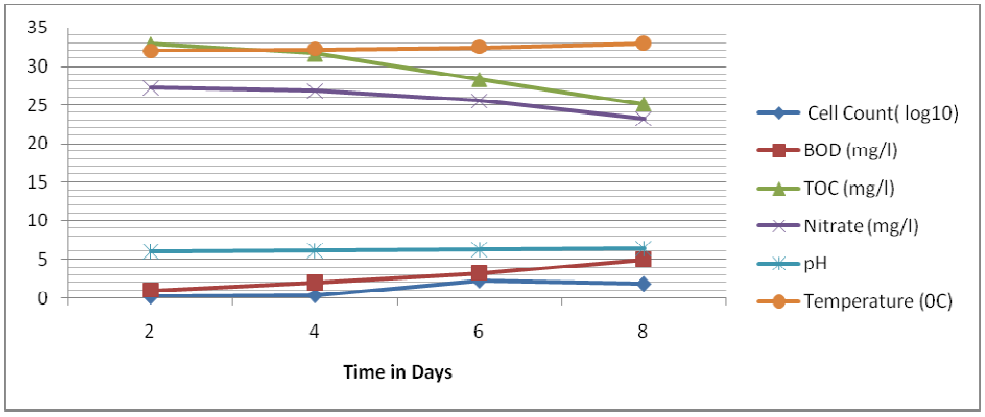

Figure 3: Measurement of growth of *Hanseniasporaopuntiae MT366875* on paraeforce medium

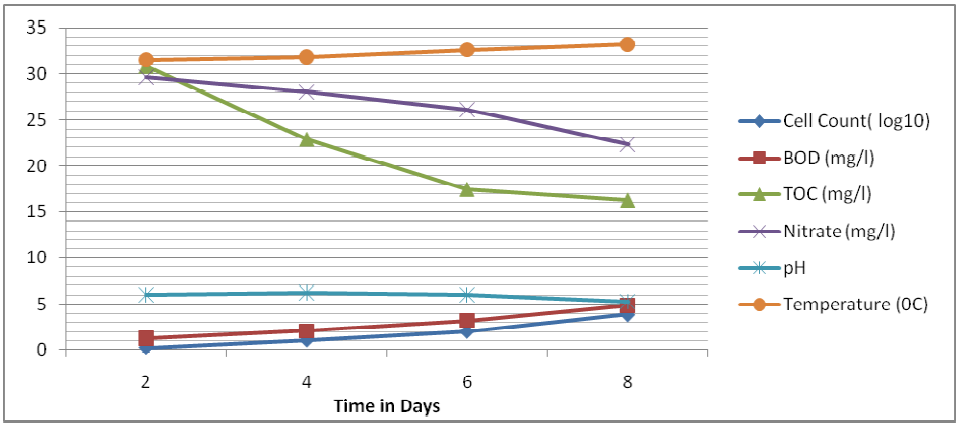

Figure 4: Measurement of growth of *Pichiacecembensis MT566876* on paraeforce medium

temperature was 32^{0}C. Day four recorded a cell count of 0.37 while BOD was 1.99mg/l, TOC was 31.6mg/l and Nitrate concentration was 26.8mg/l. The pH was 6.15 and temperature was 32.2^{0}C. On the sixth day, the cell count was 2.27, BOD was 3.25mg/l, TOC was 28.3mg/l and Nitrate was 25.5mg/l. The pH value was 6.26 and temperature of 32.5^{0}C. Day eight recorded a decrease in cell count of 1.85. The BOD was 5.01mg/l, TOC was 25.1mg/l and Nitrate was 23.2mg/l. the pH of the medium was 6.4 and temperature was 32.8^{0}C.

Figure 4 above illustrated the growth of *Pichia cecembensis MT566876* and its effect on the physicochemical parameters of the paraeforce medium. On the second day of incubation, the cell count was 0.23, BOD was 1.26mg/l, TOC was 30.8mg/l and Nitrate was 29.6mg/l. The pH was 6.05 while the temperature was 31.5^{0}C. In day four, the cell count was 1.04, BOD was 2.09mg/l, TOC was22.9mg/l, Nitrate was 28.0mg/l, pH was 6.21 and temperature of 31.8^{0}C. Day six recorded a cell count of 1.99, BOD of 3.22mg/l, TOC of 17.5mg/l, Nitrate concentration of 26.1mg/l and pH of 6.0 while the temperature was 32.6^{0}C. In day eight, the cell count was 3.86, BOD was 4.9mg/l, TOC was16.3mg/l and concentration was 22.3mg/l. The pH and temperature were 5.3 and 33.2^{0}C.

