PRODUCTION OF *TRAMETES GALLICA* LIGNOCELLULASES FOR WHEAT STRAW DEGRADATION

Jun Xie,[a*] Sishi Luo,[a] Lei Feng,[a] Ning Xu,[a] Yuqi Wang,[a] Xiaoli Xu,[a] and Shiyu Fu [b*]

Trametes gallica (*T. gallica*) is a high producer of lignin-degrading enzymes including laccases, lignin peroxidases (LiPs), manganese-dependent peroxidases (MnPs), and hemicellulases (Hcels). The enzyme activities could peak at an early stage of fermentation. The activities of laccases and LiPs were high in high-nitrogen, low–carbon, and high inorganic salt media, while the activities of MnPs, and Hcels were the high in low-nitrogen, high–carbon, and high inorganic salt medium. It was found that *T. gallica* caused 34.4% mass losses after 20 days, 46.7% after 30 days, and 70.1% after 60 days, and at the same time *T. gallica* was able to degrade lignin at an early stage of solid fermentation. Based on these results, *T. gallica* may be a strain candidate for biopulping in the paper industry.

Keywords: Trametes gallica; Enzymes; Plant Biomass; Degradation

*Contact information: a: Institute of Applied Biotechnology, South China Agricultural University, Biomass Energy Key Lab of Guangdong Regular Higher Education Institutions, Guangzhou 510642, PRC; b: State Key Laboratory of Pulp and Paper Engineering, South China University of Technology; *Corresponding authors: xiejun@scau.edu.cn; shyfu@scut.edu.cn*

INTRODUCTION

Plant biomass, synthesized by photosynthesis, mainly consists of lignin, cellulose, and hemicellulose. Among them, lignin accounts for about thirty percent of the biomass, and the degradation of lignin is a key process to obtain cellulose in the paper industry. In the plant fiber, lignin is combined intimately with cellulose and hemicellulose to form ligninocelluloses. This is a complicated, stereochemically complex, heterogeneous aromatic renewable biopolymer that is very stable, and very difficult to degrade (Aihara 1992; Dong et al. 2005; Higuchi 1982).

Paper's consumption correlates directly with the development of the world economy. However, excessive deforestation to facilitate economic development is unacceptable in regions lacking sufficient wood resources, so we must look for other cellulosic materials. For example, China, as an agriculturally rich country that has abundant crop stalks that contains cellulose suitable for pulp preparation. Generally, high temperatures, high pressure, and strong alkali are the main means to obtain pulp from lignocelluloses. We know that pulping with such chemicals can produce a waste stream that is the main source of water pollution from the pulping industry. The method of biodegradation of lignin may solve such problems, because it has the potential to be an environmentally friendly process (Huang et al. 2006; Messner and Srebotnik 1994; Paice et al. 1995; Sun et al. 2004). Biopulping of crop stalks can substitute for wood pulping,

and it can protect the environment by utilizing natural resources more reasonably, and in this way reduce environmental pressure.

Trametes gallica is a high producer of lignocellulases (Huang et al. 2006; Sun et al. 2004). In the present article, an optimal liquid media for *T. gallica* producing lignocellulases was defined, and the behavior of their enzymes is discussed. The solid-state fermentation with this strain was investigated for the capacity and behavior of its enzyme production, and the results are compared with those enzymes obtained in liquid state fermentation. Finally, the activity of its degradation of wheat-straw biomass was investigated.

EXPERIMENTAL

Fungal Species

T. gallica isolated from mycelium that grew on decayed wood could also be cultivated on plant straw and crop wastes, and it showed powerful ligninocellulolytic activity. *Phanerochaete chrysosporium* 1767 was a model strain for studying lignin degradation.

Culture Conditions

Potato Dextrose Agar (PDA) culture medium and Kirk's low nitrogen medium were used as the culture media (Tien et al. 1984).

To investigate whether nutrition elements affect the behaviors of enzymes production, four kinds of media were prepared, applying orthogonal trial statistics. In Table 1, LM1 was the low-nitrogen (N), low-carbon(C), and low inorganic salt (IS) medium; LM2 was the low N, high C, and high-IS one; LM3 was the high N, low C and high-IS one; and LM4 was the high N, high C, and low-IS one.

