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Production and characterization of ligninolytic enzymes of *Bjerkandera adusta* grown on wood meal/wheat bran culture and production of these enzymes using a rotary-solid fermenter

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Manganese peroxidase (MnP) and lignin peroxidase (LiP) were produced by growing a white-rot fungus *Bjerkandera adusta* statically, on a wood meal/wheat bran culture in flasks. MnP and LiP reached their maximum activity after 6 and 19 days of inoculation, respectively. Both MnP and LiP are thought to be important enzymes in lignin biodegradation by *B. adusta*. Ion exchange chromatography showed that *B. adusta* produced a single LiP and a single MnP enzyme in wood meal/wheat bran culture. These enzymes were separated and characterized. The molecular weight of MnP was 46,500 with a p*I* of 3.9. The molecular weight of LiP was estimated to be 47,000 with a p*I* of 3.5. Spectral analysis demonstrated that both enzymes are heme proteins. Production of these enzymes was also achieved using a rotary-solid culture fermenter. MnP, LiP and veratryl alcohol oxidase were produced by *B. adusta* in the fermenter.

Key Words——*Bjerkandera adusta*; lignin peroxidase; manganese peroxidase; rotary-solid type fermenter; solid culture.

White-rot fungi are known to produce extracellular ligninolytic enzymes, such as laccase, manganese peroxidase (MnP) and lignin peroxidase (LiP) (Kirk and Farrell, 1987; Gold et al., 1989). LiP and MnP catalyze the H_2O_2-dependent oxidation of lignin and its related compounds, halogenated phenolic compounds, polycyclic aromatic hydrocarbons and other aromatic compounds by a one-electron oxidation mechanism followed by a series of non-enzymatic reactions that yield various types of degradation products (Kirk and Farrell, 1987; Gold et al., 1989; Hammel, 1989). Laccase is known to oxidize various phenolic compounds as well as lignin (Thurston, 1994) via the phenoxy radical formed by one-electron oxidation. Thus, the ligninolytic enzymes may be widely applicable to various oxidation bioprocessing systems such as wood pulping, bleaching, conversion of lignin to useful products and paper mill effluent treatment. Development of large-production methods of the ligninolytic enzymes makes it possible to operate these bioprocessing systems at a moderate price.

The production and characterization of ligninolytic enzymes have been thoroughly studied in liquid cultures using the white-rot fungus *Phanerochaete chrysosporium* Burds. (Kirk and Farrell, 1987; Hatakka, 1994). Recently, ligninolytic enzymes of other white-rot fungi including *Pleurotus ostreatus* (Jacq.: Fr.) Kummer (Kofujita et al., 1991), *Lentinus edodes* (Berk.) Singer (Forrester et al., 1990), *Phlebia radiata* Fr. (Niku-Paavola et al., 1988), *Coriolus versicolor* (L.: Fr.) Quél. (Dodson et al., 1987) and *Bjerkandera adusta* (Willd.: Fr.) Karst. (Kimura et al., 1990) were isolated and characterized. The results demonstrated that fungi produce multiple ligninolytic enzymes depending on culture conditions and that in many cases the enzymes were produced as a mixture of isozymes.

In vitro degradation of lignin and its related compounds as well as production of ligninolytic enzymes have been examined mainly using liquid cultures. However, in solid substrate culture, MnP was found to be produced dominantly by *P. chrysosporium* (Datta et al., 1991), *P. ostreatus* (Kofujita et al., 1992), *L. edodes* (Kofujita et al., 1992), *P. radiata* (Vares et al., 1995) and *Ceriporiopsis subvermispora* (Pil.) Gilbn. et Ryv. (Lobos et al., 1994), whereas no or very little laccase or LiP activity was detected. Therefore, more information about the production of ligninolytic enzymes in solid culture is necessary to understand the mechanisms of lignin and lignocellulose degradation in the natural environment.

The development of techniques for the production of ligninolytic enzymes is thought to be necessary for the potential application of these enzymes. Large-scale production of ligninolytic enzymes using liquid cultures grown in reactors has been attempted. Jäger et al. (1985) first reported the production of LiP in submerged agitated of cultures of *P. chrysosporium* to which nonionic surfactants had been added. However, agitation of

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such cultures has been reported to suppress the production of ligninolytic enzymes (Faison and Kirk, 1985). Air-lift type fermenters have also been used for the production of LiP and MnP (Bonnarme et al., 1993; Laugero et al., 1996). Immobilization of the mycelium of *P. chrysosporium* on polyurethane foam or nylon-web were also reported to increase the production of LiP and MnP (Kirkpatrick and Palmer, 1987; Capdevila et al., 1989; Laugero et al., 1996). Rotary surface culture using a disk to immobilize the mycelia also achieved production of LiP (Kirk et al., 1986).

