Note

Improvement of Lignin Peroxidase Production by *Phanerochaete chrysosporium* in Shaking Culture in the Presence of Polyurethane Foam Cubes

Dengbo MA*1, Takefumi HATTORI*1, Mikio SHIMADA*1
and Takayoshi HIGUCHI*1

(Received August 31, 1990)

Abstract——Effects of polyurethane foam cubes and $MnSO_4$ on lignin peroxidase (LiP) production by the white-rot fungus *Phanerochaete chrysosporium* was investigated by use of shaking culture. Addition of both polyurethane cubes and $MnSO_4$ greatly enhanced the production of LiP activity up to 950 U/l culture medium. The increase in the enzyme activity is about twice as compared with that obtained from the stationary culture cultivated in the absence of these additives.

Key words: white-rot fungi, *Phanerochaete chrysosporium*, lignin peroxidase, production, effects, polyurethane, $MnSO_4$

1. Introduction

Lignin, next to cellulose, is the second most abundant natural polymer and is a complex optically inactive phenylpropanoid matrix that makes up 20~30% of most woods and 5~15% of most agricultural crop residues[1]. Current lignin biodegradation research has been receiving a keen attention toward fundamentals and applications of ligninolytic fungi and enzyme systems for processing these lignocellulosic materials. In practice, biomechanical pulping, microbial and enzymatic conversion of lignins to useful chemicals, upgrading of feeds by removal of lignin, and treatments of lignin-derived wastes are extensively being investigated.

Since discovery of the first ligninase or hemoprotein lignin peroxidase (LiP) from the culture fluid of a white-rot fungus, *Phanerochaete chrysosporium*[2,3], many innovative techniques were introduced and the conventional stationary culture method has actually been modified to produce LiP preparation in much greater quantity in consideration of the following factors and methods: a) use of mutant strain (INA-12)[4,5], b) use of agitation or shaking[6~8], c) a disc fermentor of RBC (rotating biological contactor) with a strain of SC 26[9], d) a polyurethane-immobilized method[10], e) nylo-fiber- and nylon-web-immobilized methods[11~13], f) a pilot scale fer-

*1 Research Section of Lignin Chemistry

mentor[14], g) addition of detergents such as Tween 80[5,15], h) addition of veratryl alcohol into the culture media[16], i) the effect of carbon sources such as glucose and glycerol[4,17], j) the effect of nitrogen concentrations in the culture media[4,18], k) the effect of 100% O_2[8,18], and l) the effects of the concentrations of trace metals, including Mn ion[9,19].

In the earlier studies reported, nitrogen-limited media were used most commonly for both cultivation of the fungus and production of the LiP enzyme[4,20]. However, the nitrogen-limited media are not advantageous for production of the enzyme protein in larger quantity. In our laboratory, Kurosaka et al.[21] reported that the LC/MN culture enhanced the LiP activity under stationary culture conditions in the presence of 100% oxygen, 1.5 mM veratryl alcohol and 0.1% Tween 80.

We report here the further improvement of the LiP production by use of polyurethane foam cubes (PU cubes) and $MnSO_4$ added in the culture system but without using either a special mutant strain or 100% O_2; the LiP activity has been icreased from the previous value 356 U/l to 950 U/l culture medium.

2. Materials and Methods

2.1 Chemicals

All the chemicals used in this experiment were in a reagent grade. Veratryl alcohol, $MnSO_4$ and MnO_2 (99.5% pure) were purchased from Wako Chemicals. Polyurethane foam was obtained from commercial source.

2.2 Culture conditions for production of LiP

The white-rot fungus *Phanerochaete chrysosporium* (BKM-F 1767. ATCC 24725) obtained from Dr. Kirk (Forest Products Laboratory, Madison) was maintained at 4°C on 2% malt agar slants. The inoculum consisting of conidial suspension was prepared from the stock agar slants according to Kirk et al.[20]. For production of LiP, the LC/MN culture (50 ml/200 ml Erlenmeyer flask) containing 0.2% glucose and 6 mM ammonium tartrate, 10 mM *trans*-aconitate buffer (pH 4.3), 1.5 mM veratryl alcohol and 0.5% Tween 80 in Kirk's basal medium with 7 fold minerals was inoculated and cultivated at 38°C under shaking culture conditions. Normally, PU cubes (size, 1.5 cm) were added into the culture flask. The cultures were agitated at 200 rpm/2.5 cm radius on a rotary shaker unless otherwise stated. For comparison, stationary culture was carried out with the same medium according to Kurosaka et al.[21] except that veratryl alcohol was added into the medium before autoclaving.

