G.J.B.A.H.S.,Vol.5(2):135-138 *(April-June,2016)* **ISSN: 2319 – 5584**

GLOBAL JOURNAL OF BIOLOGY, AGRICULTURE & HEALTH SCIENCES
*(**Published By**: Global Institute for Research & Education)*

www.gifre.org

Bioremediation of Polycyclic Aromatic Hydrocarbons (PAHs) contaminated environment: Laccase Activity of *Penicillium freii* and *Aspergillus niger* at different pH

E. Omaka[1,2], & A. Kalu[2]

[1]University of Hertfordshire, Hatfield
School of Life Sciences, Hertfordshire, AL10 9AB, United Kingdom
[2]National Biotechnology Development Agency, Lugbe, Abuja. FCT. P.M.B 5118

Abstract

The environment faces tremendous challenges particularly oil pollution that pose significant threats to lives. Polycyclic aromatic hydrocarbons (PAHs), which are natural constituents of petroleum hydrocarbons, are ubiquitous organic compounds with known and suspected carcinogenic potentials. PAHs have the tendency of bioaccumulation and are recalcitrant in nature due to their biochemical stability. Effective bioremediation requires in-depth understanding of the pollutants, the microorganisms and the parameters involved. White rot fungi through the activities of their ligninolytic enzymes have shown abilities in degrading these PAHs. Fungal cultures were isolated by enrichment culture obtained from road-side soil in University of Hertfordshire College Lane Campus, Hatfield, United Kingdom and identified as *Penicillium freii* and *Aspergillus niger*. After identification, *Penicillium freii* and *Aspergillus niger* were cultured in malt extract broth adjusted to range of broth pH 5.5, 7.0 and 8.5, incubated on a rotary shaker and harvested after seven days. Laccase activity (µmol/ml/min) was determined by oxidation of ABTS. Laccase activity (µmol/ml/min) of *Penicillium freii* and *Aspergillus niger* was optimum at pH 5.5.

1.0 Introduction

Increased industrialization in the 21st century has necessitated the need for a cleaner environment, which is a prerequisite for a general healthier life (Kollmuss & Agyeman, 2002). Achieving a clean environment entails strict adherence to environmental policies as several countries have designed strategic environmental policies to regulate environmental matters such as disposal of household waste, disposal of industrial waste, petroleum refining, transportation of petroleum products, use of fungicide and pesticide, building of structures and other relevant activities in order to safeguard the lives of humans, animals and plants; the violation of these environmental regulations usually results in penalties (Walter & Ugelow, 1979).

With the evolution of biotechnology, bioremediation has emerged out of the need to utilise the potentials of biological processes for the treatment of pollutants in contaminated environments (Balba, et al., 1998). Biological scientists have explored the use of microorganisms in the degradation of different organic waste products (Balba et al., 1998). Polycyclic aromatic hydrocarbons are recalcitrant organic compounds with suspected and known mutagenic and carcinogenic potentials. They are formed by the fusion of two or more benzene rings (Kanaly & Harayama, 2000).

Bacteria and white rot fungi have been used in several studies to degrade PAHs in vitro successfully; but demonstration of this biodegradation in vivo remains uncertain (Balba et al., 1998; Novotny et al., 2004). Some physical conditions such as temperature, pH, oxygen availability, moisture content, redox potential and substrate bioavailability influence the biodegradation rate of PAHs (Balba et al., 1998).

The presence of PAHs in aquatic habitats is no doubt a source of concern due to the danger it poses to aquatic lives, and leakages from underground natural deposits is one of the main routes through which petroleum oil makes its entry into marine habitats and such leakages poses a long lasting threat to different forms of life (Balba et al., 1998). The introduction of PAHs into the water of Puget Sound has been implicated in the development of carcinomas in bottom fish (Laliberte & Ewing, 2006). PAHs are suspected to be associated with the disruption of reproductive events in salmonids (Laliberte & Ewing, 2006). The activities of two PAHs, napthoflavone and 20-methylcholanthrene has been suggested to inhibit aromatase in ovarian follicles which would lead to mutation of the male sex chromosome from XY to XX with a negative impact on the population by reduction in the number of males and increase in the number of females (Laliberte & Ewing, 2006). PAHs are also thought to facilitate a large extent of damage to the olfactory tissues of fish (Laliberte & Ewing, 2006).

