CHARACTERIZATION AND LIGNIN DEGRADATION PROPERTIES OF THE LIGNINOLYTIC ENZYMES IN THE EXTRACELLULAR FLUIDS OF *PHANEROCHAETE CHRYSOSPORIUM 1038*

Nelly V. Georgieva[1*], Lyubov K. Yotova[1], Tinka G. Kolusheva[2], Nadezhda G. Rangelova[3]

University of Chemical Technology and Metallurgy,
8 Kl. Ohridski str., Sofia 1756, Bulgaria
[1]Department of Biotechnology,
[2]Department of Analytical Sciences,
[3]Department of Pulp, Paper and Printing Arts

*Corresponding author: nelly.georgieva@yahoo.com, Tel.: +359 2 8163 307

Received: 07/05/2009
Accepted after revision: 30/06/2009

Abstract: The effect of ligninolytic enzymes in the extracellular fluids of the cultivated cells of basidiomycetic fungi of *Phanerochaete chrysosporium* strain *1038* on hydrolyzed lignin samples *in vitro* was investigated. The growth conditions of the strain were optimized in regard to receive higher activities of ligninolytic enzymes. The influence of inorganic nitrogen, Mn^{2+} ions, temperature and *pH* of the medium on the growth and enzymes activity was also studied. It was found that extracellular fluids of the cultivated strain showed activities of lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac). The addition of 2.2 mM nitrogen and 3.5 mg/L Mn^{2+} to the medium kept constantly at 37 ^{o}C and *pH* = 5 increased significantly the quantity of exuded by cells soluble protein with considerable activities of LiP, MnP and Lac. The extracellular fluids, concentrated by ultrafiltration were incubated

for 24 h with samples of hydrolysis lignin. Evidence for the delignification processes was provided by FTIR spectroscopy, which showed decrease of free carboxylic and increase of hydroxylated and methoxylated aliphatic groups in the treated samples. The changes were regarded as result of oxygenation processes caused by the extracellular ligninolytic enzymes.

Keywords: *Laccase, Lignin peroxidase, Manganese peroxidase, Phanerochaete chrysosporium*

INTRODUCTION

Lignin is an extremely complex, three dimensional heteropolymer, made up primarily of phenyl propane units, which serves as a structural component of the cell walls of higher plants [1, 2]. It is chemically very strong and hardly degradable waste product. Except the chemical cleavage of lignin, recently, an increased interest to the biologically mediated process of lignin degradation has been regarded. Among the microorganisms capable to cleave lignin metabolically, the fungi species belonging to the Basiodiomycetes group occupy leading place. It was found that among this group the species of genera *Phanerochaete* possess strong ligninolytic activity [3]. Due to the large size of the lignin molecules it is believed that some part of degradation processes occurs predominately outside of the cells [4]. Lignin molecules are not degradable under anaerobic conditions, so as a rule aerobic conditions of growth of these strains are considered necessary. Because of the complexity of the lignin molecules, the combined action of lignolitic enzymes and several highly active but non-specific to substrate enzyme agents is needed for the effective degradation of different bonds in the lignin molecules [5].

The strain *1038* of *Phanerochaete chrysosporium* secretes in the medium specific oxidoreductases and some non-specified extracellular factors, which are involved in the metabolic cleavage of different phenolics including lignin, and wood pulp [3, 6]. The ligninolytic system in *Ph. chrysosporium 1038* consists of several enzymes: manganese peroxidise (MnP), lignin peroxidise (LiP) and laccase (Lac). Among this group MnP and LiP have a leading role [7]. Inorganic nutrients, organic compounds, temperature, *pH* of the medium and some other factors have been proved to play important role in elicitation of the enzymes with high ligninolytic activity. It was reported that the strain *1038* of *Ph. chrysosporium* showed considerable activities of these enzymes under optimal growth conditions [8]. The addition of Mn^{2+} ions in growth medium of the strain was proved to be especially effective in the induction of high MnP activity outside of cells. It was found that in the presence of Mn^{2+} MnP oxidize the unsaturated acids of lipid molecules [5]. The formed peroxide intermediates were capable to oxidize non-phenolic type of the model lignin compounds. The addition of veratryl alcohol (3,4-dimethoxybenzyl alcohol, VA) to the culture medium of the strain lead to the increase of other enzyme of ligninolytic complex - LiP [9, 10]. LiP can catalyze the oxidation of phenols and aromatic amines, aromatic ethers and polycyclic aromatic hydrocarbons. Laccase – another enzyme of lignolytic complex produced by this strain are multi-copper-containing enzymes that oxidize different phenolic compounds. The laccases are

