KATHMANDU UNIVERSITY JOURNAL OF SCIENCE, ENGINEERING AND TECHNOLOGY
VOL. 9, No. I, July, 2013, pp 60-67

ENZYMATIC COMBUSTION BY LIGNINOLYTIC ENZYMES OF LIGNICOLOUS FUNGI

[1]Praveen Kumar Nagadesi[*], [2]Arun Arya

[1]Department of Botany, P.G. section, Andhra Loyola College, Vijayawada -520008, Andhra Pradesh, India.
[2]Department of Botany, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara – 390002, Gujarat, India.

*Correspondence author: nagadesipraveenkumar@yahoo.com
Received 10 January, 2013; Revised 23 April, 2013

ABSTRACT
Lignicolous fungi are wood degrading organisms, which were able to decompose all wood polymers; lignin, cellulose and hemicellulose etc. by producing ligninolytic, cellulolytic and hemicellulolytic enzymes respectively. The complex plant polymer like lignin was biodegraded by a unique "enzymatic combustion," i.e. a nonspecific enzyme-catalyzed burning. Enzymatic combustion, involves oxidative extracellular enzymes. The selective lignicolous fungi that decompose preferentially wood lignin by lignin peroxidase, Manganese peroxidase, and laccases in wood polysaccharides leaving cellulose were *Lenzites sterioides*1, 2, *L. betulina, L. exima, Phellinus gillvus, P. nilgheriensis, P. robustus, Flavodon flavum, Ganoderma lucidum, Shizophyllum Commune*. The soft rot fungi mainly cause degradation of cellulose by producing cellulolytic enzyme were *Phoma multirostrata, Theliviopsis* stste of *Ceratocystis paradoxa, Fusarium palidoroseum, Alternaria Alternata, Chaetomium globosum, Curvularia lunata, Rhizopus stolonifer, Trichoderma Viride.* In order to determine the lignin degrading capability of different lignicolus fungi from Ratanmahal Wildlife Sanctuary, the fungi were screened for production of extracellular wood degrading enzymes on solid media by providing appropriate substrates. The results obtained revealed that 10 fungi were white rot producing microbes with both ligninolytic and cellulolytic ability. Lignocellulolytic behaviour of lignicolus fungi makes them better equipped to degrade different wood in forest area. The white rot fungi showing highest ligninolytic activity was *Lenzites exima* and lowest cellulolytic activity was recorded in case of *L. betulina.* The soft rot fungi *Phoma multirostrata Fusarium palidoroseum Alternaria Alternata Chaetomium globosum were* producing ligninolytic enzyme was reported for the first time.

Key words: Ligninolytic, Enzymatic Combusion, cellulolytic, Lignicolous fungi. Soft rot fungi, *Fusarium palidoroseum Chaetomium globosum*

INTRODUCTION
Lignin was the most abundant renewable aromatic material on earth. It was found in higher plants, including ferns, but not in liverworts, mosses, or plants of lower taxonomic ranking. Wood and other vascular tissues generally are 20-30% lignin. Most lignin is found within the cell walls, where it is intimately interspersed with the hemicelluloses, forming a matrix that surrounds the orderly cellulose microfibrils. In wood, lignin in high concentration is the glue that binds contiguous cells, forming the middle lamella. Biosynthetically, lignin arises from three precursor alcohols: *p*–hydroxycinnamyl (coumaryl) alcohol, which gives rise to *p*-hydroxyphenyl units in the polymer; 4-hydroxy-3-methoxycinnamyl (coniferyl) alcohol, the guaiacyl units; and 3,5-dimethoxy-4-hydroxycinnamyl (sinapyl) alcohol, the syringyl units. Free radical copolymerization of these alcohols produces the heterogeneous, optically inactive, cross-linked, and highly

