Vol. 27 (no. 1) 33–38 31 March 2015

Biokemistri

An International Journal of the Nigerian Society for Experimental Biology

Research Article

Partial characterization of lignin peroxidase expressed by bacterial and fungal isolates from termite gut

Evans C. Egwim[1]*, Adamu Y. Kabiru[1] and Adeshola J Tola[2].

[1]Department of Biochemistry and Global Institute for Bioexploration (GIBEX), Federal University of Technology, PMB 65, Minna, Niger State, Nigeria.

[2]Department of Biochemistry, Federal University Ndufu Alike Ikwo, PMB 1010, Abakaliki, Eboyin State, Nigeria.

*Correspondence: Evans C Egwim; c.egwim@futminna.edu.ng; +2347065809474

Received 11 March 2015: Revised 28 March 2015; Approved 29 March 2015.

ABSTRACT: Lignin peroxidase producing microorganisms were isolated from the gut of *Macrotermes nigeriense* (Soldier and worker termite). The microorganisms isolated were *Staphylococcus aureus, Staphylococcus epidermis, Bacillus subtilis, Micrococcus luteus, Epidermophyton flocussum, Microsporum distortum, Trichophyton megininii, Trichophyton mentagrophytes and Aspergillus niger.* When the isolates were incubated for 24 hours in the presence of 0.5 w/v pyrogallol to test for the expression of peroxidase activities, *B. subtilis* produced the highest activity among the bacterial isolates, with optimum temperature of 35 °C, and optimum pH of 6.5. Similar screen among the fungal isolates found *M. distortum* to express the most activities, with optimum temperature of 40 °C, and optimum pH of 5.0, after 72 hours incubation. The results from this study suggest that *B. subtilis* and *M. distortum* can be a cheap source of lignin peroxidase for large scale commercial production, biotechnological and industrial applications.

KEYWORDS: *Macrotermes nigeriense*, lignin peroxidase, microorganisms, enzyme Activity.

BKM.2015.017
This article is available online in PDF format at http://www.bioline.org.br/bk

INTRODUCTION

Lignin is a complex, three-dimensional aromatic polymer consisting of dimethoxylated, monomethoxylated and non-methoxylated phenylpropanoid subunits (Martinez *et al.,* 2005). It is found in the secondary cell wall of plants, where it fills the spaces between the cellulose, hemicellulose and pectin components, making the cell wall more rigid and hydrophobic. Lignin provides plants with compressive strength and protection from pathogens (Kumar *et al.,* 2008; Rubin, 2008). The three major roles of lignin are to protect plants against microbial attack, to give them the stiffness needed for structural stability, and to provide the hydrophobic capillary surface needed for the transport of aqueous nutrients from the roots to the leaves (Matti, 2012). It is the major industrial obstacle to converting plant carbohydrate polymers via simple sugars to biofuels like ethanol, butanol, or biodiesel, or to chemicals (e.g. organic acids, rare sugars) (Crawford, 1981). Although lignin has long been recognized as a potential source of a wide range of chemicals, attempts to make use of this potential have more or less failed.

Lignin can be depolymerized by thermochemical methods such as pyrolysis (thermolysis), chemical oxidation, hydrogenolysis, gasification, and hydrolysis under supercritical conditions (Pandey and Kim, 2002). However, many of these processes are environmentally harsh and

occur under severe conditions requiring large amounts of energy (Ward and Singh, 2002), therefore these processes are not adequate for efficient lignin valorization. Enzymes could provide a more specific and effective alternative for lignin depolymerization. Furthermore, biocatalytic processes generally take place under mild conditions, which lowers the energy input and reduces the environmental impact (Perez *et al*, 2002; Sun and Cheng, 2002; Luaine, 2011).

Termites are considered the smallest and most efficient decomposing bioreactors of wood on earth (Brune, 1998; João *et.,al,* 2011). They are well known for their contribution to efficient degradation of lignin. However, it has long been recognized that most termites do not produce their own endogenous digestive enzyme; instead they depend on lignin peroxidase from their resident gut microorganism (Leith and Raj, 2005). All higher termites lack the eukaryotic ligninolytic symbionts, such as protozoa, that are associated with the lower termites. Instead they have evolved complex hind gut communities entirely composed of bacteria or in some cases fungi (Eggleton and Tayasu, 2001; Donovan *et.,al,* 2001)

