IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB)
ISSN: 2455-264X, Volume 10, Issue 5 Series 1 (Sept. – Oct. 2024), PP 09-18
www.iosrjournals.org

Isolation And Identification Of Enzymes Associated With Pesticide Tolerance And Degradation

Ononuga, Olanrewaju O, Okosesi Ebunoluwa A, Ude Emea O, Njoku Kelechi L
Department Of Cell Biology And Genetics, University Of Lagos
Department Of Biotechnology, Federal Institute Of Industrial Research, Oshodi

Abstract
The environmental microbial composition and diversity are sensitive key indicators of the impact of organic pollutants on the environment. The inherent characteristics give microbes the ability to bioremediation polluted environments provided favourable environmental and nutritional conditions are ensured in bringing about their optimal activities. The study aims to isolate and identify enzymes associated with pesticide tolerance and degradation. Pure cultures of bacteria and fungi were isolated from soil contaminated with glyphosate in Ikorodu farm. The pure cultures were grown on minimal salt agar media amended with glyphosate. The best isolates exhibiting good tolerance to the glyphosate in the presence of this pesticide were molecularly characterized and examined in relation to enzyme expression, environmental conditions (pH and temperature), metal ion influence and nutritional conditions. Molecular characterization identified five bacteria and six fungi species which phylogenetically clustered into two orthorlogous groups. The results shows that the test isolates have the ability for expression of a range of enzymes. Laccase was produced in higher quantity than tyrosinase and peroxidases. The expressions of these enzymes were best at temperature of 40^0C and pH of 7.0 and 8.0 for tyrosinase and laccases respectively. Mercury ion caused the highest inhibition of tyrosinase activity while Fe ion inhibited laccase activity most. The test isolates successfully expressed the enzymes more in glucose media compared to others for tyrosinase. Starch used as a common carbon source induced a higher activity in laccase compared to tyrosinase. Tryptone was found to be the best nitrogen sources for tyrosinase while urea was the best for laccase production. The results of this study suggest that nutritional supplement that effectively enhances the enzyme production depends on the individual microbes and specific growth conditions. Therefore the expression of these range of enzymes under glyphosate treatment suggests their potential applications in the remediation of soil polluted with glyphosate pesticides.
Keywords: *Microorganisms, Enzymology, Synthesis, Extract, Concentration, Optimal Activities*

Date of Submission: 01-09-2024 Date of Acceptance: 11-09-2024

I. Introduction

A pesticide is any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating any pest (insects, mites, nematodes, weeds, rats, etc.), including insecticide, herbicide, fungicide, and various other substances used to control pests (EPA, 2012). Pesticides belong to a category of chemicals used worldwide as herbicides, insecticides, fungicides, rodenticides, molluscicides, nematicides, and plant growth regulators to control weeds, pests and diseases in crops as well as for health care of humans and animals (Bohmont, 2002). One traditional classification of pesticides places them in one of two groups: organic and inorganic. Organic pesticides are based on chemicals having carbon as the basis of their molecular structure. The chemicals in organic pesticides are more complex than those of inorganic pesticides and usually do not dissolve easily in water. Inorganic pesticides are simpler compounds. They have a crystalline, salt-like appearance, are environmentally stable, and usually dissolve readily in water. The earliest chemical pesticides were inorganic and included substances such as sulfur and lime (Ortiz-Hernandez et al. 2013). The vast majority of modern pesticides contain an organic chemical. There have been hundreds of pesticides developed based on organic chemicals, often with oxygen, phosphorus, or sulfur in their molecules, in addition to their basic carbon structure (Ortiz-Hernandez et al. 2013). Organic pesticides can be subdivided into two additional groups: natural organics, and synthetic organics. The natural organic pesticides (sometimes just called "organics") are derived from naturally occurring sources such as plants. Rotenone and pyrethrum are examples of natural organic pesticides (Ortiz-Hernandez et al. 2013).

Synthetic organic pesticides (usually just called "synthetics") are produced artificially by chemical synthesis. This group comprises most "modern" pesticides (i.e., discovered or used as insecticides post-World

War II), and includes DDT, permethrin, malathion, 2, 4-D, glyphosate, and many, many others (Ortiz-Hernandez et al. 2013). The primary benefits are known as consequences of the direct pesticides effects such as protection of people, animal and crop health and protection of recreational turf. The secondary benefits arise from primary and these are the less immediate, less intuitively obvious, or longer term consequences. Table 2 summarizes the effects, primary and secondary benefits, and their interactions.

