ISSN 2348-313X (Print)
International Journal of Life Sciences Research ISSN 2348-3148 (online)
Vol. 5, Issue 1, pp: (62-68), Month: January - March 2017, Available at: www.researchpublish.com

Optimizing Bioremediation of Phenol Polluted Soil Using Laccase from Soil Fungi

Balogu[1], V. Tochukwu, Mohammed[1], G. Ramatu, Mohammed[2], S. D Sani, Yewodo[1], J. Clement

[1]Department of Microbiology, Ibrahim Badamasi Babangida University, Lapai
[2]Department of Microbiology, Kaduna State University, Kaduna

Abstract: **Prevalence of laccase (LAC) producing soil fungi and bioremediation potentials of LAC crude extracts on phenol-polluted soil were assessed. LAC producing isolates screened with mineral salt agar (supplemented with 0.02% guaiacol) at 30 ^{0}C for 7 days and propagated in potato dextrose broth for 14days prior soil treatment. Twelve opaque plastic buckets were loaded with 1 kg of farm soil each and grouped into 4 sets (triplicate per set). Two sets designated treated soils were TS1 and TS2, and two sets as control soils (CS1 & CS2). TS1 and TS2 polluted with 100 mL and 150 mL of phenol respectively, were treated with 100 mL of LAC crude extracts and compared with untreated soils CS1 (100 mL phenol) and CS2 (0 mL phenol) within 5 weeks. Among the isolated fungi, only 62% were LAC producers (*Fusarium sp, Absidia sp* and *Rhizopus* sp). LAC treatments (TS1 &TS2) significantly reversed the adverse effect of phenol on soil pH and bioactivities (BOD and TOC) by 60-100% but insignificant to soil temperature. Phenol removal were optimized by TS2 (46%) and TS1 (21%), while natural degradation by CS2 was only 6% in 5 weeks. Thus, these findings are recommendable as cheap and ecofriendly alternative for managing industrial waste (phenol) pollutions.**

Keywords: **Laccase, Bioremediation, Soil fungi, optimize, Phenol.**

I. INTRODUCTION

Bioremediation is the use of biological agents to eliminate harmful environmental toxicants. Most cheap and eco-friendly detoxifying processes were achieved with microbial cells or their metabolites, which maybe indigenous or introduced. Series of degradation mechanisms by microbes are depended on enzymatic activities that form part of their metabolic processes. Microorganisms possess different enzymes in their system that enables them to carryout bioremediation effectively and one of such enzymes that have been subject of many studies including this study is laccase [1].

Laccases are multicopper enzymes belonging to the group of blue oxidases that use molecular oxygen to oxidize various aromatic and non-aromatic compounds by a radical-catalyzed reaction mechanism [2]. Laccases are found in bacteria, higher plants, fungi and insects. Among the fungal group, Laccase have been isolated from *Basidiomycetes*, Ascomycetes and Deuteromycetes [3]. Quinine and phenoxy radicals are the major metabolites of *Ascomycetes* and *Deuteromycetes* during phenol oxidative degradation [4]. Owing to the high nonspecific oxidation capacity of laccases, they are useful biocatalysts for diverse biotechnological applications. Recently, laccase have been applied in various industries such as textile, food, pulp and paper, synthetic chemistry, cosmetics, pesticides, biofuel cells, medical diagnostic tool and have also been used in the detoxification of environmental pollutants e.g. phenols and endocrine disruptors [5, 6].

Phenolic (hydroxybenzene) compounds are natural or synthetic aromatic compounds that pose a high environmental impact risk even at small concentration. Due to this fact, industries with phenolic effluents face huge economic and environmental management issues. Phenol degrading microbes (bacteria and fungi) utilize high oxidative phenol hydroxylase enzymes to form catechol [7] that could either be within aerobic or anaerobic conditions [8].

Environmental pollution which mostly arises from industrial waste, human activities and constant use of pesticide that utilize phenols as solvents, has been a major problem faced by the society. This poses a deleterious effect on the soil ecosystem and man, consequently reducing agricultural crop yield. Thus, the essence of this study was to isolate crude extract of laccase from soil fungi to optimize bioremediation of phenol contaminated farm soil.

