Journal of Applied Biological Sciences 4 (3): 69-78 2010
ISSN: 1307-1130, E-ISSN: 2146-0108 www.nobel.gen.tr

Enhanced Production and Stability of Laccase Using Some Fungi on Different Lignocellulosic Materials

Firdevs ÖZŞÖLEN[1] Pınar AYTAR[1] Serap GEDIKLI[1] Meltem ÇELIKDEMIR[1] Murat ARDIÇ[2] Ahmet ÇABUK[2*]

[1]Graduate School of Natural and Applied Sciences, Eskişehir Osmangazi University, 26480, Eskişehir, TURKEY
[2]Department of Biology, Faculty of Arts and Science, Eskişehir Osmangazi University, 26480, Eskişehir, TURKEY

[*]**Corresponding Author**
e-mail: acabuk@ogu.edu.tr

Received : August 10, 2010
Accepted : November 23, 2010

Abstract

The potentials of thirteen lignocellulosic wastes wheat bran, barley bran, cone of Pinus nigra, sawdust, corn bran, oat, rice bran, canola, dried tea, ground clover straw, sunflower stalk, soybean bagasse, dried distillers grains with soluble (DDGS) as solid substrates in respect of laccase production by the white-rot fungi *Trametes versicolor* (ATCC 200801), *Phanerochaete chrysospsorium* (ME 446) and *Pleurotus sajor-caju* under solid-state conditions were assessed. The highest laccase activity was detected when *T. versicolor* was grown on ground clover straw, showing a maximum value of 22.36 U ml^{-1}. The high enzyme activity was observed to correspond with chemical composition of substrate evaluated in respect of holocellulose, lignin, carbon and nitrogen contents. In addition, FTIR analysis was performed to chemical changes of substrate before and after using in solid state culture. Furthermore, pH and thermal stability of enzyme obtained from *T. versicolor* ATCC200801 grown on clover straw were studied. Also, effects of different inhibitors on the same enzyme activity were investigated.

Keywords: Solid state fermentation, laccase, FTIR, white rot fungi, stability

INTRODUCTION

Lignin is an aromatic polymer of phenylpropane units connected by different C-C and C-O-C linkages [1]. Since lignin is highly oxidized, it is difficult to oxidize further. Lignin is a complex heteropolymer without stereochemical regularity, due at least in part to the free radical mechanism of synthesis [1].

The most abundant biological polymer on earth, cellulose, can be hydrolyzed to soluble sugars either chemically or enzymatically. The chemical composition of cellulose is simple, consisting of D-glucose residues linked by ß-1,4-glycosidic bonds to form linear polymeric chains [1].

Hemicellulose is a complex combination of relatively short polymer made of xylose, arabinose, galactose, mannose, and glucose with acetyl and uronic side-groups. The major hemicellulose is the O-Acetyl-(4-O-methylglucurono)-xylan, also named glucuronoxylan or briefly xylan [1]. A basidiomycetes group, white rot fungi having hydrolytic enzymes such as cellulase, pectinase, xylanase and ligninase enzyme complex composed of lignin peroxidase (LiP) [2], manganese dependent peroxidase (MnP), laccase is able to degrade effieciently lignin, cellulose and hemicelluloses [3]. Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) are extracellular, multicopper enzymes that use molecular oxygen to oxidize various aromatic and nonaromatic compounds by a radical-catalyzed reaction mechanism [4]. Laccase catalyzes the four electron reduction of molecular oxygen to water with one-electron oxidation of reducing substrate, without producing hydrogen peroxide [4].

In recent years, there has been an increasing trend toward using of agricultural and industrial residues with the solid state fermentation (SSF) technique to produce several enzymes synthesized from filamentous fungi [5]. SSF involves the growth of fungi on moist solid particles, in situations in which the spaces between the particles contain a continuous gas phase and a minimum of visible water [6]. Advantages of SSF process are higher yields in a shorter time period than submerged fermentation applications [7], resembling the natural habitat for filamentous fungi, reducing energy and cost requirements [8]. Besides, the substrates used in SSF processes are often products or byproducts of agriculture, forestry or food processing [6].

The present work has been designed to determine activities of laccase produced from *Trametes versicolor* (ATCC200801), *Phanerochaete chrysosporium* (ME 446) and *Pleurotus sajor-caju* on grown different lignocellulosic substrates. Then, it was found out that growing of evaluated fungi may cause how many decrease in holocellulose, carbon contents.

MATERIALS AND METHODS

Microorganisms and experimental set-up

The microorganisms used were the three members of ligninolytic fungi, *Trametes versicolor* (ATCC200801), *Phanerochaete chrysosporium* (ME 446) and *Pleurotus sajor-caju. P. chrysosporium* ME446 was provided by Dr. T.K. Kirk (U.S. Dept. of Forest Products Agriculture Lab., Madison, Wisconsin 53705, U.S.A). P. sajor-caju was kindly supplied by Dr. I. F. Zadrazil (Weisdrangveg 4, 3300 Braunschweing, and Federal Republic of Germany). Also, *T. versicolor* (ATCC200801) were obtained from ATCC.

The fungi were first grown on agar slants using a malt extract-agar (Fluka) medium. The fungi were subcultured in malt extract-broth (Merck) and incubated at 30 °C for 4 days. 5 g of dried, shredded or ground lignocellulosic material and 15 ml of distilled water as moisturizing liquid were placed into a 250 ml-flask and autoclaved. Substrates used were wheat bran, barley bran, cone of *Pinus nigra,* sawdust, corn bran, oat, rice bran, canola, dried tea, ground clover straw, sunflower stalk, soybean bagasse, dried distillers grains with solubles (DDGS) and these natural substrates were procured from the local market, dried and was ground to 0.2–0.4 mm using mechanical blender (Retsch).

After subcultured mycelia were taken from an actively growing fungus they were homogenized and inoculated 4 ml per Erlenmeyer including solid media. The Erlenmeyer flasks were incubated at 30°C statically. Three parallel cultures were made. At the end of twelve days, 40 ml of distilled water was added to medium and agitated in shaking incubator at the rate of 200 rev min^{-1} (Edmund Bühler-Labortechnik-Materialtechnik Johanna Otto GmbH). The contents were filtered through preweighed Whatman filter paper No. 1. The filtrate was centrifuged at 5000 rev min^{-1} for 15 minutes. These culture filtrates were used to determine enzyme activities.

