Journal of Environmental Sciences Vol.17, No.4, pp.620—622, 2005

ISSN 1001—0742
CN11—2629/X

Article ID: 1001-0742(2005)04-0620-03 **CLC number**: X172 **Document code**: A

Cooperation between ligninolytic enzymes produced by superior mixed flora

WANG Hai-lei[1,*], LI Zong-yi[1], GUO Wei-yun[1], WANG Zhen-yu[2], PAN Feng[3]

(1. College of Life Sciences, Henan Normal University, Xinxiang 453002, China. E-mail: whllly@163.com; 2. Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China; 3. Henan Key Laboratory for Environmental Pollution Control, Henan Normal University, Xinxiang 453002, China)

Abstract: Since the ability to degrade lignin with one kind of white-rot fungi or bacteria was very limited, superior mixed flora's ability to degrade lignin was investigated by an orthogonal experiment in this paper. The results showed that superior mixed flora reinforced the ability to degrade lignin, the degradation rates of both sample 9 and 10 were beyond 80% on the day 9. The cooperation between lignin peroxidase(LiP), Mn-dependent peroxidase(MnP) and laccase (Lac) for lignin degradation was also studied. By examining the activities of three enzymes produced by superior mixed flora, it was found that Lac was a key enzyme in the process of biological degradation of lignin but Lip was not; the enzyme activity ratios of Lac/MnP and Lac/LiP were significantly correlative with the degradation rate of lignin at the 0.01 level; and the ratio of MnP/LiP was an important factor affecting the degradation rate of lignin.

Keywords: white-rot fungi; lignin peroxidase; Mn-dependent peroxidase; laccase; orthogonal experiment

Introduction

Lignin is the second most abundant biopolymer in nature. It is also a major component of woody plants. Up to 30% of plant material is composed of lignin, which gives plants structural integrity. Lignin is a random heterogeneous three-dimensional polymer consisting of nonrepeating phenylpropanoid units linked by various carbon-carbon and other bonds. It is difficult to degrade because of its structural complexity. But degradation of lignin is an important step in the process of pulping, papermaking and for other future applications of the biomass from woody plants. Since lignin is extremely resistant to attacks by most microorganisms, chemical degradation of lignin has been the only applicable method for the pulping process, and plenty of environmental pollutants are produced in this process.

Considerable research has been focused on the application of white-rot fungi in environmental pollution control in the past two decades. White-rot fungi, most belonging to basidiomycetes, is one kind of higher filamentous fungi capable of degrading lignin as well as other persistent environmental pollutants. It can secrete extracellularly ligninolytic enzymes, and is the only known organism that is capable of degrading lignin extensively to CO_2 and H_2O in pure culture, playing a very important role in carbon cycle in nature. *Phanerochate chrysosporium* is the white-rot fungi used extensively as model organism for study. Because the time of culturing fungi and the cycle of degrading lignin are very long, there is a long way to go before the utilization of white-rot fungi in environmental pollution control on a large scale in reality. A lot of research(Zhang, 2002) shows that bacteria can degrade lignin. But bacteria's ability to degrade lignin is very limited.

The ligninolytic enzymes consist mainly of three enzymes, i.e. lignin peroxidase(LiP; EC1.11.1.14), Mn-dependent peroxidase (MnP; EC1.11.1.13) and laccase (Lac; EC1.10.3.2). These enzymes are secondary metabolites triggered by nitrogen, carbon or sulphur limitation. LiP was discovered by Tien (Tien, 1983) and Glenn(Glenn, 1983) in *Phanerochate chrysosporium*. It has been intensively studied and the gene for this enzyme has been cloned. This hydrogen peroxide-dependent enzyme is presented as a series of glycosylated isoenzymes with molecular masses ranging from 38 to 43 kDa, and can degrade a wide variety of nonphenolic lignin model compounds in vitro (Tünde, 2001). Mn-dependent peroxidase appears to play an important role in biological oxidation as well as in lignin biodegradation. It specifically oxidizes Mn(II) ions to Mn(III) ions in the presence of H_2O_2 and appropriate Mn(III)-chelating agents, and the resulting Mn (III) complexities can substantially oxidize a broad spectrum of phenolic and related compounds, including a variety of synthetic lignins. MnP exists as a series of glycosylated isoenzymes with pIs ranging from 4.2 to 4.9 (Lobos, 1994), and molecular masses ranging from 45 to 47 kDa. Lac is a copper-containing phenoloxidases with molecular masses of 50 – 300 kDa and acidic pIs (Xiao, 2004; William, 1999), this enzyme can oxidize phenolic substrates and aromatic amines with concomitant reduction of O_2 to H_2O, the polyphenol oxidase is widely distributed among plants and fungal species (Blanchette, 1991), and first discovered in the exudates of a lacquer tree in 1883.