2. MATERIALS AND METHODS

Sample collection/preparation:

Four bulks (4kg each) of farm soil samples collected from different locations within the environment of Ibrahim Badamasi Babangida Univsersity, Lapai. Samples (2 -5cm soil depth) were transported (< 4^0C) in clean cellophane bags to the laboratory, and refrigerated prior to analysis. Soils for bioremediation assessment were sieved in 2mm mesh, loaded into stainless steel containers and steam sterilized (121 ^{0}C, 15psi for 15mins).

Fungal Isolation and characterization:

Samples for isolation and characterization of LAC producing fungi were collected from the bulk soil before sieving. three different sampling of 1 g each were randomly collected from each bulk, diluted serially and 1 mL of the 2nd and 3rd folds were pour plated on sabouraud dextrose agar (SDA) incorporated with streptomycin. Culture plates were incubated upright at 25 ^{0}C for 5-7 days. Pure isolates were characterized with relevant morphological and biochemical assays [9].

Screening for Laccase production:

Pure fungal isolates were screened for LAC production using plates of mineral salt agar composed of 0.1 ml $ZnSO_4$, 1ml ethanol, 1.5g peptone, 5g glucose, 0.3g KH_2PO_4, 0.2g K_2HP0_4, 0.5g $FeSO_4$, 0.3g $MnSO_4$, 0.3g $MgSO_4$ and 20g Agar agar inside a flask made upto 1000mL with distilled water. Culture pates were seeded with 0.02% guaiacol and incubated at 30^{0}C for 7 days. LAC activity was visualized on plates with reddish brown zones in the medium due to polymerization of guaiacol by LAC activities [10]. The plates with this reddish brown zones were designated as LAC positive isolates.

Production and crude extract of Laccase:

LAC positive isolates (consortium) were propagated in flasks containing 1000 mL potato dextrose broth, incubated (25 ^{0}C) for 14days, and agitated periodically every 2 days. Culture suspensions were filtered through membrane filters and filtrates were centrifuged at 5000rpm for 10mins. The resultant supernatant were designated as LAC crude extract and stored (refrigerated) prior to bioremediation assessment [11]. LAC crude extracts were assigned arbitrarily unit of LAC activity/mL

Protocol for assessing the bioremediation prospect of crude LAC activity:

Twelve uniform plastic opaque buckets (10 Litres), grouped to into 4 units (TS1, TS2, CS1 & CS2) with 3 replicates for each unit were designed as:

CS1 = 1kg of soil only;

CS2= 10ml of phenol + 1kg of soil

TS1 = 10ml of phenol+100ml of LAC+1kg of soil,

TS2= 15ml of phenol+100ml of LAC+1kg of soil.

Each vessel was periodically agitated every 2 days for five weeks prior to sample collection.

Determination of soil physiochemical parameters (pH and temperature):

Temperature and pH were determined using a pH meter and glass mercury thermometer respectively. The device probes dipped into the soil samples (1.5-2.0 cm depth) for 2 minutes and the values were read and recorded [12].

Determination of Total organic carbon (TOC) and Biochemical oxygen demand (BOD):

The total organic carbon content (TOC) was determined using walkley-black procedure with slightly modification by using spectrophotometric (400nm) method for quantification. This is to avoid pigments of indicators and titrants interference as one of the pitfall of titration method [12, 13].

$\mathrm{TOC} = 0.060\ (\mathrm{ABS}) + 0.247$ This was obtained from glucose standard curve of 5Kg/C

ISSN 2348-313X (Print)
International Journal of Life Sciences Research ISSN 2348-3148 (online)
Vol. 5, Issue 1, pp: (62-68), Month: January - March 2017, Available at: www.researchpublish.com

$$BOD = \frac{\text{TOC } (4.8)}{1.5}$$

Statistical analysis:

Data were subjected to ANOVA and means were separated with Duncan multiple range test (P: 0.05) using SPSS statistical software version 20.