2.3 Enzyme assay

LiP activity from each culture medium was determined spectrophotometrically

at 30°C by measurement of the increase in absorbance at 310 nm due to the formation of veratraldehyde product from veratryl alcohol substrate. Three culture were used for each assay of LiP activity during the cultivation period and the average values of the triplicate cultures were obtained as shown in Figs 1~4 and Table 1. The reaction mixture (2.5 ml) normally contained 10 mM veratryl alcohol substrate, 0.5 mM H_2O_2, 0.3 ml of the culture filtrate and 1.9 ml of 0.1 M tartrate buffer solution (pH 3.0). The reaction was started by addition of 50 μl of 25 mM hydrogen peroxide solution. One unit of the enzyme activity (U) was defined as the amount of the enzyme protein which produces 1 μmole of veratraldehyde (ε, 9.3×10^3)/min. Molecular activity (μkat) was obtained by divition of U by 60.

2.4 Instrumental apparatus used

A Hitachi model 200—20 spectrophotometer was used for measurement of the absorbance at 310 nm. A G24 environmental incubator shaker was used for agita -tion of the fungal culture.

3. Results and Discussion

3.1 Effects of PU cubes on production of lignin peroxidase

During the course of experiments we found that the agitation of culture does

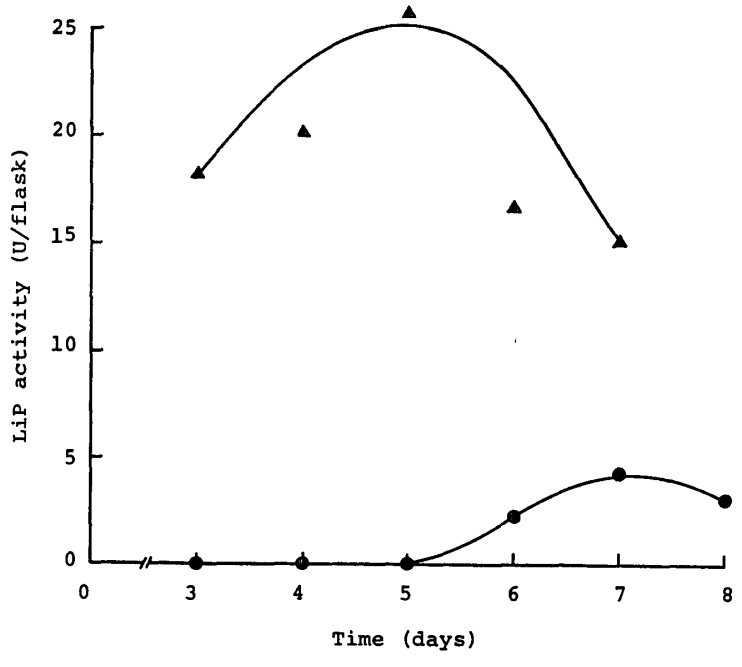

Fig. 1. Effect of polyurethane foam cubes on LiP production by *Phanerochaete chrysosporium* under shaking culture conditions, which are described in Materials and Methods.
—▲—, Cultures with 4 PU cubes/flask; —●—, Control cultures without PU cubes.

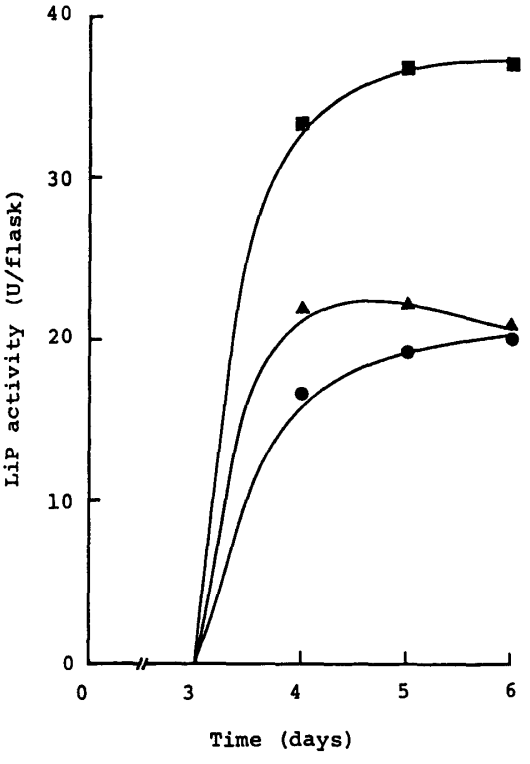

Fig. 2. Effects of the number of PU cubes per flask on LiP production by *P. chrysosporium* under shaking culture conditions (at 200 rpm for the first 60 hr and then at 125 rpm).
—●—, 2 cubes added; —▲—, 4 cubes added; —■—, 6 cubes added.

not completely inhibit the production of LiP when Tween 80 was added in the shaking culture. As shown in Fig. 1, however, the yield of LiP activity was not satisfactory and the LiP was produced at the later stage of cultivation. The result clearly shows that the PU cubes is obviously effective for production of LiP and shortening the period of the enzyme harvest. The 5-day old culture with the PU cubes yielded a 6-fold increase in the enzyme activity as compared with the maximal activity of the 7-day oid control culture without the PU cubes. Fig. 2 shows that the use of 6 cubes per flask greatly enhanced the LiP production as compared with less number of cubes used for the cultures.