Analytical analyses

Screening of solid substrates in respect of laccase activities

Laccase activity was assayed according to the protocol described by Coll et al. (1993) [9]. To determinate enzyme activity, 0.1 ml of (culture filtrate) enzyme source was added to 4.9 ml of 0.1 M sodium acetate buffer (pH 4.6) and 1 mM guaiacol as substrate. The reaction mixture prepared was incubated at 37ºC for 15 minutes. Enzyme activity in the tubes was measured by reading optical density in the UV-Visible spectrophotometer adjusted to 465 nm wavelength. (Schimadzu UV-2550). 1 U of enzyme activity was defined as the amount of enzyme that elicited an increase in A_{465} of 0.1 absorbance unit

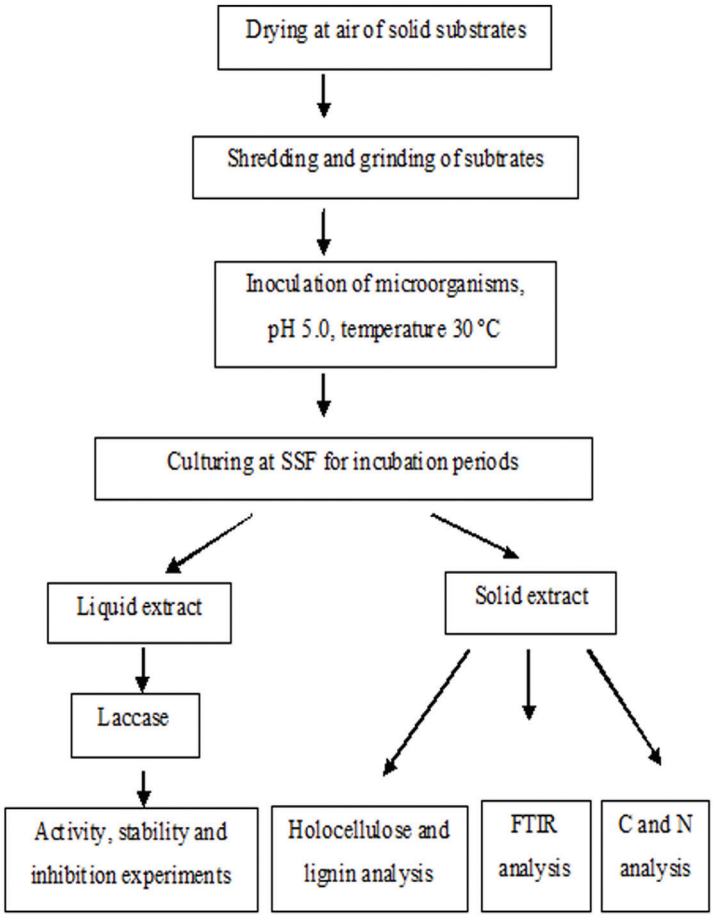

Fig. 1 Schematic flow diagram of experimental design for SSF of microorganisms

Table 1 Contents of holocellulose, carbon and weight loss of ground clover straw as substrate before and after SSF process

	Amount of holo-cellulose (%)	Amount of lignin (%)	Content of carbon (%)	Content of nitrogen (%)	Weight loss (g)
Before SSF	73.15	35.60	44.00	3.02	5.00
After SSF	57.37	34.40	39.82	1.20	4.393

per minute. Incubations with denatured laccase served as a control. Enzyme measurements were carried out in triplicates and the average values were presented.

Evaluation of enzyme activity in extended incubation periods

After substrate screening studies, ground clover straw as solid substrate and *T. versicolor,* the most efficient fungus in the way of laccase production were selected. The chosen microorganism was incubated at the selected solid media for 21 days and changes of enzyme activities were followed at 30 ºC.

Contents of holocellulose, carbon and nitrogen before and after SSF culture

T. versicolor was tested for its ability to utilize ground clover straw in terms of loss in holocellulose (cellulose and hemicellulose) and carbon contents. gravimetric determination of lignin in cultivated substrate was estimated according to Adsul et al. (2005) [10]. 5 g of substrate was suspended in 200 ml 1.0 % (wt vol^{-1}) aqueous solution of NaOH. After the mixture was autoclaved at 121 °C for 1 h, the residues were washed by tap water until reaching neutral pH; then dried at 80 °C for 48 h and weighted. The loss of weight is correspond to lignin content. Besides, nitrogen quantities of the best solid substrates including wheat bran, barley bran, and ground clover straw at the end of screening of substrate were determined. Analysis of holocellulose was performed at Wood Chemistry Laboratory of Institute of Central Anatolia Forestry Research (Ankara). Other analyses including nitrogen and carbon contents were done with elemental analyzer at Institute of Forest, Soil and Ecology Research (Eskişehir).

pH-thermal stability and effects of inhibitors on crude laccase obtained from *T.versicolor* ATCC200801 grown on clover straw

The experiments of stability and inhibition were performed using crude laccase whose activity was 20 U ml^{-1}. The effect of pH on enzyme stability was

Table 2 Effect of inhibitors on crude laccase obtained from *T.versicolor* ATCC200801 grown on clover straw

Inhibitor	Concentration of inhibitor				
Na-azide	0.01 mM	0.05 mM	1 mM	10 mM	250 mM
% inhibition[a]	88.243	90.493	90.68	90.816	91.139
EDTA	1 mM	10 mM	25 mM	50 mM	100 mM
% inhibition[a]	5.363	0.981	9.624*	7.736*	6.39*
KCN	0.1 mM	1 mM	5 mM	10 mM	100 mM
% inhibition[a]	57.326	84.958	93.181	94.09	93.776
L-cysteine	0.01 mM	0.02 mM	0.05 mM	0.5 mM	1 mM
% inhibition[a]	11.526	12.013	84.523	89.548	100

[a] All values are the averages of triplicate measurements, and the coefficients of variation were less than 5%; 100% activity was the activity obtained when 20 U ml^{-1} assay mixture at optimized conditions containing 50 mM guaiacol as a substrate was used.