The pattern of white-rot fungi ligninolytic enzymes consists of LiP-MnP, LiP-MnP-Lac, MnP-Lac and LiP-Lac. Each of white-rot fungi can secrete more than one kind of enzyme; even acking of anyone of these three enzymes, lignin could still be degraded efficiently. Considerable research showed that the cooperation among these three enzymes existed in the process of lignin degradation. This phenomenon attracts many interests. These enzymes, in particular, the cooperation among them are widely considered to have potentiality for industrial applications, such as biodegradation of environmental pollutants (Ahn, 2002), biobleaching and biopulping of wood (Machii, 2004; Toshiya, 2001), water pollution control and soil bioremediation(Yateem, 1998). Although many studies have been published concerning these enzymes, little work has been published on the cooperation among them.

Because of the limitation of degrading lignin by one kind of white-rot fungi or bacterium, superior mixed flora's ability to degrading lignin was investigated in this paper. Moreover, to obtain a proper evaluation on the cooperation between three ligninolytic enzymes, a statistical analysis was carried out with the software SPSS for Windows. The results may offer the references to the directly industrial application in environmental engineering.

1 Materials and methods

1.1 Microorganisms

Superior mixed flora consist of *Coriolus versicolor*,

Foundation item: The Henan Province Science and Technology Department of China(No. 001200217); * Corresponding author

Phanerochate chrysosporium and W10. *Coriolus versicolor*, a strain of white-rot fungi, was isolated from a tree full of rot from the pine hurst of Taihang Mountain in Henan Province, China; *Phanerochate chrysosporium* is provided from Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing, China. W10 was isolated from wastewater from the Paper Mill of Xinxiang, Henan Province, China. It is a strain of *Azotobacter* sp., and was demonstrated by considerable studies that it could secrete Lac and MnP. Fig. 1 is the micrograph of W10 using bio-microscope(Nikon HFX-IIA, Japan).

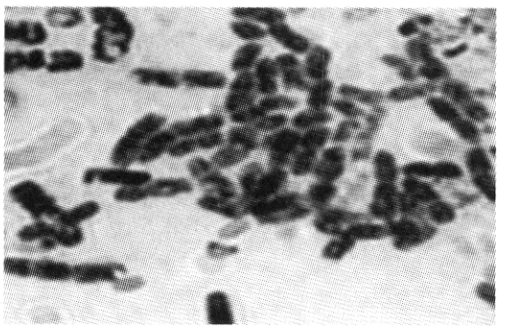

Fig.1 The micrograph of W10(400×)

1.2 Culture conditions

W10 was cultured in solid medium at 37℃, solubilized and mixed in sterilized water. *Phanerochate chrysosporium* and *Coriolus versicolor* were maintained at 33℃ on PDA plate. The liquid growth medium was based on that described by Tien and Kirk(Tien, 1988). Mycelia were collected from PDA plate on the day 7 and then were introduced into liquid growth medium. After 5 d, many of mycelia and W10 suspension were introduced into each 250 ml flask containing 100 ml lignosulfonate solution and the concentration of lignosulfonate is 2016.8 mg/L. Cultures were incubated at 33℃ in a rotary shaker with agitation at 110 r/min.