effective in demethylation and delignification of Kraft pulps when the mediator of the process is 2,2`-azinobis-(3-ethyl-benzthiazoline-6-sulphonate) (ABTS) [8, 9, 11]. Fungi and their ligninolytic enzymes are considered a prospective tool for processing pulp or bleaching of Kraft pulp, but also can be used successfully for bioremediation of waste waters from aromatic compounds [5, 12, 13].
The present paper describes results obtaining after the incubation *in vitro* of chemically defined lignin samples with the extracellular fluids from cultivated cells of strain *1038* of *Ph. chrysosporium.* The fluids showed high activities of MnP, LiP and Lac enzymes. To increase the ligninolytic activities of the enzymes in the extracellular fluids the growth of cells were optimized by adding Mn^{2+}, inorganic nitrogen and adjusting the temperature and *pH* of the medium. In addition the fluids were separated and concentrated by ultrafiltration with the aim to increase specific activities of studied enzymes. The changes in treated lignin samples were determined after the Fourier-transform infrared (FTIR) spectroscopy analysis.

MATERIALS AND METHODS

Fungal Strain

The *Phanerochaete chrysosporium 1038* strain was obtained from the National Bank of Industrial Microorganisms and Cell Cultures collections, Bulgaria.

Growth conditions

The fungus strain *Ph. chrysosporium 1038* was maintained at 4 ^{0}C on solidified medium containing yeast-malt-peptone-glucose agar slants with concentrations (in g.L^{-1}): glucose – 10.0; Peptone – 2.0; Malt extract – 10.0; Yeast extract – 2.0; KH_2PO_4 – 2.0; $MgSO_4.7H_2O$ – 1.0; agar – 20.0. The fungus was cultivated in a defined liquid mineral medium containing (in g.L^{-1}): KH_2PO_4 – 1.00; NaH_2PO_4 – 0.20; $MgSO_4.7H_2O$ – 0.50; sodium tartrate – 1.46; $CaCl_2$ – 0.001; $CuSO_4.5H_2O$ – 0.001; $FeSO_4.7H_2O$ – 0.001; $ZnSO_4$ – 0.001; thiamine hydrochloride – 0.001. Nitrogen (N) as diammonium tartrate salt was added to the media at final concentrations 2.2 mM. Glucose (as single energy and carbon source) was added to the medium at concentration of 10 g.L^{-1} (56 mM) after preliminary sterilization. The concentration of Mn^{2+} (as $MnSO_4$) was 0.35 and 3.5 mg.L^{-1}. All chemicals were purchased from Merck Inc. (Germany). *Ph. chrysosporium* strain *1038* was cultivated (as a batch culture) on a rotary shaker at 140 rpm for 120 h at 30 ^{0}C. To enhance production of MnP and LiP in the extracellular fluid, $MnSO_4$ and VA (2.5 mM) were added respectively 48 hours after the start point of cultivation [14]. At the end of cultivation the extracellular fluid was separated by centrifugation at 4000 rpm and concentrated (30 times) by ultrafiltration using YM10 (YM 10, Amicon Corp.,) membrane. Further, the concentrated solution was dialyzed against 5 mM sodium tartaric buffer (*pH* = 4.5) for 24 h at 4 ^{0}C [3].

Protein assay

Total protein was determined according Bradford [15] using bovine serum albumin (Sigma Co) as a standard protein.

