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Full Length Research Paper

Analysis of Enzymes and Metabolites of Paraeforce Degradation Using Living Yeast Cells and *Aspergillus spinosus*

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This study was carried out in the Department of Microbiology, Michael Okpara University of Agriculture, Umudike in Abia State. The aim of the study is to analyze enzymes and metabolites associated with fungal degradation of paraeforce. Soil samples for fungal isolation were collected from impacted sites and inoculated on potato dextrose agar (PDA). . the isolates used in this research were Pichia kudriavzevii, Hansenspora opuntiae, Aspergillus spinosus and Pichia cecembensis. The isolates were screened for enymes production on 50mg/l concentration of paraefoce medium. Qualitative and quantitative assay for enzyme production in hydrogen peroxide, methyl red, guaiacol and hydrogen peroxide-pyrogallol complex proved potential of all the fungi for catalase, lignin peroxidase, laccase and manganese peroxidase production. The results showed that these four fungi have great potential for catalase, peroxidases and laccase production after six days aerobic incubation in paraeforce and these enzymes facilitated the utilization of the paraeforce. In terms of formate and oxalate production, greater yields were observed in the mixed culture, natural attenuation (positive control) and *Aspergillus spinosus* MT366879. In the mixed culture, synergy was found to be responsible for the great yield. The production of these metabolites is linked to availability of the paraeforce degrading enzymes and the ability of the degrading fungi to tolerate, utilize and grow on the paraeforce medium.

Keywords: Living Yeast Cells, Enzymes*, Aspergillus spinosus*

INTRODUCTION

The use of herbicides in agriculture has over the years contributed immensely to food and cash crop production. The wrong application of these herbicides has resulted in the contamination of soils, streams, rivers and ground water which are important natural resources (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 herbicides' degradation such as chemical precipitation, electrophoresis/ electrochemical treatment, solvent extraction, membrane technology, evaporation recovery, and chemical oxidation or reduction are quite cumbersome and expensive (Rhodes, 2012) and sometimes leave behind toxic metabolic intermediate products that further contaminate the soil (Onianwah, 2014). According to Belal *et al* (2008), most microorganisms can detoxify these compounds, mineralize

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them or use them 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. Baldwin *et al* (1977) found that the most effective organisms for decomposing herbicides are fungi, isolated mainly from several soils hence the choice of these species.

The objective of this research is to determine the potential of *Pichia kudriavzevii, Hansenspora opuntiae* and *Pichia cecembensis* to produce paraeforce degrading enzymes and to determine the various enzymes activity. This study was done in Department of Microbiology, Michael Okpara University of Agriculture, Umudike in Abia State.

MATERIALS AND METHODS

Isolation of fungal species

Soil samples were used for the isolation of the test fungal species. The collected soil samples was homogeneously mixed and carefully sorted to remove stones and other unwanted soil debris using a 2.5 mm sieve. The PDA media was autoclaved at 121^oC for 15 minutes, allowed to cool and 20ml dispensed aseptically on the sterile disposable Petri dishes (Oxoid, 2007). One gram of each sorted soil sample was homogenously mixed with 1 drop of Tween 80 to enhance growth and was sprinkled onto the potato dextrose agar (PDA) and incubated for 7 days at 30^oC. Ampiclox 25mg/l was added to the media after autoclaving to prevent contamination by bacteria.

To purify the fungal isolates, the cultures was carefully and aseptically sub-cultured on potato dextrose agar (PDA) and stored on slants for future use (Cheesbrough, 2001). The fungal isolates were characterized on the basis of cultural and morphological characteristics (Aneja, 2005) in a lactophenol cotton blue wet mount on a microscope at x10 and x40 objective lenses and on the basis of their gram reaction. Observed characteristics was recorded and compared with the established identification key by Barnett and Hunter (1972).

Assay of the Fungal Associated Enzymes and Metaboliets of Paraeforce Degradation

Screening of fungal species for enzymes' production

Extracellular enzymes assay was conducted to investigate the production of enzymes by the isolated fungi. The fungi were screened for the production of the following enzymes:-

Catalase production

To determine catalase production potential of the isolated fungi, small inoculums of the culture will be mixed into 3% H_2O_2 solution (Shivani and Jain, 2015). Effervescence due to breakdown of H_2O_2 into O_2 and H_2O shows catalase production. Catalase activity will be evaluated by using scale to indicate the degree of reaction. Thus, -, +, ++, +++ to indicate "no reaction, weak, moderate and strong reactions respectively.