Table 1. Components of the Liquid Media (mL)

Media	Ammonium tartrate	Glucose	Large amount element	Trace element	Vitamin b_1	dd H_2O
LM1	1	2.5	5	5	1	35.5
LM2	1	7.5	15	15	3	8.5
LM3	10	2.5	15	15	3	4.5
LM4	10	7.5	5	5	1	21.5

Ammonium tartrate (22.0 g·litre^{-1}) as nitrogen source, Glucose (200 g·litre^{-1}) as carbon source; the concentration of Vitamin b_1 was 100 mg ·litre^{-1} and was adjusted to pH 4.2.

Static liquid-state incubation was done as follows: the strain was inoculated in 50 mL of LM1, LM2, LM3, and LM4, respectively, in triplicate; they were incubated at 28°C for 24 days. The activities of Laccase, Lips, MnPs, Cellulases (Cels), and Hcels were determined every other day.

Static solid-state incubation (Camarero et al. 1998) was done as follows: Solid-state media SM2 and SM3 were composed of one portion 60-mesh wheat straw powder and four portions LM2 and LM3 (but not containing glucose) (wt/wt), respectively. After inoculation, the incubating cultures were controlled statically at 28°C. Experiments were carried out in triplicate, and sterilized straw powder was included as a control. After

every 10th day of incubation, the treated straw powder was recovered, and the enzymes activities, weight loss, and chemical analyses were carried out as described below.

Enzymatic Activities

The treated wheat straw powder was immersed in 12 mL double-distilled water at 4°C overnight, and then stirred for 2 hours at 200 rotations per minute (rpm); the extracted liquid was separated from the solid-state medium by filtration. 4.0 mL distilled water was added and stirred for another 2 hours at 200 rpm; and the combined extracted liquid was used to determine the activities of Laccase, LiPs, MnPs, Cels, and Hcels (Camarero et al. 1999).

Laccase activity was determined with ABTS (2, 2'-azinobis-3-ethylbenzthiazo-line-6-sulfonate) as the substrate (Childs et al. 1975). In a reaction system containing the same volume ABTS ($0.5 mM \cdot L^{-1}$), enzyme solution, the increase of optical density (OD) value at 420nm within three minutes of the reaction was measured. The formula used was

$$\text{Activity of laccase (U/L)} = n \times \frac{\Delta A \times 10^6}{23{,}300 \times 3}, \quad (1)$$

where n is the dilution multiple, and ΔA is the increase of OD value at 420 nm within three minutes.

Veratryl alcohol was used as the substrate for the detection of LiPs (Tien and Kirk 1984). The reaction system contained tartaric acid buffer ($0.1 M \cdot L^{-1}$, pH 3.0), veratryl alcohol ($2.0 mM \cdot L^{-1}$), H_2O_2 ($0.4 mM \cdot L^{-1}$), 0.1% Tween 80, and some enzyme solution. The increase of OD value at 310nm within three minutes of the reaction was measured. The LiP activity (U/L) is calculated as

$$\text{LiP activity (U/L)} = n \times \frac{\Delta A \times 10^6}{9300 \times 3}. \quad (2)$$

MnPs activity was measured by the method described by Gold and Glenn (1988). The reaction system contained natrium lacticum buffer ($0.1 M \cdot L^{-1}$), $MnSO_4$ solution ($0.1 mM \cdot L^{-1}$), and H_2O_2 ($0.1 mM \cdot L^{-1}$), and the increase of OD value at 240nm within one minute of the reaction was measured.

$$\text{MnP activity (U/L)} = n \times \frac{\Delta A \times 10^6}{7800 \times 3}. \quad (3)$$

Hcels activity was measured with xylan as the substrate (Namori et al. 1990). Enzyme solution (0.5 mL) was added to 2% xylan suspension (0.5 mL) in 0.1 $M \cdot L^{-1}$ acetate buffer, pH 6.0, and the mixtures were incubated at 55°C for 30 min. The mixtures were cooled rapidly in ice water and centrifuged (10,000 × g) to removed insoluble xylan. 1 ML of 3,5-dinitrosalicylate (0.5%) solution was added to 0.5 mL of the supernatant. The mixture was cooked in boiling water. After cooling, the absorbance at 535 nm was measured on a spectrometer. One unit of xylanase activity was defined as releasing 1

μmol of xylose by enzyme in 1 min.