Enzymes such as peroxidase from microorganisms in the gut of these termites are needed to break down the component of lignin into simple sugars that can then be fermented. Lignin peroxidases were the first ligninolytic enzymes to be discovered (Tien and Kirk, 1983). Lignin peroxidase [EC 1.11.1.14] are glycosilated enzymes of about 340 amino acids with a molecular weight between 38 and 50 kDa, a single heme, and two calcium ions (Hammel & Cullen, 2008; Sinclair et al., 1992). They degrade lignin through H2O2 using a mechanism that resembles that used for other peroxidades (Hammel & Cullen, 2008; Martinez et al., 2005; Wong, 2009). Lignin peroxidase can be isolated, extracted and produce locally, thereby saving the cost of importation into the country. This enzyme is very useful in biotechnological and industrial applications.

The objectives of this study were to isolate microorganism from the gut of *Macrotermes nigeriense* (Soldier and woker termite) for their potential for lignin peroxidase production and to partially characterize these enzymes.

MATERIALS AND METHODS

Collection of sample

Ten soldier and worker termites were aseptically collected from a termitarium located at Bosso campus, Federal University of Technology, Minna, Niger State, Nigeria in June 2012. They were kept in a sterile container and transported to the Microbiology Laboratory for further analysis.

Isolation of Microorganisms from the gut of termites (*Macrotermes nigeriense*)

The termites were sterilized in ethanol for one minute and were air-dried. The gut was cut open using sterile fin forceps and wire loop was used to collect a loop full portion of exudates from the gut. A portion of the gut was streaked onto nutrient agar plates, using inoculating loop and incubated for 18-24 hours at 37 ^{o}C to support optimal bacterial growth. Samples were also streaked on Saubourand dextrose agar (SDA) plates and incubated at 25 ^{o}C for 3-5 days to allow fungal growth. Individual colonies observed were sub-cultured on Nutrient agar and Saubourand dextrose agar plates for another 18-24 hours and 3-5 days respectively, and finally grown on agar slants to preserve the pure culture.

Table 1: Screening of bacterial isolates for lignin peroxidase activity.

Microorganism	Absorbance (450 nm)	Enzyme Activity U/ml (x10^{-5})
Staphylococcus aureus	0.170	1.15
Staphylococcus epidermis	0.180	1.21
Bacillus subtilis	0.220	1.48
Micrococcus luteus	0.150	1.01

Table 2: Screening of fungal isolates for lignin peroxidase activity.

Microorganism	Absorbance (450 nm)	Enzyme Activity U/ml (x10^{-5})
Epidermophyton floccusum	0.05	3.37
Microsporum distortum	0.14	9.45
Trichophyton megininii	0.06	4.05
Trichophyton mentagrophytes	0.07	4.72
Aspergillus niger	0.10	6.74

Identification of microorganism

Identification of bacteria colonies was done based on Bergeys manuals and by their growth pattern, gram staining, and catalase test. Different fungi colonies were identified morphologically and by their microbiological appearance including the pigmentation, shape, presence of special structure and characteristics of spores as described by Oyeleke and Manga (2008). A small portion of each mycelia growth was carefully picked with the aid of a sterile needle and placed on a drop of lactophenol cotton blue that has been dropped on a slide and covered with cover slip. The slide was then observed under the microscope at (X10) objective lens and (x40) to detect the spores and some special structures of the fungi.

Screening for lignin peroxidase producers

Sodium chloride (0.1%) solution (10 ml) were measured into different test tubes, and were sterilized, after which the loopfull of grown culture of isolated colonies (for bacteria and fungi) were inoculated into the test tubes. Each isolate (1 ml) was inoculated into each petri dish for bacteria and fungi separately. Nutrient agar and Saubourand dextrose agar containing 0.5 w/v pyrogallol was poured onto the plate and carefully stirred. The plates were incubated for 24 hours (bacteria) and 72 hours (fungi) to observe the expression of peroxidase activities. A reddish hollow zone in the plates indicated a lignin peroxidase producer as described by (Savitha *et al*., 2011).

All the isolated organisms that were inoculated on petri dishes containing Saubourand dextrose agar SDA and Nutrient agar containing pyrogallol were observed and development of reddish hallow zone was assessed on daily basis. The organisms showing faster growth were selected as potent strains. The strains were identified by gram staining and biochemical tests for bacteria and lactophenol cotton-blue method microscopic observation for fungi (Cappuccino and Sherman 2010).