3. RESULTS AND DISCUSSION

LAC producing fungi were *Fusariums* sp (39%), Absidia sp (15%) and *Rhizopus* sp (8%), while non-LAC producing fungi were *Aspergillus niger* (15%), *Aspergillus flavus* (15%) and *Penicillium* sp had (8 %). The overall prevalence of LAC fungi from soil was 62% and non-LAC fungi was 38 %(Fig 1). Spectrum of fungi isolated from this study were natural habitats of soil environments. This concurred with the studies of [10, 14], which isolated similar groups of fungi from soil samples. Previous studies on Laccase production by fungi have inferred that culture medium rich in carbon and nitrogen content have significant effect on laccase production [15 - 17]. This perhaps account for relative high prevalence of Laccase producing fungi observed in this study, due to special mineral salt medium rich in carbon and nitrogen used in this study.

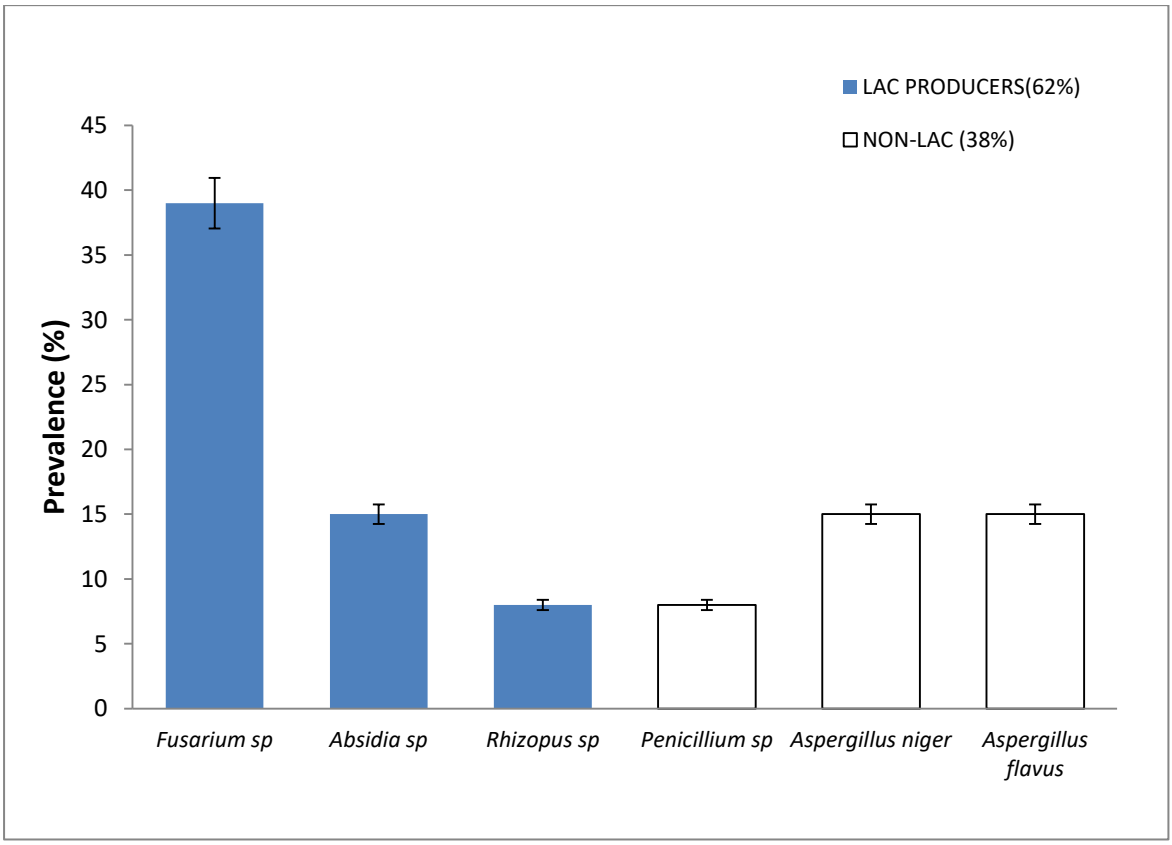

Figure 1: Prevalence of LAC and Non-LAC producing fungi isolated from soil *Key: LAC = Laccase*