In the present experiment, the ligninolytic enzymes produced by *B. adusta* grown on wood meal/wheat bran culture were separated and characterized. This report also describes the production of ligninolytic enzymes using a solid culture fermenter.

Materials and Methods

Preparation of extractive-free wood meal Beech (*Fagus crenata* Blume) chips were ground with a Wiley mill and then extracted with ethanol-benzene mixtures (1/2, v/v) using a Soxhlet's extractor.

Strains and culture conditions *Bjerkandera adusta* (K-2679) was precultured on 3.9% (w/v) potato-dextrose agar (Nissui) plate at 28°C for 6 d. Two pieces of the precultured agar (1 cm × 1 cm) were cut off and inoculated on a wood meal/wheat bran medium containing 4.5 g of extractive-free beech wood meal (42–60 mesh), 0.5 g of wheat bran and 15 ml of distilled water. Cultures were grown statically at 30°C in 200-ml Erlenmeyer flasks.

Enzyme assay MnP activity was determined by the method of Kofujita et al. (1991) using a reaction mixture containing 0.4 mM guaiacol, 50 mM Na-lactate buffer (pH 4.5), 0.2 mM $MnSO_4$ and 0.1 mM H_2O_2 in a total volume of 1 ml. The reaction was measured by monitoring the increase in absorbance of the reaction product at 465 nm. MnP activity was calculated by subtracting the activity determined in the absence of Mn (II) from that in the presence of Mn (II). One unit of MnP was defined as the amount of enzyme that increased the absorbance at 465 nm by 1.0 per min. LiP activity was measured spectrophotometrically as described by Tien and Kirk (1984) using a reaction mixture containing 0.8 mM veratryl alcohol, 0.1 M Na-tartrate buffer (pH 3.0), 0.25 mM H_2O_2 and enzymes solution in a final volume of 1.0 ml. Veratryl alcohol oxidase (VAO) activity was measured using a reaction mixture containing 0.8 mM veratryl alcohol, 0.1 M Na-phosphate buffer (pH 7.0) and enzyme solution in a final volume of 1.0 ml (Kimura et al., 1990). One unit of LiP and VAO activity was defined as 1 nmol veratryl alcohol oxidation to veratraldehyde per min. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Electrophoresis IEF was carried out with a precast gel (Phast Gel, pH range 4–6.5, Pharmacia) on a Pharmacia LKB-Phast System. A set of standard proteins (Low p*I* Kit, p*I* 2.8–6.5, Pharmacia) was used for the calibration of p*I*. Active staining was performed with 1.4 mM 3,3′-diaminobenzidine tetrahydrochloride as a substrate in the presence of 0.3 mM H_2O_2. SDS-PAGE was also carried out with a precast gel (Phast Gel, Gradient 10–15, Pharmacia) on the Phast System. Molecular weights were estimated with standard markers (Dai-ichi Kagaku).

Preparation of crude enzymes Wood meal cultures were extracted with 20 ml of 20 mM Na-succinate buffer (pH 4.5) per flask at 28°C for 1 h with periodical shaking (60 rpm). The extract was filtered through a double gauze by gravity and centrifuged (9,000 rpm, 4°C) for use as the crude enzyme preparation.

Determination of wood components The amount of lignin in the wood meal/wheat bran culture was determined as Klason lignin by the method of Yoshihara et al. (1984).

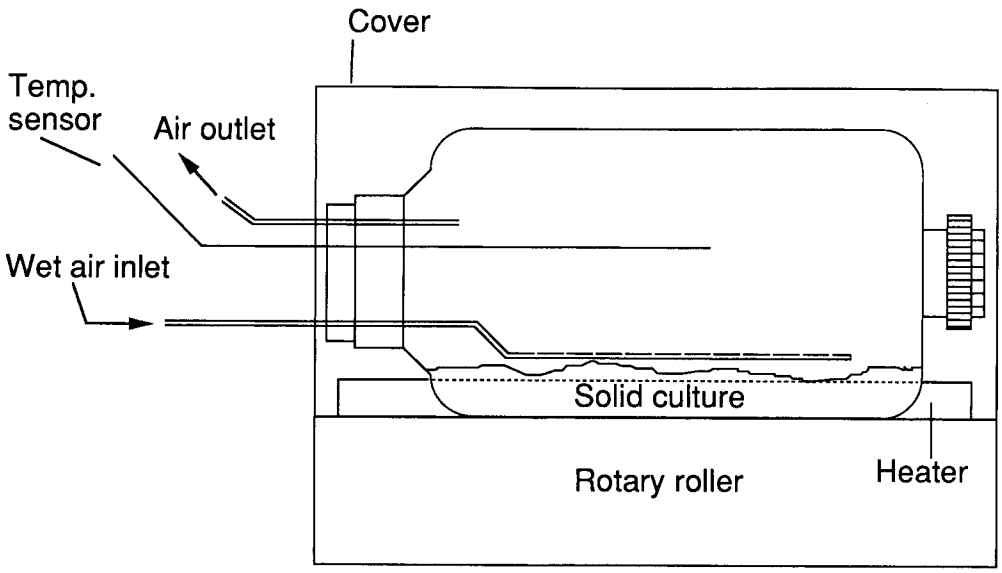

Fig. 1. Rotary solid fermenter system.

