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Evaluation of Enzymes Production Activity of *Trichoderma*, *Aspergillus* and *Rhizopus* Species in Paraeforce (Herbicide) Degradation

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Authors' contributions

This work was carried out in collaboration among all authors. Authors FIO and HOS designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors VCE and VOI managed the analyses of the study. Author CJU managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The study aims to evaluate enzymes that facilitate fungal degradation of paraeforce. Soil samples for fungal isolation were collected from impacted sites and inoculated on potato dextrose agar (PDA). The isolates were screened for growth and tolerance to paraefoce in 50 mg/l concentration of the test herbicides. *Trichoderma*, *Aspergillus* and *Rhizopus* species were found to grow in paraeforce supplemented PDA. Qualitative and quantitative assay for different 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.

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1. 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 groundwater which are important natural resources [1]. These contaminations do not pose danger to only the non-target organisms and the environment but expose 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 oxidetion or reduction are quite cumbersome and expensive, and sometimes leave behind toxic metabolic intermediate products that further contaminate the soil.

According to Belal et al. [2], most microorganisms can detoxify these compounds, mineralize them or use them for microbial growth. Biodegradation is achieved through microbial complex enzyme systems and their ability to withstand adverse environmental conditions [3]. Fungi feature among nature's most vigorous agent of wastes' decomposition and are an essential component of the soil food web. Baldwin et al. [4] 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 *Trichoderma* sp*., Aspergillus* sp. and *Rhizopus* sp. species to produce paraeforce degrading enzymes and to determine the various enzymes activity.

2. MATERIALS AND METHODS

2.1 Isolation of Fungal Species

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

To purify the fungal isolates, the cultures were carefully and aseptically sub-cultured on PDA and stored on slants for future use. The fungal isolates were characterized based on cultural and morphological characteristics [5] 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 were recorded and compared with the established identification key by Barnett and Hunter [6].

2.2 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:-

2.2.1 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 [7]. Effervescence due to the breakdown of H_2O_2 into O_2 and H_2O shows catalase production. Catalase activity will be evaluated by using a scale to indicate the degree of reaction. Thus, -, +, ++, +++ to indicate "no reaction, weak, moderate and strong reactions respectively.

2.2.2 Determination of catalase activity

Extracellular catalase activities were measured in culture filtrates using the method described by Caridis et al. [8]. Catalase activity was measured spectrophotometrically by observing the decrease in light absorption at 240 nm during decomposition of H_2O_2 by the enzyme.

2.3 Laccase Production

Qualitative method: The ability of the fungal strains to secrete extracellular laccase was assayed according to the method of Kiiskinen et al. [9]. 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 an 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 [10]. This was repeated each day for six (6) days. Enzyme activity was expressed as International Units (IU/ml), [11]. 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. [12] 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°C. Growth was followed for 2 weeks. A 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.

A quantitative method of lignin Peroxidase activity: Lignin peroxidase activity was determined by the method described by Arachibald [13]. This method is based on the oxidation of dye azure B. The reaction mixture (1 ml) contained 50mM sodium tartrate buffer (pH 3.0), 32 mM azure B, 1 mM hydrogen peroxide and culture filtrate. The mixture is incubated for 10 min at 30°C. The reaction was initiated by adding hydrogen peroxide and absorbance is immediately measured at 651 nm in one-minute intervals after addition of H_2O_2. One unit of enzyme activity is expressed as a 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 [14] as reported by Lopez et al. [12]. Isolates were inoculated into nutrient agar and incubated at 30°C for 48hours. Thereafter, 30 ml 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.

A quantitative method of Manganese Peroxidase activity: Manganese peroxidase (MnP) activity was measured following the method described by Paszczynski, et al. [15]. This method is based on the oxidation of guaiacol.

2.4 Statistical Analysis

Statistical Package for the Social Sciences (SPSS) statistics 20 software was used to determine the statistically significant differences in the pattern of enzyme activities of the fungi.

3. RESULTS

Table 1 shows three species of paraeforce degrading fungi isolated and characterized from previously exposed soil samples. These are *Trichoderma* sp., *Aspergillus* sp. and *Rhizopus* sp. these isolates are illustrated in Plate 1. Plates 1 and 2 illustrate the qualitative enzymes assay of the various species of the isolated paraeforce fungal degraders.