The analysis of variance done on cell counts and the relationship with the physicochemical parameters measured showed that there is no significant difference at 0.05 level ($p>0.05$) for cell counts, BOD, Nitrate, pH, and temperature for all the tested fungi. This implies that the differences observed in the data were not statistically different. The TOC was significantly different at 0.05 level ($p<0.05$).

Assessment of the effect of different paraeforce concentration on fungal growth.

The effect of different concentrations of paraeforce (1/10, 1/20, 1/40 and 1/80 dilutions) on the fungal growth was determined by the measurement of the absorbance of the broth culture on mineral salt medium over an incubation period of eight days. Figure 6 showed that in 1/10 dilution treatments, *Pichia kudriavzevii MT366877*had an absorbance of 0.002, *Hanseniaspora opuntiae MT366875*

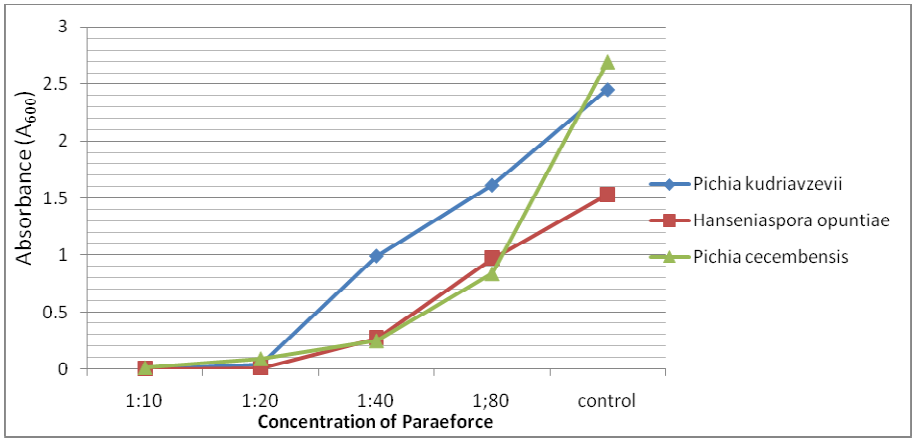

Figure 6: Effect of paraeforce concentration on fungal growth

(0.001) and *Pichia cecembensis MT566876* (0.015). In 1/20 dilution, the absorbance were *Pichia kudriavzevii MT366877* (0.045), *Hanseniaspora opuntiae MT366875* (0.013) and *Pichia cecembensis MT566876* (0.093). The absorbance recorded in 1/40 treatments showed relative increase in all fungal growth. Thus, *Pichia kudriavzevii MT366877*was 0.991, *Hanseniaspora opuntiae MT366875* (0.270) and *Pichia cecembensis MT566876* (0.250). In 1/80 dilution treatments, there was increase in growth in all the treatment options with recorded absorbance of *Pichia kudriavzevii MT366877* (1.613), *Hanseniaspora opuntiae MT366875* (0.968) and *Pichia cecembensis MT566876* (0.836).

Fungal degradation of paraeforce in unamended sample

In day zero, the concentration of paraeforce in the samples was 50mg/kg of soil except in option A (negative control), where paraeforce was not added. Option B (positive control) was allowed for natural attenuation to take place where none of the test fungi was added. Fourteen days after treatment, paraeforce reduction in the various treatment options B, C, D, E and F (mixed culture of the five test fungi) were 2.81, 0.03, 0.006, 0.01, and 3.95mg/kg. In day 28, the reduction rate increased to 5.9, 1.87, 0.96, 1.24, and 10.3mg/kg for B, C, D, E and F respectively. There was observed increase in reduction rate in day 42. On the 42 second day, treatment options B, C, D, E, and F recorded degradation rate of 11.45, 3.86, 1.86, 3.09, and 17.9mg/kg of treated soil samples respectively. On the 56 day, the reduction rate increased to 23.8, 9.34, 3.67, 7.9, and 23.0mg/kg for B, C, D, E and F respectively. On the 70th day, the rate of paraeforce reduction for the various treatment options were 29.5, 13.6, 8.21, 10.6, and 33.8mg/kg for options B, C, D, E and F. The degradation rate increased to 36, 18.6, 15.3, 15.9, and 41.6mg/kg for treatment options B, C, D, E and F respectively on the eighty-fourth day. In day ninety, the degradation rate were 41.37, 27.5, 22.4, 23.1, and 46.3 representing 82.7%, 55%, 44.8%, 46.2%, and 72.6% respectively. The data were represented in Figure 6. The one way analysis of variance done showed a significance at 0.05 level ($p<0.05$)