Enzymes assay

Lac (EC 1.10.3.2) activity was determined as a rate of oxidation of ABTS as described previously [16]. The oxidation of ABTS was determined by the increase in absorbency at 420 nm ($\varepsilon_{420} = 3.6\times10^6$ $M^{-1}cm^{-1}$) at 30 ^{0}C. The enzyme activity was expressed in enzyme units (U = μmol substrate/min).
MnP **(**EC 1.11.1.13**)** activity was assayed based on the oxidation of guaiacol as a substrate. The reaction was initiated by adding hydrogen peroxide and was monitored by the increase in absorbance at 465 nm ($\varepsilon_{465} = 26600$ $M^{-1}cm^{-1}$) [16]. One unit of MnP was defined as the amount of enzyme needed to oxidize 1 μmol of Mn^{2+} into Mn^{3+} for 1 min.
LiP (EC 1.11.1.14) activity was measured using VA as a substrate [16]. The reaction was initiated by H_2O_2 and the formation of veratryl aldehyde was monitored at 310 nm (ε_{310} – 9300 $M^{-1}cm^{-1}$). The amount of LiP required for the oxidation of 1 μmol VA into veratryl aldehyde per min was defined as 1 enzyme unit. The enzyme activity was expressed as a number of enzyme units per liter. All measurements related with the determination of enzyme activities were carried out in a UV-VIS spectrophotometer Secoman S 750.

Lignin treatment

The hydrolyzed lignin samples were obtained from softwood and hardwood (50/50) and taken from the Dept. of Pulp, Paper and Printing Arts – University of Chemical Technology and Metallurgy – Sofia, Bulgaria. Each reaction mixture (10 mL) contained: 5 mM ABTS; 20 mM VA, enzyme solution (culture supernatant) and 0.25 g lignin in 0.1 M sodium acetate buffer (*pH* = 5.0). After a 24 h reaction on a rotary shaker at 140 rpm at 30 ^{0}C, the lignin samples were filtrated and washed with distilled water (DW). The control sample was treated with DW instead of culture supernatant. Collected and washed with DW lignin samples after the treatment were subjected to FTIR spectroscopic analysis.

FTIR spectroscopy

FTIR spectra were recorded on a Perkin-Elmer Spectrum 1000 FTIR spectrophotometer, resolution 2 cm^{-1}. All spectra were normalized between absorbance of 0 and 1 in the region of 1710 – 1600 cm^{-1}. Each spectrum was analyzed by second derivatization and curve fitting. The spectra were transferred to a personal computer and subjected to a line shape analysis by the Grams-software [17].

RESULTS AND DISCUSSION

Effect of Mn^{2+} ions on the activities of extracellular ligninolytic enzymes

The enzymatic assay of the supernatant collected from the cultivated cells of *Phanerochaete chrysosporium* strain *1038* showed presence of LiP, MnP and Lac activities (Table 1). The addition of Mn^{2+} ions to the culture medium of the strain increased significantly the extracellular activity of MnP. The maximal activity of extracellular MnP was assayed when the addition of 3.5 $mg.L^{-1}$ Mn^{2+} in the medium was done. The 10 fold increase of Mn concentration ions in the medium influenced positively the activity of MnP and Lac but not altered significantly LiP activity. These results indicate that Mn ions are not strictly necessary for the elicitation of extracellular LiP activity (Table 1). The effect of inorganic nitrogen supplied to the culture medium was also strong positive elicitor of the enzyme activities. It has been found in previous experiments that the increase of inorganic nitrogen in the medium from 0.35 to 3.5 mM N in the medium increased strongly MnP activity of strain while 35 mM N acted inhibitory [16]. The addition of 2.2 mM N in the medium was optimal for the cell growth and for the exudation of extracellular protein with high LiP, MnP and Lac activities as well.