Table 1. Primary and Secondary Benefits of Pesticides (Cooper J. and Dobson H., 2007)

PRIMARY BENEFITS	SECONDARY BENEFITS
1. Controlling pests and plant disease vectors	**Community benefits**
Improved crop/livestock quality Reduced fuel use for weeding Reduced soil disturbance Invasive species controlled	Community benefits Nutrition and health improved Food safety/security Life expectancy increased Reduced maintenance costs
2. Controlling disease vectors and nuisance organisms	**National Benefits**
Human lives saved Human disturbance reduced Animal Suffering reduced Increased livestock quality	National agricultural economy Increased export Revenues Reduced soil erosion/moisture loss
3. Prevent or control of organisms that harm other human activities and structures	**Global benefits**
Tree/bush/leaf hazards prevented Recreational turf protected Wooden structures protected	Less pressure on uncropped land Fewer pests introduced elsewhere International tourism revenue

Pesticide use raises several environmental concerns, including human and animal health hazards. Food contaminated with toxic pesticides is associated with severe effects on human health because it is a basic necessity of life. Over 98% of sprayed insecticides and 95% of herbicides reach a destination other than their target species, including non-target species, air, water and soil (Miller, 2014). Pesticide toxicity can result from ingestion, inhalation or dermal absorption. Continued exposure to these chemicals for a long period may result in various diseases some of which are listed below: neurological, psychological and behavioural dysfunctions, hormonal imbalances, leading to infertility, breast pain, immune system dysfunction, reproductive system defects, cancers, genotoxicity, blood disorders. Pesticides can contaminate soil, water, turf, and other vegetation. In addition to killing insects or weeds, pesticides can be toxic to a host of other organisms including birds, fish, beneficial insects, and non-target plants. Recent articles and reports review toxicological and epidemiological evidence for various health effects associated with pesticides (Lushchak *et al*., 2009; Kubrak *et al*., 2012; Maksymiv *et al*., 2015). Extensive toxicological studies in animals demonstrate that several pesticides to which the general population may be chronically exposed are potential carcinogens, neurotoxins, reproductive toxins, and immunotoxins (Baker and Wilkenson, 2010; Bolognesi and Merlo, 2011). Gonzalez *et al.,* (2005) showed DNA damage under 2,4-D exposure in Chinese Hamster ovary cells (CHO). There are evidences on the involvement of pesticides in the development of neurodegenerative diseases (Franco *et al*., 2010; Gupta P.K., 2011). Many scientists reported the impact of pesticides on biochemical parameters, in particular on protein metabolism (Li *et al*., 2011), endocrine (Cooper *et al*., 2000; Goldman *et al*., 2012; Stoker *et al*., 2015) and reproductive systems (Abarikwu *et al*., 2009).

Pesticide contamination of both surface and ground waters can affect aquatic fauna and flora, as well as human health when water is used for public consumption (Cerejeira *et al*., 2003). Aquatic organisms are directly exposed to chemicals from agricultural production via surface run-off or indirectly through trophic chains. This project aimed to produce the following enzymes; laccase, tyrosinase, lignin peroxidase, and manganese peroxidase from pesticide-degrading microorganisms.

II. Materials And Methods

Study Area

The study area is a food crop farm located in Gberigbe, Imota, Ikorodu town, Lagos state. Its geographical coordinates are 6° 40' 0" North, 3° 40' 0" East. It has a mean annual rainfall of 1670mm. It is an evergreen area with slightly dark humus and loamy soil, good for planting and harboring soil micro-organisms like molds and bacteria that promote soil aeration, and nitrogen fixation.