3.2 Effect of shaking speed on LiP production

We found that the shaking speed in the first 60 hr incubation (mycelial growth phase) was very important for LiP production. The results (Figs. 1 and 2) show that the decrease in the shaking speed from 200 rpm to 125 rpm 60 hr after the inoculation did not significantly affect the LiP activity for the cultures with 4 PU cubes/flask. However, the cultures cultivated at 125 rpm from beginning to end

produced much lower LiP activity (data not shown). In the latter case, mycelium grew on the outer surfaces of PU cubes as thick mycelial mat, which might be responsible for the lower LiP production.

3.3 Effects of carbon sources on LiP production

Since the earlier investigations reported that glycerol is a much better carbon source than glucose for greater production of LiP activity in some cases[4,17], we examined the effectiveness of these two carbon sources. Fig. 3 shows that glucose was more effective in rapid production of LiP than glycerol, although the culture grown on glycerol produced somewhat higher LiP activity than that grown on glucose on day 6.

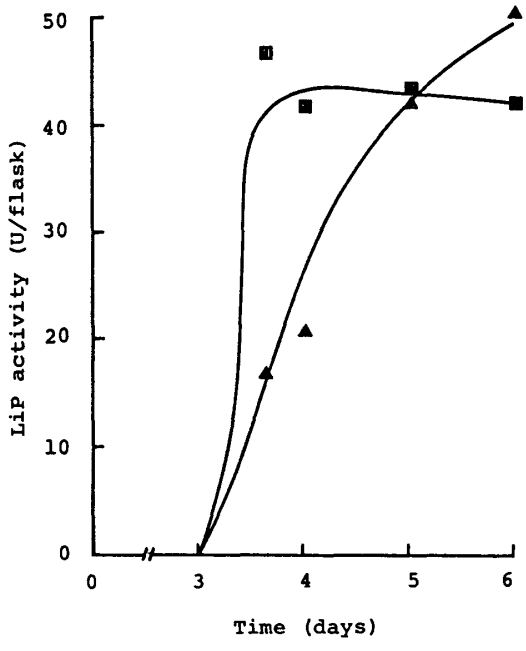

Fig. 3. Effects of carbon sources on LiP production by *P. chrysosporium* in the shaking culture with 6 PU cubes/flask (at 200 rpm for the first 60 hr and then at 125 rpm).
—■—, Glucose (0.2%) was used; —▲—, Glycerol (0.175%) was used.

3.4 Effects of $MnSO_4$ and MnO_2 on the LiP production

Kirk et al.[9] first reported that the addition of 6 times more basal level of Mn^{2+} increased the LiP activity up to 180% under the culture conditions of the disc fermentor. Quite recently, Brown et al.[22] demonstrated that the increase in Mn^{2+} concentration also led to the increase in Mn-peroxidase activity of the same white -rot fungus. Alternatively, Kern[19] reported that MnO_2 was effective for greater production of LiP activity.

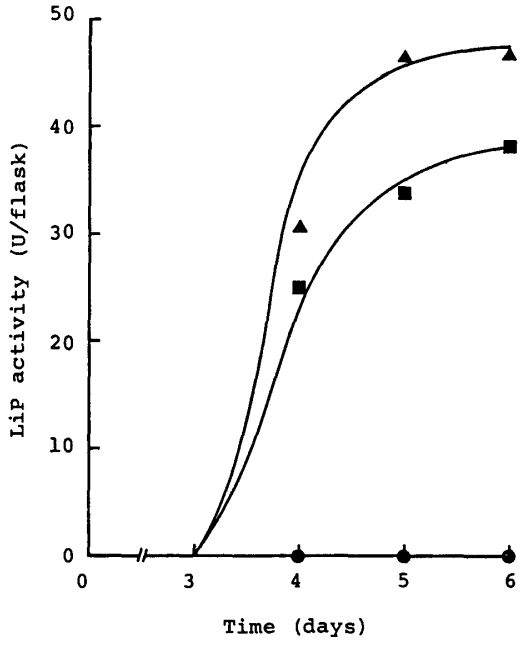

Fig. 4. Effects of $MnSO_4$ and MnO_2 on LiP production by *P. chrysosporium* under the shaking culture conditions (6 PU cubes/flask, at 200 rpm for the first 60 hr and then at 125 rpm).
—▲—, addition of 180 μM $MnSO_4$; —●—, addition of MnO_2, —■—, no addition of Mn species.

Table 1. The results of LiP productions by *P. chrysosporium* under the various conditions studied.