* refers % induction rather than inhibition.

investigated in the pH range 2-8 (HCl-KCl buffer, pH 2; sodium acetate buffer, pH 3-5; potassium phosphate buffer, pH 6-8) by measurement of the activity remaining after incubation until 24 hours at +4 °C. After incubation for the desired time period, 100 µl samples of enzyme solution were taken for determination of enzyme activity according to Coll et al. (1993) [9].

The effect of temperature on enzyme stability was determined from 20 to 90 ºC in sodium acetate buffer, pH 4.5. After each time period, 100 µl aliquots were taken from samples, and the laccase activity was measured as described above. The estimations were carried out in triplicate. The results of pH and thermal stability studies were investigated as the residual laccase acitivity with the guaiacol assay method.

The effects of potential inhibitors including sodium azide, EDTA, KCN, and L-cysteine at different concentrations on enzyme activity were monitored with guaiacol as substrate in sodium acetate buffer after 15 min of preincubation of the enzyme with various inhibitors. A laccase solution of the same concentration without additional inhibitors served as a control. All chemicals used in this study were purchased from Merck and were of analytical purity. Measurements were made in triplicate.

FTIR spectroscopy analysis of ligninocellulose evaluated before and after SSF

Fourier transform infrared spectrophotometer was used for evaluating the change in surface functional groups of ground clover straw as substrate after SSF. FTIR spectra were recorded on solid samples in KBr pellets by means of a Perkin Elmer FTIR 100 spectrometer with a resolution of 4 cm^{-1}. The concentration of the sample in the tablets was constant of 3mg / 500 mg KBr.

The spectra were subjected to base line correction and the bands were studied to quantify the changes in the chemical structure of ligninocellulose matrix.

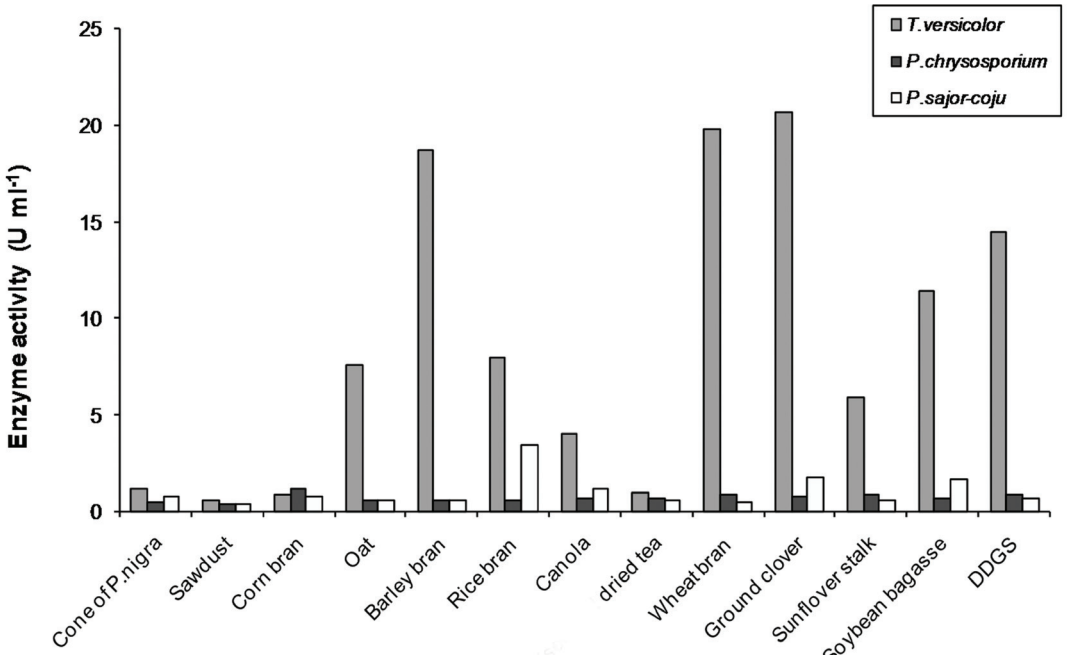

Fig. 2 Evaluation of laccase activities produced from *T.versicolor, P.chrysosporium* and *P.sajor-caju* grown on different solid substrates (Working conditions: 30 ºC, 5 g of solid substrate, 4 ml of inoculum, 12 days of incubation time under static conditions)

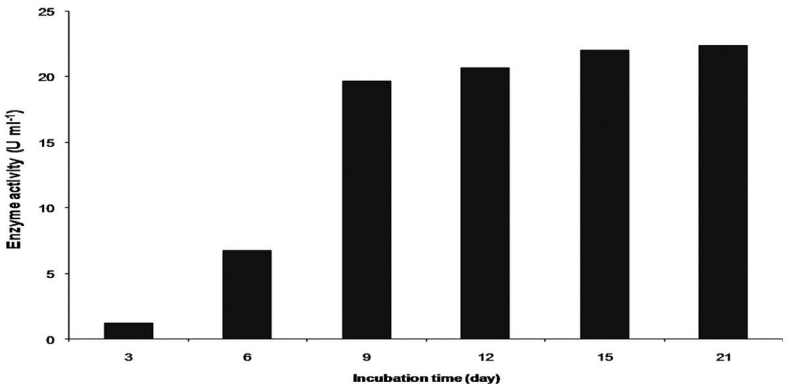

Fig. 3 A daily activity of laccase (U ml^{-1}) produced from *T.versicolor* ATCC200801 grown on ground clover straw, the best solid substrate (Working conditions: 30 ºC, 5 g of solid substrate, 4 ml of inoculum, 21 days of incubation time under static conditions).