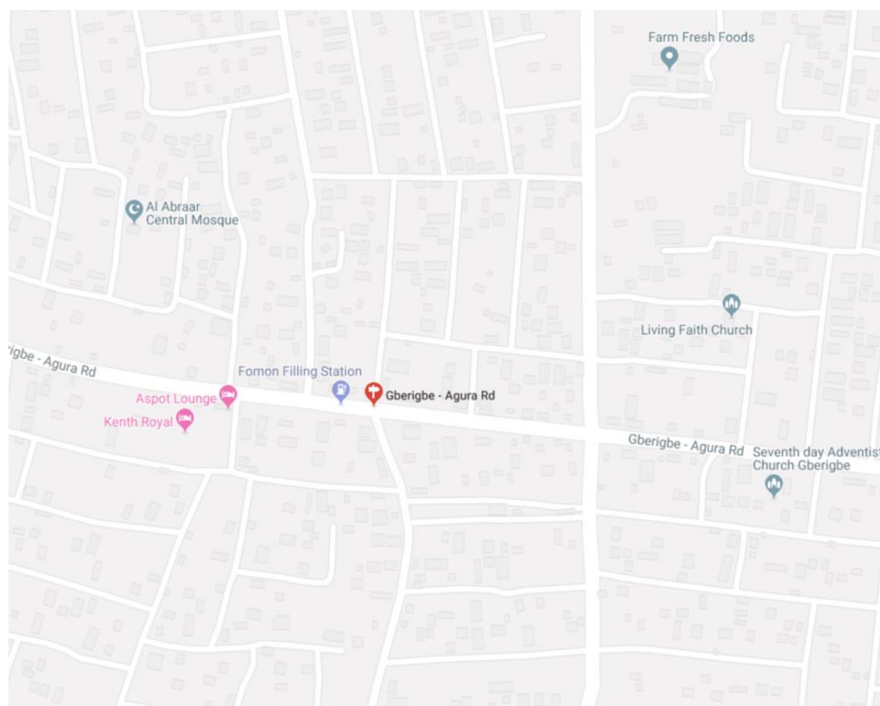

Figure 1. Map of Ikorodu Local Government showing Gberigbe, Imota – Study Area

Sample Collection

Pesticide-contaminated soil samples of 250 grams each were collected in sterile containers using a spatula from four designated points at the crop farm located in Imota, Ikorodu. The soil samples were stored appropriately in plastic bags and transported to the laboratory where they were kept at 4^0C till analysis. The soil samples were later air-dried and sieved with 10mm mesh.

Isolation of Bacterial and Fungal Colonies

The microorganisms were isolated by serial dilution technique on Minimal Salt Agar Media. A sample suspension was prepared by adding 1.0 g sample to 10 ml distilled water. It was mixed well for 15 min and thereafter vortexed. Each suspension was serially diluted from 10^{-1} to 10^{-7}. 0.1 ml of the suspension was pipetted onto plates with minimal salt media and spread with a glass spreader. A sterile filter paper was incorporated with the herbicide and placed on the lid of the plate to create a mobile phase and the plates were incubated at 37°C for 8 days. After the incubation period, the colonies were counted and multiplied by the reciprocal of the dilution factor reported as colony forming unit (Cfu/g). The isolates were maintained on nutrient agar slants and kept in a refrigerator for further use (Hiren *et al.,* 2017).

Identification of Bacterial and Fungal Isolates

Identification of bacterial isolates was carried out by routine bacteriological methods i.e., by the colony morphology, preliminary tests like Gram staining, Motility test, oxidase test, catalase test, Methyl red and Vogues-Proskauer tests (Hiren *et al*., 2017).

Screening

Bacteria and fungi were screened based on degradation capability using glyphosate. Bacteria and fungi which showed higher degradation activity were selected and further study was carried out using it. The screening of isolates degrading glyphosate was measured as a decrease in optical density using a spectrophotometer. Degradation of glyphosate was done by promising isolate and the effect of the incubation period was optimised (Hiren *et al.,* 2017).

Substrate Specificity

The ability of force-up degrading bacterial isolates to utilize pure force-up (glyphosate) substrate was tested in the minimal salt medium. Herbicide - Force up was used at 100ppm and introduced into 100ml Erlenmeyer flasks containing 10ml basal medium. The flasks were inoculated with the test bacteria isolates (except one which served as control) and were subsequently incubated in a gyratory shaker incubator at 150 revolutions per minute and 30^0C for 7 days according to (Nwaogu *et al.,* 2017). Turbidity was checked at initial stage (0 hours), 4th day and 7th day using visual and UV/viable spectrophotometers. (Nwaogu *et al.,* 2017).

Molecular Characterization of Bacterial and Fungal Isolates

PCR amplification of bacterial and fungal small-subunit rDNA (18S rRNA gene) was carried out using the primer set ITS4 and ITS 5 (Smit et al., 1999). The ITS 4 and ITS 5 primers amplified a 1.5-kb section of the 18S rRNA gene. Primer sequences were as follows: ITS4 TCCTCCGCTTATTGACATGS and ITS5 GGAACTAAAAGTCGTAACAAGG. PCR amplification was performed in a 25 µL reaction containing 5 U of Taq DNA polymerase (Sigma), a 10 X dilution of the manufacturer's buffer (Sigma), 200 µM concentrations of each deoxynucleoside triphosphate (dNTPs), and 20 pM of primers EF4 and EF3 and 50 ng of genomic DNA. The reaction conditions were as follows: initial denaturation at 94 °C for 5 min, 36 amplification cycles of denaturation at 94 °C for 30 seconds, annealing at 54 °C for 30 seconds and primer extension at 72 °C for 45 seconds; followed by a final extension at 72 °C for 7 minutes. PCR amplifications were carried out using a Thermo-Hybaid PCR thermal cycler (Thermo Fisher Scientific USA). Aliquots of the PCR products (5 µL) were analyzed in 1% (w/v) agarose gels (Sigma, USA) by horizontal gel electrophoresis. DNAs were visualized by UV excitation after staining with ethidium bromide (0.5 mg/L). The PCR product was purified using Bangalore Genie PCR purification kit following the manufacturer's instruction. The 18S rRNA nucleotide sequence was determined by PCR-direct sequencing done by Chromous Biotech Pvt. Ltd. Phylogenetic analysis of the 18S rRNA gene sequences was performed with CLC DNA workbench version 6. The phylogenetic trees were inferred using the neighbour-joining method (Saitau and Nei, 1987) and bootstrap analyses were performed. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004).