Table 1. Colonial and cell morphological of the fungal isolates

Code	Colonial morphology	Cell morphology	Presumptive organism
F1	Grey, rough edged and dry	Spherical, budding cells, single and occasional paired and elongated	*Trichoderma* sp.
F2	Dry black, flat colonies with rough edge on PDA	Septate and branched mycelia. Conidia were in chains.	*Aspergillus* sp.
F3	Whitish fluffy colonies covering the entire plate brown/orange spores and whitish cotton like structures	Non septate hyphae, sporangiospores and black hemispherical columella	*Rhizopus* sp.

***Trichoderma* sp.**

***Aspergillus* sp.**

***Rhizopus* sp.**

Plate 1. Fungal isolates from paraeforce exposed soils

3.1 Enzyme Assay

Table 2 shows the qualitative assay of enzymes production by the different microorganisms. The stronger potential was seen with *Rhizopus* sp., followed by *Aspergillus* sp. and *Trichoderma* sp. Quantitative enzyme activity was measured Spectrophotometrically over six days. On the first day, the three fungal isolates showed a low-level enzymes activity thus; 0.06, 0.12 and 0.01 U/ml of catalase enzyme for *Trichoderma, Aspergillus* and *Rhozopus* respectively. This low activity changed after the second day as represented in Table 3. There was a lag phase between the third and fourth day with respect *Trichoderma* production of catalase enzyme followed by exponential phase. The fungi presented the same picture in peroxidase production except in manganese peroxidase where there is a sharp decline on the fifth day from 4.77 U/ml to 2.9 U/ml in *Aspergillus* sp. Similarly, there was sharp decline in lactase production on the fifth day by *Trichoderma* sp. from 5.2 to 4.4 U/ml. Generally, *Rhizopus* showed greater potential for the production of catalase, manganese peroxidase, lignin peroxidase and laccase with unit values of 6.1, 6.6, 6.88 and 5.56 U/ml respectively when compared with *Aspergillus* and *Trichoderma* (4.91, 2.9, 6.08 , 4.9 and 4.2, 2.21, 4.4, 2.94 U/ml respectively) on the sixth day as shown on Tables 3, 4, 5 and 6. Analysis of variance on data generated showed that there is no significant difference ($p<0.05$) on the pattern of enzyme activities of these fungi.

Table 2. Qualitative assay of associated fungal enzymes

Organism	Catalase	Mn peroxidase	lignin peroxidase	Laccase
Trichoderma sp.	+	+	+	++
Aspergillus sp.	++	++	++	++
Rhizopus sp.	++	++	+++	+++

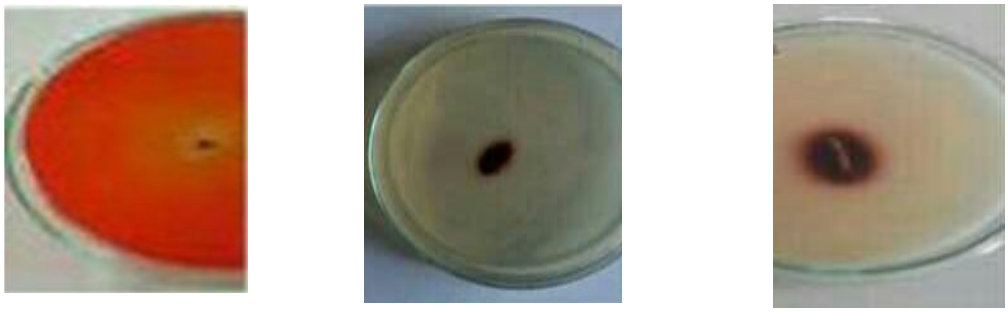
Key: (-) = no reaction, (+) = weak reaction, (++) = moderate reaction, (+++) = strong reaction

Table 3. Catalase production during growth of isolates (Extracellular enzyme activity)

Time (days)	Catalase activity (U/ml)		
	***Trichoderma* sp.**	***Aspergillus* sp.**	***Rhizopus* sp.**
1	0.06	0.21	0.01
2	0.18	0.56	0.32
3	2.35	3.43	2.43
4	2.0	4.96	3.12
5	3.15	5.38	5.7
6	4.25	4.91	6.1

Table 4. Manganese Peroxidase production during growth of isolates (Extracellular enzyme activity)