Table 1. *Effect of Mn^{2+} and inorganic nitrogen added to the growth medium of cultivated Ph. chrysosporium 1038 cells on the extracellular MnP, Lip and Lac activity*

Growth medium	Protein content ($mg.L^{-1}$)	LiP ($U.mg^{-1}$ protein)	MnP ($U.mg^{-1}$ protein)	Lac ($U.mg^{-1}$ protein)
Control: 2.2 mM N without Mn^{2+}	2±0.1	20.18±1.0	nd*	nd*
2.2 mM N plus 0.35 $mg.L^{-1}$ Mn^{2+}	5±1.0	48.12±4.0	57.32±3.0	9.76±1.0
2.2 mM N plus 3.5 $mg.L^{-1}$ Mn^{2+}	7±1.5	52.24±4.5	134.64±5.0	21.52±2.0

(*nd – not detected); data are means of 3 independent measurements ± SD

Effect of temperature and *pH* on the activities of extracellular ligninolytic enzymes produced by the strain *1038*

Among the important external factors influencing the extracellular enzymes activities of microorganisms are temperature and *pH* of the growth medium [18]. It was demonstrated that incubation temperature influenced significantly enzyme activities of the strain *1038* although the optimal temperature is differed among the studied enzymes. The temperature optimum of LiP and MnP was 37 ^oC while for the Lac it was 30 ^oC (Fig.1).

The *pH* optimum of Lac and MnP were 5.0 at 37 ^oC while LiP activity was maximal at *pH* = 3.0 (Fig. 2). Hence, the optimal conditions for growth and enzyme activities of the strain *1038* defined *pH* 5.0 and temperature 37 ^oC. The samples of chemically processed lignin were incubated for 24 hours with the defined volumes of concentrated extracellular fluids collected from the supernatant of growth medium of the strain *1038* cultivated under optimal conditions (*pH* 5, inorganic nitrogen 2.2 $mg.L^{-1}$ N, temperature 37 ^oC and 3.5 $mg.L^{-1}$ of Mn ions).

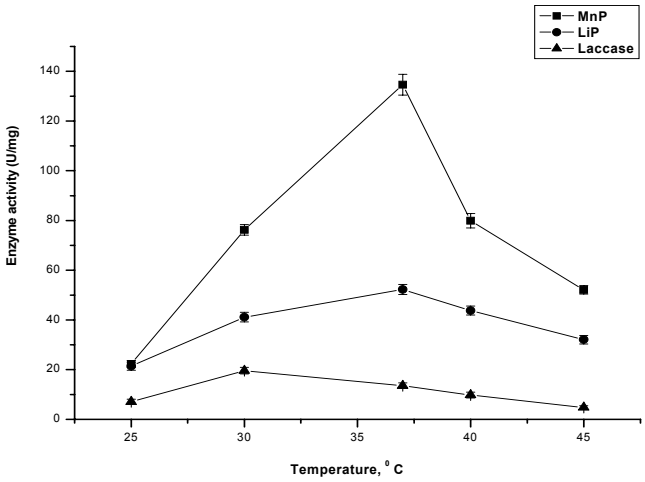

Figure 1. *Temperature profiles of MnP, LiP and Lac activity of* ***Ph. chrysosporium 1038*** *at pH = 5.0.*
Every point represents the mean of 5 independent measurements ± SD.

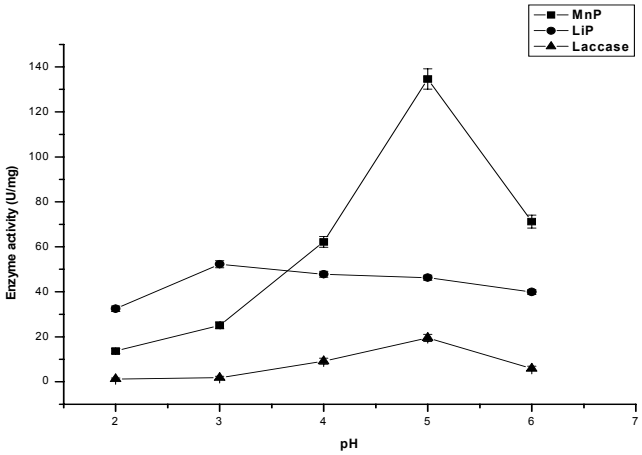

Figure 2. *pH profiles of MnP, LiP and Lac activity of* ***Ph. chrysosporium 1038*** *at T = 37 ^{0}C. Every point represents the mean of 5 independent measurements ± SD.*