Among the control samples, only CS2 significantly retarded the organic activities (-17.3%) and there was no change in CS1 (0%). However, after 5 weeks of treatment with LAC, the negative effect on organic activities due to phenol pollution, were restored to 17.3% and 10.8% by TS1 and TS2 respectively (Fig 2). Based on the positive observed changes in organic activities in soil, it was concluded that 100mL of LAC crude extract would optimize removal of phenol (10 - 15mL) from 1kg of polluted soil by 62 - 100%. This was obvious in the uniformity of TOC values of TS1, TS2 and CS1 after 5 weeks. Within the 1st week, both TOC (2.78 Kg/C) and BOD (8.89mg/L) of CS2 were adversely affected by phenol compared to CS1 values of 3.26Kg/C and 10.43mg/L respectively. However, LAC treated soils TS1 and TS2 completely restored TOC and BOD activities to the original status of 3.26Kg/C and 10.43mg/L respectively (Fig. 3).

ISSN 2348-313X (Print)
International Journal of Life Sciences Research ISSN 2348-3148 (online)
Vol. 5, Issue 1, pp: (62-68), Month: January - March 2017, Available at: www.researchpublish.com

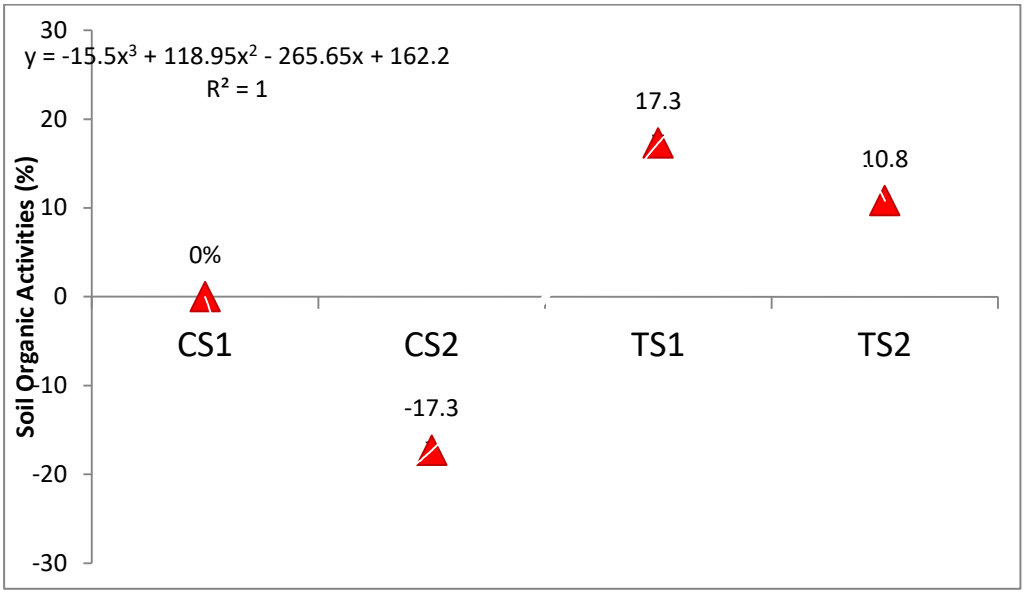

Figure 2: Effect of phenol on the overall soil Organic Activities (TOC and BOD)

CS1= 1kg of soil only
CS2= 10ml of phenol+ 1kg of soil
TS1= 10ml of phenol+100ml of laccase+1kg of soil
TS2= 15ml of phenol+100ml of laccase+1kg of soil