Fungal degradation of paraeforce in amended sample (optimization)

The degradation rate increased with the amendment of the treatment options using sterilized poultry wastes as shown in Figure 7. Treatment options B, C, D, E and F showed reduction rate of 10.98, 0.9, 0.03, 0.98, and 6.7mg/kg of the treated soil samples on the 14th day. On the 28th day, B, C, D, E and F recorded 26.3, 2.2, 2.06, 3.12, and 23.9mg/kg respectively. Also, treatment option B, C, D, E and F recorded 32.6, 4.98, 4.1, 23.6, 4.87, and 43.96mg/kg after 42 days of incubation. In day 56, treatment option F was able to degrade all the incorporated paraeforce (50mg/kg representing 100%) into the soil samples. Treatment options B, C, D and E recorded reduction rate of 43.2, 15.67, 6.78 and 10.2mg/kg respectively. In day 70, treatment option B achieved hundred percent degradation (50mg/kg) leaving the treatment option frees of paraeforce. Other treatment options C, D and E recorded 20.1, 11.54 and 17.3mg/kg respectively. In day eighty four, treatment options C, D and E recorded 28.9, 18.39 and 19.67mg/kg respectively. On the 90th day, C, D and E recorded 73.4%, 58.2% and 52.2% reduction representing 36.7, 29.5 and 26.08mg/kg. The one way analysis of variance done showed that there is no significant difference the data

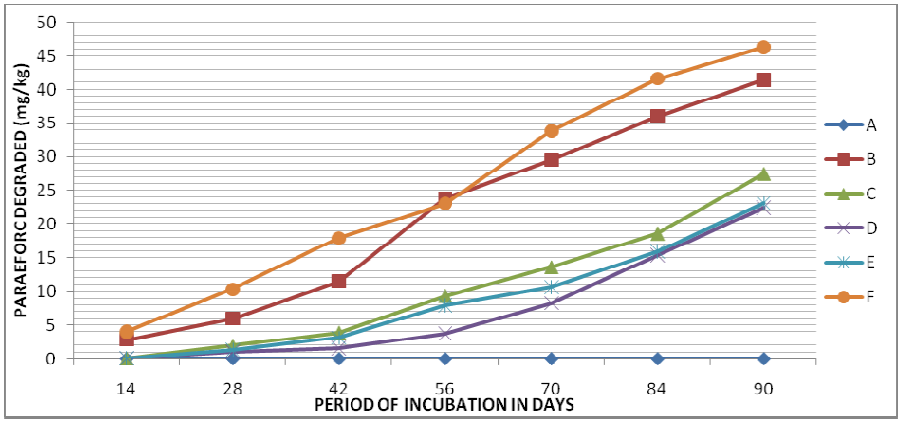

Figure 7: Rate of Paraeforces' degradation by fungal isolates

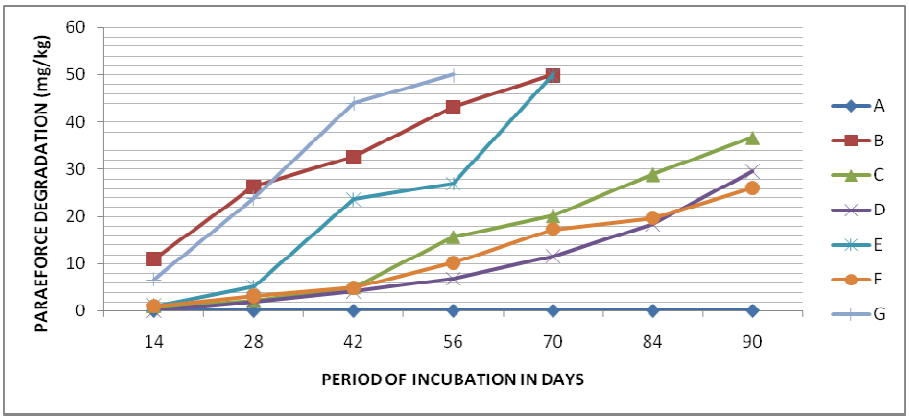

Figure 8: Optimization of the fungal degradation process using poultry wastes

generated at 0.05 level ($p<0.05$). Comparison of the unamended and optimized treatments using t-test showed there was no significant difference since the p-value is greater than 0.05 ($p>0.05$)