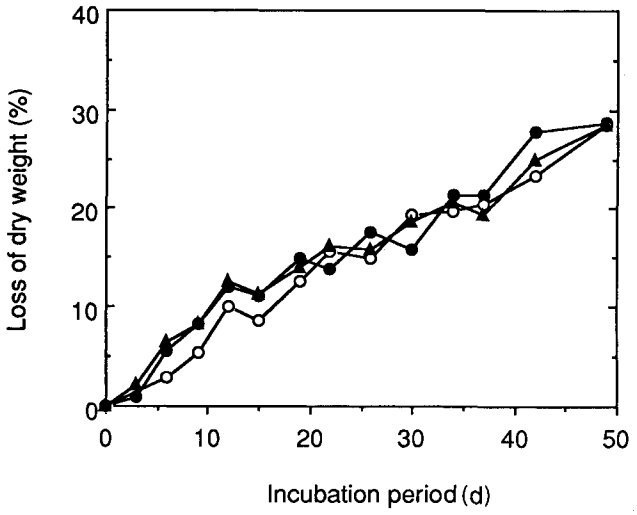

Fig. 2. Degradation of wood components by *B. adusta*. Loss of lignin (–●–), holocellulose (–○–) and culture dry weight (–▲–) were measured as described in Materials and Methods.

The amount of holocellulose (cellulose and hemicellulose) was determined by the method of Wise et al. (1946), using sodium chlorite as an oxidant.

Production of ligninolytic enzymes using a fermenter *Bjerkandera adusta* was incubated in a rotary-solid fermenter (Fig. 1) using wood meal/wheat bran medium containing 162 g of extractive-free beech wood meal, 18 g of wheat bran and varying amounts of added water. A suspension of ground mycelia prepared according to Kimura et al. (1990) was used as an inoculum. Incubation was carried out at 30°C with the culture vessels kept stationary, except for a 10 min period of rotation at 0.6 rpm, once a day. The culture was flushed with air for 10 min, once a day (250 ml/min).

Results

Time course of loss of wood components *Bjerkandera adusta* grew well on the wood meal/wheat bran culture medium, with an approximately 30% decrease in the total weight of the culture recorded during the 50 days of cultivation. Loss of holocellulose and lignin proceeded equally, indicating that the degradation of the wood components by this fungus was nonselective. The degradation of lignin is strong evidence for the production of ligninolytic enzymes in the culture (Fig. 2).

Production of ligninolytic enzymes by *B. adusta* grown on wood meal/wheat bran culture As shown in Fig. 3, *B. adusta* produced both MnP and LiP in the wood meal/wheat bran medium. MnP activity was reached maximum in the early period of mycelial growth, i.e. on the 6th day of the culture, and then was maintained at a low level. Production of LiP started on day 10 with maximum activity reached at day 19 of the cultivation. Laccase activity was negligibly low throughout the culture period. Similar results were also obtained with four other cultivations which were independently carried out.

Separation and characterization of MnP of *B. adusta* MnP produced in the wood meal culture was separated by the following steps.

Step 1. Wood meal/wheat bran cultures of *B. adusta* grown on for 6 d (30 cultures) were extracted with 20 mM Na-succinate buffer (pH 4.5) as described in Materials and Methods. Extract of each culture was combined (800 ml) and concentrated by ultrafiltration using Amicon PM-10 membranes (molecular cut-off:

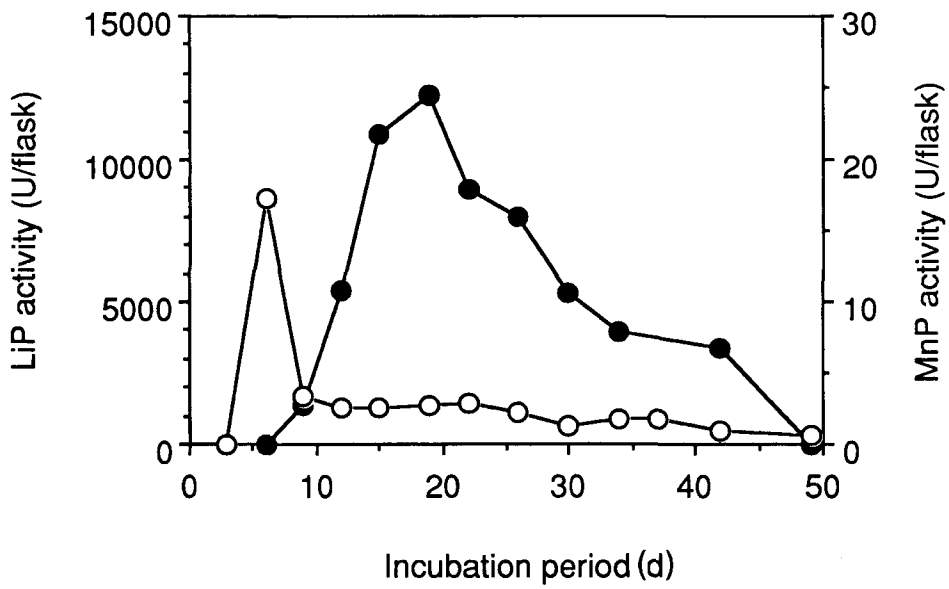

Fig. 3. Activity of ligninolytic enzymes produced by *B. adusta* grown on wood meal/wheat bran culture. MnP (–○–) and LiP (–●–) activity was measured as described in Materials and Methods.

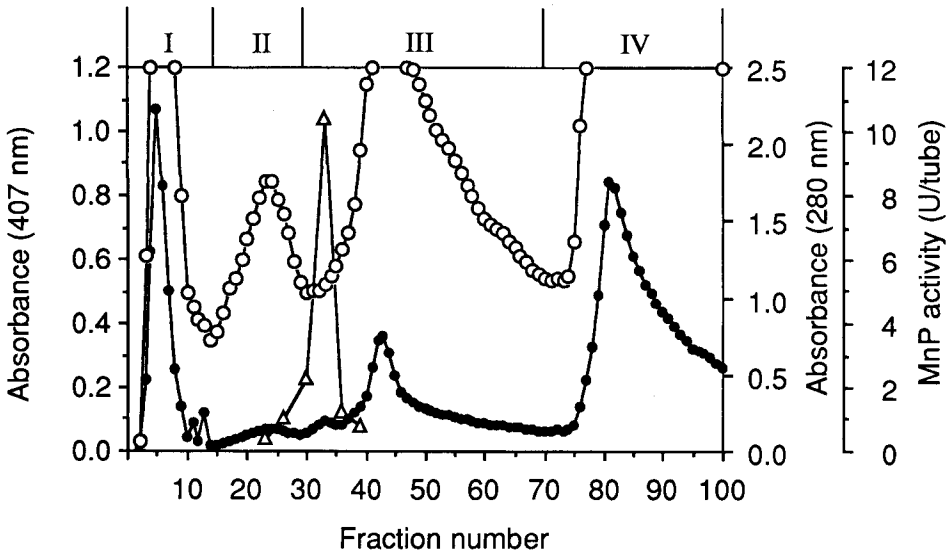

Fig. 4. DEAE-Sepharose chromatogram of the crude extracts of the culture of *B. adusta* grown on wood meal/wheat bran. MnP was separated from the extract of 6-d-old culture on a DEAE-Sepharose CL-6B column as described in the text. Elution was carried out with a stepwise gradient of NaCl 0 (I), 0.15 (II), 0.25 (III) and 0.5 M (IV).

10,000). The concentrate was dialyzed five times against 1 L of 20 mM Na-succinate buffer (pH 4.5) every 2 h. The dialysate contained 47.8 mg of protein with a specific activity of 10.2 U/mg and a total of 489 U.

Step 2. The dialyzed enzyme solution was applied to a DEAE-Sepharose CL-6B (Pharmacia) column (25 mm internal diam × 11 cm long) previously equilibrated with 20 mM Na-succinate buffer (pH 4.5) (Fig. 4). Elution was carried out with a stepwise gradient of NaCl to 0.5 M, and 5 ml fractions were collected. The eluent of protein was measured by monitoring absorbance at 280 nm and 407 nm based on the spectrum of heme protein, and MnP activity. Fractions which showed MnP activity were pooled and concentrated through a Centriprep-10 microconcentrator (Amicon, Denver, USA), then dialyzed against 20 mM Na-succinate buffer (pH 4.5). The dialysate contained 6.5 mg of protein with a total of 337 U.

Step 3. The dialysate was subjected to gel-filtration chromatography with a Pharmacia HiLoad 16/60 Superdex 200 prep grade. The elution was carried out at a flow rate of 1.0 ml/min using 10 mM Na-acetate buffer (pH 5.5) containing 0.1 M NaCl. Active fractions were pooled and dialyzed against 20 mM succinate buffer (pH 4.5). The dialysate contained 3.1 mg protein with a total of 218 U.