Inoculum and Fermentation Medium

The medium was prepared by weighing the following medium composition in grams per litre; 6g - Bacteriological peptone, 5g –glucose, 2g - NH_4NO_3, 0.8G - KH_2PO_4, 0.5g $MgSO_4.7H_2O$, 0.2g - K_2HPO_4, 0.25g – $CUSO_4$.5H20, 2g – yeast extract, 0.25 – $MnSO_4.7H_2O$, substrate-1.0g. The above medium composition was dissolved in 1000ml of distilled water after which 100ml of the medium was measured into a conical flask (250ml capacity each) heated on hot plate to homogenize and then sterilized in an autoclave at 121℃ for 15 minutes after which they were removed and allowed to cool before the organism was inoculated (Bertrand *et al*., 2004). After incubation, the production medium was centrifuged at 6000rpm for 30 min to separate the cells. The supernatant was collected and subjected to estimate the enzyme activities (Bertrand *et al*., 2004).

Effect of Temperature on the Production of the Enzymes

To study the effect of temperature on enzyme production the submerged fermentation was carried out at different temperatures (5°C - 80°C) (Bertrand *et al*., 2004).

Effect of pH on the Production of the Enzymes

The fermentation medium was prepared by varying the pH values (3.0 – 11.0) for the production of the enzymes (Bertrand *et al*., 2004).

Enzyme Activity Assay

Laccase

Laccase activity was measured by the MBTH-DMAB assay (Castillo *et al*., 1994). The assay is based on the oxidative coupling of 3-methyl-2- benzothiazolinone hydrazone (MBTH) and 3-(dimethylamino) benzoic acid (DMAB). Laccase catalyses the formation of a deep purple compound with a peak absorbance at 590 nm in the presence of 3-methyl-2- benzothiazolinone hydrazone, 3-(dimethylamino) benzoic acid and $MnSO_4$. A reaction mixture was prepared in a cuvette containing lactate succinate buffer 73.5 mM (pH 4.5), DMAB 0.99 mM, MBTH 0.07 mM, $MnSO_4$ 0.3 mM and 100 µl of the prepared laccase solution. The reaction was started with addition of the enzyme and absorbance at a wavelength of 590nm was measured for 60min at 30ºC using a UV-Vis spectrophotometer**.**

Lignin Peroxidase

The Lignin Peroxidase (LiP) activity was assayed via the oxidation of veratryl acohol to veratrylaldehyde at 310 nm (Tien and Kirk 1988). Two milliliters of enzymatic assay consisted of 0.4 ml of citrate – phosphate buffer (100 mM, pH 2.7),0.1 ml of veratryl alcohol (20mM) and 0.5 ml of fluid sample . Forty microliters of H_20_2 (20mM), which was freshly prepared daily, was added to start the reaction. The conversion to veratrylaldehyde was monitored in 1 ml quartz cuvette at 310 nm wavelength using UV-Vis spectrophotometer. One unit of enzyme activity corresponded to the oxidation of 1 micromolecule veratrylaldehyde converted from veratryl alcohol per minute under the assay conditions with a molecular coefficient of € 310=9300 M-1cm-1.

Manganese Peroxidase

Manganese Peroxidase (MnP) activity was measured via the oxidation of guaiacol to a coloured product using the UV-Vis spectrophotometer at 465 nm (Li *et al*., 2008). The enzymatic assay of 1.0 ml consisted of 0.4 ml of sodium lactate buffer (100mm, pH 4.5), 0.1 ml of guaiacol (1mm), 0.1 ml of $MnSO_4$ (1mm), and 0.4ml of supernatant of the sample. The reaction was started by the addition of 30µl of H_20_2 (0.1mm), which was prepared freshly daily. The formation of the coloured product was measured in 1ml quarts cuvette at 465nm wavelength. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 macromole of colored product per minute at 30^0C under the assay conditions with a molar extinction coefficient of € 465=12100 M-1cm-1.

Tyrosinase

A reaction cocktail of 9ml deionized water 10ml buffer and 10ml tyrosinase was prepared in a suitable container. The mixture was mixed and adjusted to pH 6.5 at 25ºC with 1m HCl. The mixture was oxygenated by bubbling 99.9% pure O_2 through the reaction cocktail for 5 minutes. The mixture was pipetted in milliliters into suitable quartz cuvettes. The reaction cocktail was equilibrated to 25^0C. The A_{280nm} was monitored until constant using UV-Vis spectrophotometer, then 0.1ml buffer and 0.1ml enzyme solution was added immediately and mixed by inversion and the increase in A_{280nm} was recorded for approximately 10 minutes (Castillo *et al.,* 1994). The r $A_{280nm/}$ minute was obtained using the maximum linear rate for both the test and blank.