Avicel stained by Remazol Brilliant Blue R was used as the substrate to determine Cels (Leisola and Linko 1976). Cels activity was measured with CMC-Na as the substrate; one unit of Cels was defined as the activity forming 1μmol Glucose in 1 min.

Chemical Analysis

The mass of cellulose in the sample was determined after digestion with nitric acid-alcohol mixture and being dried to constant weight 105°C (The National Standard of the P.R. of China GB745-78). After hydrolysis with hydrochloric acid, hemicelluloses (as polypentoses) were calculated by titration with 1 M NaOH, using acetate aniline to signal the end point. Lignin was detected after the other components were sequentially degraded with acid detergent and 72% H_2SO_4, and the residue was dried to constant weight at 105°C (The National Standard of the P.R. of China GB2677.8-81).

RESULTS AND DISCUSSION

Production of Enzymes in Different Liquid Culture Media

In order to obtain the optimal media for producing enzymes, orthogonal trial statistics experiments were applied. The activity profiles of enzymes produced by *T. gallica* in the 4 liquid media during the cultivation process are shown in Fig. 1.

The results showed that all four enzymes produced by *T. gallica* had two or more activity peaks in the four kinds of liquid media, which indicated that the strain had two or more physiological activity periods in these media., Different media only affected Laccase outputs; but for Hcels, the outputs and activity peaks time were all affected, which indicated the metabolic patterns and the gene expressive regulation of *T. gallica* were related to the environmental conditions (Feng and Zhang 1999). The nutritional conditions changed the production of Laccase, Lips and MnPs, indicated that the expression of different enzymes in the strain required different nutritional conditions.

T. gallica is a high producer of lignocellulases, including laccase, MnPs, and Hcels. The optimal medium to produce laccase was LM3, and its activity was able to reach as high as 1824 U/L after the 12th day. Thus, the laccase peak activity of *T. gallica* was 4.5 times more than that of *Phellinus torulosus* (Pelaez et al. 1995), 5.5 times more than that of *Trametes versicilor* (Paice et al. 1995), and approximately 30 times as much as *P. chrysosporiun* (Srinivasan et al. 1995). For MnPs, the optimal medium was LM2, in which MnP reached 64.4 U/L after the 6th day, and the MnP peak activity of *T. gallica* was 16.2 times more than that of *Pleurotus erynggi* (Paice et al. 1995). For Hcels production, the optimal medium was LM2, in which Hcel reached 865.9 U/L after the 18th day. It was well documented that different strains are optimized under different conditions; the differences of the four enzymes produced by *T. gallica* to those reported before can provide useful insight. In detail, Laccase achieved its maximum activity six days earlier than Hcels, indicating that the strain degraded lignin first, and then hemicellulose. The short time for reaching the peaks can be considered to be a benefit relative to the pretreatment process for biopulping. Therefore, *T. gallica* may be an acceptable strain for biopulping.