Determination of catalase activity

Extracellular catalase activities were measured in culture filtrates using the method described by Caridis *et al.*, (1991). Catalase activity was measured spectrophotometrically by observing the decrease in light absorption at 240 nm during decomposition of H_2O_2 by the enzyme.

Laccase production

Qualitative method

The ability of the fungal strains to secrete extracellular laccase was done according to the method of Kiiskinen *et al.* [2004]. The assay plate contained 15 ml potato dextrose agar amended with 0.02% of guaiacol. The plates will be incubated at 30 C for 1–5 days. The presence of brown color around the colony will be considered as guaiacol oxidizing laccase secreting organism.

Quantitative method

Guaiacol has been reported as efficient substrate for laccase assay. The intense brown color development due to oxidation of guaiacol by laccase was correlated to its activity and read at 450 nm (Jhadav et al, 2009). This was repeated each day for six (6) days. Enzyme activity was expressed as International Units (IU/ml), (Adiveppa and Basawanneppa, 2015). The laccase activity in U/ml is calculated using the extinction coefficient of guaiacol (12,100 M-1 cm-1) at 450 nm by the formula:

$E.A = (A^* V) / (t^* e * v)$,

where E.A = Enzyme Activity (U/ml), A = Absorbance at 450nm, V = Total volume of reaction mixture (ml), v = enzyme volume (ml), t = Incubation time (min) and e = Extinction Coefficient (M-1cm-1).

Qualitative method of lignin Peroxidase assay

Methyl Orange Dye Decolorization Plate Assay of Lopez et al (2006) was used to primarily screen isolates for their lignolytic potential. Culture was inoculated onto methyl orange agar plates (0.5% methyl orange in PDA) and

incubated at 25^oC. Growth was followed for a period of 2 weeks. Positive reaction is indicated by the formation of a clear zone around the colony. Positive results indicate the production of lignin degrading enzymes which decolorize the polymeric dyes.

Quantitative method of lignin Peroxidase activity

Lignin peroxidase activity was determined by the method described by Arachibald (1992). This method is based on the oxidation of dye azure B. The reaction mixture (1ml) contained 50mM sodium tartrate buffer (pH 3.0), 32mM azure B, 1mM hydrogen peroxide and culture filtrate. The mixture is incubated for 10 min at 30ºC. The reaction was initiated by adding hydrogen peroxide and absorbance is immediately measured at 651nm in one-minute intervals after addition of H_2O_2. One unit of enzyme activity is expressed as decrease in absorbance of 0.1 units per minute.

Qualitative method of Manganese Peroxidase assay

This was determined qualitatively using the method of Rayner and Boddy (1988) as reported by Lopez et al (2006). Isolates were inoculated into nutrient agar and incubated at 30^0C for 48hours. Thereafter, 30ml of 0.4% (v/v) H_2O_2 and 1% pyrogallol in water will be added to colonies. Those with yellow-brown colour will be recorded as positive.

Quantitative method of Manganese Peroxidase activity

Manganese peroxidase (MnP) activity was measured following the method described by Paszczynski, *et al* (1988). This method is based on the oxidation of guaiacol.

Analysis of some associated metabolites

Experimental design
A = 50g of untreated soil sample only (negative control)
B = 50g of untreared sterilized soil sample with to which is seeded 10ml broth culture of mixed culture of *Pichia kudriavzevii*, *Hanseniaspora opuntiae* and *Pichia cecembensis MT566876* (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). oxalate was determined using the methods described by Munir,et al (2001) and Vishal et al. (2014) while formate was determined using the method of (Denning et al, 2015) .

Determination of oxalate

Soil extract was used to determine oxalic acid content. Extraction was carried by following the traditional method described by Munir,et al (2001). A 0.5g of soil sample will be transferred to 50ml capacity volumetric flask. A 30ml 0.25 N HCl was added and kept in boiling water bath for about 15 minutes, cooled to room temperature and volume made up with 0.25 N HCl to 10ml. This solution was used as extract for determination of oxalic acid.