There are two sub-divisions of PAHs; low molecular weight PAHs that have fewer than four benzene rings with some adverse effects on the reproductive and death frequency of aquatic animals and high molecular weight PAHs that has four and above benzene rings suspected to be carcinogenic and mutagenic (Boonchan et al., 2000).

PAHs are generated continuously as a result of the nature of the sources through which they enter the environment (Abou-Arab et al, 2010). PAHs contaminated soil also pose a threat to microorganisms, invertebrates and plants resident in such area due to its toxicity (Andreoni, et al., 2004). The intense generation of interest in the bioremediation of PAHs contaminated ecosystem is due to their ubiquitous nature coupled to their suspected deleterious effects on human lives amongst other factors (Habe & Omori, 2003; Johnsen, Wick & Harms, 2005; Kanaly & Harayama, 2000). The molecular stability and hydrophobicity of PAHs are two basic factors contributing to the recalcitrance of high molecular weight (HMW) PAHs in environments (Kanaly & Harayama, 2000). For the bioremediation of PAHs, white rot fungi (which include *Trametes versicolor, Phanerochaete chrysosporium, Irpex lacteus*, amongst others) have demonstrated the ability to secrete extracellularly at least one of the three major ligninolytic enzymes; manganese peroxidase (EC 1.11.1.13), lignin peroxidase (EC 1.11.1.14) and laccase (EC 1.10.3.2) together with H_2O_2 producing oxidases (Heinkill et al.,1998; Kalmis et al., 2008; Novotny et al., 2004; Vyas, Volc & Sasek, 1994). Glyoxal oxidase (E.C. 1.2.3.5) and superoxide dismutase (E.C. 1.15.1.1) are known to provide the H_2O_2 requirement of lignin peroxidase and manganese peroxidase (Pointing, 2001; Vyas et al., 1994).

Environmental factors such as pH, temperature, water availability, oxygen supply/level, and PAHs bioavailability, nutrient requirements of microbes and adaptation of the microorganisms' population influences the degradation of PAHs in contaminated soils. (Balba et al., 1998). Laccase activity of different white rot fungi have been studied (Heinzkill et al., 1998; Patrick et al., 2011). This study focused on the effect of pH on the laccase activity of *Penicillium freii* and *Aspergillus niger*. Understanding this biodegradation process with pH parameter will be useful in optimising bioremediation of PAHs.

The structure of this study centres around culturing of *Penicillium freii* and *Aspergillus niger* in malt using extract broth at different pH, extraction of extracellular Laccase enzyme from *Penicillium freii* and *Aspergillus* niger and Laccase assays using the required substrates and reagents.

2.0 Materials and Methods

Fungal cultures were isolated by enrichment culture obtained from road-side soil in University of Hertfordshire College Lane Campus, Hatfield, United Kingdom. They were identified microscopically and with molecular techniques using PCR amplification and sequencing of 18S rDNA and identified as *Penicillium freii* and identified as *Aspergillus niger*.

2.1 Fungal Growth

900ml Malt extract broth solution was prepared the solution was divided equally in 3 parts of 300ml each and the pH of 300ml each of the solution was adjusted to 5.5, 7.0 and 8.5 each. 100ml Malt extract broth solutions was added to nine conical flasks; with each set of three conical flasks containing the different pH ranges, inoculated with fungal cultures and incubated in a rotary shaker at 25^0C and 120 rpm. The fungal cultures were harvested after seven days.