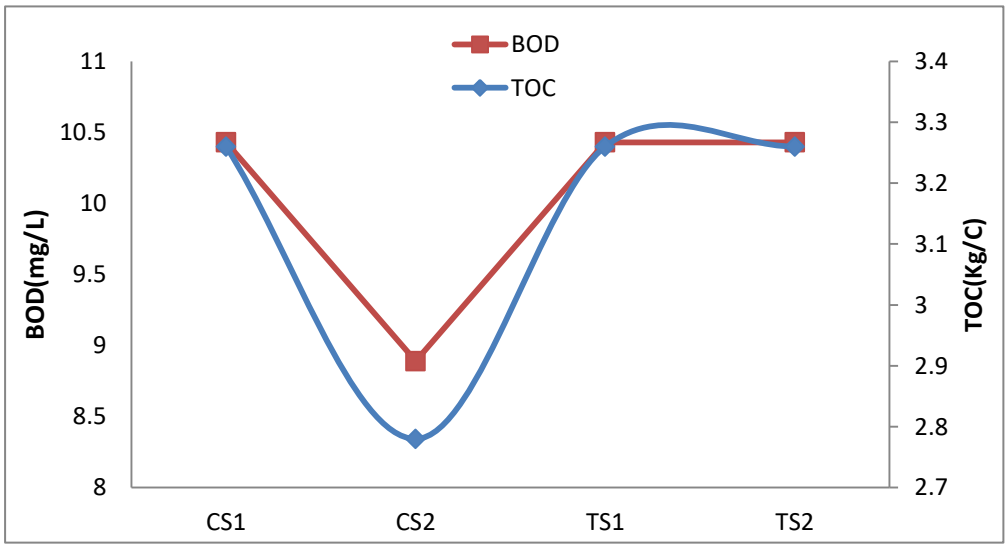

Figure 3: TOC and BOD profile of treated soil after five (5) weeks

CS1= 1kg of soil only
CS2= 10ml of phenol+ 1kg of soil
TS1= 10ml of phenol+100ml of laccase+1kg of soil
TS2= 15ml of phenol+100ml of laccase+1kg of soil

Phenol pollution reduced the pH of CS2, TS1, and TS2 to 3.5, compared to the pH of 4.0 observed in CS1, which contain no phenol. However, after 5 weeks assessment, pH of treated soils (TS1 and TS2) and control soils (CS1 and CS2) were 3.7 and 3.9 respectively. (Fig. 4). Among the environmental factors (such as temperature, nutrient availability and bioavailability), pH is one of the major parameter that influence enzymatic activity of the impacted soil [18]. The pH of treated soils were significantly different ($p<0.05$) from the control values. This may be attributed to acid-base interaction and chemical derivatives of phenol, that is as a result of degradations impact of LAC that invariably affects the pH. However, optimum pH for laccase activity depends on the substrate and environmental factors. The observed pH range of this study coincides with the findings of [19], thereby validates the stability of LAC activity in this study.

ISSN 2348-313X (Print)
International Journal of Life Sciences Research ISSN 2348-3148 (online)
Vol. 5, Issue 1, pp: (62-68), Month: January - March 2017, Available at: www.researchpublish.com