polydisperse polymer. Most gymnosperm lignins contain primarily guaiacyl units. Angiosperm lignins contain approximately equal amounts of guaiacyl and syringyl units. Both types of lignin generally contain only small amounts of *p* –hydroxyphenyl units.
Fungi that cause decay of wood are of great biotechnological importance since wood and other lignocellulosic materials are renewable resources for the production of paper, fuel and chemicals. Lignicolous fungi are wood degrading organisms, which are able to decompose all wood polymers; lignin, cellulose and hemicellulose etc. by producing ligninolytic, cellulolytic and hemicellulolytic enzymes respectively. Lignicolous fungi are the most efficient ligninolytic microorganisms in nature. The white rot fungi degrade lignin more rapidly and extensively than brown-rot fungi. They bring about lignin decay through an oxidative process that is thought to involve enzymes such as lignin peroxidase (LiP), manganese-dependent peroxidase (MnP), and laccase, all of which have broad substrate specificities [15] LiP attacks both phenolic and nonphenolic aromatic residues, and the latter give rise to cation radicals that fragment spontaneously [14]. MnP catalyzes the oxidation of Mn(II) to Mn(III). which in turn can oxidize phenolic substrates [7]. Laccase abstracts one electron from phenolic compounds, although in the presence of primary substrates it can also oxidize nonphenolic aromatic compounds as well as Mn(II) [1, 3]. Both LiP and MnP are able to depolymerize synthetic lignin in vitro [11, 22]. Due to the participation of peroxidase in lignin breakdown, the extracellular production of hydrogen peroxide by white rot fungi is essential to the process. Several oxidases have been proposed to be enzymes which accomplish this task; these oxidases include, among others, pyranose oxidase [5], methanol oxidase [17], aryl alcohol oxidase [9], and glyoxal oxidase (GLOX) [12, 13]. The fact that GLOX is secreted by *Phanerochaete chrysosporium* and is activated by LiP and its corresponding aromatic substrate [16] strongly suggests that GLOX plays a key role in regulation as well as production of extracellular hydrogen peroxide by *Phanerochaete chrysosporium.*

The lignin degrading basidiomycetes which cause white rot in wood samples were able to produce wood decay enzymes which have the capacity to degrade the cell wall components into final products of H2O and CO2. The ligninolytic activity and cellulolytic activity showing Basidiomycetes fungi were screened. The above mentioned ligninolytic enzymes degrade the lignin polymer into simpler compounds with low molecular weight compounds > 1 Kd as intermediate products during the "Enzymatic combustion" processes. Brown-rot decay (BRD) is the most destructive and costly Form of decay of softwoods in service (1). The brown-rot mechanism can be by: i) Acidic pH during colonization: ii) diffusion of low molecular weight, nonenzymatic decay agents (i. e., Fe^{+++}/H_2O_2) into the wood cell wall; iii) extensive depolymerization of polymeric polysaccharides and oxidation of lignin; and iv) measurable strength loss (modulus of rupture/modulus of elasticity: MOR/MOE) prior to significant weight loss [6].

MATERIALS AND METHODS

Study area

Ratanmahal Wildlife Sanctuary (RWLS) is a relatively small area of 55.65 sq km consisting of dry deciduous forest. The total existing sanctuary area lies between the river Panam and Orsang. The 11 villages of Ratanmahal forest are situated at the southernmost part of Limkheda taluka of Dahod district of Gujarat state. It is situated between 70º 37' to 74º 11" East Longitude and between 22º 32" to 22º 35' North Latitude. The climate is

sub-tropical arid, which turns damp and humid during monsoon. Minimum and maximum rainfall ranges between 957mm to 2101mm. A survey was undertaken in RWLS between October 2008 to January 2011 for collection of samples from living trees and fallen branches.

Isolation and identification of fungi

The fungi associated with the samples were isolated. Wood chips measuring 5×5×1mm were aseptically removed from the samples and transferred to Petri plates containing 2% malt extract agar medium with 250µg streptomycin sulphate per ml and in another petri plate PDA medium containing with 250µg streptomycin sulphate per ml. Eight pieces of wood were removed from each disc and placed in 2 plates. The plates were incubated at 25 °C for 7days. Each colony thus obtained was transferred to a new agar slant. Identification of these fungi was based on colony character and their microscopic examination. It was not possible to identify some fungal species and they were put into an unknown group.