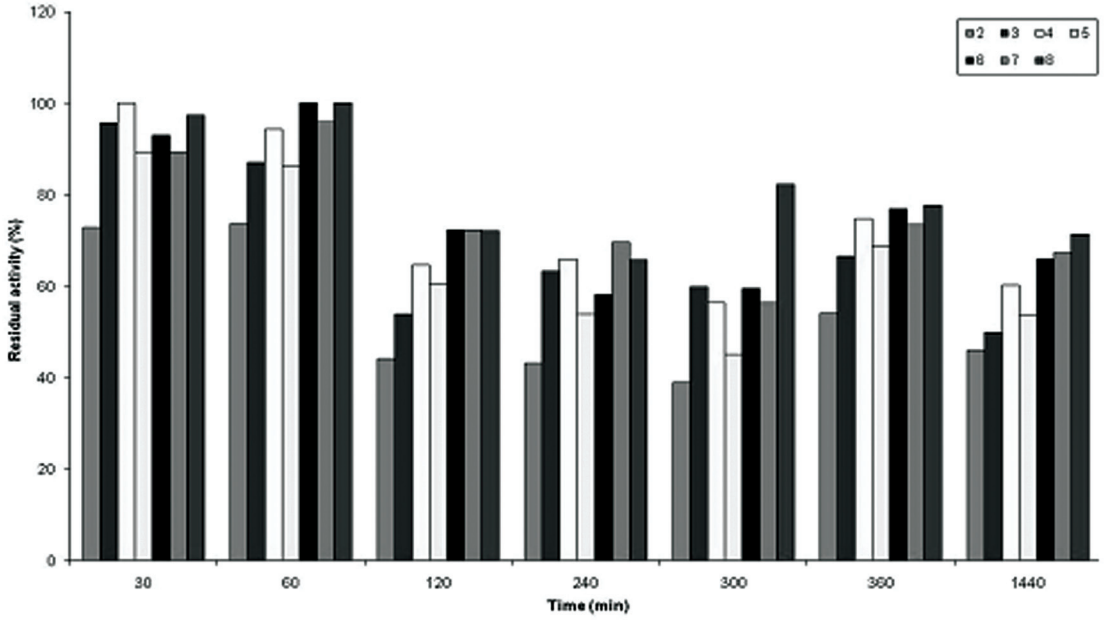

Fig. 4 Enzyme stability after preincubation at +4 °C in different pHs (2-8)

RESULTS AND DISCUSSION

Screening of solid substrates and microorganisms in respect of laccase activities

Fig. 1 shows schematic flow diagram of experimental design for SSF at pH 5 and, 30 ºC. Several supports such as wheat bran, barley bran, cone of Pinus nigra, sawdust, corn bran, oat, rice bran, canola, dried tea, ground clover straw, sunflower stalk, soybean bagasse, DDGS were tested in order to determine the most suitable one for production of laccase for *T.versicolor, P. chrysosporium* and *P. sajor-caju*. While fungal mycelium penetrated the clover straw at least partially covering their surface, fenolic compounds liberated such as ferulic, vanillic, and p-coumaric acids known to present in wheat bran, might induce laccase activity [11]. A larger surface area was provided by this penetration of the mycelium on the surface of the solid particles and possibly decreased mass transfer constraints, thereby, induced activity [12]. Laccase is considered to be the most common ligninolytic enzyme in white-rot fungi [13]. Gupte et al. (2007) studied ligninolytic enzyme production under solid-state fermentation by white rot fungi [14]. They found maximum laccase activity about 60 U ml^{-1} by *P. ostreatus* on 8th day of fermentation with wheat straw among various substrate (wheat straw, corncobs, coconut

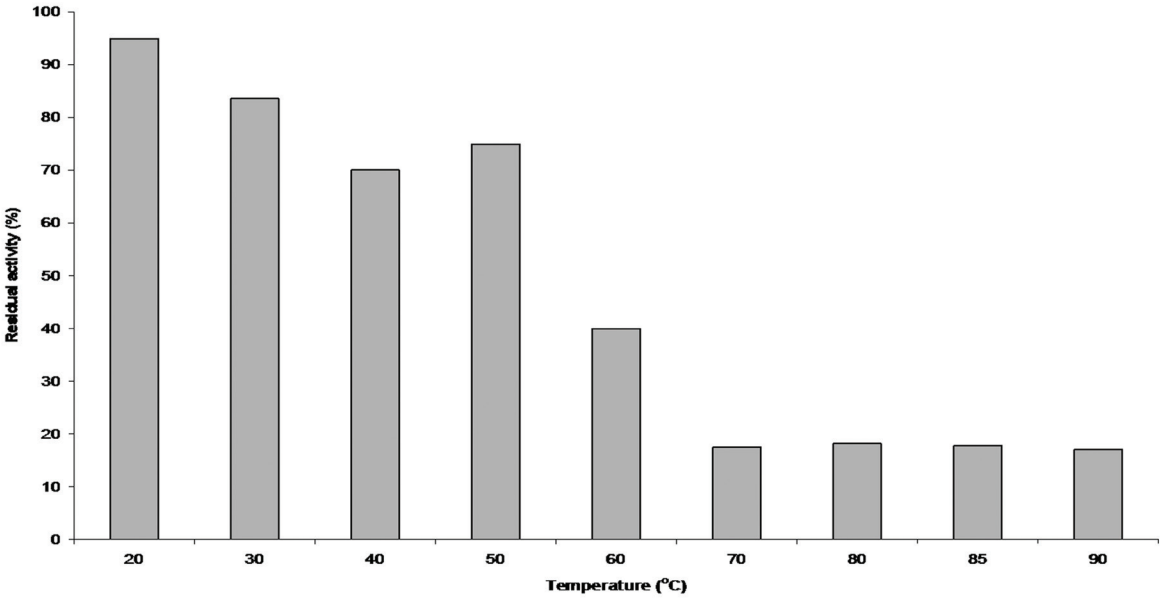

Fig. 5 Enzyme stability after preincubation for 30 min at 20-90°C

coir, wheat bran and rice bran) under solid conditions. Kurt and Buyukalaca (2010) investigated yield performances of enzyme acitivities of *P. ostreatus* and *P. sajor-caju* cultivated on wheat bran and produced higher mushroom yields than substrates without bran as wheat bran contained low molecular and soluble carbohydrates which were easily used by fungus [15]. The parallel results were also found in many studies [16]. Chawachart et al. (2004) detected extracellular laccase activity of *Coriolus versicolor* strain RC3 as 0.22 U ml^{-1} with rice bran, while 0.09, 0.01 and 0.01 U ml^{-1} obtained from wheat bran, glucose and rice straw meal, respectively after 15 days cultivation [17].