Lignin peroxidase production and assay

Lignin peroxidase was assayed by measuring the rate of H_2O_2-dependent oxidation of pyrogallol to purpurogallin (Chance and Maehly, 1955). The reaction progress was measured spectrophotometrically. The standard reaction mixture contained 1 ml of pyrogallol (2 mM) in 10 mM Sodium acetate buffer pH 5.0 and 1 ml of culture supernatant. The reaction was initiated by the addition of 0.15 M H_2O_2 and the linear increase in absorbance was read at 450 nM at 30 ^{o}C for 30 minutes. The reaction blank contained 1 ml of distilled water instead of the enzyme source and the mixture was incubated at 30 ^{o}C for 30 minutes, after which the absorbance was read at 450 nm (Jhadav *et al.,* 2009). Enzyme activity was expressed as International units (IU), where one unit of lignin peroxidase was define as amount of enzyme required to oxidize 1 µmol of pyrogallol. The lignin peroxidase activity in U/ml is calculated using the molar coefficient of pyrogallol (2470 $M^{-1}cm^{-1}$) at 450 nm.

Effect of Temperature on Lignin peroxidase activity

The culture was filtered using Whatman filter paper and the filtrate was centrifuged at 100 rpm for 10 minutes. The supernatant collected was used as enzyme source. The cell free extract of the fungus and bacteria were taken as crude lignin peroxidase preparations while studying each parameter, the other reaction conditions were kept constant. The effect of temperature on lignin peroxidase activity was assayed as described earlier while the incubation temperature was varied from 20 to 50 ^{0}C for 30 minutes. The temperature at which the enzyme showed maximum activity was noted as the optimum temperature.

Figure 1: Colony morphology of Bacteria isolated strain, E3 (*Bacillus subtilis).* Reddish hallow zone signifies growth region.

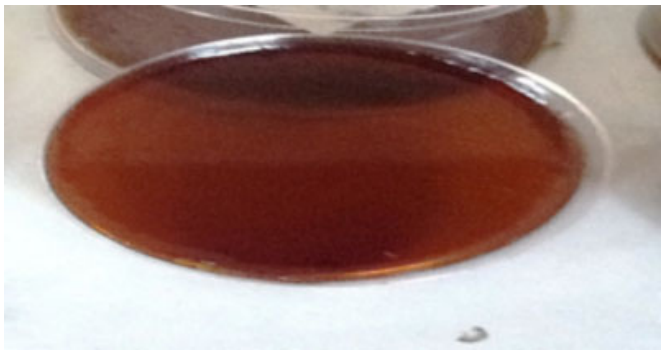

Figure 2: Colony morphology of fungi isolated strain, I2 (*microsporum distortum).* Reddish hallow zone signifies growth region.

Effect of pH on Lignin peroxidase activity

The influence of pH on lignin peroxidase activity was assayed as described above with different pH range of 4.0 to 7.0, and incubated at 30 ^{o}C for 30 minutes and absorbance values were recorded at 450 nm.

RESULTS AND DISCUSSION

Termites can be viewed as ecological engineers that contribute to the digestion of lignin. However, endogenous lignin peroxidase alone cannot accomplish lignin digestion; rather, a suite of additional microbial lignin peroxidase, in the gut of these termites is needed.

In this work, nine microorganisms were isolated from termite gut as lignin peroxidase producers. All of these organisms oxidize pyrogallol present in the screening medium. It was observed that *Staphylococcus aureus, Staphylococcus epidermis, Trichophyton megininii, Trichophyton mentagrophytes, Micrococcus luteus, and Epidermophyton floccusum,* showed low for peroxidase activities.

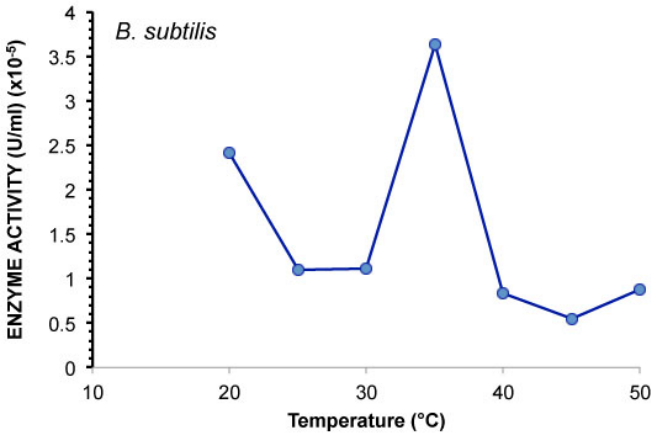

Figure 3. Effect of temperature on lignin peroxidase activity produced by *B. subtilis*. The optimum temperature of *B. subtilis* was at 35 °C with activity of 3.63x10^{-5} U/ml, temperature above and below 35 °C showed low lignin peroxidase activity.

