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Research Article
Enzymatic Recovery of Crude Oil Polluted Soil Enhanced by Treatment using *Mariscus alternifolius* Vahl. and *Fimbristylis ferruginea*

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Abstract

Background and Objective: Soil extracellular enzymes activities can reflect the metabolic need and nutrient availability of soil and as well possess the propensity to restore polluted soil towards normalcy. This study investigated the ability of some soil extracellular enzymes to recover crude oil polluted agricultural soil. **Materials and Methods:** Standard potentiometric method of pHKCl determination, loss of weight on ignition and culture methods were employed for all enzymatic and respiratory assays, organic matter and microbiological analyses, respectively. **Results:** Ninety days after planting, *M. alternifolius* and *F. ferruginea* treated soil groups recorded between 8.53 and 673.24% recovery as regards to dehydrogenase, protease, acid and alkaline phosphatase activities. **Conclusion:** Generally, the enzymes restored the polluted soil towards normalcy, probably necessitated by the treatment plants which aided in the abatement of the pollution and restoration of polluted soil.

Key words: Recovery, crude oil, polluted soil, soil extracellular enzymes, *Mariscus alternifolius* Vahl., *Fimbristylis ferruginea*

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Soil extracellular enzymes (SEES) are proximal drivers of decomposition[1] and activities of these enzymes in soils can be altered by crude oil pollution. Soil enzyme activities are related to heavy metal contamination and reduced by 10-50 times with an increase in heavy metal concentration. This increase may lead to heavy metal toxicity thereby affecting the microbial density, diversity and activity[2].

Soil micro-organisms produce extracellular enzymes to execute various biogeochemical processes involving several inorganic and redox reactions. Hydrolytic enzymes are needed for decomposition and mineralization of nutrients which are significant for the function of ecological unit[3]. In fertile soils, heterotrophic microbes are presented with detritus from plants and other biomasses that are rich in carbon[4] and other nutrients essential for their cell growth and maintenance. However, micro-organisms cannot transport these large macro-molecules directly into their cytoplasm[5]. They require extracellular hydrolytic enzymes (EHES)[4] to depolymerize them and generate soluble, low-number oligomers and monomers that are then recognized by cell-wall receptors and transported across the outer membrane and into the cell[5].

Extracellular enzymes such as proteases, dehydrogenases and phosphatases are engrossed for the recycling of carbon, nitrogen and phosphorus[6]. The activities of these enzymes can unswervingly mirror metabolic need and the nutrients available to soil micro-organisms[7].

Soil proteases (EC 3.4) are enzymes that perform proteolysis (protein catabolism) by hydrolyzing peptide bonds[8]. They are fundamental in nitrogen mineralization in a process which controls the quantity of plant available nitrogen[9,10]. Synthesis and activities of proteases in the soil are regulated by many factors such as climate, soil properties and the presence of organic compounds of plant and microbial basis[11].

Soil dehydrogenases (EC 1.1.1) are the main representatives of the oxidoreductase enzymes class[12]. Amid all enzymes in the soil environment, dehydrogenases are one of the central and are used as a marker of overall soil microbial activity[12,13]. This is due to its intracellular occurrence in virtually all viable living microbial cells[14,15] and function as a measurement of the the metabolic state of soil micro-organism[16]. It is one of the most adequate, vital and sensitive indicators relating to soil fertility[17]. The activities of dehydrogenases depend on the same factors, which influence the microbial abundance and activity[18]. They are closely related with microbial oxidoreduction processes[14]. Dehydrogenases play a momentous task in the biological oxidation of soil organic matter (SOM) by transferring hydrogen from organic substrates to inorganic acceptors[19]. However, the association between an individual biochemical property of soil dehydrogenases and the total microbial activity is not always apparent since microbial disintegration is highly diverse[20].