In our experiments, ground clover straw led to the highest activity levels, reaching maximum values of about 20.68 U ml^{-1}. Besides, laccase activity was observed in *T. versicolor* grown on wheat bran (19.77 U ml^{-1}), and barley bran (18.66 U ml^{-1}) (Fig. 2). According to results obtained, *T. versicolor* as laccase producer, ground clover straw as substrate were selected for measurement of daily enzyme activity.

Evaluation of enzyme activity in extended incubation periods

Following substrate screening studies, the most efficient fungus and the best solid substrates were determined. Then *T. versicolor* was incubated at the

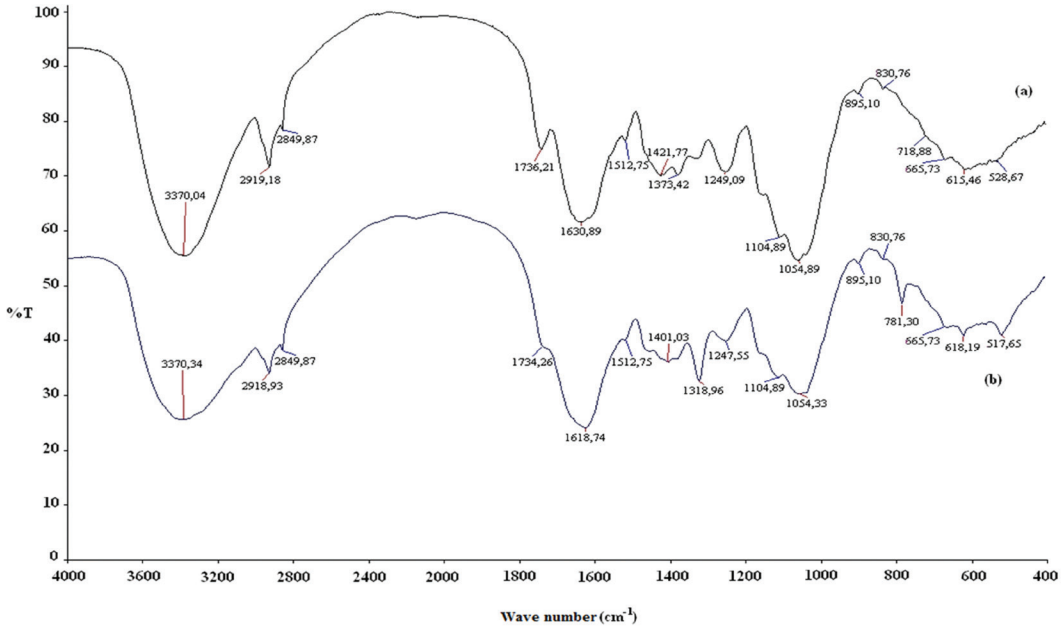

Fig. 6 The FTIR spectra of the solid sample, ground clover straw before (a) and after (b) SSF (Working conditions: 30 ºC, 5 g of solid substrate, 4 ml of inoculum, 15 days of incubation time under static conditions).

Rosales et al. diverse food wastes, apple, orange and potato, screened for laccase production, under solid state fermentation conditions, by the white rot fungus Trametes *hirsuta* [18]. Potato peelings gave the highest activity, reaching about 5 U l^{-1} within 8 days. Mazumder et al. also developed a SSF system for production of laccase by *P. ostreatus* using potato dextrose yeast extract medium and polyurethane foam as a supporting material [19]. The maximum laccase production in the SSF system was as high as 30 U ml^{-1}. Rosales et al. focused on the obtaining of high laccase activity levels by optimizing several variables affecting laccase production under solid-state conditions [20]. The highest activities (31.786 U l^{-1}) were obtained operating at the following conditions: 2.5 g ground orange peelings and 5mM copper sulphate. The process was also scaled to 250 ml fixed-bed and 1.8 l tray reactors. The former produced maximal activities of about 3000 U l^{-1}, whereas the latter exhibited much higher values of around 12.000 U l^{-1}.

selected solid media which was ground clover straw up to 21 days. Clover straw as solid substrate (growth medium) and *T. versicolor* as laccase producer were used for further experiments. Optimum laccase activity was observed that grown on ground cslover straw for 15 days (Fig. 3).

Amount of weight loss and chemical analyses of clover straw before and after SSF culture

Contents of holocellulose, lignin, carbon, nitrogen and amount of weight loss of ground clover straw as substrate before and after grown in the SSF medium were presented in Table 1. The enzyme activity significantly may depend on the lignocellulosic material content. Whilst the carbon content of substrate was decreased, laccase synthesis was induced by phenolic compounds including in clover straw, which might serve to degrade this lignocellulolytic compound providing carbon and nitrogen nutrients [17]. Decrease of nitrogen and carbon sources is well known a major factor in

triggering ligninolytic system of white rot fungi [21]. Cellulose consumption (22%) and lignin decrease (1.2%) after 15 days incubation may be due to the ability of *T. versicolor* ATCC200801 for different cellulases (endoglucanase, β-glucosidase and cellobiohydrolase) and ligninase production. *T. versicolor* is known to secrete cellulases, hemicellulases as well as delignifying enzymes due to its large striated fruiting body [22]. The biodegradation variety of lignocellulosic materials and enzyme production that bring about hydrolysis of the macromolecules of cellulose, hemicelluloses and lignin components, respectively may depend on insoluble lignocellulosic substrate experimented [23]. Differences of nitrogen content of the most efficient solid substrates including ground clover straw (3.02%), wheat bran (2.26%), and barley bran (1.69%) may affect its laccase production. According to our results, fungus utilized solid substrate as carbon and nitrogen source. These results are in agreement with the results reported by some researchers [24]. Moreover, it agrees with the results obtained by Rodriguez Couto et al. (2002) [25]. They found barley bran as the best support among several supports tested for laccase production with 1200 U l^{-1} by SSF cultures of *T. versicolor*.