Calculations:

$$\text{Units/ml enzyme} = \frac{(\text{rA280nm/min Test} - \text{rA280nm/min Blank})(\text{df})}{(0.001)(0.1)}$$

df = Dilution factor

0.001 = The change in A280nm/minute per unit of tyrosinase at pH 6.5 at 25°C in a 3 ml reaction mix 0.1 = Volume (in milliliters) of enzyme used

$$\text{Units/mg solid} = \frac{\text{Units / ml enzyme}}{\text{mg solid/ml enzyme}}$$

$$\text{Units/mg protein} = \frac{\text{Units / ml enzyme}}{\text{mg protein/ml enzyme}}$$

Protein Assay

Protein Extraction

To 0.2g of the sample, 10 ml of chilled phosphate buffer was added. The mixture was centrifuged for 20 minutes at 5000rpm. It was further centrifuged with 10ml of phosphate buffer solution for another 10 minutes. The supernatant was pooled together and 10ml of supernatant was mixed with 6ml of 10% TCA solution and thereafter left in ice for precipitation to occur. The precipitate was harvested by centrifuging at 5000rpm for 30 minutes after which the supernatant was decanted. The precipitate was dissolved in 5ml of 0.2m NaOH, it was then used as protein extract (Lowry *et al*., 1951).

Protein Determination

Protein extract of 0.2ml was measured into tubes and 0.8ml of distilled water was added to it. Meanwhile, 1.0ml of distilled water was used as blank, while the bovine serum albumin standard curve was set up (Lowry *et al*., 1951).

Determination of Specific Activity

The Specific activity of an enzyme gives the measurement of the activity of the enzyme.

This is the activity of an enzyme per milligram of total protein (expressed in units/mg).

It is the amount of product formed by an enzyme in a given amount of time under a given condition per milligram of protein. The specific activity of the tyrosinase, laccase, lignin peroxidase and manganese peroxidase was determined using the formula below (Lowry *et al*., 1951).

$$\text{Specific activity} = \frac{\text{Enzyme activity (units/ml)}}{\text{Protein concentration (mg/ml)}}$$

Statistical Analysis

All data obtained were subjected to descriptive analysis, Dunette's test and Tukey's multiple T-test using Graphpad Prism^{TM}, version 7.0. Where applicable, the data were subjected to two-way analysis of variance (ANOVA) and differences between samples were determined. Level of significance was set at $p<0.05$.

III. Results

microbial colony counts for the different soil samples collected from the farm

The microbial colony counts of the soil from the farm are shown in Tables 1. Soil collected from farmland four had the highest bacteria load (33.0X 10^6 **CFU**) followed by farmland three (14.0 X 10^6) and the least was the soil from the farmland one (2.5X 10^6 **CFU**). However, soil 1 that had the lowest bacteria counts highest fungi count (23.6X 10^6 **CFU**) while soil 4 that had the highest bacteria count had the lowest fungi count (0.5X 10^6 **CFU**).

Table 2. Microbial (bacteria and fungi) count of soil collected from farm (10^6CFU)

Soil type	1	2	3	4
Bacteria Count	**2.5±0.5**	**11.5±0.5**	**14.0±1.0**	**33.0±3.0**
Fungal count	3.6±**0.**6	0.6±**0.3**	1.2±**0.**1	0.5±**0.**6

Cultural characteristics of the various isolates

The characteristics of the culture media of the various isolates subjected to various test is shown in table 4.2. All the bacteria were creamy in colour (table 1a) while fungi were greenish yellow (isolate S11a and S33b), black (isolate S11d and S22a), Brown (isolates S22b), white (isolate S33c) and green (isolate S44a). Gram reactions shows that they ere all gram positive. The cellular morphology of bacteria were rod in shape. Biochemical test detected presence of catalase, tyrosinase and laccase except in $S2_4$ and $S4_1$ where oxidase was absent. In the bacteria culture tyrosinase hydrolysis was highest in $S3_1$ (22) and lowest in $S2_4$ and $S1_2$ (16). For laccase hydrolysis it ranged from 12 µmol/ml in $S2_4$ to 16 µmol/ml in $S4_1$. All the fungi isolates showed presence of tyrosinase and laccase. Tyrosinase hydrolysis was higher in fungi ranging from 27 µmol/ml in isolate S44b to 24 µmol/ml in isolates S33b, S11a and S33b. Laccase hydrolysis was also higher in fungi ranging from 26 in isolates S22a and S33b to 30 in isolate S11a.

Table 3: Bacteria characteristics of the culture media of the various isolates

ISOLATE CODE	COLOUR	GRAM REACTION	CELLULAR MORPHOLOGY	CATALASE TEST	OXIDASE TEST	TYROSINASE	TYROSINASE HYDROLYSIS	LACCASE	LACCASE HYDROLYSIS
$S1_1$	Cream	G+ve	Rods	+	+	+	20	+	17
$S1_2$	Cream	G+ve	Rods	+	+	+	16	+	14
$S2_1$	Cream	G+ve	Rods	+	+	+	19	+	14
$S2_4$	Cream	G-ve	Rods	+	-	+	16	+	12
$S3_1$	Cream	G+ve	Rods	+	+	+	22	+	15
$S3_4$	Cream	G+ve	Rods	+	+	+	20	+	13
$S4_1$	Cream	G-ve	Rods	+	-	+	16	+	16
$S4_3$	Cream	G+ve	Rods	+	+	+	18	+	14