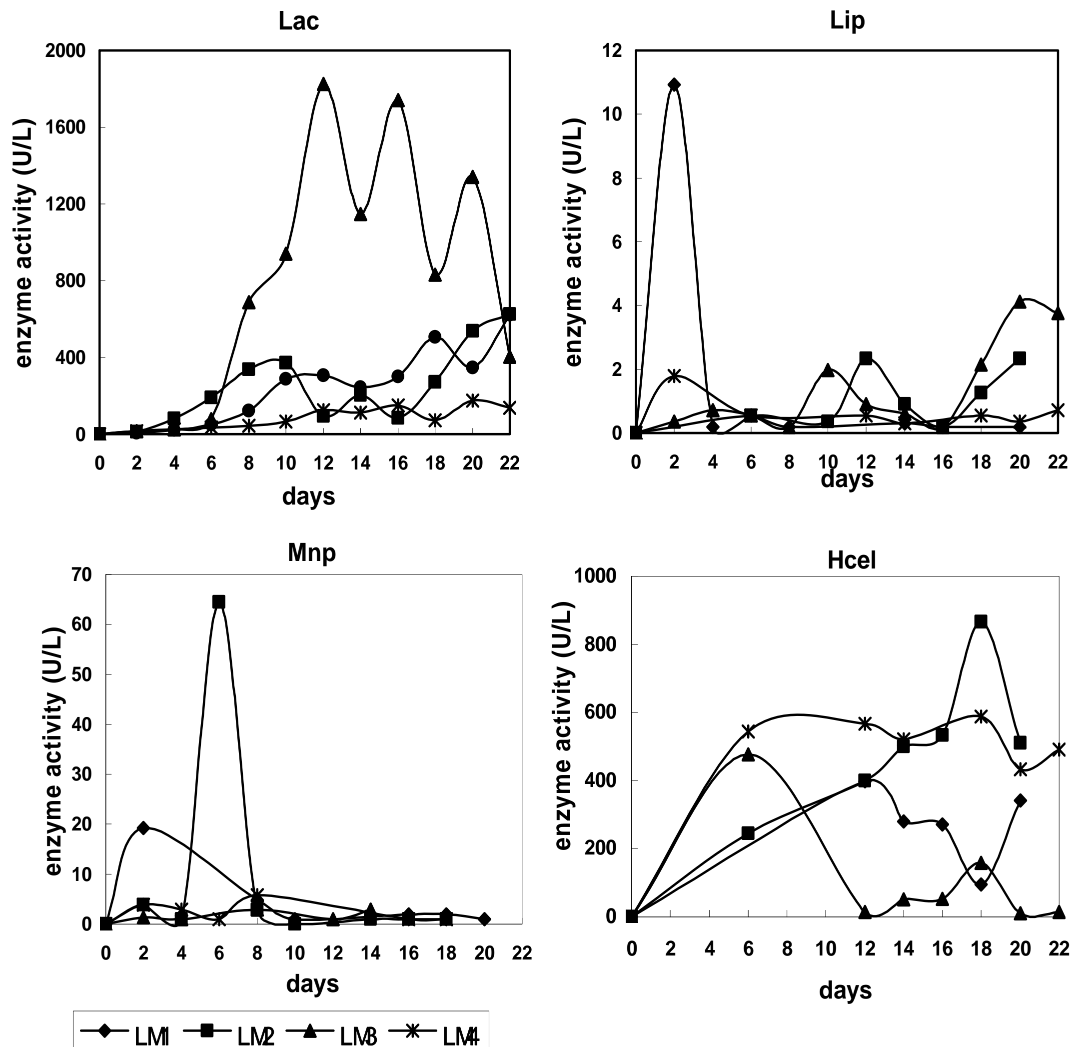

Fig. 1. Changes of lignocellulases production in different liquid media by *T.gallica*

The Avicel method (Leisola and Linko 1976) did not find Cels activity. This indicated that *T. gallica* did not produce Cels, or that it was too low to detect. Here the strain was statically incubated without oxygen charge, and all enzyme activities were detected under 20°C, so the measured values of enzyme activities were on the low side.

Production of Enzymes in Solid Culture Media

Lignin degradation might be inhibited by glucose added to the solid media. When the ratio of wheat straw powder and liquid medium was 1:4 (wt/vol.), the lignin degradation efficiency was best. Because LM2 was the optimal medium for *T. gallica* to produce MnPs and Hcels, and LM3 was the optimal one for producing laccase and Lips, *T. gallica* was incubated in SM2 and SM3, respectively. The four enzymes produced by this strain in solid media per 10 days are shown in Figs. 2, 3, and 4.