Colour reaction with Indole reagent

This method is based on the reaction described by Vishal et al. (2014). Indole reagent was freshly prepared by dissolving 100 mg of indole in 100ml of concentrated sulfuric acid. Assay mixture contained 2 ml standard oxalic acid solution at various concentrations, ranging from 0.100 to 1.00 mg per ml, prepared in 1 N H_2SO_4. Blank was prepared with 2 ml of 1 N sulfuric acid instead of oxalic acid solution. Then 2 ml of indole reagent was added in each test tube including blank, allowing the reagent to run down the side of the tube to minimize heat development. All test tubes were placed in water bath at 80 to 90^0C for 45 minutes. Cooled to room temperature and absorbance was measured at 525 nm on spectrophotometer (Shimadzu, UV-1900). The formation of pink-coloured compound is due to the reaction between indole and oxalic acid.

Determination of formate

The soil extract above was used for formate determination thus; A 3.5 mL of 100% acetic anhydride, 50mL of 30% (w/v) sodium acetate, and 1mL of 2-propanol solution containing 0.5% (w/v) citric acid and 10% (w/v) acetamide were added in a 10 mL vial. Then 0.5mL of sample was added to this assay solution and incubated for 90 minutes at 25^0C. Finally, the absorbance was determined at 515nm using a UV–vis spectrophotometer. The formate standard calibration curve was prepared against the appropriate concentrations of formate.

The assumption is that a complex compound, recorded at 515nm using UV–vis spectroscopy, was formed by the reaction of formate with a mixture of citric acid, isopropanol, acetic anhydride, sodium acetate in an appropriate ratio (Denning et al, 2015).

Table 1: Colonial and Cell Morphological of the fungal isolates

Code	Colonial morphology	Cell morphology	presumptive organims
F1	Grey, rough edged and dry colonies	Spherical, budding cells, single and occasional paired and elongated	*Pischia kudriavzevii*
F2	Whitish, convex, smooth and entire edged colonies	Spherical, sometimes oval and budding yeast cells in pairs or single	*Hansenspora opuntiae*
F3	Dry black, flat colonies with rough edge on PDA	Septate and branched mycelia. Conidia were in chains.	*Aspergillus spinosus*
F4	moderate colonies, whitish and smooth with entire edge	single, paired and short chain yeast cell, gram positive	*Pichia cecembensis*

RESULTS

Assay of the Fungal Associated Enzymes and Metaboliets of Paraeforce Degradation

Assay of the Fungal Enzymes

The assay of catalase production was done using hydrogen peroxide. The result showed that *Pichia kudriavzevii MT366877*, *Hanseniaspora opuntiae MT366875*, *Aspergillus spinosus MT366879 a*nd *Pichia cecembensis MT566876* had the potential of catalase production at different degrees as shown in Table 2. The highest production level was reported in *Aspergillus spinosus MT366879* that showed the highest degree of cleared zone around its colonies followed by *Pichia kudriavzevii MT366877* with moderate degree of cleared zone. *Hanseniaspora opuntiae MT366875* and *Pichia cecembensis MT566876* showed small cleared zone around their colonies. In peroxidase production, *Hanseniaspora opuntiae MT366875*, *Aspergillus spinosus MT366879*, and *Pichia cecembensis MT566876* showed moderate reaction while weak reaction was observed in *Pichia kudriavzevii MT366877*. In laccase production, *Aspergillus spinosus MT366879* was moderate while *Pichia kudriavzevii MT366877*, *Hanseniaspora opuntiae MT366875*and *Pichia cecembensis MT566876* demonstrated a very weak reaction.

In quantitative analysis of catalase activity in paraeforce degradation, *Pichia kudriavzevii MT366877* demonstrated the ability to generate catalase in day 2, 4, 6, 8 and 10 at 0.06, 0.18, 2.35, 2.10 and 2.15 activity levels respectively. *Hanseniaspora opuntiae MT366875* recorded activity levels of 0.006, 0.05, 0.17, 0.99 and 1.57 in day 2, 4, 6, 8 and 10 while *Aspergillus spinosus MT366879* recorded activity levels of 0.21, 0.56, 3.43, 4.96 and 5.38 in day 2, 4, 6, 8 and 10. In day 2, 4, 6, 8 and 10, and *Pichia cecembensis MT566876* recorded activity levels of 0.01, 0.09, 0.93, 1.68 and 2.59 in day 2, 4, 6, 8 and 10 respectively. The catalase activity of the various fungal species was illustrated in Figure 1 below.