Table 4: Fungi characteristics of the culture media of the various isolates

ISOLATE CODE	COLOUR	TYROSINASE	TYROSINASE HYDROLYSIS	LACCASE	LACCASE HYDROLYSIS
S11a	Greenish yellow	+	24	+	30
S11d	Black	+	26	+	27
S22a	Black	+	24	+	26
S2 2b	Brown	+	26	+	28
S33b	Greenish yellow	+	24	+	26
S33c	White	+	25	+	27
S44a	Green	+	25	+	28
S44b	Greenish gray	+	27	+	29

DNA Sequence Analysis/ Molecular Identification Of Bacterial And Fungi Isolate

Basic Local Alignment Search Tool (BLAST) analysis identified five bacteria strains and six fungi strains which were characterized (Table 6). The identified bacteria had five strains of pseudomonas species (*Pseudomonas aeruginosa* strain MZ4A 94% identity and GC content of 54.55; *Pseudomonas aeruginosa* strain 22 98% identity and GC content of 55.04 and *Pseudomonas aeruginosa* strain HS-D38 having 99% identity and GC content of 54.38) as well as *Bacillus subtilis* strain VBN01 (94% identity and 55.01 GC) and *Rhizobium*

huautlense strain RA14. The fungi species identified were *Galactomyces geotrichum* strain CBS 774.71, *Aspergillus niger* strain YMCHA 73, *Aspergillus flavus* strain JN-YG-3-5 18S, *Aspergillus fumigatus* strain FJAT-31052 , *Aspergillus flavus* strain JN-YG-3-5, *Penicillium simplicissimum* strain SNB-VECD11G and *Trichoderma gamsii. Their sequences length were lower than the bacteria ranging 579.00 in Trichoderma gamsii* to 612.00 in *Aspergillus niger* strain YMCHA 73. S

Table 5. Microbial sequence characteristics

Organism type	Sample ID.	Organisms identified by BLAST	IDENT (%)	Sequence length (Bp)	% GC
BACTERIA	1	*Rhizobium huautlense* strain RA14	85	770.00	54.55
	2	*Pseudomonas aeruginosa strain* MZ4A	94	891.00	54.55
	3	*Pseudomonas aeruginosa* strain 22	98	863.00	55.04
	4	*Bacillus subtilis* strain VBN01	94	849.00	55.01
	5	*Pseudomonas aeruginosa* strain HS-D38	99	879.00	54.38
FUNGI	a	*Trichoderma gamsii*	99	579.00	53.54
	b	*Aspergillus flavus* strain JN-YG-3-5 18S 99%	95	589.00	56.71
	c	*Aspergillus niger* strain APBSDSF96	99	612.00	58.66
		Aspergillus fumigatus strain FJAT-31052	99	590.00	58.31
		Aspergillus flavus strain JN-YG-3-5	99	588.00	56.80
		Penicillium simplicissimum strain SNB-VECD11G		583	57.98

Phylogenetic Analysis

Phylogenetic analysis based on the nucleotide sequence of the bacteria and fungi were compared with each other (Fig. 1). The Neighbor-Joining distance analysis with sequence difference and topology showed two major clusters (fungi and bacteria). In the fungi group, *Aspergillus* formed a cluster showing their close relationship while in the bacteria group, the *Pseudomonas* species formed a single cluster showing also their relationship and evolutionary origin.

Figure 2: Phylogenetic relationship among all microbial isolates observed in the study.

Enzyme Production

The effect of duration on the specific enzyme activity (tyrosinase, laccase, manganese peroxidase, lignin peroxidase) is shown in table 6. Tyrosinase activity significantly differed with respect to day of examination and organism under investigation. It was noticed that *Penicillium simplicissimum* produced the highest tyrosinase activity (2.144 µmol/ml) on the 4th day of investigation while the Uncharacterized bacteria 1 produced the lowest tyrosinase activity (0.44 µmol/ml) on the first day of examination. The activities of tyrosinase between the first day and second day did not significantly increase on the second except for the third uncharacterized bacteria. All the characterized microbes had higher activities of tyrosinase compared to the uncharacterized. All orgarnisms tyrosinase increased significantly on the third day. More so, increase in laccase activity were not significant in all the organisms except for *Pseudomonas aeruginosa* strain MZ4A increase significantly from 4.47 µmol/ml on the first day to 14.65 µmol/ml on the second till the fourth day. The highest laccase activity was 14.24 µmol/ml in *Pseudomonas aeruginosa* strain MZ4A (day 4) and the lowest was 2.99 µmol/ml in the the second uncharacterized bacteria for day. However, manganese peroxidase decreased significantly ($p>0.05$) from the day two till the fourth day. The lowest manganese peroxidase activity was the uncharacterized bacteria 1 (1.44 µmol/ml) while the highest manganese peroxidase was in *Aspergillus fumigatus* (12.09 µmol/ml) on day 0ne. Similarly, lignin peroxidase decreased significantly ($p>0.05$) from the first day to the fourth day. *Penicillium simplicissimum* had the highest peroxidase activity (4.83 µmol/ml) while the uncharacterized bacteria 1 had the lowest peroxidase activity (0.43 µmol/ml).