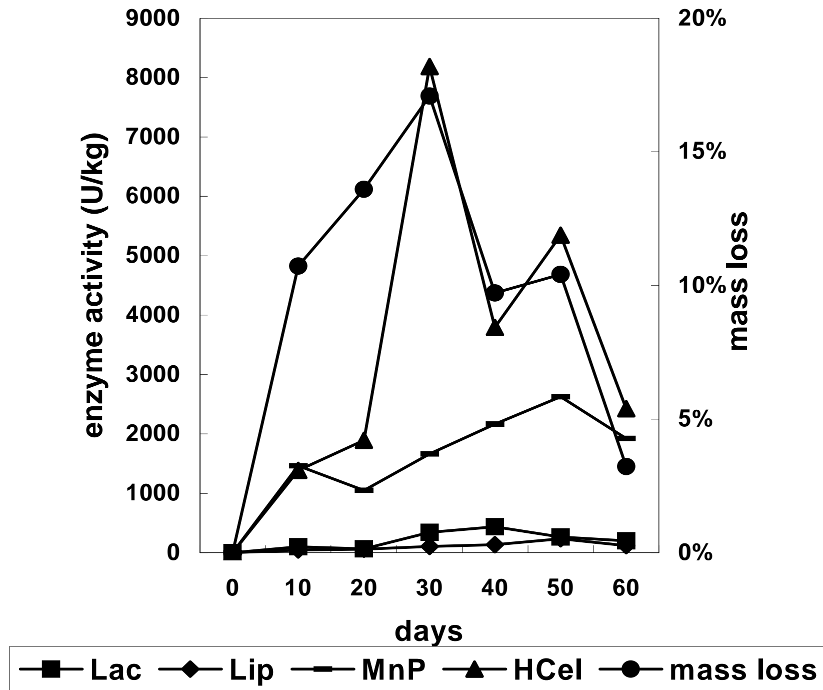

Fig. 2. Weight loss and activities of the 4 enzymes produced by *T. gallica* in SM2

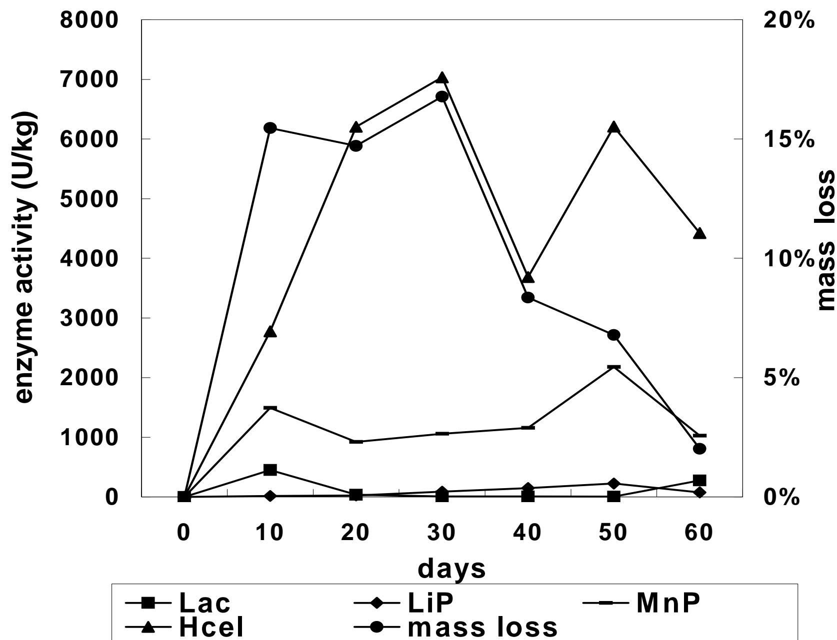

Fig. 3. Weight loss and activities of the 4 enzymes produced by *T. gallica* in SM3