Manganese peroxidase activity of the fungal species was illustrated in Figure 2. The manganese peroxidase activity of *Pichia kudriavzevii MT366877* in day 2, 4, 6, 8 and 10

Table 2: Qualitative assay of associated fungal enzymes

Code	catalase	M. peroxidase	L. peroxidase	laccase
Pichia kudriavzevii	++	+	+	+
Hanseniaspora puntiae	+	++	++	+
Aspergillus spinosus	+++	++	++	++
Pichia cecembensis	+	++	+	+

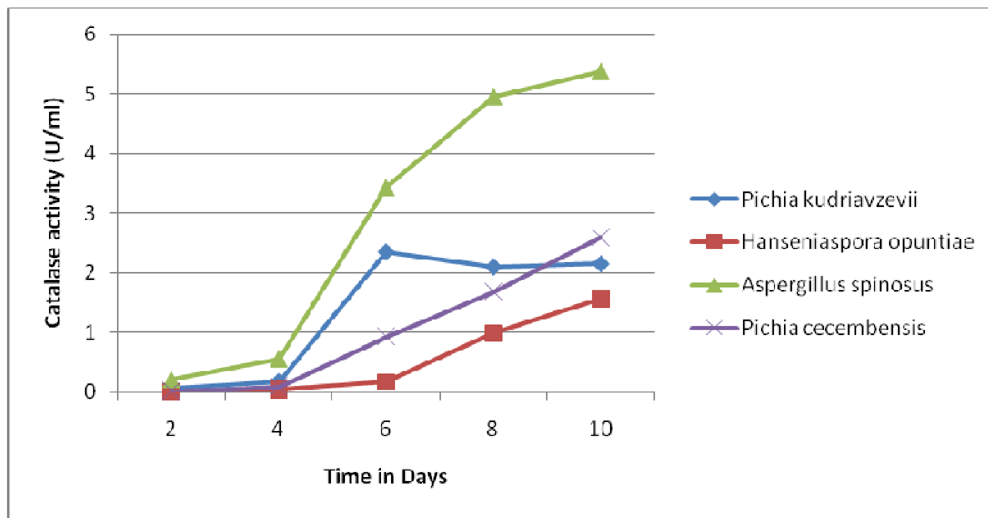

Figure 1: Catalase production by the fungal isolates

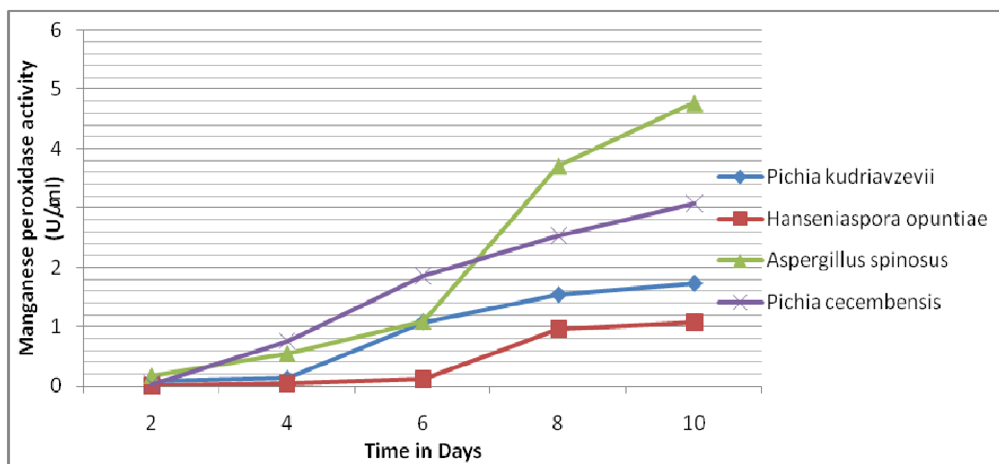

Figure 2: Manganese peroxidase production by the fungal isolates

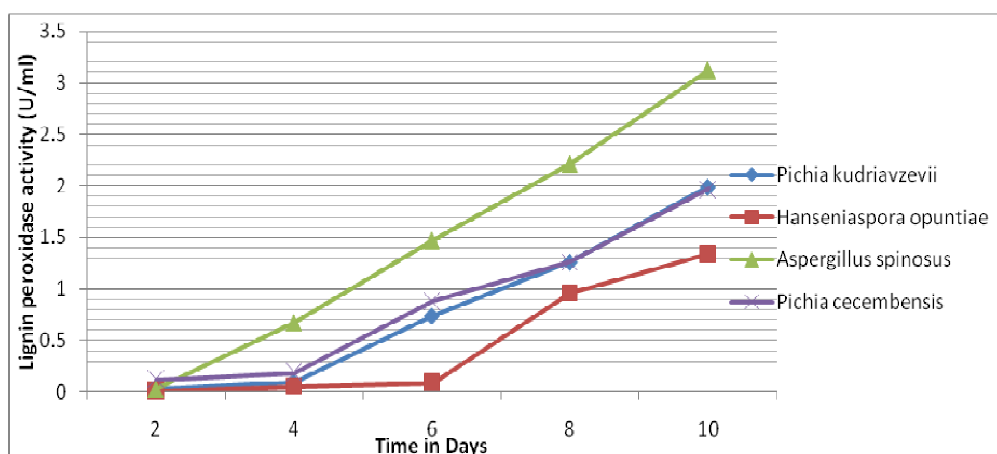

Figure 3: Lignin peroxidase production by the fungal isolates

were 0.08, 0.13, 1.08, 1.54 and 1.73 while that of *Hanseniaspora opuntiae MT366875* recorded 0.001, 0.04, 0.12, 0.96 and 1.08 respectively. *Aspergillus spinosus MT366879* had activity of 0.17, 0.55, 1.98, 3.71 and 4.77 for day 2, 4, 6, 8 and 10 respectively. In *Pichia cecembensis MT566876*, the activity readings were 0.03, 0.76, 1.86, 2.53 and 3.08 for day 2, 4, 6, 8 and 10 respectively.

The lignin peroxidase activity of the various fungal species was illustrated in Figure 3 above. It showed that *Pichia kudriavzevii MT366877* recorded activity of 0.03, 0.09, 0.74, 1.26 and 1.99 on day 2, 4, 6, 8 and 10 respectively while *Hanseniaspora opuntiae MT366875* had 0.01, 0.05, 0.09, 0.96 and 1.34 for day 2, 4, 6, 8 and 10 respectively. *Aspergillus spinosus MT366879* showed lignin peroxidase activity of 0.25, 0.67, 1.47, 2.21 and 3.12 in day 2, 4, 6, 8 and 10 while in *Pichia cecembensis MT566876*, the lignin peroxidase activity in day 2, 4, 6, 8 and 10 were 0.12, 0.19, 0.88, 1.26 and 1.97 respectively.