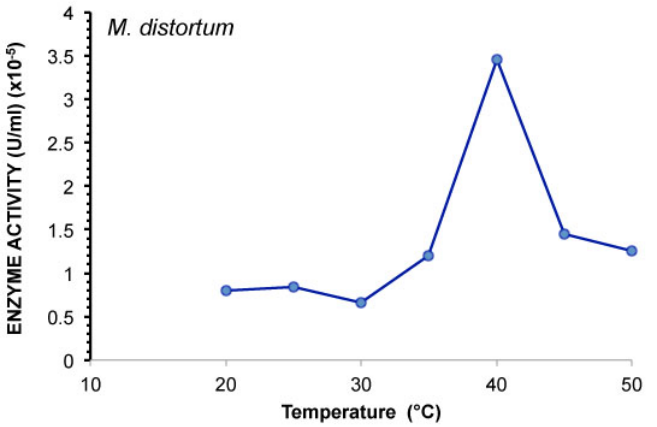

Figure 4: Effect of temperature on lignin peroxidase activity produced by *M. distortum*. The optimum temperature of *M. distortum*, was at 40 °C with activity of 3.45 U/ml(x10), temperature above and below 40 °C showed low lignin peroxidase activity.

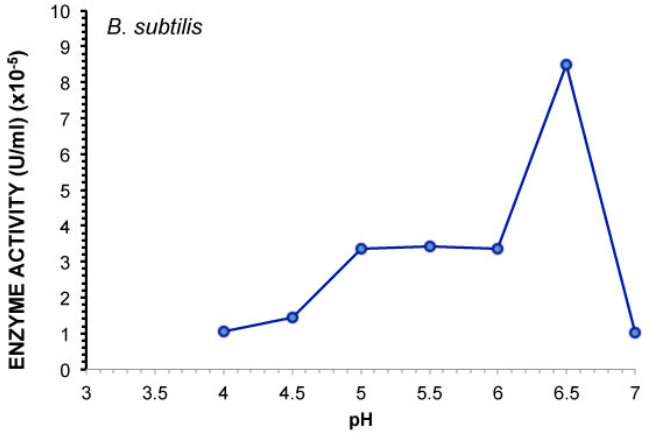

Figure 5: Effect of pH on lignin peroxidase activity produced by *B. subtilis*. The optimum pH of lignin peroxidase enzyme produce by *B. subtilis* was at pH 6.5 with activity of 8.50 x10^{-5} U/ml. pH values below or above this optimum values showed reduced lignin peroxidase activity.

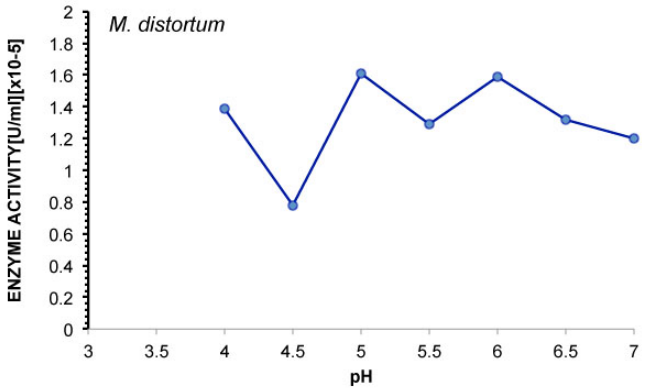

Figure 6: Effect of pH on lignin peroxidase activity produced by *M. distortum*. The optimum pH of lignin peroxidase enzyme produced by *M. distortum* was at pH 5 with activity of 1.61 U/ml (x10). pH values below or above this optimum values showed reduced lignin peroxidase activity.

The activity of lignin peroxidase produced by isolates from Termite gut was measured in an assay and the maximum activity of Lignin peroxidase was observed in *Bacillus subtilis and Microsporum distortum* in bacterial and fungal isolates respectively.