The phosphatase enzyme (EC 3.1.1) is used to explain a wide group of enzymes that hydrolyze organic phosphorus compounds, pyrophosphates, inorganic polyphosphates and metaphosphates in soils. It is generally accepted that plants utilize only inorganic phosphorus and since a huge proportion of soil phosphorus is organically bound, the mineralization of this organic fraction can be vital in plant nutrition[21]. The C-O-P ester bonds present in soil organic phosphorus compounds are hydrolyzed by phosphatase. Phosphatase activity is, hence, a vital aspect in sustaining and controlling the rate at which phosphorus is recycled through soils[22]. This study was therefore carried out to determine the soil extracellular enzyme activities in crude oil polluted soils undergoing treatment using *M. alternifolius* and *F. ferruginea*.

MATERIALS AND METHODS

Experimental design: This study was carried out at the University of Port Harcourt Ecological Center for 10 months between May, 2017 and February, 2018.

Crude oil polluted agricultural soil obtained from a spill site located in Ogoniland, Nigeria was randomly collected using sterile plastic bags sealed with rubber bands. Likewise, unpolluted soil was collected from an agricultural farmland located in the University of Port Harcourt. The soil samples were transported to Ecological Centre of the University of Port Harcourt for pot experimental study. Viable and mature seeds of *M. alternifolius* and *F. ferruginea*, collected from the wild after identification of the species from the spill site were used for nursery and afterward transplanted into polluted soil samples for treatment. Soil enzymatic, respiratory and microbial activities were determined in the soil samples prior to the commencement of the treatment and subsequently every 30 days over a 90 day period. The soil extracellular enzymes and respiratory activities and microbial density in crude oil polluted soils undergoing treatment were ascertained.

Soil enzymatic and soil microbial respiratory activities: The Alef and Nannipieri[23] potentiometric method of pHKCl determination as adapted by Chukwuma *et al.*[2] was employed for all enzyme assays while substrate induced method was employed for respiratory assays.

Protease activities: Protease activities were determined based on amino acids released after incubation of soil with sodium caseinate for 2 h at 50°C. The supernatant containing 1.4 M $NaSO_4$ and Folin-Ciocalteu (diluted 3 times) was read at 578 nm.

Dehydrogenase activities: Dehydrogenase activities were determined by estimating the rate of reduction of 2, 3, 5- triphenyl tetrazolium chloride (TTC) to triphenyl formazan (TPF) after incubation at 30°C for 24 h. Aliquots of the supernatants were thereafter read at 485 nm.

Acid and alkaline phosphatase activities: Acid and alkaline phosphatase activities assay relied on p-nitrophenol phosphate (PNP) determination for 1 h at 37°C. The supernatants containing aliquots of 0.5 M $CaCl_2$ and 0.5 M NaOH were read at 485 nm.

Respiratory activity: The substrate (glucose) induced method was employed for soil microbial respiratory activity estimation where screened (2 mm sieve) soil sample was introduced into the respiratory flask in addition to a glucose solution. After 24 h at 25°C, the respiratory activity was determined by titrating the CO_2 captured using NaOH with HCl and phenolphthalein indicator.

Soil microbial analyses: Total Culturable Heterotrophic Bacteria Count (TCHBC) and Total Heterotrophic Fungi (THF) TCHBC and TFC were determined by spread plate on nutrient agar (NA) and potato dextrose agar (PDA) methods respectively, as well as plate count agar (PCA) method as adopted by Seeley and VanDemark[24] and Ogunmwonyi *et al.*[25]. Following decimal dilutions (5 fold) of soil suspensions, TCHBC were plated out on NA medium and incubated at 30°C for 24 h while fungal isolates were plated out on PDA medium and incubated for 3 days at 28±2°C.

Physicochemical analysis

Soil Organic Matter (SOM): Organic matter was determined by loss of weight on ignition method as reported by Motsara and Roy[26] where sieved (2 mm) soil was dried at 105°C for 4 h, weighed, re-dried at 400°C and reweighed.

Statistical analysis: Results are expressed as means±standard deviation of triplicate determination. One way analysis of variance was employed to determine the significant difference between the groups. Data between groups were ascertained by the Bonferroni test using Statistical Package for the Social Science (SPSS®) Version 20 statistics software at 95% ($p = 0.05$) confidence level, while data between periods were ascertained by the Student t-test.

RESULTS

Protease activities: Protease activities of the various treated soil groups as shown in Table 1 revealed an increase in the absolute values of *M. alternifolius* treated soil over time, while those of *F. ferruginea* treated soil increased 30 DAP but subsequently decreased 60 and 90 DAP.