Screening of lignin degrading enzymes

The lignicolus fungi like *Lenzites sterioides*1, 2, *L. betulina, L. exima, Phellinus gillvus, P. nilgheriensis, P. robustus, Flavodon flavum, Ganoderma lucidum, Shizophyllum Commune, Phoma multirostrata, Theliviopsis* state of *Ceratocystis paradoxa, Fusarium palidoroseum, Alternaria Alternata, Chaetomium globosum, Curvularia lunata, Rhizopus stolonifer, Trichoderma Viride* isolated from RWLS, Gujarat, India were selected for enzymatic studies. Ligninolytic and cellulolytic ability was evaluated by substituting the malt extract agar medium (2%) with tannic acid for ligninase (0.5%) [18] and carboxymethyl cellulose for cellulose (0.5%) (Bains *et al* 2006). On solidification, the plates were inoculated at the center with 1cm^2 mycelial disc of different fungal cultures under study and incubated at 28±1˚C for a week. The replicates were maintained for each set of observations. The respective enzyme activities were evaluated by measuring the zone of clearance if any, found by flooding the plates with dye (0.25% Congo red) for 15min [21] for detection of cellulolytic activity while the ligninolytic activity was assessed by observing brown coloured zone around respective fungal colonies.

RESULTS AND DISCUSSION

The lignin degrading lignicolous fungi were *Lenzites sterioides*1, 2, *L. betulina, L. exima, Phellinus gillvus, P. nilgheriensis, P. robustus, Flavodon flavum, Ganoderma lucidum, Shizophyllum commune,* soft rot fungi like *Phoma multirostrata, Alternaria Alternata, Chaetomium globosum* and Pink mold *Fusarium palidoroseum,* which have ability to produce both ligninolytic and cellulolytic activity. The lignicolous fungi mainly cause degradation of cellulose by producing cellulolytic enzyme were soft rot fungi like *Phoma*

Table 1: Lignicolous fungi showing types of rots with ligninolytic and cellulolytic activity

Plant	fungus	Isolate number	Type of rot	Ligninolytic hallozone in cm	Cellulolytic hallozone in cm
Tectona grandis	*Lenzites sterioides*1	RS2b	white	4.6±0.6	9±1.6