Four bacteria strains were isolated from the gut of the termite. Of the four bacteria isolated and screened for lignin peroxidase activity production, *B. subtilis* showed the highest lignin peroxidase activity (1.48x10^{-5} U/ml) while *M. luteus* showed the least (Table1). Five fungi were isolated from the gut of the termites. Of the five fungi isolated and screened for lignin peroxidase activity production, *M. distortum* showed the highest lignin peroxidase activity (9.45x10^{-5}U/ml) while *E. floccusum* showed the least (Table1)

High activities were observed by very strong reddish hallow zone on plates of *Bacillus subtilis, and Microsporum distortum*. *B. subtilis* lignin peroxidase showed its highest enzymatic activity of $3.63x10^{-5}$ U/ml at 35°C (optimum temperature). In the experiment on the effect of pH on enzyme activity, the highest activity of $8.50\ x10^{-5}$ U/ml was observed at pH 6.5. Lignin peroxidase from the most active fungal isolate (*M. distortum*) had the highest activity of 3.45 U/ml (x10) at 40 °C in the experiment designed to test its temperature dependence. When the activities of *M. distortum* lignin peroxidase was tested for pH dependence, the highest activity of $1.61x10^{-5}$ U/ml was seen at pH 5.

Different experiments, such as effect of temperature and pH were carried out to optimize the culture conditions for the lignin peroxidase produced. *B. subtilis* had an optimum pH of 6.5 while *M. distortum* had an optimum pH of 5. These were slightly lower than the values reported by Ismat (2012) who recorded optimum pH of purified lignin peroxidase oxidation of veratryl alcohol as 6.8. These are in contrast to previous studies by Asgher *et al.* (2007) who reported optimum pH of 4.0, for free lignin peroxidase using veratryl alcohol as substrate. The optimum temperature for *M. distortum* lignin peroxidase was 40 ^{o}C. This is in agreement with the findings of Asgher et al. (2007) who reported an optimum temperature of 40 °C for lignin peroxidase using Veratryl alcohol as substrate. However this value was slightly lower than the findings by Ismat (2012) who recorded optimum temperature of 45 ^{o}C for purified lignin peroxidase oxidation of veratryl alcohol. The optimum temperature of *B. subtilis* lignin peroxidase was at 35 ^{o}C. This result is in contrast with an earlier work by Ekrem *et. al.,* (2008) who reported that lignin peroxidase had maximum activity at 25 °C for pyrogallol, 30 °C for guaiacol and 45° C for 4-methyl cathecol. The same pattern of results was reported by Zadrazil *et. al.* (1999) who found that lignin peroxidase from *Pleurotous* sp. and *Dichromotus squelen* showed optimum temperature of 30 °C.

It is clear that the results obtained from the present work should be interpreted within the context of the nature of enzyme sources used. It is quite likely that the enzymes would behave more like the ones reported in the literature if they were purified. Further understanding of the properties of the enzyme from the different isolates will become available when we characterize the purified enzymes.

The result obtained from this study indicates that *Staphylococcus aureus, Staphylococcus epidermis, Bacillus subtilis, Micrococcus luteus, Epidermophyton floccosum, Microsporium distortum, Trichophyton megininii, Trichophyton mengatrophytes and Aspergillus niger* isolated from termite gut are lignin peroxidase producers. Of these*, Bacillus subtilis and Microsporum distortum* from bacteria and fungi isolates showed the highest activity. This work has demonstrated that both bacterial and fungal isolates from termite gut can potentially be sources of lignin peroxidase. These microorganisms can be used as potential source for the commercial production of this enzyme for application in different industries such as food, paper, textiles, cosmetics biotechnology industries and chemical industries.

In order to fully explore its ligninolytic potential, the peroxidases from these sources need to be purified and characterized. Full sequencing and characterization of the genes for the isolated lignin peroxidase will open the way for its modification in order to optimize the production of enzymes for local industries. We have ongoing work to explore these further aspects to optimize the use of the enzymes for industrial applications.

REFERENCES

Asgher, M., Asad, M. J., Bhatti, H. N. (2007). Hyperactivation and thermostabilization of Phanerochaete chrysosporium lignin peroxidase by immobilization in xerogels. *World Journal of Microbiology and Biotechnology* 23: 525-531

Bibi, I. and Bhatti, H.N. (2012) Kinetic and Thermodynamic Characterization of Lignin Peroxidase Isolated from Agaricus bitorqus A66. *International Journal of Chemical Reactor*. 11:1-9

Brune A: Termite guts: the world's smallest bioreactors. Trends Biotechnol 1998, 16:16-21.

Cappuccino, J., Sherman, N. (2010). Microbiology: A Laboratory Manual, Benjamin Cummins. Pp.560

Crawford RL: Lignin Biodegradation and Transformation.: Wiley-Interscience; 1981.