Dehydrogenase activities: The dehydrogenase activities (Table 2) of both treated soil groups significantly ($p<0.05$) increased 30 and 60 DAP albeit no significant difference ($p<0.05$) was recorded 90 DAP.

Acid phosphatase activities: Acid phosphatase activities of the treated soil groups are shown in Table 3. Results revealed that 90 DAP, a significant ($p<0.05$) increase in acid phosphatase activities of *M. alternifolius* treated soil was recorded while a significant ($p<0.05$) decrease in activities was recorded in *F. ferruginea* treated.

Alkaline phosphatase activities: Alkaline phosphatase activities are presented in Table 4. There was no significant difference ($p<0.05$) recorded in the alkaline phosphatase activities of *M. alternifolius* and *F. ferruginea* treated soil groups.

Soil respiratory activities: The soil respiratory activities of the treated soil groups are presented in Table 5. There was a significant ($p<0.05$) decrease in soil respiratory activities of *M. alternifolius* treated soil group while no significant difference ($p<0.05$) was recorded in *F. ferruginea* treated soil, 90 DAP.

Soil organic matter (SOM): The result of soil organic matter content (SOM) of the treated soil groups as presented in Table 6. There was a significant ($p<0.05$) decrease in SOM over time.

Total culturable heterotrophic bacteria count (TCHBC): The results of total heterotrophic bacteria count of the treated soil groups are presented in Table 7. There was a significant ($p<0.05$) increase in the TCHBC over time.

Table 1: Protease activities (tyrosine mg kg^{-1} dry matter h^{-1}) of the soil groups

Groups	BP	30 DAP	60 DAP	90 DAP	R 30 DAP (%)	R 60 DAP (%)	R 90 DAP (%)
Unpolluted control	12.02±2.0[a]	6.48±3.58[a]	32.49±3.61[a]*	22.99±3.66[a]*	NA	NA	NA
Polluted control	12.28±1.01[a]	23.87±16.10[a,b]	23.87±17.71[a]	13.06±4.74[b]	NA	NA	NA
M. alternifolius	12.28±1.01[a]	13.30±4.37[a,b]	24.90±12.03[a]	25.14±17.22[a,b]	60.78	11.88	121.65
F. ferruginea	12.28±1.01[a]	39.56±3.16[b]*	18.42±7.88[a]	13.91±1.90[a,b]	-90.20	-63.37	8.53

Values are Mean±standard deviations of triplicate determinations, values in the same column with different letters (a,b) are significantly different at p<0.05, *p<0.05 compared to the corresponding values before planting, BP: Before planting, DAP: Day(s) after planting, NA: Not applicable

Table 2: Dehydrogenase activities (TPF mg kg^{-1}/day) of the soil groups

Groups	BP	30 DAP	60 DAP	90 DAP	R 30 DAP (%)	R 60 DAP (%)	R 90 DAP (%)
Unpolluted control	8.56±0.89[a]	14.82±2.11[a]	26.08±40.26[a,b]	0.13±0.02[a]	NA	NA	NA
Polluted control	1.24±0.11[b]	6.84±5.06[b]*	4.05±0.15[a,b]	1.02±0.50[b]	NA	NA	NA
M. alternifolius	1.24±0.11[b]	4.86±1.06[b]*	2.92±0.73[a]*	0.24±0.11[b]	-24.85	-5.15	88.26
F. ferruginea	1.24±0.11[b]	3.60±0.49[b]*	4.44±0.55[b]*	1.34±1.11[a,b]	-40.73	1.79	-35.88

Values are Mean±standard deviations of triplicate determinations, values in the same column with different letters (a,b) are significantly different at p<0.05, *p<0.05 compared to the corresponding values before planting, BP: Before planting, DAP: Day(s) after planting, NA: Not applicable

Table 3: Acid phosphatase activities (PNP mmol kg^{-1} dw h^{-1}) of the soil groups