Step 4. The dialyzed enzyme solution was subjected to ion-exchange chromatography on a Pharmacia

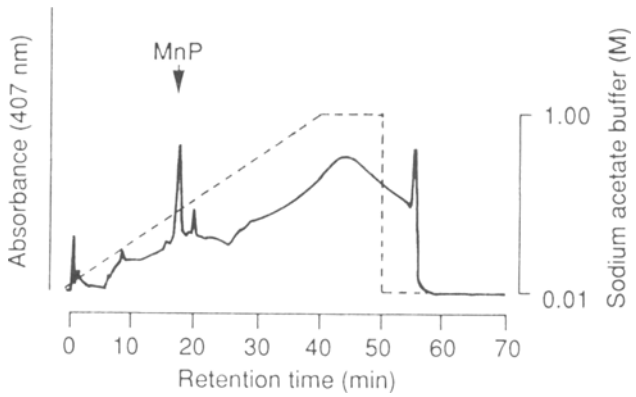

Fig. 5. Mono-Q chromatogram of MnP. MnP was separated on Pharmacia Mono-Q (5/5) with Na-acetate buffer (pH 6.0) using a linear gradient of NaCl from 10 mM to 1 M. The elution rate was 1.0 ml/min and the eluent was monitored at 407 nm.

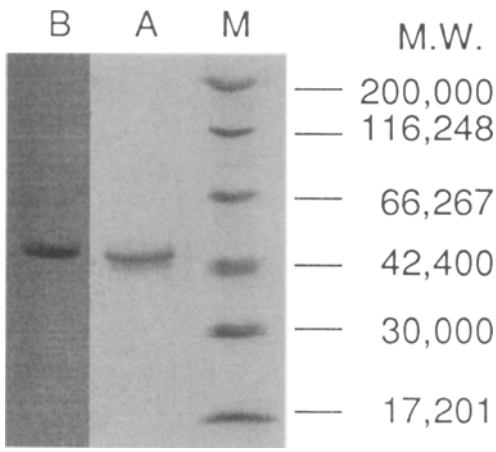

Fig. 6. SDS-PAGE of separated ligninolytic enzymes produced by *B. adusta* grown on wood meal/wheat bran culture. Three μg of protein was loaded for the both enzymes and was stained with Coomassie brilliant blue R-250. M: Standard marker, A: MnP, B: LiP.

Mono-Q column (5/5). The elution was carried out with Na-acetate buffer (pH 6.0) using a linear gradient of 10 mM to 1 M at a flow rate of 1.0 ml/min. The elution of protein was measured by monitoring absorbance at 407 nm (Fig. 5). Active fractions obtained from 5 runs of chromatography were pooled and concentrated through a Centriprep-10 microconcentrator, then dialyzed against 20 mM Na-succinate buffer (pH 4.5). The final MnP preparation contained 0.2 mg of protein with a total of 11.4 U and a specific activity of 51.8 U/mg. The preparation gave a single band with a trace impurity in SDS-PAGE (Fig. 6A). The trace impurity was also observed by staining with Coomassie brilliant blue in IEF. However, only single band was observed by active staining with 3,3′-diaminobenzidine tetrahydrochloride in IEF and the impurity was not stained by the active staining. Therefore, this impurity is not thought to be peroxidase. In this study, this enzyme was used as MnP in following experiments. Further purification of this enzyme is now under trying.

SDS-PAGE against standard proteins of known molecular weight demonstrated MnP of *B. adusta* to have a molecular weight of 46,500. The p*I* of the protein was 3.9 in IEF. An absorption maximum at 407 nm, as well as weak maxima at 502 and 636 nm in the UV/VIS spectrum (data not shown) indicated that the enzyme was a heme protein, as is MnP of *P. chrysosporium* (Wariishi et al., 1988). MnP of *B. adusta* exhibited an optimum pH and temperature of 4.5 and 60°C, respectively, and was stable below 60°C and in the pH range of 4.0 to 6.0. Activity of MnP was completely inhibited by 1 mM NaN_3. Addition of 1 mM EDTA, KCN and dithiothreitol (DTT) resulted in a 98, 84 and 81% decrease in activity, respectively.

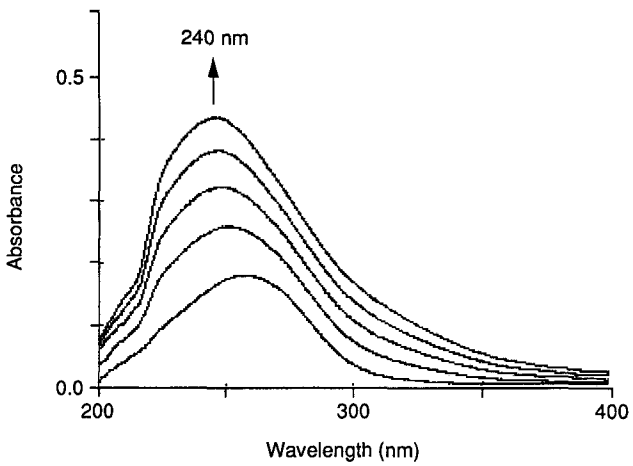

Fig. 7. Oxidation of Mn (II) to Mn (III) by MnP produced by *B. adusta*.
Oxidation of Mn (II) by MnP was measured in the reaction mixture containing 2 mM $MnSO_4$, 50 mM Na-lactate buffer (pH 4.5), 50 μM H_2O_2 and 1.1 μg of MnP in a final volume of 1.0 ml. The reaction was started by the addition of H_2O_2 at room temperature and the spectra recorded every 30 s.