Figure 4 below showed laccase activity in paraeforce biodegradation process. In day two, the enzymes' production measured in U/ml were 0.35 for *Pichia kudriavzevii MT366877, Hanseniaspora opuntiae MT366875* (0.03), *Aspergillus spinosus MT366879* (0.12), and *Pichia cecembensis MT566876* (0.08). The increased production continued on day four, six and eight for all the studied fungal isolates. On the tenth day which was the pick day of the investigation, laccase activity was recorded as *Pichia kudriavzevii MT366877* (5.9), *Hanseniaspora opuntiae MT366875* (2.71), *Aspergillus spinosus MT366879* (5.38), *and Pichia cecembensis MT566876* (0.96).

Determination of metabolites' concentration

In the estimation of oxalate, standard concentrations of 0.001 to 1.0mg were prepared and the absorbance of the different concentrations measured. The absorbance was used to prepare a calibration graph as in Figure 5 below. This graph was used to estimate the concentration of various treatments in milligram.

In oxalate production as shown in Table 20, the concentration (mg/l) of the treatments A to G in day 5 were 0, 0.39, 0.04, 0.07, 0.12, 0.09, and 0.56 while on the 10th day, the absorbance readings were 0, 0.9, 0.44, 0.33, 0.7, 0.3, and 0.96 for treatments A to G. This was illustrated in Figure 6 below.