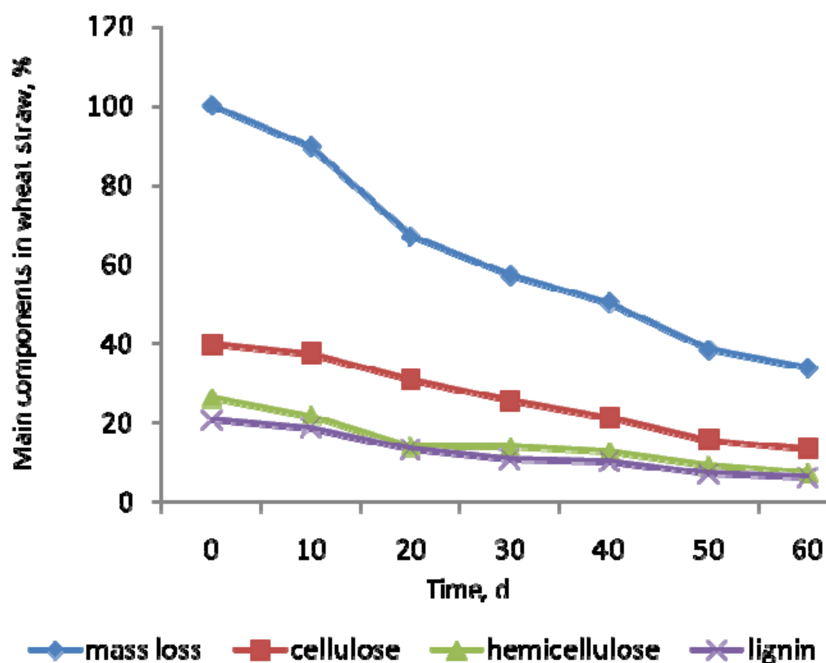

Fig. 4. Relationship between the incubation time and the contents of main components in SM2

In solid media, the activities of Lips gradually increased until the 50th day, when it decreased. The activity of laccase reached the highest level on the 10th day in solid media SM3. And MnPs went to the first activity peak on the 10th day, then decreased to the minimum on the 20th day, and then increased steadily and reached its second peak on the 50th day.

The activities of Hcels both in SM2 and SM3 already reached peaks on the 30th day, but the highest activity peak was achieved in SM2.

Cels activities were not detected by the Avicel method (Leisola and Linko 1976), which indicated that *T. gallica* did not produce Cels, or that it was too low to detect.

The Effects on *T. gallica* Producing Lignocellulases in Incubation Mode

From the results of enzyme production, the activity peaks and yields of Laccase, Lips, and MnPs produced by *T. gallica* were obviously different in solid and liquid media, which indicated that lignocellulases were not connected, but the production of Hcels was connected by these two modes of incubation. So it is believed that the enzyme-producing mechanisms of the two incubation modes are different.

T. gallica Degrading Plant Biomass

Figures 2 and 3 show that *T. gallica* reached a degradation kinetics peak during the period between 20 and 30 days. The peak was 10 days earlier than that of the strain producing Laccase, and was 20 days earlier than that of the strain producing Lips, MnPs, and Cels. It was interesting that the time of the peak was identical to that of the strain producing the Hcels peak, and this indicated that Hcels were critical and closely related to degradation kinetics of biomass. It is necessary that hemicelluloses are degraded first if biomass is to be degraded effectively.

Figures 2 and 3 also show that the ability of *T. gallica* to degrade wheat straw in SM2 was higher than in SM3; therefore, SM2 was used for incubating the strain to determine the dry weight of wheat straw, as well as the contents of lignin, cellulose and hemicelluloses per 10 days. The results shown in Fig. 4 indicate that *T. gallica* could effectively degrade wheat straw lignin, causing a 34.4% loss during 20 days, 46.7% loss during 30 days, and 70.1% loss during 60 days. In addition, it caused 64.8% of biomass loss in SM2 and 64.1% in SM3 during 60 days, which was about 1.5 times as much as *P. chrysosporiun* (Camarero et al. 1998). These data indicate that *T. gallica* is very capable of degrading lignocelluloses.

Figure 4 shows that *T. gallica* degraded more lignin and hemicellulose than cellulose in the same time period, and the reduction curve of cellulose paralleled those of hemicelluloses and lignin. This indicated that the degradation of lignin accompanied the disappearance of cellulose and hemicelluloses, or *T. gallica* degraded lignin on the premise that it would make use of hemicelluloses and cellulose first. In other words, the degradation of cellulose and hemicelluloses was an indispensable supplementary process during the degradation of lignin.

CONCLUSIONS

T. gallica was able to produce lignocellulases with high activity, and its peak was reached at an early stage of fermentation. These results suggest that it might degrade plant biomass and lignin preferentially in solid media, so it might be an excellent strain for biological pulping.

An environmentally friendly process using *T. gallica* in biopulping can be shown as follows: wheat straw → splitting wheat straw → inoculating and incubating this strain to the material at 26°C for 20 days → treating the material again with Hcels → pulp.