Table 1. Effect of organic acids on activity of MnP produced by *B. adusta*.

Organic acids	Relative activity (%)[a]
Lactic	100
Malonic	100
Citric	66
Tartaric	65
Acetic	41
Formic	18
Succinic	14
None (water)	27

a) The activity was measured as described in the Materials and Methods. Organic acid was added to final concentration of 50 mM.

Oxidation of Mn (II) to Mn (III) by MnP was confirmed spectrophotometrically by measuring the spectrum of the Mn (III)-lactate complex (Glen and Gold, 1985) (Fig. 7). This indicated that the separaed enzyme preparation was manganese peroxidase. The initial rate of guaiacol oxidation in the presence of various organic acids is shown in Table 1. High MnP activity was observed in the presence of lactic and malonic acids, which for MnP of *P. chrysosporium* are reported to bind to Mn (III) as the chelators (Wariishi et al., 1992).

The oxidation rate of various substrates by MnP was determined. As shown in Table 2, MnP of *B. adusta* oxidized various phenolics and aromatic amines including guaiacol, 2,6-dimethoxyphenol, catechol, *o*-aminophenol and *o*- and *p*-phenylenediamines. 2,2′-Azino-bis-(3-ethylbenzothiazoline-6-sulphonate) (ABTS) was also oxidized by MnP of *B. adusta*, whereas veratryl alcohol, a standard substrate for LiP, was not.

Purification and characterization of LiP of *B. adusta* LiP produced in the wood meal/wheat bran culture was purified by the following steps.

Step 1. Wood meal/wheat bran cultures of *B. adusta* grown on for 19 d (20 cultures) were extracted with 20 mM Na-succinate buffer (pH 4.5) as described in Materials and Methods (643 ml). Extracts of the culture

Table 2. Substrate specificity of MnP pruduced by *B. adusta*.

Substrates	Wavelength (nm)	Specific activity[a] (U/mg)
o-Phenylenediamine	440	534
p-Phenylenediamine	494	1000
o-Aminophenol	440	602
Catechol	398	1147
Guaiacol	465	193
2,6-Dimethoxyphenol	468	1727
Veratryl alcohol	310	0
ABTS[b]	415	546

a) The activity was measured as described in the Materials and Methods. Substrate was added to final concentration of 0.4 mM.
b) 2,2′-Azino-bis-(3-ethylbenzothiazoline-6-sulphonate).

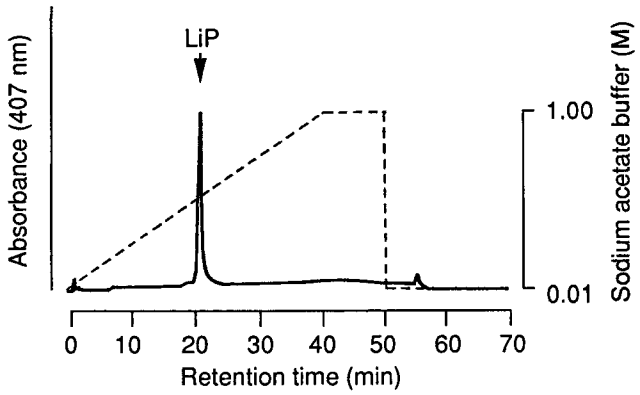

Fig. 8. Purification of LiP from crude extracts of the culture of *B. adusta* grown on wood meal/wheat bran culture. LiP was purified from filtrates of 19-d-old culture on Pharmacia Mono-Q (5/5) with Na-acetate buffer (pH 6.0) using a linear gradient of NaCl from 10 mM to 1 M. The elution rate was 1.0 ml/min and the eluent was monitored at 407 nm.

were concentrated and dialyzed five times every 2 h in a similar to that described for the separation of MnP. The dialysate contained 13.7 mg of protein with a specific activity of 4,600 U/mg and a total of 63,400 U.

Step 2. The dialyzed enzyme solution was subjected to ion-exchange chromatography on a Pharmacia Mono-Q column (5/5) in a manner similar to that described for the purification of MnP (Fig. 8). Active fractions obtained from 7 runs of chromatography were pooled and concentrated through a Centriprep-10 microconcentrator, then dialyzed against 20 mM Na-succinate buffer (pH 4.5). The final LiP preparation contained 1.2 mg of protein with a total of 25,600 U and a specific activity of 22,300 U/mg. The preparation gave a single band in SDS-PAGE (Fig. 6B).