2.2 Chemicals and Reagents

2-2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH) was supplied by Sigma-Aldrich UK. Other reagents of analytical grade include sodium phosphate, sodium chloride, sodium acetate and sodium bicarbonate were used.

2.3 Extraction of Fungal Extracellular Enzymes

Laccase enzymes were extracted with reference to Vyas et al., 1994. Conical flasks containing fungal cells were harvested after seven days of growth. One hundred ml sodium phosphate buffer (0.1mol/L, pH 6.5) containing 0.1mol/L NaCl, was added to each flask, the contents beaten gently with a glass rod and agitated on a rotary shaker at room temperature for 45 min. the enzyme extracts were filtered through a nylon cloth and spun (15 000g, 20 min, 4^0C). Supernatants were collected and stored at -20^0C

2.4 Laccase Activity

Majcherczyk et al., 1997 method with modifications was used. Laccase activity was determined by oxidation of ABTS. The reaction mixture (2ml) contained 1ml of 5 mM ABTS, 0.5ml of 0.1 M sodium acetate buffer pH 5.0 and 0.5ml of fungal samples supernatant. Oxidation of ABTS was followed by an absorbance increase at 405 nm. Laccase activity was terminated with addition of 1ml of cold 0.4M $NaHCO_3$ and 100 µl of test samples were loaded onto micro titre plate in five replicates. The enzyme activity is defined as the amount of enzyme capable of oxidizing one µmol of ABTS min^{-1}. Enzyme activity rate is calculated as shown below (see equation 1) and expressed as µmol/ml/min.

$$\mu mol/ml/min = \frac{\text{O D value X Total reaction volume}}{\text{E X ml of supernatant X incubation time}} \quad \ldots\ldots\ldots\ldots (1)$$

Where OD value = Optical density of ABTS oxidized by colour change
Total reaction volume = (2ml)
E = extinction coefficient of ABTS (0.0844)
incubation time = 45 minutes

3.0 Results

Table 1. Absorbance at 405nm in five replicates, average absorbance at 405nm and standard deviation of *Aspergillus niger* cultured at different pH for Laccase activity.

ASPERGILLUS SPP								
SAMPLES	CONTENTS	1	2	3	4	5	Average	Standard deviation
pH 5.5	Supernatant + substrates	0.139	0.143	0.144	0.133	0.128	0.137	0.007
pH 7.0	Supernatant + substrates	0.133	0.116	0.138	0.149	0.105	0.128	0.018
pH 8.5	Supernatant + substrates	0.123	0.124	0.14	0.125	0.103	0.123	0.013
Control 1 - pH 5.5	Supernatant only	0.065	0.066	0.067	0.068	0.064	0.066	0.002
Control 2 - pH 7.0	Supernatant only	0.068	0.069	0.068	0.067	0.066	0.068	0.001
Control 3 - pH 8.5	Supernatant only	0.071	0.069	0.069	0.068	0.067	0.069	0.001
Control 4	Buffer + substrates	0.065	0.063	0.063	0.066	0.06	0.063	0.002

Table 2. Laccase Activity (µmol/ml/min) of *Aspergillus niger*

pH	Laccase Activity (µmol/ml/min)
5.5	0.144
7.0	0.135
8.5	0.130

Table 3. Absorbance at 405nm in five replicates, average absorbance at 405nm and standard deviation of *Penicillium freii* cultured at different pH for Laccase activity

PENICILLIUM SPP								
SAMPLES	CONTENTS	1	2	3	4	5	Average	Standard deviation
pH 5.5	Supernatant + substrate	0.177	0.201	0.188	0.183	0.172	0.184	0.011
pH 7.0	Supernatant + substrate	0.168	0.16	0.12	0.135	0.181	0.153	0.025
pH 8.5	Supernatant + substrate	0.148	0.189	0.169	0.176	0.165	0.169	0.015
Control 1 - pH 5.5	Supernatant only	0.086	0.083	0.086	0.083	0.087	0.085	0.002
Control 2 - pH 7.0	Supernatant only	0.065	0.066	0.07	0.072	0.067	0.068	0.003
Control 3 - pH 8.5	Supernatant only	0.077	0.078	0.086	0.077	0.071	0.078	0.005
Control 4	Buffer + substrates	0.059	0.064	0.064	0.067	0.068	0.064	0.004