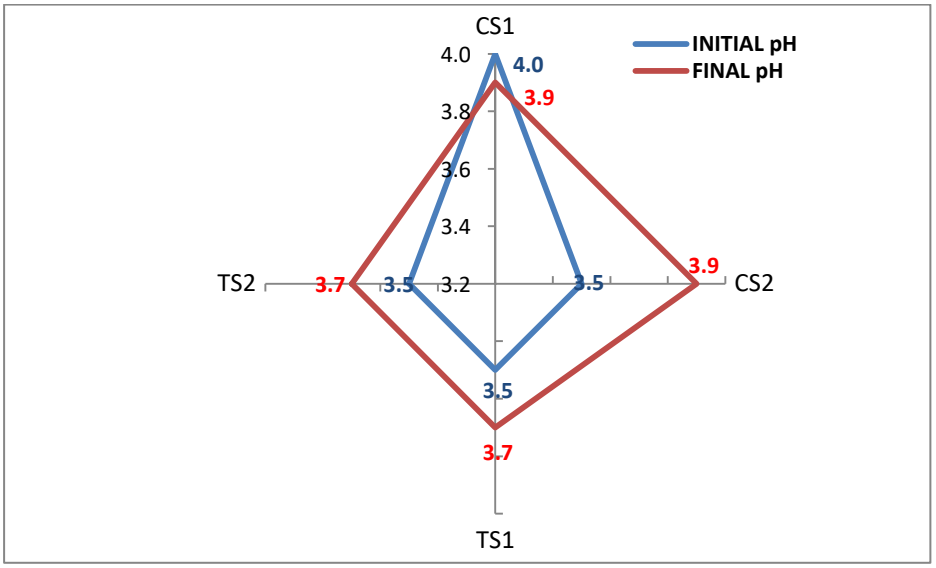

Figure 4: Effect of LAC on pH of phenol polluted soil after five (5) weeks

CS1= 1kg of soil only
CS2= 10ml of phenol+ 1kg of soil
TS1= 10ml of phenol+100ml of laccase+1kg of soil
TS2= 15ml of phenol+100ml of laccase+1kg of soil

Temperature (26.0 ^{0}C - 26.9 ^{0}C) were relatively stable irrespective of the treatments and were not significantly different ($p<0.05$) from the control values throughout the period of study (Fig. 5). This was unconnected to the effect of ambient environmental temperature. Perhaps, the metabolic activities during the 5 weeks assessment accounts for the slight increase in temperature from 26.0 ^{0}C to 26.9 ^{0}C. Similarly, the study of [20] reported that laccases were active over a range of temperature (20-55^{0}C), and this concurred with this study's temperature range. This further validates the stability of LAC used during this study.

There was direct correlation of phenol degradation and amount of LAC applied as treatments ($R^2 = 0.74$). However, the correlation was not perfect but definitely a prospective model that could optimize phenol degradation efficient of LAC. Phenol degradation in CS2 was 6%, due to natural phenomenon, while TS1 and TS2 optimized phenol removal by 24% and 46% respectively (Fig. 6). Higher phenol degradation rate observed in TS2 than TS1 validates the proximity theory of enzyme catalysis, which stipulates that high substrate concentration increase the rate of enzyme activity. This means that increase in phenol induced an increase in biodegradation rate of phenol.

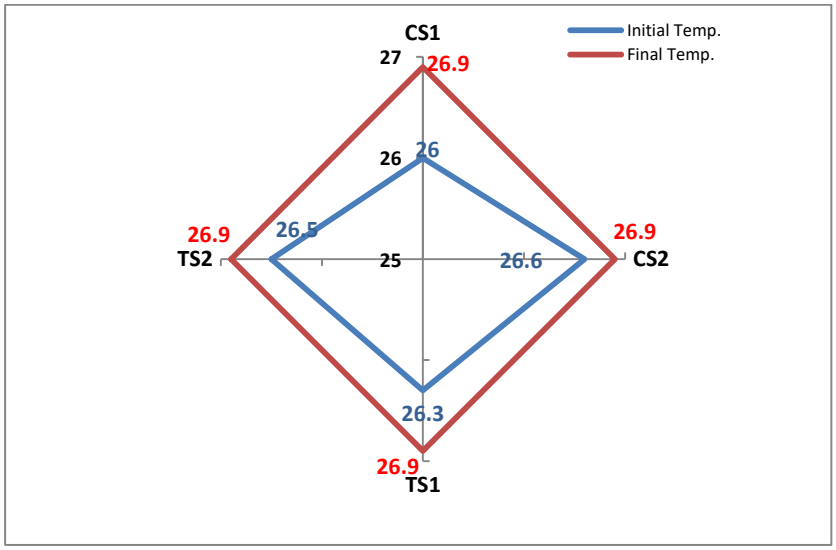

Figure 5: Effect of LAC on temperature (^{0}C) of phenol polluted soil after five (5) weeks

ISSN 2348-313X (Print)
International Journal of Life Sciences Research ISSN 2348-3148 (online)
Vol. 5, Issue 1, pp: (62-68), Month: January - March 2017, Available at: www.researchpublish.com