Groups	BP	30 DAP	60 DAP	90 DAP	R 30 DAP (%)	R 60 DAP (%)	R 90 DAP (%)
Unpolluted control	1.64±1.01[a]	0.66±0.07[a]	1.34±0.22[a]	7.40±1.21[a]*	NA	NA	NA
Polluted control	4.49±1.11[b]	5.71±0.73[b]	4.25±0.93[b]	12.55±2.16[b]	NA	NA	NA
M. alternifolius	4.49±1.11[b]	2.51±1.25[a,c]	2.60±0.70[b,c]	7.54±1.27[a]*	63.32	56.77	97.33
F. ferruginea	4.49±1.11[b]	5.71±1.06[b,c]*	2.60±1.23[b,c]*	1.15±0.13[c]*	0.16	56.87	221.30

Values are Mean±standard deviations of triplicate determinations, values in the same column with different letters (a,b,c) are significantly different at p<0.05, *p<0.05 compared to the corresponding values before planting, BP: Before planting, DAP: Day(s) After planting, NA: Not Applicable

Table 4: Alkaline phosphatase activities (PNP mmol kg^{-1} dw h^{-1}) of the soil groups

Groups	BP	30 DAP	60 DAP	90 DAP	R 30 DAP (%)	R 60 DAP (%)	R 90 DAP (%)
Unpolluted control	1.22±0.21[a]	2.71±1.20[a,c]*	0.33±0.06[a]	4.34±1.00[a]*	NA	NA	NA
Polluted control	3.12±2.11[a]	3.56±0.70[a]	3.35±1.00[b]	5.21±1.13[a]	NA	NA	NA
M. alternifolius	3.12±2.11[a]	4.99±0.65[b]	2.56±0.80[b]	0.26±0.10[b]	-167.66	26.34	573.24
F. ferruginea	3.12±2.11[a]	0.86±0.61[c]	0.45±0.21[a]	4.62±1.11[a]	316.77	96.13	67.72

Values are Mean±standard deviations of triplicate determinations, values in the same column with different letters (a,b,c) are significantly different at p<0.05, *p<0.05 compared to the corresponding values before planting, BP: Before planting, DAP: Day(s) after planting, NA: Not applicable

Table 5: Soil microbial respiratory activities (mg) of the soil groups

Groups	BP	30 DAP	90 DAP
Unpolluted control	2.76±0.01[a]	1.08±0.12[a]*	1.12±1.13[a,b]
Polluted control	1.80±0.10[b]	2.13±0.58[a]	1.47±0.39[a]*
M. alternifolius	1.80±0.10[b]	3.26±0.12[a]	0.24±0.21[b]*
F. ferruginea	1.80±0.10[b]	1.92±1.85[a]	0.72±0.63[a,b]

Values are Mean±standard deviations of triplicate determinations, values in the same column with different letters (a,b) are significantly different at p<0.05, *p<0.05 compared to the corresponding values before planting, BP: Before planting, DAP: Day(s) after planting

Table 6: Soil Organic matter (SOM) content (%) of the soil groups

Groups	BP	30 DAP	90 DAP
Unpolluted control	2.82±0.01[a]	2.76±0.61[a]	2.34±0.26[a]*
Polluted control	5.27±0.01[b]	4.64±0.40[b]	3.88±0.30[b]*
M. alternifolius	5.27±0.01[b]	4.77±0.40[b]	4.59±0.72[b]*
F. ferruginea	5.27±0.01[b]	4.75±0.13[b]*	3.79±0.35[b]*

Values are Mean±standard deviations of triplicate determinations, values in the same column with different letters (a,b) are significantly different at p<0.05, *p<0.05 compared to the corresponding values before planting, BP: Before planting, DAP: Day(s) after planting

Total fungi count (TFC): The result of total fungi count as shown in Table 8 revealed significant (p<0.05) increase in the HUF over time.