The molecular weight of LiP was estimated to be 47,000 by SDS-PAGE. The p*I* of the enzyme was measured to be 3.5 by IEF. The absorption spectrum of the enzyme had a maximum at 407 nm with weak maxima at 500 and 630 nm (data not shown), indicating that this enzyme is a heme protein. This spectral property is similar to that of LiP of *P. chrysosporium* (Renganathan and Gold, 1986). The optimum pH and temperature of purified LiP was 2.5 and 35°C, respectively.

Production of ligninolytic enzymes by *B. adusta* using a solid fermenter Production of ligninolytic enzymes of *B. adusta* was examined using a rotary-solid fermenter (Fig. 9). The activity of three ligninolytic enzymes was measured periodically. The moisture concentration of the medium is thought to be important for the growth of mycelia and the production of ligninolytic enzymes. The incubation of *B. adusta* was independently carried out with medium containing three different starting moisture concentrations, 37%, 66% and 74%. The enzyme activity was calculated as the activity per 5 g dry weight of medium.

As shown in Fig. 9, MnP activiy was the highest in the culture in which the initial moisture concentration of the medium had been 74%, and the activity was reached

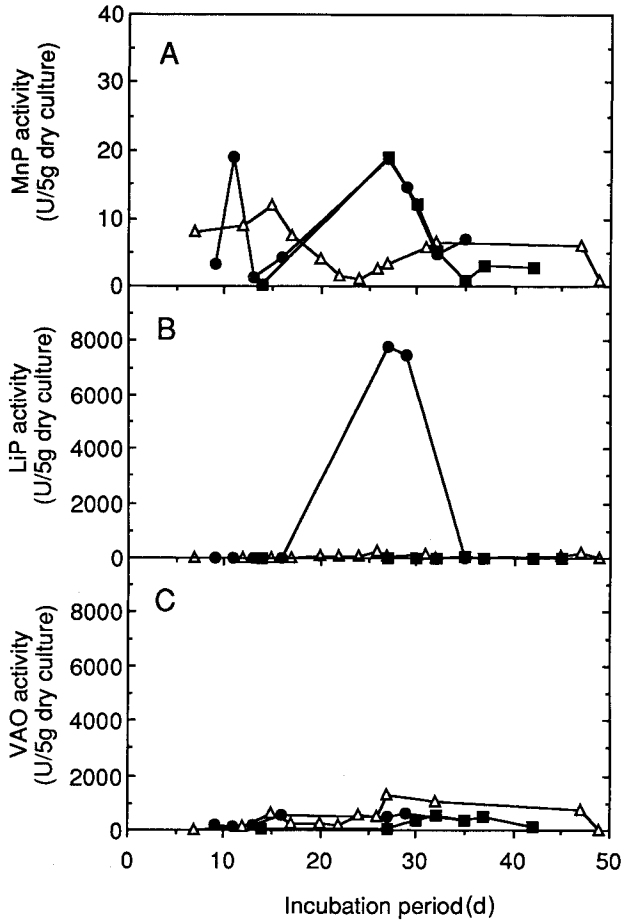

Fig. 9. Effect of starting moisture concentrations on production of LiP and MnP by *B. adusta* in a rotary solid fermenter. Cultivation was carried out in wood meal/wheat bran culture containing 74% (–●–), 66% (–△–) and 37% (–■–) water. The activities of MnP (A), LiP (B) and VAO (C) were measured periodically as described in the Materials and Methods.

maximum on days 11 and 19. Interestingly, the activity of MnP in 37% of moisture (19.5 U/5g) was found to be almost the same as that in 74% of moisture (19.0 U/5g). LiP activity was found to be 2,000 U/5 g of dry culture in 27-d-old cultures in which the initial moisture concentration had been 74%, whereas activity was negligibly low in 27-d-old cultures that initially had 66% and 37% moisture concentrations. These results suggest that the moisture concentration of the culture affects the production of LiP by *B. adusta*.

However, moisture concentration is not thought to significantly affect the production of MnP by *B. adusta*. Furthermore, the highest VAO activity, 1,123 U/5 g of dry weight, was observed in 27-d-old cultures in which the initial moisture concentration had been 66%.

Discussion

Ligninolytic enzymes produced by *B. adusta* grown on wood meal/wheat bran culture It is not clear whether the production of ligninolytic enzymes in solid wood cul-

ture is similar to or different from production in conventional liquid cultures.