Culture conditions	Day of maximal LiP activity	LiP activities*		
		U/l	(μkat/l)	Ratio
Stationary culture	6	417	(7.0)	1.0
Shaking cultures				
Control culture	7	87	(1.5)	0.2
〃 +4 cubes	5	500	(8.3)	1.2
〃 +6 cubes	5	750	(12.5)	1.8
〃 +6 cubes+50 μM $MnSO_4$	5	822	(13.7)	2.0
〃 +6 cubes+180 μM $MnSO_4$	5	950	(15.8)	2.3

*, LiP activities were assayed as described in the text and calculated for the initial culture volume after correction of the volume decreased due to drying during the cultivation.

We investigated effects of $MnSO_4$ and MnO_2 on LiP production under the present shaking culture conditions with 6 PU cubes/flask. Results are shown in Fig. 4, which clearly indicates that the cultures with both 180 μM $MnSO_4$ and the PU cubes yielded significantly greater LiP activities than those lacking $MnSO_4$.

Surprisingly, in contrast to Kern's results, MnO_2 completely inhibited the LiP production under the present experimental conditions.

The results of LiP production by *P. chrysosporium* under various conditions studied are summarized in Table 1. By use of PU cubes we successfully improved LiP production in shaking culture.

The major reason for effectiveness of the PU cubes is probably due to the sponge structures of this material within which the filamentous mycelia grew uniformly and did not form pellets. As a result, the increased subfaces of mycelia facilitated not only uptake of nutrients and dioxygen but also excretion of the enzyme proteins into the extracellular liquid.

In conclusion, the highest LiP activity was obtained from the culture with both 6 PU cubes/flask and 180 μM $MnSO_4$ under shaking culture conditions (Table 1).

References

1) T.K. KIRK: *Phil. Trans. R. Soc. Lond.* A**321**, 461 (1987)
2) J.K. GLENN, M.A. MORGAN, M.B. MAYFIELD, M. KUWAHARA and M.H. GOLD: *Biochem. Biophys. Res. Commun.*, **114**, 1077 (1983)
3) M. TIEN and T.K. KIRK: *Science*, **221**, 661 (1983)
4) J.A. BUSWELL, B. MOLLET and E. ODIER: *FEMS Microbiol. Lett.*, **25**, 295 (1984)
5) M. ASTHER and A. CORRIUE: *Enzyme Microb. Technol.*, **9**, 245 (1987)
6) A. JAGER, S. CROAN and T.K. KIRK: *Appl. Microbiol. Lett.*, **50**, 1274 (1985)
7) M. LEISOLA and A. FIECHTER: *FEMS Microbiol. Lett.*, **29**, 33 (1985)
8) M. LEISOLA, D.U. THANEI-WYSS and A. FIECHTER: *J. Biotechnol.*, **3**, 97 (1985)
9) T.K. KIRK, S. CROAN, M. TIEN, K.E. MURTAGH and R.L. FARRELL: *Enzyme Microb. Technol.*, **8**, 27 (1986)
10) N. KIRKPATRICK and J.M. PALMER: "Lignin enzymic and microbial degradation" (E. ODIER, ed., INRA Publ., Paris,) No. **40**, 191 (1987)
11) S. LINKO, L.-C. ZHANG, M. LEISOLA, Y.-Y. LINKO, FIECHTER and A. LINKO: *ibid.*, 209 (1987)
12) S. LINKO: *Enzyme Microb. Technol.*, **10**, 410 (1988)
13) S. LINKO: *J. Biotechnol.*, **8**, 163 (1988)
14) H. JANSHEKAR and A. FIECHTER: *J. Biotechnol*, **8**, 97 (1988)
15) A. HATTAKKA, A. KANTELINEN, A. TERVIA-WILO and L. VIRIKARI: p.185 (1987) in ref. 10
16) F. TONON, E. ODIER, M. ASTHER, L. LESAGE and G. CORRIEU: p. 165 (1987) in ref. 10
17) H. WILLERSHAUSEN, H. GRAF and A. JAEGER: p. 203 (1987) in ref. 10
18) B.D. FAISON and T.K. KIRK: *Appl. Environ. Microbiol.*, **49**, 299 (1985)
19) H.W. KERN: *Appl. Microbiol. Biotechnol.*, **32**, 223 (1989)
20) T.K. KIRK, E. SCHULTZ, W.J. CONNORS, L.J. LORENZ and J.G. ZEIKUS: *Arch. Microbiol.* **117**, 277 (1978)
21) H. KUROSAKA, K. UZURA, T. HATTORI, M. SHIMADA and T. HIGUCHI: *Wood Research* **76**, 17 (1989)
22) J.A. BROWN, J.K. GLENN and M.H. GOLD: *J. Bacteriol.*, **172**, 3125 (1990)