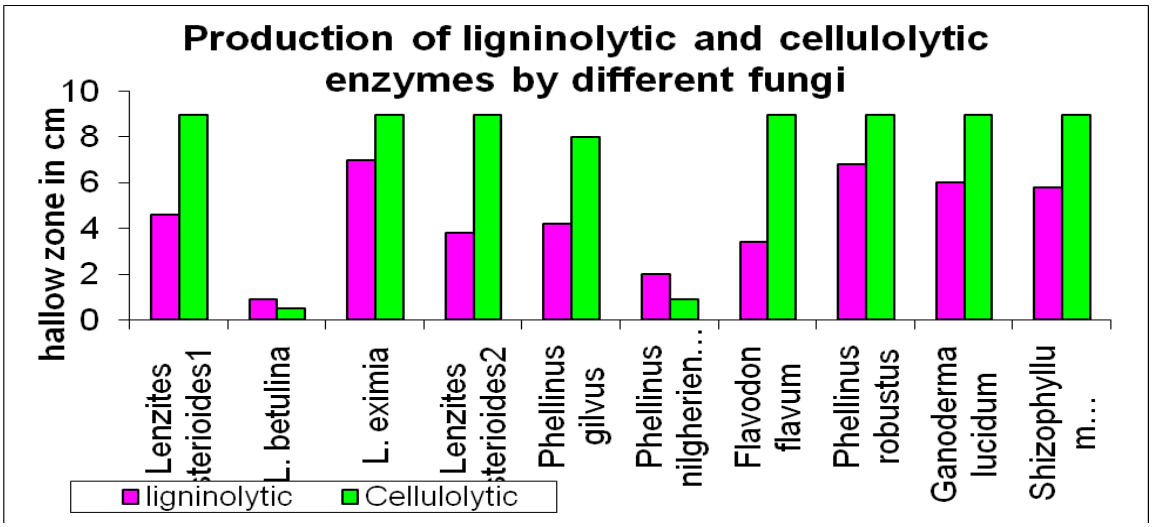
T. grandis	*L. betulina*	DS2c	white	1.7±0.6	1.5±3.6
Callia arborea	*L. exima*	AS5a	white	7.0±1.8	9.0±2.5
Alangium salvifolium	*Lenzites sterioides* 2	RS3b	white	3.8±2.6	9.0±1.4
Terminalia bellerica	*Phellinus gillvus*	RS10a	white	4.2±1.2	8.0±0.8
Cassia fistula	*P.nilgheriensis*	RS17b	white	2.0±0.8	0.9±1.0
Terminalia crenulata	*Flavodon flavum*	RS5b	white	3.4±2.5	9.0±2.2
Cassia fistula	*P.robustus*	RS17d	white	6.8±2.6	9.0±1.6
T. grandis	*Ganoderma lucidum*	RS2e	White	6.0±2.3	9.0±0.4
Tamarindus indica	*Shizophyllum Commune*	AS1a	White	5.8±3.4	9.0±0.9
Madhuca indica	*Phoma multirostrata*	RS4c	soft	5.4±1.0	6.7±3.6
Emblica officinalis	*Theliviopsis* stste of *Ceratocystis paradoxa*	RS15b	UN	--	7.5±2.6
Holarrhena antidysenterica	*Fusarium palidoroseum*	RS1e	Pink mold	4.5±2.5	3.0±1.3
Mangifera indica	*Alternaria Alternata*	AS2b	soft	1.9±1.6	2.8±2.8
Calotropis	*Curvularia lunata*	DS1b	soft	--	5.2±1.8
Garuga pinnata	*Chaetomium globosum*	RS13c	soft	3.2±1.3	4.5±1.4
T. grandis	*Rhizopus stolonifer*	RS2f	Grey mold	--	9.0±1.2
Holarrhena antidysenterica	*Trichoderma Viride*	RS1a	Green mold	--	9.0±1.2

-- activity not detected

∗ indicates each component values are based on the three replicates.
± Results were significant at P <.05 level by one way ANOVA.

multirostrata, Alternaria Alternata, Chaetomium globosum, Curvularia lunata, Pink mold fungi *Fusarium palidoroseum,* green mold fungi like *Rhizopus stolonifer, Trichoderma Viride* and unknown rot fungi *Theliviopsis stste of Ceratocystis paradoxa,*. (Table I). The differentiation of white rot fungi and soft rot fungi was tested by Bavendham depending on the reddish browning of tannic acid agar medium. From these fungi 10 were white rot fungi and other soft rot fungus collected from Rathanmahal Wildlife Sanctuary, Gujarat. The highest ligninolytic activity (hallow zone of clearance 7cm) was shown by *L. eximia* and lowest ligninolytic activity (hallow zone of clearance 0.9cm) was shown by *L. betulina.* The highest cellulolytic activity (hallow zone of

clearance 9cm) was shown by 6 white rot fungi (Plate I). The lowest cellulolytic activity (hallow zone of clearance 0.5cm) was shown by white rot fungi *L. betulin* (Histogram 1). The soft rot fungi *Phoma multirostrata Fusarium palidoroseum Alternaria Alternata Chaetomium globosum were* producing ligninolytic enzyme was reported for the first time.