In solid lignocellulosic medium, MnP was reported to be the major phenol oxidizing enzyme produced by *P. chrysosporium* (Datta et al., 1991). MnP was also produced dominantly by *L. edodes* and *P. ostreatus* in wood meal/wheat bran cultures, whereas no or negligibly low LiP and laccase activity was detected (Kofujita et al., 1992). These results suggest that MnP plays an important role in lignin biodegradation in solid substrates (Kofujita et al., 1992). Interestingly, however, the present study showed that *B. adusta* produced both MnP and LiP in solid culture, reflecting the important role of LiP, in addition to MnP, in lignin degradation by *B. adusta*. The period of cultivation in which this fungus produced the enzymes was shorter than that of previous experiments involving *L. edodes* and *P. ostreatus*, in which the maximum MnP activity was achieved after 80 to 90 days of culture (Kofujita et al., 1992). These results indicated that *B. adusta* can be used for large-scale production of ligninolytic enzymes.

The ligninolytic enzymes produced by *B. adusta* grown on wood meal/wheat bran culture were separated and characterized. Datta et al. (1991) reported that the dominant MnP isozyme from lignocellulosic cultures of *P. chrysosporium* was different from that of liquid cultures. Similar results were also obtained in wood meal cultivation of *P. chrysosporium* (Kofujita et al., 1992). Kimura et al. (1994) reported that *B. adusta* produced 4 LiP isozymes in liquid culture. In the present analysis, anion exchange chromatography followed by Mono-Q HPLC showed that the LiP produced in the wood meal/wheat bran culture of *B. adusta* consisted of a single protein. IEF analysis suggested that the LiP produced in the solid culture corresponded to LPO-4 of the 4 isozymes produced in liquid culture (Kimura et al., 1990). Kimura et al. (1990) reported that southern hybridization analysis indicated the presence of multiple LiP genes in the chromosomal DNAs of *B. adusta*. In addition, different types of gene and cDNA of MnP of *B. adusta* were also cloned (Kimura et al., 1991). Therefore, *B. adusta* is thought to have multiple genes for LiP isozymes. These results suggest that the mechanism of expression of genes encoding LiP depends on growth conditions, as discussed by Datta et al. (1991).

Production of ligninolytic enzymes using a fermenter

For the industrial utilization of ligninolytic enzymes, development of large-scale production techniques is indispensable.

Previously, various approaches have been tried in an attempt to increase ligninolytic enzymes production using *P. chrysosporium*. For example, addition of the detergents Tweens 20 and 80 and oleic acid to stationary surface cultures was found to enhance the production of these enzymes (Jäger et al., 1985; Asther et al., 1987). Flushing of the culture with molecular oxygen was found to promote the production of enzymes (Bar-Lev and Kirk, 1981; Faison and Kirk, 1985). Production of LiP by immobilized mycelia has also been examined (Kirkpatrick and Palmer, 1987; Capdevila et al., 1989; Laugero et al., 1996). Several types of bioreactors have been used to prepare ligninolytic enzymes from liquid cultures (Kirk et al., 1986; Linko, 1988; Laugero et al., 1996).

The production of various hydrolase enzymes including amylases, proteases and others has been traditionally performed using solid substrate cultures of *Aspergillus oryzae* (Ahlburg) Cohn and other Aspergilli (Narahara et al., 1982; Madamwar et al., 1989). Cellulases are also produced in solid substrate culture fermenters (Dueñas et al., 1995). However, there have been no attempts at large-scale production of ligninolytic enzymes in solid substrate culture fermenters. In this study, *B. adusta* was cultivated in a rotary-solid fermenter, and the enzyme activity produced was compared with that produced in stationary flask cultures.

As shown in Fig. 9, the activity of MnP was found to be significantly higher than that of LiP and VAO. The production of LiP was affected by the starting moisture concentration in the medium, whereas MnP and VAO production was not. The activity of MnP produced in solid statically grown flask cultures was 17 U/flask (5 g culture), while that in the rotary fermenter, which contained 162 g of wood meal culture, was 19 U/5 g culture. In total 616 U of MnP was produced per fermenter. Therefore, the MnP activity produced in one fermenter operation corresponds to that produced in 32 flasks. These results indicate that this type of fermenter culture could be employed for the production of MnP.

Rotation of the culture vessel, which may result in the shear of growing mycelia, must be limited. On the other hand, rotation is thought to be important to homogenize the growth of the mycelium and to distribute the enzyme into the medium. In the present experiment, the culture vessel was rotated periodically in short time intervals, in order to avoid mechanical inhibition of the produced enzymes (Venkatadri and Irvine, 1990; Laugero et al., 1996), effects on intracellular metabolism of mycelium (Shimada et al., 1981; Bonnarme et al., 1993) or liberation of inhibitory substances by morphological damage to the growing mycelia (Bonnarme et al., 1993). Further investigations to optimize the parameters of the operation of the fermenter, such as rotation speed and duration, as well as optimizing the culture medium will be required.

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