In formate production, the absorbance for the various treatments A to G was recorded in was illustrated in Figure 7. On the 5th day, the concentration of different treatments were 0, 0.096, 0.05, 0.03, 0.07, 0.04, 0.09 and 0.2 respectively. On the 10th day, the readings were 0, 0.98, 0.6, 0.3, 0.9, 0.7, 0.97 and 1.07mg/l for A to G respectively.

DISCUSSION

The assay of enzymes done in this study showed the activities of extracellular enzymes catalase, manganese peroxidase, lignin peroxidase and laccase (Table 2). These enzymes are secreted more by *Aspergillus spinosus MT366879* as shown in Table 2. The ability to produce these enzymes account for the optimum performance of the test fungi in the biodegradation of paraeforce. The vigorous mycelia and great biomass formation of *Aspergillus spinosus MT366879* boast its ability to produce large quantity of these enzymes (Mukherjee, and Mittal, 2005) more than *Pichia kudriavzevii, Hanseniaspora opuntiae MT366875*and *Pichia cecembensis.* This is also supported by the work of Onianwah *et al.* (2019) which stated that the great potential of fungi in biodegradation process is by virtue of their aggressive growth, great biomass production and extensive hyphae in the environment. All the four fungal isolates had initial delay

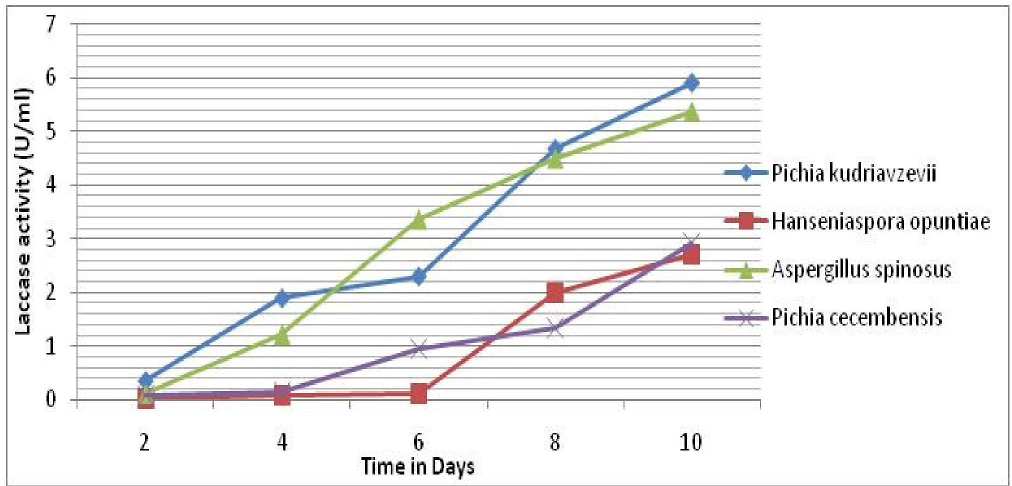