FTIR analysis

Recently FTIR spectroscopy has become a preferred technique for rapid in situ analysis of lignin structure [19, 20]. Faix and co-workers have discussed the utility of FTIR spectroscopy for detection of free phenolic hydroxyl content of lignin [21]. Some changes in the structure of the incubated with the concentrated extracellular fluids hydrolyzed lignin samples appeared after the incubation procedure. In Fig.3 the differences between the spectra of control (1) and treated lignin samples (2 and 3) can be observed. Our data from FTIR spectra showed significant differences in the region 1800 – 1400 cm^{-1} assigned to aromatic rings absorptions of lignin. At the band around 1710 cm^{-1} was found decrease of the absorbance of peaks in the samples 2 and 3

compared to the control sample, due to C=O stretching vibrations. This result relates to the process of transformation of carboxylic groups in ketonic and ester groups [22]. Since in lignin β-O-alkyl-aryl bonds constitute, the most common linkage between lignin subunits (40-60%) and they are well detected by different spectroscopic methods, it is generally considered that changes in this region of absorbance is main basis for the identification of alteration in the lignin polymer. Other linkages commonly ascribed to the lignin polymer are approximately to an order lower. The increase of hydroxyl and methoxylated groups in the lignin samples can be due to coupled action of MnP and LiP considered different in their catalytic mechanism [21].

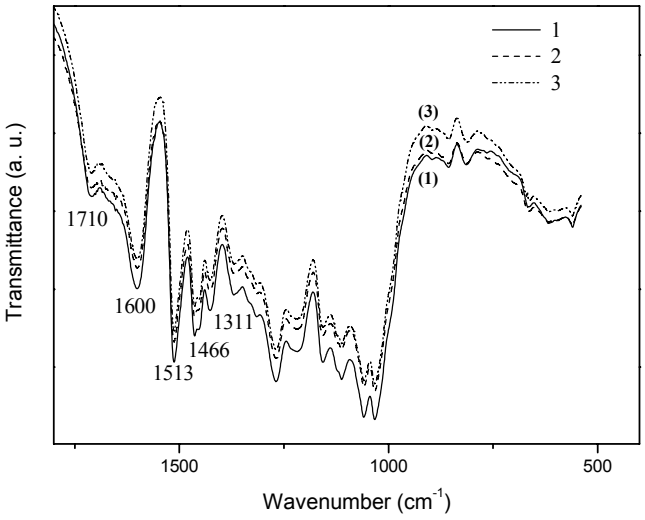

Figure 3. *FTIR spectrum of lignin treated samples (1- control, 2 and 3 - lignin treated with culture supernatant)*

The presence of band 1600 cm^{-1} in the spectra of control lignin sample characterizes the absorption of the carbonyl groups in the p-substituted aromatic ketons [20]. The occurrence of this peak in the sample 2 and sample 3 was found to decline in stepwise way. These results rely to some changes in the number of carbonyl groups substituted on para-state of phenyl rings. It was found the presence of band at 1513 cm^{-1} in the spectra of control sample (1), having considerably lower intensities than those observed in the spectra of treated samples (2 and 3), which is attributed mostly to the C=C aromatic skeletal vibrations. It was also detected weak signal from the bands at 1513 cm^{-1} and at 1466 cm^{-1} in the control sample, which decreased slowly in the treated samples. These bands are mostly assigned to C–C ring skeletal vibrations and C–H vibrations in $–CH_3$ or $–OCH_3$, respectively.