Table 4. Laccase Activity (µmol/ml/min) of *Penicillium freii*

pH	Laccase Activity (µmol/ml/min)
5.5	0.194
7.0	0.161
8.5	0.178

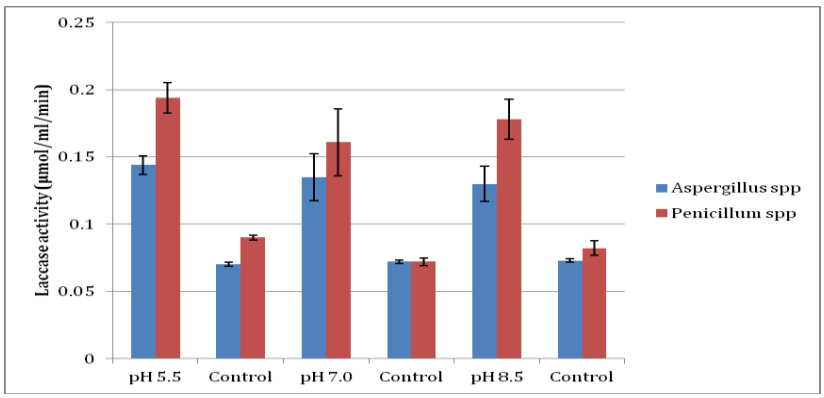

Figure 1. Laccase activity (µmol/ml/min) of fungal samples at pH 5.5, 7.0 and 8.5. Colour symbols represent the different fungal samples. Vertical bar represents standard deviation of mean.

Discussion

Table 2 is the calculated laccase activity of *Aspergillus niger* obtained from table 1, while table 4 is the calculated laccase activity of *Penicillium freii* obtained from table 3. Table 2 showed a steady decrease in laccase

activity of *Aspergillus niger* from pH 5.5 through 7.0 to 8.5. While in table 4, the laccase activity decreased from pH 5.5 to 7.0, but slightly increased at pH 8.5. In both organisms, laccase activity was optimum at pH 5.5. In the study of Jordaan et al., 2004, the oxidation of ABTS by the laccase of an unidentified wild fungal isolate was monitored over pH range of 2 to 6.8 and the optimum pH was found to be 4. Also in the study of Schlosser, Grey & Fritsche, 1996, on *Trametes versicolor*, laccase oxidation of ABTS had optimum pH of 3.5, while laccase oxidation of 2,6-dimethoxyphenol (DMP) and L-DOPA had an optimum pH of 4.5. The study on *Panaeolus papilionaceus* and *Panaeolus sphinctrinus* by Heinzkill et al., 1998 showed the enzyme activity of laccase purified from these organisms had an optimum pH range of 3 to 5. In the study of Patrick, Mtui, Mshandete & Kivaisi, 2011, the optimum pH for laccase activity was pH 6.0 after seven days of incubation and pH 5.5 after nine days of incubation. Most fungal laccases usually have optimum activity in acidic pH ranges (Heinzkill et al., 1998; Patrick et al., 2011). The optimum pH of 5.5 for laccase activity of fungal samples used in this study is in agreement with the above documented studies cited indicating that maximum laccase activity of ligninolytic fungi occurs in acidic pH ranges.