Stability and inhibition experiments

In our study on pH stability performed at 4 °C, showed a 45-60% of inactivation of laccase in the pH range 2.0-5.0 after 24 h, whereas 65-70% of the initial activity remained at pH 6.0-8.0 (Fig. 4). Phosphate buffer enhanced laccase activity compared to the activities in both KCl-HCl and acetate buffers. These results are similar to those reported with the immobilized *L. edodes* laccase from solid state fermentation cultures [26]. Due to the higher acidity, the pH 2.0 may partially inactivate the enzyme [27]. In a study on pH stability, carried out at room temperature, showed a substantial inactivation of free laccase in the pH range 3.0-5.0 after 24 h, whereas ≥80% of the initial activity remained at pH 7.0-10.0 [28].

In a previous study, optimum pH differed markedly according to the substrate used; the optimum pH was 6.2 with syringaldazine and 2.6 with ABTS. This has been clarified by the difference in redox potential between a reducing substrate and the type 1 copper in the active site of the enzyme [29]. In our study, guaiacol was as substrate and this substrate exhibited a good activity in pH range tested. Preliminary experiments of D'Annibale et al. (1996) showed that the enzyme reached maximum stability in the pH range 5-7, rather than at more acidic pH values [30]. The difference in pH optima for ABTS and phenolic substances such as guaiacol and syringaldazine is typical for laccases and reflects the different oxidation mechanisms depending on the substrate [31].

The enzyme in this study was stable at the temperature range from 20 to 50 °C for 30 min in the pH 4.5 (Fig. 5). The residual activity–temperature profile is reported in Fig. 5. The laccase showed the highest activity at 50 °C. The enzyme started the activity loss from 60 °C, showed 40% activity at 60 °C, and only 17.1% activity retained at 90 °C. These results could suggest that laccase was more stable against thermal denaturation. Similar results were obtained in other studies [32]. As shown in Fig. 5, the thermal stability of crude laccase was maximal at 50 ºC and decreased sharply after 50 °C because the enzyme was obtained from eukaryotic organism. The loss of enzyme activity above 50 °C may be due to the release of copper ions from the enzyme laccase as suggested by Palonen et al. (2003) [33]. Regarding the thermal stability, the soluble enzyme was swiftly inactivated between 50 and 80 °C [28]. The optimum temperature for substrate oxidation by fungal laccases spans a range of 25-80 °C, with most enzymes having optima at 50-70 °C. The different purified isoenzymes of *P. ostreatus* laccase exhibit optimal temperature of 35-60 °C [34]. Our results are consistent with these reports.

The significant advantages of the laccase from *T. versicolor* ATCC200801 are the high activity obtained in the solid state culture via clover straw, as well as its high thermal and pH stability; therefore, it can be easily used for environmental applications. The enhanced resistance to thermal and pH denaturation could be a good potential in wastewater treatment applications. Especially, the thermal stability is one of the most noteworthy characteristics for the application of the biocatalyst.

The effects of several laccase inhibitors were determined with guaiacol (50 mM) as a substrate in 2 M sodium acetate buffer (pH 4.5). Table 2 shows the inhibitory effect of different compounds on laccase activity. According to these results, inhibition of laccase activity was observed with all the inhibitors used except with EDTA. The order of inhibition was L-cystein > sodium azide > KCN > EDTA at 1 mM concentrations (Table 2). Comparison of the observed values suggests that the incubation of enzyme with 1mM sodium azide resulted in 90% inhibition of the enzyme activity while the metal ion chelator EDTA (1 mM) showed only partial inhibition (5.3%). However, higher concentrations of EDTA were not inhibited; whereas, it induced enzyme activity. Similar results were also observed in study of Murugesan et al. (2006) [35]. Inhibition studies performed with some putative laccase inhibitors revealed that EDTA was not an efficient inhibitor of the crude laccase, whereas sodium azide, L-cysteine, and KCN showed a strong inhibition toward enzyme activity. The result was similar to that seen with laccase from *Daedalea quercina* [36], *Pycnoporus sanguineus* [37] and *Lentinula edodes* [38].

FTIR spectroscopy analysis of ligninocellulose evaluated before and after SSF

FTIR spectra of fungal treated for 15 days and untreated samples of ground clover straw are shown in Fig. 6. The FTIR spectra in the region of water absorption 3000–3700 cm-1, and also those were is possible the interaction with different functional groups 1550–1800 cm^{-1} and 900–1550 cm^{-1} are given in Fig. 6. The FTIR

spectra of the lignin in the region from 1800 cm^{-1} to 4000 cm^{-1} did not provide much information other than broad hydroxyl and aliphatic CH absorptions. The absorption band centered at about 3400 cm^{-1} (Fig. 6) is assigned to -OH groups of the water and also -OH in to aliphatic and aromatic groups. For this later group of lignin the position of the peak is shifted at higher wavelengths denoted stronger interactions with of the water with lignin molecule or between functional groups. In the 1618 cm^{-1} region appear many bands, that of 1630 cm^{-1} being assigned to the valence vibration of the carbonyl group in ketone or carboxyl groups. Kumar et al. (2006) studied solid state fermentation *Achras zapota* ligninocelluloses by *P. chrysosporium* and the degradation of lignin and other structural moieties of *A. zapota* ligninocellulose were confirmed by FTIR [39]. According to FTIR spectra, a strong hydrogen bonded (O–H) stretching absorption was seen at 3400 cm^{-1} and a prominent C–H stretching absorption around 2960 cm^{-1}. Furthermore there were many well-defined peaks in the finger print region between 1800 and 600 cm^{-1} . The peaks in the finger print were determined by researchers: 1100 cm^{-1} for aromatic skeletal and C–O stretch in cellulose and hemicellulose, 1320 cm^{-1} for C–H vibration in cellulose and C–O vibration in syringyl, 1380 cm^{-1} for C–H deformation in cellulose and hemicellulose, 1440 cm^{-1} for C–H deformation in lignin, 1460 cm^{-1} and derivatives 1640 cm^{-1} for absorbed O–H and conjugated C–O [40, 41].

Our data of FTIR spectra referred change of lignin band and decrease of holocellulose and carbon contents attributed to use of cellulose and lignin of solid substrate during SSF.