Generally, the analysis of FTIR spectra of the treated lignin evidenced the occurrence of some de-lignification processes. The FTIR spectra of treated and control samples of hydrolysis lignin show some decrease of absorbance due to the availability of free carboxylic groups and less pronounced increase of the amount of hydroxylated and methoxylated aliphatic groups in the lignin samples. These changes can be regarded mostly as a result of oxygenation process occurred with the participation of reactions

catalysed by ligninolytic enzymes of LiP and MnP. The LiP and MnP differ in their catalytic action [4]. LiP usually leads to formation of a cation radicals by taking single electron from the aromatic ring of lignin molecule while MnP by oxidizing Mn^{2+} to Mn^{3+}, which as highly reactive intermediate, capable to attack and oxidize the lignin structure *in situ*. The oxidation of lignin by Lac is generally considered as a process associated with the destructive role of LiP and MnP. The result of combined action of 3 enzymes has been attributed to coupling reactions between phenoxy radicals, expected products from LiP-catalysed oxidation of phenolic lignin intermediates and the oxidation of aromatic ring with the participation of H_2O_2 produced by the strain [14]. The small modifications of lignin structure, found in our samples treated with extracellular fluids of the strain *1038*, can be explained with the low specificity of action of studied enzymes but not a consequence of their low activity. This is not surprising as there are enough evidences showing that lack of some small mediator factors, produced by host strain, can limit the efficiency of the process. However, encouraging results have been achieved only when fungus was incubated with kraft pulp for long period of time. Most of authors have considered that live fungus secretes a number of molecules and redox factors which in cooperative way with lignin degrading enzymes destruct lignin slowly but continuously. So, the reasons for the small changes in the structure of lignin in our experiments, first, the duration of incubation of isolated enzymes with lignin samples and, second, lack of mediator factor produced by live species.

CONCLUSIONS

The laboratory cultivation procedure for optimal growth and production of lignotlytic enzymes in the extracellular fluids of strain *1038* of *Ph. chrisosporum* was developed. The conditions of cell growth were lower nitrogen (2.2 mM NH_4^+), higher Mn^{2+} content (3.5 $mg.L^{-1}$) and temperature kept at 37 ^oC and *pH* = 5. The obtained fluids showed maximal activity of LiP, MnP and Lac assayed *in vitro*. FTIR analysis of samples of hydrolyzed lignin incubated for 24 hours with supernatant, concentrated previously 30 times by ultrafiltration, gave additional evidence for the ligninlytic properties of these fluids. The spectra showed that some changes in the range of absorbance of methoxylated and hydroxylated aliphatic groups and free carboxylic groups of lignin molecules. These changes could be associated with oxidative cleavage of some side substituted groups in aromatic rings or propyl side chain of monolignol moiety of lignin molecules.

ACKNOWLEDGMENTS

The authors are grateful for the support of the University of Chemical Technology and Metallurgy, Sofia, Bulgaria, Grant. No. 2009-10599.

SYMBOLS

ABTS - 2,2`-azinobis-(3-ethyl-)benzthiazoline-6-sulphonate
DW - distilled water
FTIR - Fourier-transform infrared spectroscopy
MnP - Manganese peroxidase
Lac - Laccase
LiP - Lignin peroxidase
VA - veratryl alcohol - 3,4-dimethoxybenzyl alcohol