Figure 4: Laccase production by the fungal isolates

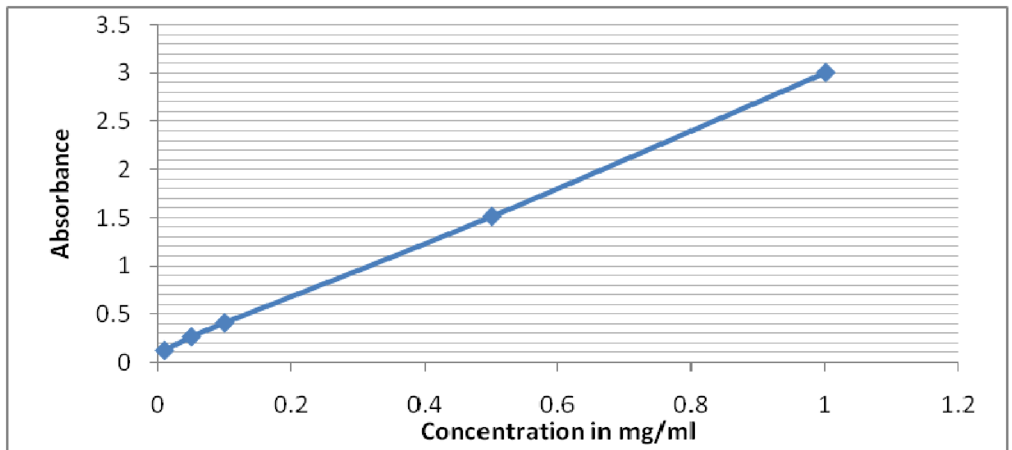

Figure 5: standard calibration curve for oxalate estimation

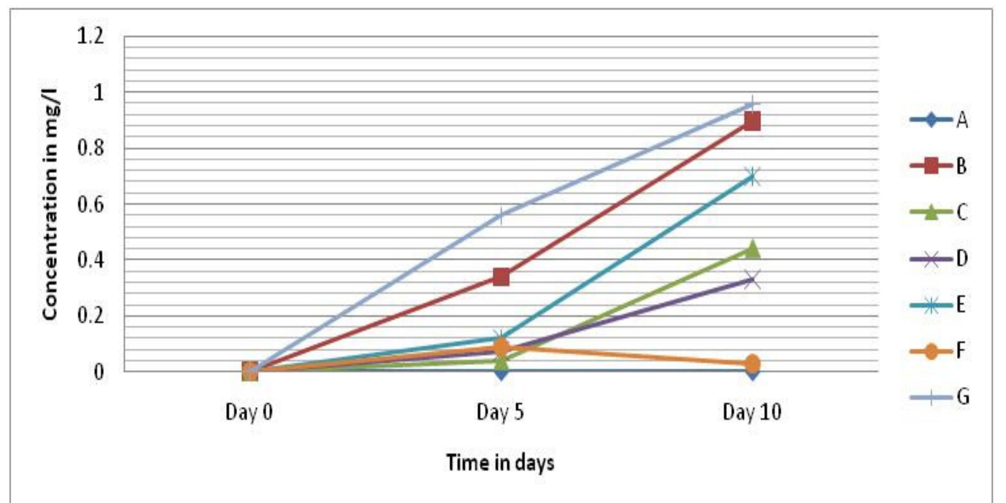

Figure 6: determination of oxalate concentration in paraefoce degradation

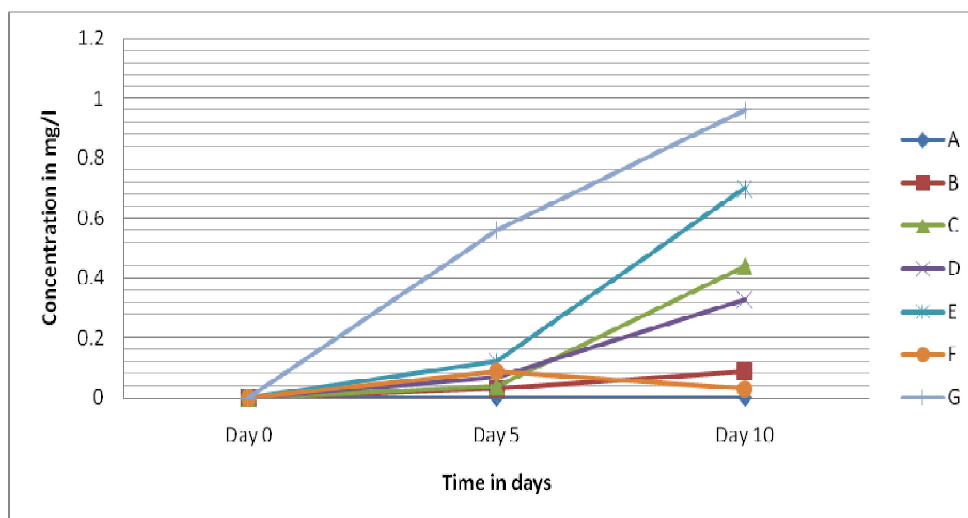

Figure 7: deteremination of formate concentration in paraefoce degradation

and low levels of catalase enzyme production as shown in Figure 1 until the fourth day when the dynamics changed. This is probably because the enzyme systems required to degrade the paraeforce have not fully been developed (Das, and Chandran, 2011). Except in the case of *Pichia kudriavzevii MT366877* treated option that had a slight decrease in level of catalase enzyme production from day six, others continued in steady rise from the fourth day to the tenth day. This may be due nutrient limitation or accumulation of metabolic products that may be toxic to *Pichia kudriavzevii MT366877* leading to cell death. Rhodes (2012) postulated that rate of biodegradation of pesticides is maximum given adequate supply of nutrient. Also, a change in the medium physicochemical conditions due to the presence of metabolites may have affected the enzymes' activities. Besides, the enzymes may be denatured by changes in some physicochemical parameters such as temperature and pH.