Histogram 1: Production of ligninolytic and cellulolytic enzymes by different lignicolous fungi

The white-rot causing basidiomycetous members degrade lignin more rapidly and extensively than other studied fungi. Like the brown-rot fungi, they invade the lumens of wood cells, where they secrete enzymes that degrade lignin and the other wood components **[**10]. In the present study the lignin degrading lignicolous fungi were studied in which the white rots degraded lignin more rapidly. During its mineralization by white-rot fungi, lignin undergoes a number of oxidative changes, including aromatic ring cleavage. Depolymerization of wood results into formation of Carboxylic acids, Oxalate, Glyoxylate, Formate, Malate, Veratryl alcohol and Unsaturated fatty acids are low molecular weight compounds involved in lignin and wood polysaccharide degradation by white-rot fungi **[**10]**.** In the present study also the production of low molecular weight compounds was possible because the ligninolytic enzymes production by lignicolous fungi.

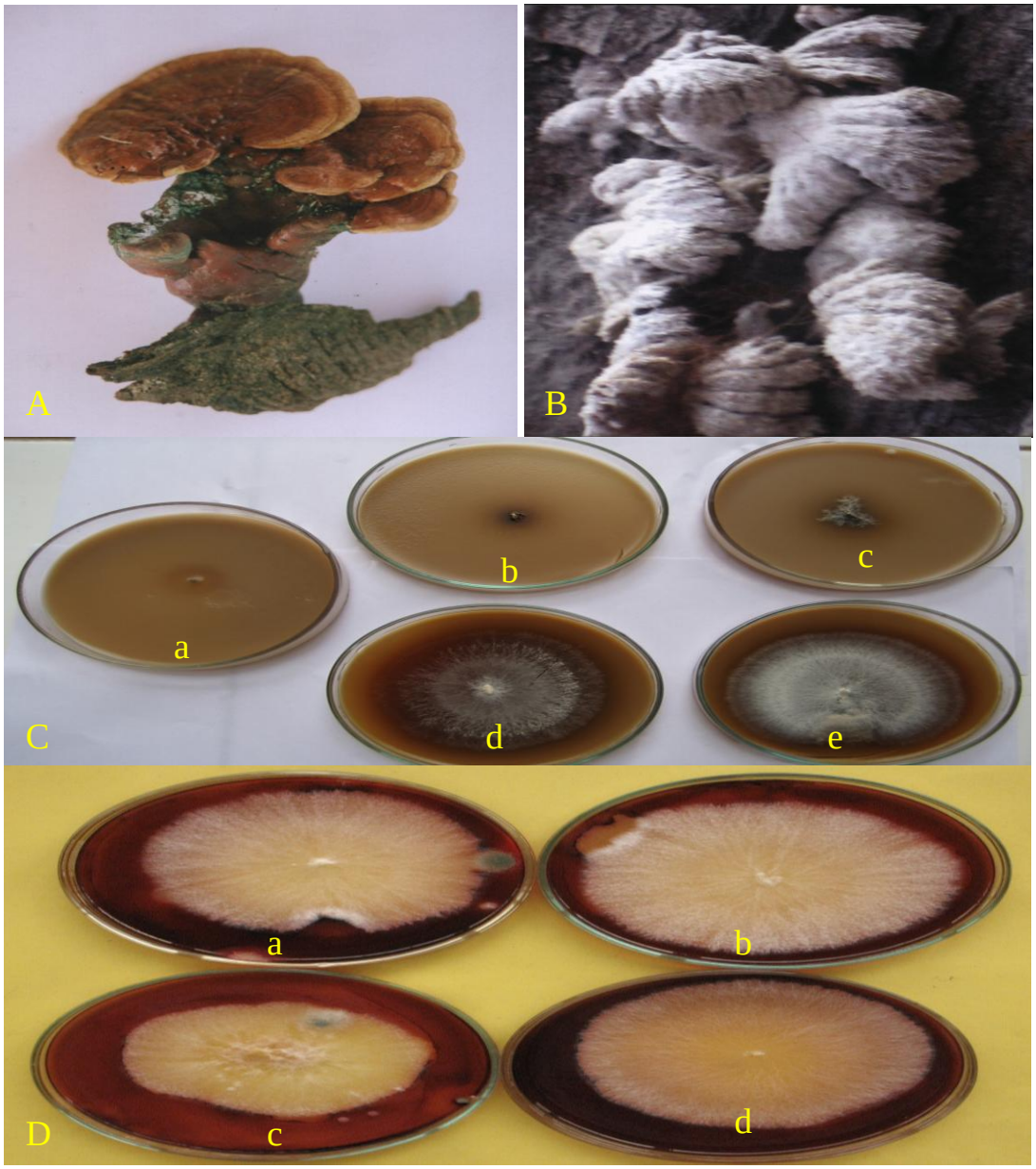


Fig. A. Basidiocarp of *Ganoderma lucidum;* Fig. B. Sporopher of *Schizophyllum commune* Fig. C. ligninolytic activity a) Control, b) *Lenzites sterioides* c) *Flavodon flavus* d) *Lenzites exima* e) *Phellinus gilvus* Fig. D. Cellulolytic activity a) *Lenzites betulina*, b) *Lenzites sterioides,* c) *Lenzites exima* d) *Flavodon flavus*

The proposed mechanisms for the nonenzymatic "exo- type" agent of brown-rot Fungi include i) production of hydroxyl radicals by Fenton chemistry [23] ii) acid-catalyzed hydrolysis by hydronium ion [19] iii)reduction of iron by cellobiose dehydrogenase plus autoxidation [20] iv) one-electron oxidation [4] v) production of low molecular weight chelators and iron-binding compounds (siderophores) [8]. In the present paper the fenton chemistry is possible because of ligninolytic enzymes production by white rot and soft rot fungi. Two peroxidases discovered in the extracellular fluid of Phanerochaete chrysosporium, lignin peroxidase (LiP) and manganese peroxidase (MnP) are known to