REFERENCES

1. Dean, J.: Lignin analysis in: *Methods in plant biochemistry and molecular biology*. Chapter 17, CRC Press LLC, **1997**, 199-215.
2. Martins, G., Pereira, A., Straccalano, B., Antunes, P., Pasquini, D., Curvelo, A., Ferreira, M., Riul Jr. A., Constatino, C.: Ultrathin films of lignins as a potential transducer in sensing applications involving heavy metal ions, *Sensors and Actuators B,* **2008**, **129**, 525-530.
3. Tien, M., Kirk, T.: Lignin degrading enzyme from *Phanerochaete chrysosporium* Purification, characterization, and catalyc properties of a unique H_2O_2-requiring oxygenase, *Proc. Natl. Acad. Sci.USA*, **1984**, **81**, 2280-2284.
4. Heinzkill, M., Mesner, K.: The ligninolytic system in fungi, in: *Fungal Biotechnology*, (Editor: Anke, T.), Chapman and Hall, Weinheim, **1997**, 213-276.
5. Breen, A., Singleton, F.: Fungi in lignocellulose breakdown and biopulping, *Curr. Opin. Biotechnol*., **1999**, **10**, 252-258.
6. Field, J.A., de Jong, E., Feijoo Costa G., de Bont, J.A.: Screening for ligninolytic fungi applicable to the biodegradation of xenobiotics, *Trends Biotechnol.,* **1993**, **14**, 44–49.
7. Hofrichter, M.: Lignin conversion by manganese peroxidise, *Enzyme Microb. Technol.*, **2002**, **30**, 454-466.
8. Hamman, O., Rubia, T., Martinez, J.: Effect of carbon and nitrogen limitation on lignin peroxidase and manganese peroxidise production by *Phanerochaete flavido-alba, J. Appl. Microbiol*., **1997**, **83**, 751-757.
9. Bourbonnais, R., Pais, M.: Demethylation and delignification of kraft pulp by *Trametes versicolor* Laccase in the presence of 2,2-azinobis(3-ethylbenzthiazoline-6-sulphonate), *Appl. Microb. Biotechnol*., **1992**, **36**, 823-827.
10. Mester, T., Jong E., Field, J.: Manganese regulation of veratryl alcohol in white rot fungi and its indirect effect on lignin peroxidise, *Appl. Environ. Microbiol*., **1995**, **61**, 1881-1887.
11. Thrston, C.: The structure and function of laccases. *Microbiology*, **1994**, **140**, 19-26.
12. Abadulla, E.: Decolorization and detoxification of textile dyes with a Laccase from *Trametes hirsute, Appl. Environ. Microbiol*., **2000**, **66**, 3357-3362.
13. Archibald, F., Bourbonnais, R., Jurasek, L., Paice M., Reid, I.: Kraft pulp bleaching and delignification by *Trametes versicolor*, *J. Biotechnol*., **1997**, **53**, 215-236.

14. Hammel, K., Moen, M.: Depolymerization of a synthetic lignin in vitro by lignin peroxidase, *Enzyme Microb Technol.,* **1991**, **13**, 15-18.
15. Bradford, M.M.: *Anal Biochem*, **1976**, **72**, 248-54.
16. Georgieva, N., Kolusheva, T.: Effect of nitrogen and manganese on the activities of ligninolytic enzymes produced by *Phanerochaete chrysosporium 1038*, *Compt. rendus de l`Acad. Bulgare des Sci*. **2007**, **60**, 1193-1198.
17. Andreeva, A., Karamancheva, I., Hendlich, M.: Secondary structural analysis of chloramphenicol acetyltransferase type I using FTIR Spectroscopy, *Bulg. Chem. Comm.,* **2001**, **33**, 9-14.
18. Perez, J., Martinez J., Rubia, T.: Purification and partial characterization of a laccase from the white rot fungus *Phanerochaete flavido-alba, Appl. Environ. Microbiol.,* **1996**, **62**, 4263-4267.
19. Ghatak, H.: Spectroscopic comparison of lignin separated by electrolysis and acid precipitation of wheat straw soda black liquor, *Ind. Crops Prod.,* DOI:10.1016/j. indcrop.02.011. **2008**.
20. Liu, Q., Wang, S., Zheng, Y., Luo, Zh., Cen, K.: Mechanism study of wood lignin pyrolysis by using TG-FTIR analysis, *J Anal. Appl. Pyrolysis,* **2008**, **82**, 170-177.
21. Faix O.: FTIR Spectroscopy in: *Methods of lignin chemistry*, (Editors: Lin, S., Dence C.), Springer-Verlag, Berlin, **1992**, Chapter 4.1.
22. Oliveira, L., Evtuguin, D., Cordeiro N., Silvestre, A.: Structural characterization of stalk lignin from banana plant, *Ind. Crops Prod.,* DOI:10.1016/j.indcrop. 04.012. **2008**.