1.3 Orthogonal experiment

We selected an $L_{25}(5^6)$ orthogonal table(Table 1) and arranged three factors, i.e. A (*Phanerochate chrysosporium*), B (*Coriolus versicolor*) and C (W10) to obtain enough information from the experiment. Since there are five levels in this orthogonal table, the interactions among three microorganisms are not considered. 1, 2, 3, 4 and 5 represent all kinds of levels of factors. Table 2 is a list of factors' levels, 1.0%, 2.5%, 5.0%, 7.5%, 10.0% (v/v) are defined as the inoculum quantity of microorganism.

1.4 Enzyme assay and analytical techniques

Table 1 Orthogonal table of the experiment

Sample		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Level	A	1	1	1	1	1	2	2	2	2	2	3	3	3	3	3	4	4	4	4	4	5	5	5	5	5
	B	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
		1	2	3	4	5	2	3	4	5	1	3	4	5	1	2	4	5	1	2	3	5	1	2	3	4
	C	1	2	3	4	5	3	4	5	1	2	5	1	2	3	4	2	3	4	5	1	4	5	1	2	3
		1	2	3	4	5	4	5	1	2	3	2	3	4	5	1	5	1	2	3	4	3	4	5	1	2
		1	2	3	4	5	5	1	2	3	4	4	5	1	2	3	3	4	5	1	2	2	3	4	5	1

Table 2 List of factors' level(%)

Level	A	B	C
1	1.0	1.0	1.0
2	2.5	2.5	2.5
3	5.0	5.0	5.0
4	7.5	7.5	7.5
5	10.0	10.0	10.0

Crude enzyme was prepared by centrifuging the samples at 4000 r/min, enzyme activities were determined by spectrophometer. LiP activity was measured as described by Brian P *et al*. (Brian, 1993), with 1 U defined as 1 μmol of veratryl alcohol oxidized to vertraldehyde per min. Lac activity was determined as described by Bourbonnais and Paice(Bourbonnais, 1990), with 1 U defined as 1 μmol of 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonate) (ABTS) oxidized per min. MnP activity was measured as described by Michel(Michel, 1991), with 1 U defined as 1 μmol of Mn^{2+} oxidized to Mn^{3+} per min. lignosulfonate was measured as described by Jiang Ting-da(Jiang, 2001).

1.5 Chemicals

Veratryl alcohol is from Sigma Chemical Co., both ABTS and nitrilotriacetate are from Aldrich Chemical Co. Other chemicals used are all analytical grade.

2 Results and discussion

2.1 Lignin degradation rates of different samples

Lignosulfonate in all the samples was degraded by superior mixed flora in this experiment. There are six samples' degradation rates surpassed 60%, and the degradation rates of sample 10 and 9 are up to 80.6% and 81.5% on the day 9. All the samples' degradation rates are in Fig. 2. The statistical analysis was carried out by the software SPSS for Windows. It was found *Coriolus versicolor* played a crucial role in the process of lignin degradation. The main enzyme secreted by *Coriolus versicolor* is Lac, so the correlation between the Lac activity and the degradation rate was studied.

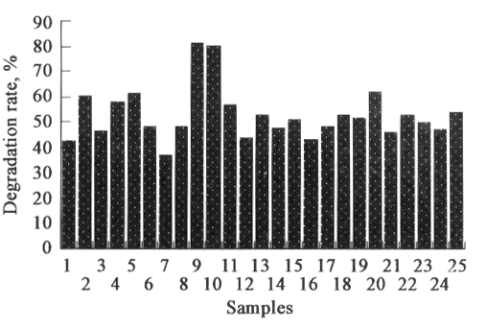

Fig.2 Lignosulfonate degradation rates of different samples

2.2 Correlative analysis between degradation rate and three enzymes

Using the software SPSS for Windows, the correlations between the degradation rates of different samples and the means of three enzyme's activities were analyzed. Results suggested that the mean of LiP activity was negatively correlative with the degradation rate. That is to say, too high LiP activity could restrain the degradation of lignin. This phenomenon proved that LiP was not an important enzyme in the lignin degradation system of superior mixed flora. But the mean of Lac activity was correlative with it. Two-tailed test indicated that correlation coefficient was 0.558, and correlation was significant at the 0.01 level. From the dot chart of Lac activity and degradation rate we could make a conclusion that Lac was directly correlative with the

degradation rate of lignin (Table 3 and Fig.3). There were considerable reports showed that Lac was a phenoloxidase. It could cleave the C—C and C—O bonds of some phenolic lignin model compounds, but in addition to the degradation of phenolic compounds, it was necessary to degrade non-phenolic structures in the process of lignin degradation. Thus higher lignin degradation rate is not the result of single enzyme, but the effect of the three enzymes's cooperation.