IV. Discussion

Colony count of soil collected from farm

The environmental microbial community composition and diversity are sensitive key indicators to the impact of organic pollutant on the microbial ecology system over time (Zhao *et al.,* 2014). The high population of microbes in the soil samples is an indication of their roles. In this study high population of bacteria was enumerated compared to fungi. Ameh and Kawo (2006) had similar report. This difference can be the ubiquitous nature of bacteria. The microbial counts differed within the sampled soil. This can be linked to soil physicochemical parameters and the level of contaminants present in the soil. This is in agreement with the result of Zhao *et al.* (2014) who noted that pollution reduced microbial diversity. The implications of these finding is that the microorganisms found in these soil can be useful in the bioremediation of soil contaminated with pesticide products and possibly other organic polluted sites.

Cultural characteristics of the various isolates

Classical microbiology uses both gross and microscopic morphology to identify microbes. Gross morphology includes colony shape, size, and surface features (). In our study, we integrated the morphological and biochemical examination to identify the microbes. The creamy color, rod shape and gram reactions confirms them to bacteria. Murch (2003) asserted that the structures assigned to bacteria are cocci (round), rods, or spirochetes (corkscrew). It is clear that in the ikorodu farmof Lagos, majority of bacteria is the Gram positive and rods. In this the dominating bacteria is the Gram positive species while the fungal species identified corroborate the work of Ameh, (2017).

With the revolution in molecular biology, the characterization and typing of microbes has become easier. The molecular analysis of the gene sequence of the pure microbial culture identified five bacteria species and six fungi species. *Pseudomonas aeruginosa* species, dominated the bacteria species while *Aspergillus* species dominated the fungi species. These abundance of *Pseudomonas aeruginosa* and *Aspergillus* species in the farm exposed to pesticides might be due to their high ability to tolerate and degrade pesticides (Darsa, *et al*., 2014). Muhammad *et al*. (2016), reported that the accumulation of strains of fungi in the contaminated pesticides soils shows characteristic pattern for degrading process. They also reported that fungal strain*s* are able to use pesticides as a carbon and energy source under aerobic conditions. Similar, Chishti *et al*. (2012) reported that bacteria strains involve in the degradation process derive energy intake from these degradation products. *Rhizobium* species was identified. It has been documented that Pesticide-degrading bacteria such as *Rhizobium* species are effective for repairing soil, polluted by organic phosphorus pesticide (Zhao *et al*., 2012). More so the strain of *Pseudomonas* has been found to have a great capacity of biodegrading permethrin and cypermethrin pesticides (Mendoza *et al*., 2012). Microbial strain screening and isolation are very effective for degradation of pesticides in mineral culture medium. Therefore, the presence of these microbial species in our study can suggest their biodegradation potential towards glyphosate.

Phylogenetic Analysis

The Phylogenetic analysis showed two distinct groups namely the bacteria and fungi. This can be the combination of selective factors, proximity and functional capacity of microbes (Ning and Beiko, 2015). Functionally, phylogenetically distant lineages can share common functional features and functions. Ning and

Beiko (2015) also opined that functional similarities exist between operational taxonomic units (OTUs) that belong to different high-level taxonomic groups. Most of microbial sequences analyzed in different taxonomic divisions could be related to representatives with known metabolic traits.

Enzyme Production

Enzymes produced during different metabolic pathways in microbes present in soil are the key for bioremediation of pesticides (Muhammad, and Muhammad, 2016). This study shows that these microbes have versatile ability to degrade glyphosate by enzymatic action laccase, tyrosinase and peroxidases. Fungi has the highest enzyme production. Research by mohammad and Muhammad (2016) asserted that Lindane is degraded by fungus Conidiobolus through enzyme action. Also in a study of atrazine (AT) and alachlor (AL), their degradation by treating them with extracellular enzyme extracted from fungi was determined (Chirnside, 2007). This finding suggest that *Aspergillus fumigates* can be hily efficient in glyphosate degradation.