Recent works have shown that some lignocellulosic wastes stimulate enzyme production by basidiomycetes [42]. There are some studies reporting that agricultural wastes were employed as nutritional supplement [43] by using ligninolytic fungi; however, there is no similar work for comparison using ground clover straw as substrate, *T. versicolor* and these cultivation conditions.

In conclusion, the important potential of agricultural and industrial with ligninocellulose wastes, large amounts are present in most countries, for enzyme production in solid state cultures of white rot fungi evaluated has been demonstrated. In this study, the utilization of such wastes/ by-products from agriculture, forestry, different industry, some of which may include considerable concentrations of soluble carbohydrates and inducers of enzyme [18, 44] both converted biomasses into value-added product such as laccase and contributed to decrease production costs. Moreover, solid substrate and water as the culture medium have been utilized in our experiments. In previous studies, ground clover straw was added to the enzyme production medium as a substrate in submerged cultures [24]. On the other hand, there is no similar work for comparison using this substrate, this organism and these fermentation conditions without adding extra carbon source. Thereby, in order to produce laccase using ground clover straw, an abundant and inexpensive solid substrate, investigation of other basidiomycetes and different culture conditions will be favorable. An advantage related to possible environmental applications will provide that the enzyme obtained is stable in terms of pH and temperature. Providing laccase by growing on clover straw of white rot fungi under solid conditions requires confirmation through measuring gene expression levels.

Acknowledgements

This study is partly based on the MSc thesis of F. ÖZŞÖLEN who is one of the authors. We would like to express our gratitude to Dr. Güneş KÜRKÇÜOĞLU at Eskişehir Osmangazi University for FTIR measurements. We also thanks to Wood Chemistry Laboratory of Institute of Central Anatolia Forestry Research affiliated Republic of Turkey Ministry of Environment and Forest. We special thanks to Salim TÜRKEL at Institute of Forest, Soil and Ecology Research affiliated Republic of Turkey Ministry of Environment and Forest.

REFERENCES

[1] Glazer AN, Nikaido H. 2007. Microbial Biotechnology Fundamentals of Applied Microbiology, New York:Cambridge University Press.

[2] Dodson PJ, Evans CS, Harvey PJ, Palmer JM. 1987. Production and properties of an extracellular peroxidase from *Coriolus versicolor* which catalyses Cα-Cβ cleavage in a lignin model compound. FEMS Microbiol. Lett. 42: 17–22.

[3] Schmidt O. 2006. Wood and tree fungi. Biology, damage, protection, and use. Springer, Heidelberg.

[4] Alcalde M. 2007. Laccases: Biological Functions, Molecular Structure and Industrial Applications. In: Poliana J., MacCabe A. (ed) Industrial Enzymes Structure, Function and Applications, Springer,. 461 pp.

[5] Pandey A, Selvakumar P, Soccol CR, Nigam P. 1999. Solid-state fermentation for the production of industrial enzymes. Curr. Sci. 77: 149–162.

[6] Mitchell DA, Krieger N, Berovic M. 2006. Solid state fermentation bioreactors: fundamentals of design and operation, Springer, Berlin, Heidelberg, New York.

[7] Viniegra-González G, Favela-Torres E, Noe Aguilar C, Jesús Romero-Gómez J. de, Díaz-Godínez G, Augur C. 2003. Advantages of fungal enzyme production in solid state over liquid fermentation systems. Biochem. Eng. J. 13: 157- 167.

[8] Robinson T, McMullan G, Marchant R, Nigam P. 2001. Remediation of dyes in textile effluent: a critical review on current treatment technologies with a proposed alternative. Bioresour. Technol. 77: 247–255.

[9] Coll PM, Fernandez-Abalos JM, Villanueva JR, Santamaria R, Perez P. 1993 Purification and Characterization of a Phenoloxidase (Laccase) from the Lignin-Degrading Basidiomycete PM1 (CECT 2971). Appl. Environ. Microbiol. 59: 2607-2613.

[10] Adsul MG., Ghule JE., Shaikh H, Singh R, Bastawde KB, Gokhale DV, Varma AJ. 2005. Enzymatic hydrolysis of delignified bagasse polysaccharides. Carbohyd. Polym. 62: 6-10.

[11] Leonowicz A, Cho NS, Luterek J, Wilkolazka A, Wojtas-Wasilewska M, Matuszewska A, Hofrichter M, Wesenberg D, Rogalski J. 2001. Fungal laccase: properties and activity on lignin. J. Basic Microbiol. 41: 185−227.

[12] Herpoël J, Asther M, Sigoillot JC. 1999. Design and scale up of a process for manganese peroxidase production using the hypersecretory strain Phanerochaete chrysosporium I-1512. Biotechnol. Bioeng. 65: 468–473.

[13] Saparrat MCN, Guillén F, Arambarri AM, Martinez TA, Martinez MJ. 2002. Induction, Isolation, and Characterization of Two Laccases from the White Rot Basidiomycete *Coriolopsis rigida.* Appl. Environ. Microbiol. 68: 1534-1540.

[14] Gupte A, Gupte S, Patel H. 2007. Ligninolytic enzyme production under solid- state fermentation by white rot fungi. J. Sci. Ind. Res. India. 66: 611-614.

[15] Kurt S, Buyukalaca S. 2010. Yield performances and changes in enzyme activities of *Pleurotus spp. (P. ostreatus and P. sajor-caju)* cultivated on different agricultural wastes. Bioresour. Technol. 101: 3164-3169.

[16] Wang D, Sakoda A, Suzuki M. 2001. Biological efficiency and nutritional value of *Pleurotus ostreatus* cultivated on spent beer grain. Biores. Tech. 78: 293-300.

[17] Chawachart N, Khanongnuch C, Watanabe T, Saisamorn L. 2004. Rice bran as an efficient substrate for laccase production from thermotolerant basidiomycete *Coriolus versicolor* strain RC3. Fungal Divers. 15:23-32

[18] Rosales E, Rodriguez Couto S, Sanroman A. 2002. New uses of food waste: application to laccase production by *Trametes hirsuta*. Biotechnol. Lett. 24: 701–704.

[19] Mazumder S, Soumendra BK, Mukherjee M. 2009. Laccase production in solid-state and submerged fermentation by *Pleurotus ostreatus*. Eng. Life Sci. 9: 45–52.