Table 7: Total culturable heterotrophic bacteria count (TCHBC) (in Log_{10} CFU g^{-1}) of the soil groups

Groups	BP	45 DAP	90 DAP
Unpolluted control	6.65±0.01[a]	6.06±0.18[a]*	7.27±0.09[a]*
Polluted control	5.43±0.02[b]	6.48±0.06[b]*	7.48±0.06[b,c]*
M. alternifolius	5.43±0.02[b]	6.54±0.13[b]*	7.56±0.29[a,c]*
F. ferruginea	5.43±0.02[b]	6.54±0.14[b]*	7.27±0.12[a,c]*

Values are Mean±standard deviations of triplicate determinations, values in the same column with different letters (a,b,c) are significantly different at p<0.05, *p<0.05 compared to the corresponding values before planting, BP: Before planting, DAP: Day(s) after planting

Table 8: Total fungi count (TFC) (in Log_{10} CFU g^{-1}) of the soil groups

Groups	BP	45 DAP	90 DAP
Unpolluted control	4.91±0.01[a]	5.81±0.05[a]*	6.73±0.10[a]*
Polluted control	4.85±0.00[b]	5.57±0.19[a]*	6.36±0.18[a,b]*
M. alternifolius	4.85±0.00[b]	5.65±0.13[a]*	6.45±0.06[b]*
F. ferruginea	4.85±0.00[b]	5.60±0.18[a]*	6.28±0.11[b]*

Values are Mean±standard deviations of triplicate determinations, values in the same column with different letters (a,b) are significantly different at p<0.05, *p<0.05 compared to the corresponding values before planting, BP: Before planting, DAP: Day(s) after planting

Pearson's correlation coefficient (PCC) of observed enzyme activities versus soil organic matter (SOM): Statistical analysis has shown a trend between organic matter, enzyme

Table 9: Pearson's correlation coefficient (PCC) of observed enzyme activities versus soil organic matter (SOM) content of the soil groups

Enzyme activity	Unpolluted control	Polluted control	*M. alternifolius* treated soil	*F. ferruginea* treated soil
Protease	-0.16	+0.68	+0.78	+0.74
Dehydrogenase	-0.96	-0.91	+0.42	-0.63
Acid phosphatase	-0.79	+0.94	+0.70	-0.45
Alkaline phosphatase	+0.26	-0.81	+0.88	-0.22
Respiratory	+0.50	+0.99	+0.82	+0.99

*Correlation is significant at the 0.05 level (2-tailed)

and respiratory activities (Table 9). Pearson's correlation coefficient (PCC, a measure of the linear correlation or dependence between two variables) of +0.68, +0.78, +0.74 (Table 9) for protease activity in polluted control, *M. alternifolius* treated and *F. ferruginea* treated soils respectively, showed a substantial positive correlation with soil organic matter while unpolluted control showed a negative correlation (-0.16 PCC) with soil organic matter. The considerable negative correlation of -0.96, -0.91 and -0.63 for dehydrogenase activity and SOM was recorded in unpolluted control, polluted control and *F. ferruginea* treated soil respectively, while a fair positive correlation (+0.42 PCC) was observed in *M. alternifolius* treated soil. Acid phosphatase activity showed a substantial positive correlation with soil organic matter for polluted control and *M. alternifolius* treated soil (+0.94 and +0.70 PCC, respectively) and a fair positive correlation (+0.45 PCC) for *F. ferruginea* treated soil while unpolluted control showed a substantial negative correlation (-0.79 PCC). On the other hand, alkaline phosphatase activity revealed a substantial negative (-0.81 PCC) and positive correlation (+0.88 PCC) with SOM for polluted control and *M. alternifolius* treated soil respectively, while a fair positive (+0.26 PCC) and negative (-0.22 PCC) correlation was observed for unpolluted control and *F. ferruginea* treated soil, respectively. Soil microbial respiratory activity showed a substantial positive correlation (+0.50, +0.99, +0.82 and +0.99 PCC) with soil organic matter for unpolluted control, polluted control, *M. alternifolius* and *F. ferruginea* treated soils.