CS1= 1kg of soil only
CS2= 10ml of phenol+ 1kg of soil
TS1= 10ml of phenol+100ml of laccase+1kg of soil
TS2= 15ml of phenol+100ml of laccase+1kg of soil

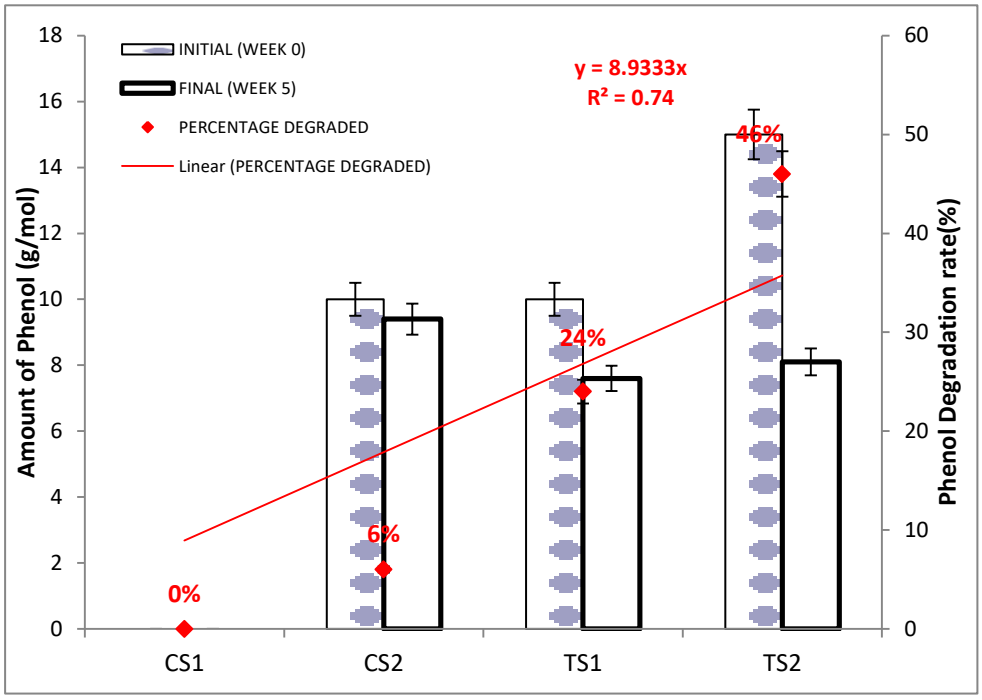

Figure 6: Effect of LAC on Phenol degradation rate of polluted soil after 5 weeks period

CS1= 1kg of soil only
CS2= 10ml of phenol+ 1kg of soil
TS1= 10ml of phenol+100ml of laccase+1kg of soil
TS2= 15ml of phenol+100ml of laccase+1kg of soil

4. CONCLUSION

Results obtained from this work would aid advancement of rehabilitation technology for agricultural soils contaminated with phenol. Within the purview of this laboratory scale assessement, LAC crude extract effectively optimized bioremediation of phenol contaminated soil. However, further studies should be geared towards on-field bioremediation assessment.

REFERENCES

[1] Matsuzawa, S., Nasser-Ali, L., and Garrigues, P. (2001). Phytolytic behaviour of polycyclic aromatic hydrocarbons in diesel particulate matter deposited on the ground. *Environmental science & technology*, *35*(15), 3139-3143.

[2] Morozova, O. V. Shumakorich, G. P. Gorbacheva, M. A., Sweev, S. V. and Yaropolov, A.I. (2007). "*Blue Laccases: Biochemistry (Moscow*)".(72):10,136- 1150.

[3] Gianfreda, I., Xu F. and Bollag, J. M. (1999). "Laccases a useful group of Oxidoreductive Enzymes". *Bioremediation Journal*, 3(1):1 – 25.

[4] Eggert, C. Temp, U. and Eriksson, K. E. L. (1996). "The Liqninolytic System of the White Rot Fungus Pycnoporus Cinnabarinus: Purification and Characterization of the Laccases*," Applied and Environmental Microbiology*, 62(4):1151 – 1158.