Different effectors

Enzymatic activities are involved in processes important to environmental function, such as organic matter decomposition and synthesis, nutrient cycling and decomposition of xenobiotics (Silvia, 2003). The results suggest that the hydrolytic and ligninolytic enzymes are are produced in such a way to induce degradation/transformation of glyphosate. Of particular interest is the capacity for production of these enzymes in the presence of glyphosate pesticide. In this study, *Aspergillus* species exhibited very high laccase activity in the presence of glyphosate pesticides. Previous studies suggest that a fungus showing high laccase activity in compost is a potential commercial source for laccase (Trejo-Hernandez *et al*., 2001). Thus, the levels of laccase produced by these test isolates may have some applications. The results also show higher production of laccase by the all isolates under glyphosate stress. Thus this enzyme might be involved in degradation metabolism. This support the works of Senthilkumar *et al.* (2011) and Bordjiba *et al*. (2001) that laccase demonstrated the ability to degrade endosulfan and methyl parathion pesticides and triazines. The result also shows to *Aspergillus* species produce higher levels of tyrosinase and peroxidases thus contributing the roles of these species in metabolism of organic contaminants. Abiotic factors that influence enzymatic activities largely determine the fate of pesticides in the environment. This work shows that production of laccase and tyrosinase were strongly inhibited by temperature above 40^0C. This is because high temperature can lead to loss of enzyme activity. Adeleye *et al*., (2018) has adduced that microbial degradation rates are generally decreased with increasing temperature which is believed to be as a result of decreased rates of enzymatic activity at such decreased temperature. The implication is that temperature range above 40^0C can inhibit microbial degradation rates of glyphosate. Adeleye *et al*., (2018) reiterated that higher temperatures inevitably increase the rates of organic compound metabolism to a maximum range of 30°C - 40°C in a typical soil environment. Moreover laccase activity was more resistant to temperature than tyrosinase probably due to their thermostability. Metabolic activities of microorganisms in a system can often be directly connected to the pH (acidity or alkalinity) of the system that is under examination. The result showed that pH played a vital role in the enzymatic activities of these microbes. The optimum pH activities for tyrosinase were 7.0 while laccase was 8.0. This is in tandem with the works of Adeleye *et al*., (2018) who reported that pH range of 7 to 8 can support optimum microbial degradation in such environment. Laccase was also found to be more resistant to pH changes compared tyrosinase. This finding implies that laccase can serve better in extreme environmental conditions. Various metal ions can influence enzyme activities in various ways. They can activate/inhibit their activities. It highly depends on the nature of the metal ions and the type of enzyme activity. Some metal ions act as part of the enzyme called allosteric compounds, which could aid in increasing the enzyme activity. In other cases, it could also affect the conformational shape of the enzyme and thus reduce the rate of enzyme activity. In this present study, mercury ion influenced tyrosinase activity negatively. This is because they compete with copper ions for the active site on tyrosinase. Similar study by Ann (2011) have shown that among other metals only mercury, silver, and gold ions inhibited tyrosinase action. On the other hand, the effects of metal ions oxidation catalyzed by laccase show that Fe^{2+} ion has obvious effect on the activity. The nature of inhibition can be competitive type. Wang *et al*. (2011) found out that inhibition of laccase by Fe^{2+} is realized through the reduction of 2,2'-azino-di(3-ethylbenzthiazoline-6-sulfonic acid). Enzyme activity may depend upon the nature and concentration of amended substrates and alternative electron acceptors (Silvia, 2005). The present study shows that glucose influenced enhanced tyrosinase activities most. This finding is not in an agreement with previous studies that adduced very low tyrosinase activity of cells cultured in medium with 5 mM glucose. The type of carbon source in the medium plays a major role in the production of ligninolytic enzymes (Gaulhaup *et al*., 2002). The carbon source of the growth medium appears to regulate the ligninolytic enzymes in microbes and the activity of these enzymes can be increased by the choice of the carbon source (Vaithanomsat *et al*., 2012). Both inorganic and organic carbon sources used in the present study supported the growth of the microbes. The bacterial strain achieved the maximum growth with glucose when compared with others.

The nature and concentration of the nitrogen source are the most important factors for the production of ligninolytic enzymes in white-rot fungi (Murugesan and Vembu, 2016). With regard to tyrosinase production, tryptone was found to be the best nitrogen sources while urea was the best for laccase production. However, Niladevi *et al*. (2009) reported yeast extract as the best nitrogen source for laccase production for the strain *Streptomyces psammoticus* whereas Kaushik and Thakur (2014) showed that tryptone induces the maximum laccase production compared to yeast extract in *Bacillus* sp. The results of the present study suggest that nutritional supplement that effectively enhances the enzyme production depends on the individual microbes and specific growth conditions.

V. Conclusion

It can be concluded from this study that pesticide degrading microorganisms have the inherent characteristics which may possibly give them the ability to bioremediation of glyphosate and other organic polluted environments provided that favourable environmental and nutritional conditions are ensured in bringing about their optimal growth. The results of the present study suggest that nutritional supplement that effectively enhances the enzyme production depends on the individual microbes and specific growth conditions. This study has provided valuable knowledge on the abilities of bacteria and fungi for glyphosate degradation. This will serve as a sound basis for the further exploitation of these species in biological remediation processes. Therefore, bioremediation technologies have got huge potentials in ensuring effective cleanup of glyphosate in polluted environments.