play important roles in the initial degradation of lignin [19]. In the present study the lignicolous fungi also produces lignin degrading enzymes like lignin peroxidase and manganese peroxidase. Laccase also has been implicated in lignin degradation in some organisms. These enzymes and the organisms that produce them are widely considered to have potential for industrial applications, such as biodegradation of environmental pollutants, bioconversion of lignin, biobleaching and biopulping of wood chips, desulfurization of petroleum and coal, and delignification of agricultural plant residues [8]. In the present study the white rot and soft rot fungi have the capacity to degrade lignocelluloses substances so they have wide application.

ACKNOWLEDGEMENT

The author is thankful to the Head, Department of Botany, The Maharaja Sayajirao University of Baroda for laboratory facilities. This study is funded by DST, New Delhi.

REFERENCES

[1] Archibald FS & Roy B, Production of manganic chelates by laccase from the lignin degrading fungus *Trametes versicolor*. *Appl. Environ. Microbiol*. 58(1992) 1496.

[2] Bains R K, Rahi D K, & Hoondal G S, Evaluation of wood degrading enzymes of some indigenous white rot fungi. *J. Mycol. Pl. Pathol*. 36 (2) (2006)161.

[3] Bourbonnais R & Paice M, Oxidation of non phenolic substrates: an expanded role for laccase in lignin biodegradation. *FEBS Lett*. 267 (1990) 99.

[4] Enoki A, Itakura S & Tanaka H, The involvement of extracellular substances for reducing molecular oxygen to hydroxyl radical and ferric iron to ferrous iron in wood degradation by wood decay fungi. *J. Biotech*. 53 (1997) 265.

[5] Eriksson KE, Pettersson B, Volc J & Musilek V, Formation and partial charectarizationof glucose 2 oxidase, A H2O2 producing enzyme in *Phaenerochaete chrysosporium. Appl. Microbiol. Biotechnol*. 23(1986) 257.