[20] Rosales E, Rodriguez Couto S, Sanroman A. 2007. Increased laccase production by *Trametes hirsuta* grown on ground orange peelings. Enz. Microb. Technol. 40: 1286-1290.

[21] Leatham GF, Kirk TK. 1983. Regulation of ligninolytic activity by nutrient nitrogen in white-rot basidiomycetes. FEMS Microbiol. Lett. 16: 65-67.

[22] Lahjouji K, Storms R, Xiao Z, Joung K, Zheng Y, Powlowski J, Tsang A, Varin L. 2007. Biochemical and molecular characterization of a cellobiohydrolase from *Trametes versicolor.* Appl. Microbiol. Biotechnol. 75: 337-346.

[23] Buswell JA, Cai YJ, Chang ST. 1993. Fungal and substrate-associated factors affecting the ability of individual mushroom species to utilize different lignocellulosic growth substates. Chang ST., Buswell JA, Chiu SW, Eds., Chinese University Press, Hong Kong.

[24] Erden E, Ucar MC, Kaymaz Y, Pazarlioglu NK. 2009. New and different lignocellulosic materials from Turkey for laccase and manganese peroxidase production by *Trametes versicolor*. Eng. Life Sci. 9: 60–65.

[25] Rodriguez Couto S, Gundın M, Lorenzo M, Sanroman A. 2002. Screening of supports for laccase production by *Trametes versicolor* in semi-solid-state conditions. Process Biochem. 38: 249–255.

[26] D'Annibale A, Stazi SR, Vinciguerra V, Sermanni GG. 2000. Oxirane-immobilized *Lentinula edodes* laccase: stability and phenolics removal efficiency in olive mill wastewater. J. Biotechnol. 77: 265-273.

[27] Tavares APM, Rodriguez O, Macedo EA. 2008. Ionic liquids as alternative co-solvents for laccase: Study of enzyme activity and stability. Biotechnol. Bioeng. 101: 201-207.

[28] Berrio J, Plou FJ, Ballesteros A, Mart nez AT, Mart nez MJ. 2007. Immobilization of pycnoporus coccineus laccase on Eupergit C: Stabilization and treatment of olive oil mill wastewaters. Biocatal. Biotransfor. 25: 130-134.

[29] Farnet AM, Criquet S, Pocachard E, Gil G, Ferre E. 2002. Purification of a new isoform of laccase from a Marasmius quercophilus strain isolated from a cork oak litter (*Quercus suber* L.). Mycologia. 94: 735-740.

[30] D'Annibale A, Celetti D, Felici M, Di Mattia E. 1996. Substrate specifity of laccase from *Lentinus edodes*. Acta Biotechnol. 4: 257-270.

[31] Xu F. 1997. Effects of redox potential and hydroxide inhibition on the pH activity profile of fungal laccases. J. Biol. Chem. 272: 924–928.

[32] Cho N, Cho H, Shin S, Choi Y, Lenowicz A, Ohga S. 2008. Production of fungal laccase and its immobilization and stability. J. Fac. Agr. 53: 13-18.

[33] Palonen H, Saloheimo M, Viikari L, Kruus K. 2003. Purification, characterization and sequence analysis of a laccase from the ascomycete *Mauginiella sp*. Enz. Microb. Technol. 33: 854–862.

[34] Baldrian P. 2006. Fungal laccases-occurrence and properties. FEMS Microbiol. Rev. 30: 215-242.

[35] Murugesan K, Arulmani M, Nam I, Kim Y, Chang Y, Kalaichelvan PT. 2006. Purification and characterization of laccase produced by a white rot fungus *Pleurotus sajor-caju* under submerged culture condition and its potential in decolorization of azo dyes. Appl. Microbiol. Biotechnol. 72: 939-946.

[36] Baldrian P. 2004. Purification and characterization of laccase from the white-rot fungus *Daedalea quercina* and decolorization of synthetic dyes by the enzyme. Appl. Microbiol. Biotechnol. 63: 560–563.

[37] Lu L, Zhao M, Zhang B, Yu S, Bian X, Wang W, Wang Y. 2007. Purification and characterization of laccase from *Pycnoporus sanguineus* and decolorization of an anthraquinone dye by the enzyme. Appl. Microbiol. Biotechnol. 74: 1232-1239.

[38] Nagai M, Sato T, Watanabe H, Saito K, Kawata M, Enei H. 2002. Purification and characterization of an extracellular laccase from the edible mushroom *Lentinula edodes* and decolorization of chemically different dyes. Appl. Microbiol. Biotechnol. 60: 327–335.

[39] Kumar AG, Sekaran G, Krishnamoorthy S. 2006. Solid state fermentation of *Achras zapota* lignocellulose by *Phanerochaete chrysosporium*. Bioresour. Technol. 97: 1521–1528.

[40] Collier W, Schultz TP, Kalasinsky TV. 1992. Infrared study of lignin fermentation of aryl–alkyl ether C–O stretching peak assignments. Holzforschung. 46: 523–528.

[41] Pandey KK, Theagarajan KS. 1997. Analysis of wood surfaces by diffuse reflectance (DRIFT) and photoacoustic (PAS) Fourier transform infrared spectroscopic techniques. Holz. Roh. Werkstoff. 55: 383–390.

[42] Elisashvili V, Penninckx M, Kachlishvili E, Tsiklauri N, Metreveli E. et. al. 2008. *Lentinus edodes* and *Pleurotus* species lignocellulolytic enzymes activity in submerged and solid state fermentation of lignocellulosic wastes of different composition. Bioresour. Technol. 99: 457–462.

[43] Gomez J, Pazos M, Rodriguez Couto S, Sanroman A. 2005. Chestnut shell and barley bran as potential substrates for laccase production by *Coriolopsis rigida* under solid-state conditions. J. Food Eng. 68: 315-319.

[44] Kapich AN, Prior BA, Botha A, Galkin S, Lundell T, Hatakka A. 2004. Effect of lignocellulose-containing substrates on production of ligninolytic peroxidases in submerged cultures of *Phanerochaete chrysosporium* ME-446. Enz. Microb. Technol. 34: 187–195.