DISCUSSION

Environmental factors influenced the growth of the test microorganisms as shown in Figures 2,3 and 4. The figures showed that paraeforce utilization and metabolism, as well favourable environmental factors enhanced the growth of these fungi hence increase in cell count. This is complemented by the works of Gadd (2001) that environmental factors trigger the growth and survival of microorganisms in the soil. The measurement of pH, temperature, nitrate (NO_3), total organic carbon (TOC) and biochemical oxygen demand (BOD) showed a relationship between these parameters and the fungal growth. With increase in cell count, there were a corresponding increase in temperature and BOD with seemingly decrease in NO_3, TOC and pH. Decrease in NO_3 and TOC may be because the paraeforce was used as N_2 and Carbon source for the growth of the fungi. Slight decrease in pH may be due to the presence of acidic intermediate metabolic products. Precisely, depletion of oxygen in the medium may account for slight increase in the observed BOD.

Assessment of effect of different concentration of paraeforce in the medium showed that higher growth rate was observed at a dilution of 1:80 (50mg/L) which is the concentration indicated by the manufacturers as requirement for the control of weeds in farms. At a high concentration of 1:10, growth of some of the fungal species was inhibited as shown in Figure 6. In the present study, the concentration of 1:80 allows the growth of all the fungal species. This is supported by the work of Wilbawa *et al.* (2009) which states that high concentration of herbicides inhibits the activities of most soil microorganisms. Similarly, Subhani *et al.,* (2000) recorded that over application of herbicides in soil can inhibit some of the natural processes in the ecosystem and may decrease the performance of the non-target organisms. It is, also clear in the work of Halimah *et al.* (2010) that herbicide's effect on fungal growth is specific with respect to the herbicide type and

dose applied. The adverse effect of paraeforce is evident when compared with control sample which does not contain the herbicide.

Figure 7 and showed the potential of these fungal isolates and the effect of optimization of the medium in degradation of paraeforce. The slow rate at which *Pichia kudriavzevii MT366877, Hanseniaspora opuntiae MT366875*and *Pichia cecembensis MT366876* to effectively degrade paraeforce may be due to inadequate production of degradation enzymes. Synergistic effect (Guillén *et al.,* 2000) was observed when mixed culture of the test fungi was seeded into the sample, degrading 46.3mg/kg (92%) of the paraeforce added into the soil sample. The mixed culture yielded greater result than the other treatment options. This result is supported by Yang Yang *et al* (2013) who opined that bioaugmentation facilitates accelerated biodegradation processes.

Optimization of the process using poultry wastes increased the rate of paraeforce degradation. By this amendment, the mixed fungal culture achieved 100% of paraeforce degradation in fifty-six days. The poultry waste increased nutrient supply to test fungi leading to microbial cell proliferation and availability of paraeforce to the microorganisms. This is in line with the work of Rhodes (2012) and Zawierucha *et al.* (2014) that the rate of degradation becomes maximal when there is a supply of nutrients to the soil. In mixed culture the slight decline after forty-two may be due to nutrient depletion and cell death. This research demonstrated the potential of *Pichia kudriavzevii MT366877, Haneniaspora opuntiae MT366875* and *Pichia cecembensis MT566876* to degrade paraeforce and is supported by the works of Singh (2014) and Zhang *et al.* (2011) that fungi can be used in bioremediation of pesticides in an impacted soil.

CONCLUSION

From this study, fungi are good degraders of Paraeforce returning the soil to almost its original safe state. The fungi; *Pichia kudriavzevii MT366877, Haneniaspora opuntiae MT366875,* and *Pichia cecembensis MT566876* utilize the herbicide as source of nitrogen and carbon and were observed to provide oxidative enzymes (catalase, peroxidases and laccase) that facilitated the degradation processes. Optimization of the process with poultry wastes stimulated the fungal growth and increased the rate of degradation of the pollutant. The high degradation potential of the isolates place them at advantage as bioremediation tools on paraeforce impacted soil and must be encouraged..

Author Contributions

O.F.I., E.C.V and I.O.V designed the research. O.F.I wrote the manuscript. O.F.I., E.C.V and I.O.V. established the field experiments. O.F.I and S.O.H did the sample collection and provided the materials needed for the research. S.O.H did the molecular and statistical analysis, O.F.I performed the experiments. All the authors revised the manuscript and approved the final version.

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