[6] Green III F & Highley T L, Brown-rot wood decay--insights gained from a low-decay isolate of *Postia placenta. Trends in Plant Pathology* 1 (1997)1.

[7] Glenn J K, Akileswaran L & Gold M H, Mn(II) oxidation is the principal function of the extracellular Mn-peroxidase from *Phaenerochaete chrysosporium*. *Arch. Biochem. Biophys.* 251(1986) 688.

[8] Goodell B, Jellison J, Liu J, Daniel G, Pascyenski A, Fekete F, Krishnamurthy S, Jun L, & Xu G, Low molecular weight chelators and phenolic compounds isolated from wood decay fungi and their role in the fungal biodegradation of wood *J. Biotechnol*. 53 (1997) 133.

[9] Guillen F, Martinez A T & Martinez M J, Production of hydogenperoxide by arylalcohol oxidasefrom the ligninolytic fungus *Pleurotua eryngii*. *Appl. Microbiol. Biotechnol* 32 (1990) 465.

[10] Hakala T K, *Characterization of the lignin-modifying enzymes of the selective white-rot fungus Physisporinus rivulosus* Helsinki University Printing House, Helsinki. (2007)1.

[11] Hammel K E, Jensen K A, Mozuch M D, Lamducci L L, Tien M & Pease E, Ligninolysis by a purifies lignin peroxidase. *J. Biol. Chem*. 268 (1983) 12274.

[12] Kersten P J & Krik T K, Involvement of a new enzyme. Glyoxal oxidase, in extracellular H2O2 production by *Phaenerochaete chrysosporium*. *J. Bactriol* 169(1987) 2195.

[13] Kersten P J, Glyoxal oxidase of *Phanerochaete Chrysosporium*: its characterization and activation by ligning peroxidase. *Proc. Natl. Acad. Sci. USA* 87 (1990) 2936.

[14] Kersten P J, Tien M, Kalyanamaran & Krik T K, The ligninase of *Phaenerochaete chrysosporium* generates cation radicals from methoxibenzenes. *J. boil. Chem.* 260 (1985) 2609.

[15] Kirk T K & Farrel R L, Enzymatic combustion: the Microbial degradation of lignin. *Annu. Rev. Microbiol*. 41 (1987)465.

[16] Kurek B & Kersten P J, Physiologial regulation of glyoxal oxidase from *Phanerochaete Chrysosporium* by peroxidase systems. *Enzyme Microb. Technol*. 1 (1995) 751.

[17] Nishida A & Eriksson K E, Formation, Purification and partial charectarization of methanol oxidase, A H2O2 producing enzyme in *Phaenerochaete chrysosporium*. *Biptechnol. Appl. Biochem*. 9 (1987) 325.

[18] Nobles M K, Identification of cultures of Wood Inhabiting Hymenomycetes *Canadian Journal of Botany* 43 (1964) 1097.

[19] Shimada M, Ma D, Akamatsu Y & Hattori T, Proposed role of oxalic acid in wood decay systems of wood-rotting basidiomycetes. *FEMS Microbiol Revs.* 13 (1994) 285.

[20] Suttie E D, Hyde S M, & Wood P M, Preliminary studies of the performances of iron chelatiors as inhibitors of brown-rot (*Cniophora puteana*) attack. The International Research Group on Wood Preservation, (1996) Documentation No. IRG/WP/96-10185.

[21] Teather R M & Wood P J, Use of Congo red olysaccharide interaction of cellulolytic bacteria from the bovine serum. *Appl. Environ. Microbiol*. 43 (1982) 777.

[22] Wariishi H, Valli K & Gold M N, *In vitro* depolymerization of lgnin by manganese peroxidase of *Phanerochaete chrysosporium*. *Biochem. Biophys. Res. Commun.* 176 (1991) 269.

[23] Wood P M, Pathways of production of fenton reagent by wood-rotting fungi. *FEMS Microbiol*. Rev. 13(1994) 313.