ACKNOWLEDGEMENTS

The authors are grateful to Professor Lucian A. Lucia for advice in formatting the manuscript. The authors are also grateful to Natural Science Foundation of Guangdong for financial support (Grant. No. 06025793).

REFERENCES CITED

Aihara, J. (1992). "Why aromatic-compounds are stable." *Scientific American* 266, 62-68.

Camarero, S., and Barrase, J. M. (1998). "Evaluation of *Pleurotus* species for wheat-straw biopulping," *J. Pulp Paper Sci.* 24, 197-203.

Camarero, S., and Sarkart, S. (1999). "Description of a versatile peroxidase involved in the natural degradation of lignin that has both mangnanese peroxiase and lignin peroxidase substrate interaction sites," *J. Biochemistry* 274, 10324-10339.

Childs, R. E., and Bardsley, W. G. (1975). "The steady-state kinetics of peroxidase with 2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid) as chromogen," *Biochem. J.* 145, 93-103.

Dong, J. L., Zhang, Y. W., Zhang, R. H., Huang W. Z., and Zhang, Y. Z. (2005). "Influence of culture conditions on laccase production and isozymes patterns in the white-rot fungus *Trametes gallica*," *J. Basic Microbiol*. 45(3), 190-198.

Feng, H., and Zhang, Y. Z. (1999). "Analysis of 5' upstream regulatory sequences of lignin peroxidase(*LIP*) genes of *Phanerochaete chrysosporim*," *Acta Biochimica et Biophysica Sinica* 31, 669-674.

Gold, M., and Glenn, J. K. (1988). "Manganese peroxidase from *Phanerochaete chrsosporium*," *Method. Enzymol*. 161B, 258-264.

Higuchi, T. H. (1982). "Biodegradation of lignin: Biochemistry and potential applications," *Expertia*. 38, 159-166.

Huang, Q. M., Xie, J., Zhang, H. F., Yang, F., and Yang, W. S. (2006). "Screening of mutant *Trametes gallica* strain with high yield of Laccase and its production condition," *Mycosystema* 25(2), 263-272.

Leisola, M., and Linko, M. (1976). "Determination of the solubilizing activity of a cellulase complex with dyed substrates," *Analytical Biochemistry* 70, 592-599.

Messner, K., and Srebotnik, E. (1994). "Biopulping - An overview of developments in an environmentally safe paper-making technology," *FEMS Microbiol Rev*. 13, 351-364.

Namori, T., and Watanabe, T. (1990). "Purification and properties of thermostable xylanase and β-xylosidase produced by a newly isolated *Bacillus stearothermophilus* strain," *J. Bacteriol.* 172, 6669-6672.

Paice, M. G., Bourbonnais, R., Reid, I. D., and Archibald, F. S. (1995). "Oxidative bleaching enzymes: A review," *J. Pulp Paper Sci*. 21, J280-J284.

Pelaez, F., and Martinez, M. J. (1995). "Screening of 68 species of basidiomycetes for enzymes involved in lignin degradation," *Mycological Research* 99, 37-42.

Srinivasan, C., and Dsouza, T. M. (1995). "Demonstration of laccase in the white rot basidiomycete *Phanerochaete chrysosporium* BKM-F1767," *Appl. Environ. Microbiol*. l61, 4274-4277.

Sun, X., Zhang, R. H., and Zhan, Y. Z. (2004). "Production of lignocellulases by *Trametes gallica* and detection of polysaccharide hydrolase and laccase activities in polyacrylamide gels," *J. Basic Microbiol*. 44(3), 220-231.

The National Standard of The People's Republic of China GB745-78.

The National Standard of The People's Republic of China GB2677.8-81.

Tien, M., and Kirk, T. K. (1984). "Lignin degrading enzyme from *Phanerochaete chrysosporium.* Purification, characterization, and catalytic properties of a unique H_2O_2-requiring oxygen," *Proc. Natl. Acad. Sci. USA*. 81, 2280-2284.

Article submitted: Revised version accepted: Nov. 9, 2009; Published: Nov. 14, 2009.