[5] Couto, S. R. and Toca Herrera, J. L. (2006) "Industrial and Biotechnical Applications of Laccases: A Review *Biotechnology Advances*, 24(5):500 – 513.

[6] Faccolo, J. and Cruz, O. (2008). Banana Skin: A Novel Material for a Bio-Cost Production of Laccases, M. S. Thesu, Universidad Rounra, I. Virgili.

[7] El-Naas, M. (2012). Aerobic Biodegradation of Phenols: A Comprehensive Review. Critical Reviews in Environmental Science and Technology 42(16):1631.DOI: 10.1080/10643389.2011.569872.

[8] Paula, M., Schie, V and Young, L. Y. (2007). Biodegradation of Phenol: Mechanisms and Applications. *Bioremediation Journal*, pp 1-18. http://dx.doi.org/10.1080/105883300 08951128.

[9] Ochei J. and Kolhatkar A. (2008). Medical laboratory science: theory and practice. Tate MacGraw Hill Publishing Company New Delhi India. Pp 818 – 824 Seventh reprint.

[10] Coll, P. M., Tabernero, C., Santamaria, R., and perez, P. (1993). " Charracterization and structural analysis of the laccase I gene from the newly isolated lignolytic basidiomycete PMI (CECT 2971),"*Applied Enviromental Microbiology* 59, 4129-4135.

[11] Stoilova, I., Krastanov, A. and Stanchev, V. (2010). Properties of crude laccase from Trametes versicolor produced by solid-substrate fermentation. *Advances in Bioscience and Biotechnology*, 1: 208-215doi:10.4236/abb.2010.13029.

[12] A.O.A.C. (1999). Official method of analysis. Vol. 11, 16th edition. Association of Official Analytical Chemists, Arlington, V.A.

[13] Yewodo, C. J. (2016). Bioremediation of Industrial Waste (Phenol) Polluted Soil Using Laccase from Soil Fungi. Unpublished. Thesis of Department of Microbiology, IBB University, Lapai, Nigeria. pp 1 - 33.

[14] Olga V.K.S., Elena V.S., Valeria P.G., Olga V.M., Natalia V.L., Aida N.D., Alexander I.J., Alexander M. (1998). Purification and characterization of the constitutive form of laccase from basidiomycete *Coriolushirsutus* and effect of inducers on laccase synthesis. Biotechnol.*"Applied Biochemistry"*.28: 47-54.

[15] Niku-Paavola ML, Karhunen E, Kantelinen A, Viikari L, Lundell T,Hatakka A (1990). The effect of culture conditions on the production of lignin modifying enzymes by the white-rot fungus *Phlebiaradiata*. "*Journal of Biotechnology"*. 13: 211- 221.

[16] Rogalski, J., wojtas-Wasilewaka, M., Apalovit, R., and Leonowicz, A. (1991)." Affinity chromatography as a rapid and convenient method for purification of fungal laccase," *Biotechnology and Biuoengeneering.* 37, 770-777.

[17] Schlosser D, Grey R, Fritsche W (1997). Patterns of ligninolytic enzymes in *Trametesversicolor*. Distribution of ectra and intracellular enzyme activities during cultivation on glucose, wheat straw and beech wood. Applied. Microbiology.

[18] Condi, L. Minussi, R. C., Freire, R. S. and Duran, N. (2007)."Fungal Laccase; Copper Induction, Semi Purification, Immobilization, Phenolic Effluent, Treatment.

[19] Rahman, K. S. M., thahira-Rahman, J., Lakshmanaperumalsamy, P., & Banat, I. M. (2002). Towards efficient crude oil degradation by a mnixed bacterial consotium. *Bioresource technology*, 85(3), 257-261.

[20] Saraiva, J. A., Tavares, A. P. and Xavier, A. M. (2012).E_ect of the inducers veratryl alcohol, xylidine, and ligninosulphonates on activity and thermal stability and inactivation kinetics of laccase from Trametesversicolor. "*Applied Biochemical and Biotechnology"* 167, 685-93.