In manganese peroxidase enzyme production illustrated in Figure 2, there was a steady rise in enzyme production for all the test fungi. *Aspergillus spinosus MT366879* presented an interesting picture on the sixth day with a rapid rise in manganese peroxidase production until the eight day followed by a slight decline. The rapid rise may be due to favourable medium conditions. Accumulation of toxic metabolites and nutrient depletion on the eight day may be responsible for the slight decline (Das, and Chandran, 2011) in manganese peroxidase enzymes' activity.

In the four enzymes studied, *Hanseniaspora opuntiae MT366875*was the weakest in terms of enzymes activity. This fungus was on lag phase until the sixth day where there was exponential rise in the enzymes' production. This was demonstrated in Figures 1, 2, 3 and 4. In all, *Pichia kudriavzevii, Haneniaspora opuntiae, Aspergillus spinosus MT366879* and *Pichia cecembensis MT566876* demonstrated efficient production capacity for the screened enzymes (Tien, and Kirk, 1984). Rao *et al*. (2014) and Kües, (2015) had argued that biodegradation of persistent compounds is enzymes' mediated and depended largely on the enzyme system of the degrading fungi; the accessibility and bioavailability of the persistent compound as stated by Elisashvili *et al.,* (2009). Tayade *et al.,* (2013) opined that pesticides could also be bioaccumulate in these fungi; same was the opinion of Onianwah *et al.* (2019). Oxalate and formate are two out of the four intermediate products of paraeforce biodegradation and were used in this study to monitor herbicide's degradation process. Figures 6 and 7 illustrated the various treatment options A to G and the rate at which each treatment produce oxalate and formate over a period of ten days incubation. The first five days witness low production of both oxalate and formate in all the treatment options except in option G which contain the four fungal isolates (mixed culture). The low level of oxalate and formate production observed could be associated with slow rate of paraeforce biodegradation within the first five days. The microorganisms seem to be developing the various enzyme systems (Zhang and Qiao, 2002) required to boast the breakdown of this pesticide. The rate of oxalate and formate production increased after the fifth day in all the treatment options as shown in Figure 6 and 7. Greater yields were observed in the mixed culture (G), natural attenuation (B-positive control) and *Aspergillus spinosus MT366879* (E). In the mixed culture, synergy may have been responsible for this great yield. The production of these metabolites is linked to availability of the paraeforce degrading enzymes and the ability of the degrading fungi to tolerate, utilize and grow on the paraeforce medium. This is supported by the works of Couto and Herren (2006), and

Haggblom (1992) which opined that microbial metabolism is the most important mechanism of pesticide degradation in soil and constitutes the basis for all bioremediation and bioaugmentation strategies. Besides, Yadav and Loper (2000) stated in their work that adequate environmental condition, nutrient availability and efficient microbial metabolism enhance biodegradation of xenobiotics. This was complemented by Khadrani *et al.* (1999) and Gadd (2001) who added that soil type, pH, organic matter and clay content are factors that can influence biodegradation of pesticides.

CONCLUSION

This study evaluated enzymes that facilitated fungal degradation of paraeforce. The fungi used for enzymes extraction were isolated from impacted soil samples on potato dextrose agar (PDA). The isolates screened showed potential for growth and tolerance to paraefoce in 50mg/l concentration of the test herbicides. Pichia kudriavzevii, Hansenspora opuntiae and Pichia cecembensisecies were found to grow in paraeforce supplemented PDA. Qualitative and quantitative assay for enzyme production in hydrogen peroxide, methyl red, guaiacol and hydrogen peroxide-pyrogallol complex proved potential for catalase, lignin peroxidase, laccase and manganese peroxidase production respectively. The results showed that these three fungi have great potential for catalase, peroxidase and laccase production after six days aerobic incubation in paraeforce and these enzymes facilitated the utilization of the paraeforce. It is recommended that these fungi isolates be used to clean up paraeforce impacted soil.

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