Table 3 Correlation between degradation rate and the mean of three enzyme activities

Kendall's tau_b		Degradation rate	Mean of MnP activity	Mean of LiP activity	Mean of Lac activity
Degradation rate	Correlation coefficient	1.000	0.144	−0.024	0.558**
	Sig.(2-tailed)	0.000	0.315	0.870	0.000
	N	25	25	25	25

Note: ** Correlation is significant at the 0.01 level(2-tailed)

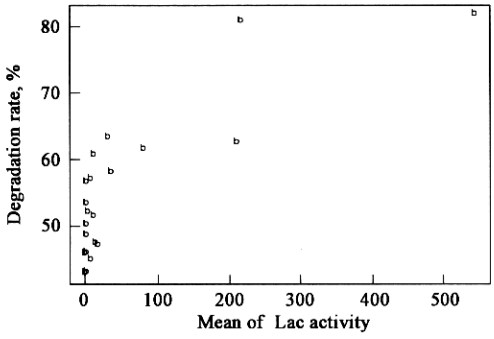

Fig.3 The dot chart of Lac activity and degradation rate of lignin

2.3 Cooperation between three ligninolytic enzymes

Several literature reported that the cooperation between two kinds of enzymes was important. For example, Wu Juan *et al*.(Wu, 2002) reported that the enzyme activity ratio of MnP to LiP was a key factor affecting the degradation rate of lignin. We investigated the relationship between the degradation rate and the enzyme activity ratios of these three enzymes, and found that the enzyme activity ratios of Lac/MnP and Lac/LiP were significantly correlative with degradation rate of lignin at the 0.01 level. Correlation coefficient is 0.491 and 0.513 respectively. From the dot chart of MnP/LiP and degradation rate a trend was observed that when the enzyme activity ratio was less than 6.97, the degradation rate increased with the enzyme activity ratio of MnP/LiP, and when the enzyme activity ratio was more than 8.55, the degradation rate was in inverse proportion to the enzyme activity ratio of MnP/LiP(Table 4 and Fig.4).

Table 4 Correlation between degradation rate and Lac/MnP, Lac/LiP and MnP/LiP

Kendall's tau_b		Degradation rate	Ratio of MnP /LiP	Ratio of Lac/ Lip	Ratio of Lac/ MnP
Degradation rate	Correlation coefficient	1.000	0.093	0.513**	0.491**
	Sig.(2-tailed)	0.000	0.513	0.000	0.001
	N	25	25	25	25

Note: ** Correlation is significant at the 0.01 level (2-tailed)

3 Conclusions

This paper has briefly reported the cooperation between ligninolytic enzymes in the process of biological degradation of lignin. According to the method of statistical analysis using the software SPSS for Windows, the conclusion can be written as follows: LiP is not an important enzyme, but Lac plays a crucial role in the process of lignin degradation; the enzyme activity ratios of Lac/MnP and Lac/LiP are significantly correlative with the degradation rate of lignin at the 0.01 level; the enzyme activity ratio of MnP/LiP is an important factor affecting the lignin degradation.

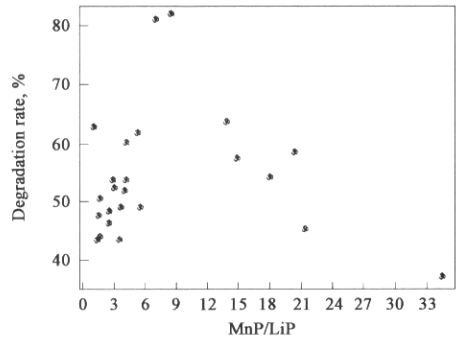

Fig.4 The dot chart of MnP/LiP and degradation rate
(MnP/LiP represents the enzyme activity mean ratio of MnP to LiP from the day 3 to the day 8)

Acknowledgements: The authors would like to acknowledge the contributions of Ms. Li Liu-yan and Ms. Wan Xia, who participated in the part of the experimental work.