Ekrem, K and Ilhami .G. (2008).Purification and characterization of Peroxidase from cauliflower (Brasica oleracea.L.var.botrytics) buds.protein and peptide letters **15**:300-320

Emmel A, Mathias AL, Wypych F, Ramos LP: Fractionation of *Eucalyptus grandis* chips by dilute acid-catalysed steam explosion. *Bioresource technology* 2003, 86(2):105-115

Hammel, K.E. and Cullen, D. (2008). Role of fungal peroxidases in biological ligninolysis. *Current Opinion in Plant Biology*. Vol. 11, No. 3, (June 2008), pp. 349-55, ISSN 1369-5266.

Jhadav, A., Vamsi K.K., Khairnar, Y. (2009*). International Journal of Microbiology Resources*, **1**:9-12

João PL, Franco C, Flávia CL, Thabata M A, Daniela A R, Fernanda B, Ana M C, Marcelo F C, Fernando F C, Adriana F P, Gonçalo AG and Fabio M S(2011). Functional characterization and target discovery of glycoside hydrolases from the digestome of the lower termite Coptotermes gestroi. *Biotechnology for Biofuels*. 4:50

Kumar R, Singh S, Singh OV: Bioconversion of lignocellulosic biomass: biochemical and molecular perspectives. *Journal of industrial microbiology & biotechnology* 2008, 35(5):377-391.

Leith A, and Raj B (2005). Isolation and Characterization of Enteric Bacteria from the Hindgut of Formosan Termite. *Journal of Bioresource Technology* 96: 1592–1598

Luaine B, Nick JP, Wierck x, Johannes HW and Harald J R (2011).Isolation and characterization of novel bacterial strains exhibiting ligninolytic potential *BMC Biotechnology*.11:94

Martinez AT, Speranza M, Ruiz-Duenas FJ, Ferreira P, Camarero S, Guillen F, Martinez MJ, Gutierrez A, del Rio JC: Biodegradation of lignocellulosics: microbial, chemical, and enzymatic aspects of the fungal attack of lignin. *Int Microbiol* 2005, 8(3):195-204.

Martinez, A.T., Speranza, M., Ruiz-Duenas, F.J., Ferreira, P., Camarero, S., Guillen, F., Martinez, M.J., Gutierrez, A. & del Rio, J.C. (2005). Biodegradation of lignocellulosics: microbial, chemical, and enzymatic aspects of the fungal attack of lignin. *International Microbiology*.3: 195-204,

Matti L, Ossi P, and Douglas D. A (2012) Lignin—Designed Randomness. *BIO-Complexity* (3):1–11.

Oyeleke, S. B. and Manga B. S. (2008). *Essentials of Laboratory Practicals in Microbiology,* First Edition, Tobest Publisher, Minna, Niger State, Nigeria, 15-34

Pandey MP, Kim CS: Lignin Depolymerization and Conversion: A Review of Thermochemical Methods.*Chem Engin Technol* 2011, 34(1):29-41.

Perez J, Munoz-Dorado J, de la Rubia T, Martinez J (2002): Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview. *Int Microbiol*, 5(2):53-63.

Rubin EM: Genomics of cellulosic biofuels. *Nature* 2008, 454(7206):841-845.

Savitha, S.D., Gururaj, B. T., Nityanand, C., Anup, A.C., Gouri, D., Azhar, Murtuza B.P. (2011) Isolation of laccase producing fungi and partial characterization of laccase *Research Article, Biotechnology. Bioinformatics Bioengineering*. **1**(4):543-549.

Sinclair, R., Yamazaki, I., Bumpus, J., Brock, B., Chang, C.S., Albo, A. & Powers, L. (1992). Structure of the active site of lignin peroxidase isozyme H2: native enzyme, compound III, and reduced form. *Biochemistry*.31:4892-9.

Sun Y, Cheng J: Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresource technology* 2002, 83(1):1-11.

Tien M and Kirk T (1983). Lignin-degrading enzyme from the hymenomycetes *Phanerochaete chrysosporium.* Science*,* 22: 661–63.

Wang, W. & Wen, X. (2009). Expression of lignin peroxidase H2 from Phanerochaete chrysosporium by multi-copy recombinant Pichia strain. *Journal of Environmental Sciences*.21: 218-22,

Ward PO, Singh A: Bioethanol technology: developments and perspectives. *Advan Appl Microbiol* 2002, 51:53-80.