Time (days)	Manganese peroxidase activity (U/ml)		
	***Trichoderma* sp.**	***Aspergillus* sp.**	***Rhizopus* sp.**
1	0.08	0.17	0.24
2	0.13	0.55	1.09
3	1.08	1.98	2.57
4	1.54	3.71	3.88
5	1.73	4.77	5.02
6	2.21	2.9	6.68



***Trichoderma* sp.** ***Aspergillus* sp.** ***Rhizopus* sp.**

Plate 2. Qualitative assay of fungal laccase production

Table 5. Laccase production during growth of isolates (Extracellular enzyme activity)

Time (days)	Laccase activity (U/ml)		
	***Trichoderma* sp.**	***Aspergillus* sp.**	***Rhizopus* sp.**
1	0.35	0.12	0.03
2	1.89	1.23	0.66
3	2.30	3.37	1.89
4	4.67	4.50	3.06
5	5.90	5.38	3.99
6	4.86	6.08	6.81

Table 6. Lignin peroxidase production during growth of isolates (Extracellular enzyme activity)

Time (days)	Lignin peroxidase activity (U/ml)		
	***Trichoderma* sp.**	***Aspergillus* sp.**	***Rhizopus* sp.**
1	0.01	0.25	0.34
2	0.09	0.67	0.78
3	0.74	1.47	2.2
4	1.26	2.21	2.89
5	1.99	3.12	3.79
6	2.94	4.9	5.56

4. DISCUSSION

Paraeforce is a recalcitrant nitrogenous based herbicide generally used in agronomy to control weeds. This herbicide is the toxic and continuous accumulation in soil poses health challenges to man and farm animals. This work strived to fungal enzymes involved in detoxification of this herbicide. The preliminary screening test done identified three species of fungi capable of degrading paraeforce. Thus, *Trichoderma* sp, *Aspergillus* sp. and *Rhizopus* sp. were selected as the most potent microorganisms for the production of the previous enzyme as contained in Table 1. Characterization in Table 1 was based on colonial and cell morphology described

by Barnett and Hunter [6]. These screened fungal species demonstrated the ability to produce enzymes catalase, peroxidases and laccase (Table 2) in line with the works of Baldwin et al. [4] which stated that fungi are the major sources of pollutant degrading enzymes. Though primary screening using indicators is a major tool for the selection of lignolytic organisms, it was observed that there was no direct correlation between enzyme activity and intensity of zone or colouration as shown in the experimental results generated.

The organisms demonstrated an exponential rate of catalase production except for *Trichoderma* sp. which on the third day showed a lag phase till the fourth day. This could be attributed to the presence of inhibitory metabolites as reported by Subhani et al. [16] and an attempt to adjust to the presence of this substance or changes in the environment such as pH conditions occasioned by these metabolites. However, the production of catalase dropped with *Aspergillus* sp. after a pick production on the fifth day. This could be attributed to nutrient depletion, increase in metabolite concentration and cell death. These factors were also responsible for a slight decline in *Rhizopus* sp. catalase activity after the fifth day.

In manganese peroxidase activity, there was a steady rise this enzyme production by the three species up to the sixth day except for *Aspergillus* sp. sharp decline on the sixth. Here, also, nutrient depletion, high concentration of metabolites, alteration of medium pH and cell death may be responsible for this decline.

Table 2 showed that *Rhizopus* has the greatest potential for the production of these enzymes. In Table 5, laccase production by these fungi followed some defined pattern over six days with slight deviation in *Trichoderma* sp. production in paraeforce supplemented medium. Maximum laccase production with *Trichoderma* sp. was on the fifth day following a gradual decline in production. All the three fungal species demonstrated great potential for the production of lignin peroxidase in paraeforce supplemented medium. Results indicated that all the three isolates could efficiently grow and produce lignolytic enzymes in both stationary and solid-state conditions of growth and thus could be exploited for enzyme production by stationary as well as solid state fermentation [17]. Conclusively, therefore, it is believed that the ability of *Trichoderma, Aspergillus* and *Rhizopus* isolates to metabolize the herbicides paraeforce is due to their ability to produce enzymes catalase, manganese and lignin peroxidases and laccase, and must be encouraged since these fungal activities are known to be ecologically friendly.

5. CONCLUSION

Trichoderma, Aspergillus and *Rhizopus* species were found to grow in paraeforce supplemented media. 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. It is recommended that these fungi isolates be used to clean up paraeforce impacted soil.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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