Conclusion

The intention of this study was to move forward the current gains in the bioremediation of contaminated environments by determining the effect of pH on the laccase activity of fungal samples and this is due to the fact that optimum pH conditions for ligninolytic enzyme activity will speed up the bioremediation process, thereby saving cost and time of bioremediation which culminates in reduction of the risk that recalcitrant environmental pollutants such as PAHs poses to humans and other forms of life. The similarity between the laccase activity results and cited documented studies shows that acidic pH conditions favours laccase activity. Understanding the pH at which most ligninolytic enzymes function optimally in different organisms will enable the development of a viable pH based model that will suit all or most of the ligninolytic enzymes of different organisms being utilised in the bioremediation process of PAHs.

References

Abou-Arab, A.A.K., Abou-Bakr, S., Maher, R.A., El-Hendawy, H.H. & Awad, A.A. (2010). Degradation of polycyclic aromatic hydrocarbons as affected by lactic acid. *Journal of American Science*, *6*(10), 1237-1246.

Andreoni, V., Cavalca, L., Rao, M.A., Nocerino, G., Bernasconi, S., Dell'Amico, E., et al. (2004). *Chemosphere*, *57*, 401-412.

Balba, M.T., Al-Awadhi, N. & Al-Daher, R. (1998). Bioremediation of oil contaminated soil: microbiological methods for feasibility assessment and field evaluation. *Journal of Microbiological Methods*, *32*, 155-164.

Boonchan, S., Britz, M. L. and Stanley, G. A. (2000). Degradation and mineralization of high-molecular-weight polycyclic aromatic hydrocarbons by defined fungal-bacterial cocultures. *Applied and Environmental Microbiology*, *66*(3), 1007-1019.

Johnsen, A.R., Wick, L.Y. & Harms. (2005). *Environmental Pollution*, *133*, 71-84.

Kalmis, E., Yasa, I., Kalyoncu, F., Pazarbasi, B. & Kocyicit, A. (2008). Ligninolytic enzyme activities in mycelium of some wild and commercial mushrooms. *African Journal of Biotechnology*, *7*(23), 4314-4320.

Kanaly, R.A. & Harayama, S. (2000). Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by bacteria. *Journal of Bacteriology*, *182* (8), 2059-2067.

Kollmuss, A. & Agyeman, J. (2002). Mind the Gap: Why do people act environmentally and what are the barriers to pro-environmental behavior? *Environmental Education Research*, *8*(3), 239-260.

Ewing, R.D. & Laliberte, D. (2006). Effect On Puget Sound Chinook Salmon Of NPDES Authorized Toxic Discharges As Permitted by Washington Department of Ecology.

Habe, H. & Omori, T. (2003). Genetics of polycyclic aromatic hydrocarbon metabolism in diverse aerobic bacteria. *Biosci. Biotechnol. Biochem*, *67*(2), 225-243.

Heinzkill, M., Bech, L., Halkier, T., Schneider, P. & Anke, T. (1998). Characterization of Laccases and Peroxidases from wood-rotting fungi (Family Coprinaceae). *Applied and Environmental Microbiology*, *64*(5), 1601-1606.

Majcherczyk, A., Johannes, C. & Huttermann, A. (1998). Oxidation of polycyclic aromatic hydrocarbons (PAHs) by laccase of *Trametes versicolor*. *Enzyme and Microbial Technology*, *22*, 335-341.

Mugica, V., Torres, M., Salinas, E., Gutierrez, M. & Garcia, R. (2010). Polycyclic aromatic hydrocarbons in the urban atmosphere of Mexico City. *Air pollution*, *4*, 76-98.

Novotny, C., Svobodova, K., Erbanova, P., Cajthaml, T., Kasinath, A., Lang. E., et al. (2004). Ligninolytic fungi in bioremediation: extracellular enzyme production and degradation rate. *Soil Biology and Biochemistry*, *36*, 1545-1551.

Pointing, S.B. (2001). Feasibility of bioremediation by white rot fungi. *Appl. Microbiol. Biotechnol*, *57*, 20-33.

Vyas, B.R.M., Volc, J. & Sasek, V. (1994). Ligninolytic enzymes of selected white rot fungi cultivated on wheat straw. *Folia Microbiol*, *39*, 235-240.