DISCUSSION

The observed increase in protease activities over time as recorded in *M. alternifolius* treated soil and 30 DAP for *F. ferruginea* treated soil may be due to growth in microbial population, while the sharp decrease in activities may have been necessitated by the inhibitory influence of the treatment plant on micro-organisms-producing proteases. The latter corresponds with the findings of Chukwuma *et al.*[2] who reported a decrease in protease activities of soils phytoremediated over a 12 week period. This assertion may be supported by Adeniyi *et al.*[27] who reported the tendency of plant extracts to inhibit certain micro-organisms. The percentage recovery given by:

$$\text{Recovery (\%)} = \frac{\text{Parameter in consideration} \times \text{Test (polluted) Control}}{\text{Normal (unpolluted) control} \times \text{Test (polluted) Control}} \times 100$$

Chukwuma *et al.*[2] showed that by 30 DAP, treatment using *M. alternifolius* restored the polluted soils towards normal value (60.78%). However, the value obtained for treatment using *F. ferruginea* nose-dived, indicating the failure in restoration, especially with regards to the activities of proteases. By 60 DAP, only treatment using *M. alternifolius* restored the polluted soil towards normal value (11.88%). Likewise, 90 DAP, treatments using *M. alternifolius* and *F. ferruginea* restored the polluted soils towards normalcy (121.65 and 8.53).

The significant ($p<0.05$) increase in dehydrogenase activities as recorded for both treated soil groups corresponds with previous report[2]. Although the microbial populations of the treated soil groups increased over time, Nath and Samanta[28] has indicated that aside the microbial population, activities of these microbes and the kind of microbes present in the soil determine the activities of enzymes. Nevertheless, it may be that the ecological equilibrium of micro-organisms was disrupted which led to the decrease in production of the enzyme. By 30 DAP, recovery of *M. alternifolius* and *F. ferruginea* treated groups nose-dived demonstrating failure in restoration particularly with regards to the activities of dehydrogenases. However, by 60 DAP treatment using *F. ferruginea* restored the polluted soil towards normal value (1.79%) albeit the value obtained for treatment using *M. alternifolius* nose-dived. Likewise, by 90 DAP, only treatments using *M. alternifolius* restored the polluted soils towards normalcy (88.26%), while values obtained for treatments using *F. ferruginea* nose-dived.

The significant ($p<0.05$) decrease in acid phosphatase activities recorded in *F. ferruginea* treated soil group may be attributed to the redundancy in microbial population and/or activity 90 DAP and this corroborates with earlier report[2] which in addition associated such a decrease to limiting

effects of nutrients in the pots posed by its depletion over time. As previously reported[29], such activities as depicted by the groups may indicate the state of the soil in terms of physicochemical properties, especially pH, which is a key determinant of soil acid and alkaline phosphatase activities. Nonetheless, the treatments restored the soil towards normal levels by 30, 60 and 90 DAP, even though treatment using *M. alternifolius* by 30 DAP nosedived indicating a failure in restoration.

The significant ($p<0.05$) decrease in soil respiration observed 90 DAP *M. alternifolius* corroborates the report[30] which previously associated such a decline in respiratory activity after attaining peak to the depletion of available carbon substrates.

The significant ($p<0.05$) decrease in SOM over time as recorded in the study corroborates the report[31] which associated such a decrease in the degradation of crude oil Nonetheless, it may, however, be an indication of microbial activities ongoing in the soil. Microbes consume organic matter and in turn release CO_2. Therefore, a decrease in SOM might be indicated high soil microbial and respiratory activity. This aligned with the report[32] that the decomposition of organic matter is largely a biological process that occurs naturally and determined by soil organisms, the physical environment and the quality of the organic matter.

The increase in TCHBC and TFC of the treated soil groups over was not surprising as similar finding[32] has been reported which shows the progressive utilization of organic matter and hydrocarbon, with the hydrocarbons functioning as primary substrates. Ebuehi *et al.*[33], such increase is indicative of increased biodegradation by the microbial community.

CONCLUSION

Microbial and enzymatic activities can reflect the metabolic need and nutrient availability of soil. The soil extracellular enzymes were found to restore the polluted soil towards normalcy, probably necessitated by the type of plant involved in the treatment. Therefore, it is noteworthy to state that the application of these plant species in the treatment of crude oil polluted agricultural soils can enhance microbial and enzyme activities thereby aiding in the abatement of the pollution and restoration of the polluted soil.

SIGNIFICANCE STATEMENT

This study discovered the influence of *M. alternifolius* and *F. ferruginea* plants species in the enzymatic recovery of crude oil polluted agricultural soil over time. This study will help researchers to uncover the critical areas of soil recovery using soil extracellular enzymes.

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