References:

Ahn M Y, Jerzy D, Jang-Eok K *et al*., 2002. Treatment of 2,4-dichlorophenol polluted soil with free and immobilized Laccase [J]. J Environ Qual, 31: 1509—1515.

Blanchette R A, 1991. Delignification by wood-decay fungi [J]. Annu Rev Phytopathol, 29: 381—398.

Bourbonnais R, Paice M G, 1990. Oxidation of non-phenolic substrates, an expanded role for laccase in lignin biodegradation [J]. FEBS Letts, 267: 99—102.

Brian P, Roy, Frederick A, 1993. Effect of kraft pulp and lignin on tramestes versicolor carbon metabolism[J]. Appl Environ Microbiol, 59(6): 1855—1863.

Glenn J K, Morgan M A, Mayfield M B *et al*., 1983. An extracellular H_2O_2-requiring enzyme preparation involved in lignin biodegradation by the white-rot basidiomycete *Phanerochate chrysosporium* [J]. Biochem Biophys Res Commun, 114: 1077—1083.

Jiang T D, 2001. Lignin[M]. Beijing: Chemistry Industry Press. 13—14.

Lobos S, Larrain J, Salas L *et al*., 1994. Isoenzymes of manganese-dependent peroxidase and laccaseproduced by the lignin-degrading basidiomycete ceriporiopsis subvermispora[J]. Microbiology, 140: 2691—2698.

Machii Y, Hirai H, Nishida T, 2004. Lignin peroxidase is involved in the biobleaching of manganese-less oxygen-delignified hardwood kraft pulp by white-rot fungi in the solid-fermentation system[J]. FEMS Microbiol Lett, 233(2): 283—287.

Michel J R F C, Dass S B, Grulke E A *et al*., 1991. Role of a manganese peroxidase and lignin peroxidase of *Phanerochate chrysosporium* [J]. Appli Environ Microbiol, 57: 2368—2375.

Tien M, Kirk T K, 1983. Lignin-degrading enzyme from the hymenomycete *Phanerochate chrysosporium*[J]. Science, 221: 661—663.

Tien M, Kirk T K, 1988. Lignin peroxidase of *Phanerochate chrysosporium*[J]. Methods in Enzymology, 161: 238—249.

Toshiya S, Tsutomu K, Bo L *et al*., 2001. New pulp biobleaching system involving manganese peroxidase immobilized in a silica support with controlled pore sizes[J]. Appl Environ Microbiol, 67: 2208—2212.

Tünde M, Katia A B, Simone C B *et al*., 2001. Oxidation of a tetrameric nonphenolic lignin model compound by lignin peroxidase[J]. J Biol Chem, 276: 22985—22990.

William A E, Tresa Q G, David D *et al*., 1999. Purification and characterization of a secreted laccase of *Gaeumannomyces graminis* var. *tritic* [J]. Appl Environ Microbiol, 65: 3071—3074.

Wu J, Xiao Y Z, Wang Y P, 2002. Research on treatment of pulp black wastewater by white-rot fungi [J]. Journal of Biology, 19(5): 17—19.

Xiao Y Z, Chen Q, Hang J *et al*., 2004. Selective induction, purification and characterization of a laccase isozyme from the basidiomycete *Trametes* sp. AH28-2[J]. Mycologia, 96: 26—35.

Yateem A, Balba M T, Awadhi N AL, 1998. White rot fungi and their role in remedition oil-contaminated soil[J]. Environment International, 24: 181—187.

Zhang J Y, Guo L P, Luo Y X *et al*., 2002. Biodegradation of lignin in wheat straw by alkaliphilic ligninolytic bacteria with compounded carbons [J]. Environmental Science, 23(1): 70—73.

(Received for review September 8, 2004. Accepted October 14, 2004)