Applied and Environmental Microbiology, Dec. 1993, p. 4031–4036 Vol. 59, No. 12
0099-2240/93/124031-06$02.00/0
Copyright © 1993, American Society for Microbiology

Stimulation of Ligninolytic Peroxidase Activity by Nitrogen Nutrients in the White Rot Fungus *Bjerkandera* sp. Strain BOS55

ERWIN E. J. KAAL, ED DE JONG, AND JIM A. FIELD*

Division of Industrial Microbiology, Department of Food Science, P.O. Box 8129, Wageningen Agricultural University, 6700 EV Wageningen, The Netherlands

Received 22 June 1993/Accepted 8 September 1993

***Bjerkandera* sp. strain BOS55, a newly isolated wild-type white rot fungus, produced lignin peroxidase (LiP) in nitrogen (N)-sufficient glucose-peptone medium, whereas no LiP was detectable in N-limited medium. The production of LiP was induced by the peptide-containing components of this medium and also by soy bean protein. Furthermore, the production of manganese-dependent peroxidase was stimulated by organic N sources, although lower production was also evident in N-limited medium. Further research showed that the induction of LiP depended on the combination of pH and the type of N source. An amino acid mixture and ammonium induced LiP only at either pH 6 or 7.3, respectively. Peptone induced LiP activity at all pH values tested; however, the highest activity was observed at pH 7.3. The results presented here indicate that *Bjerkandera* spp. are distinct from the model white rot fungus, *Phanerochaete chrysosporium*, which produces ligninolytic peroxidases in response to N limitation.**

Only a few groups of microorganisms are capable of degrading complex lignin polymers, and they are best exemplified by the white rot fungi, which cause the greatest degree of mineralization. The degradation of lignin by these fungi has aroused interest in its potential applications, such as biopulping (5) and removal of environmental pollutants (16, 17).

The white rot fungus *Phanerochaete chrysosporium* has been used extensively as a model organism to study the physiological requirements for lignin biodegradation (ligninolysis) (23). The extracellular machinery involved in lignin and xenobiotic degradation by *P. chrysosporium* is composed of lignin peroxidases (LiPs) and manganese-dependent peroxidases (MnPs), as well as H_2O_2-producing oxidases (23).

In liquid cultures of *P. chrysosporium*, lignin is degraded only during secondary (idiophasic) metabolism (5, 23), which is triggered by limitation of N (21, 25), C, or S (20). Likewise, *P. chrysosporium* has been found to produce ligninolytic peroxidases only in response to nutrient depletion (13, 19, 38). Ligninolysis and ligninolytic peroxidase production in *P. chrysosporium* are suppressed and delayed by high concentrations of N (5, 13, 15, 21, 25, 35). The fact that this fungus is ligninolytic while starving means that it produces only small amounts of LiP for commercial applications (29). The results obtained in defined cultures of *P. chrysosporium* have been widely accepted as the paradigm for the physiology of ligninolysis among most white rot fungi (23). However, lignin mineralization by some white rot fungi ([^{14}C]-lignin to $^{14}CO_2$), e.g., *Pleurotus ostreatus* (18, 27), *Lentinus edodes* (27), and an unidentified coprophilous basidiomycete (18), is not repressed by high N concentrations. With some mutants of *P. chrysosporium*, e.g., INA-12 (6, 36) and PSBL-1 (29, 40), the LiP production was even stimulated by N. Others have observed that in unsubmerged cultures of wild-type *P. chrysosporium*, immobilized on pieces of polyurethane foam, the level of LiP activity increased at very high ammonium concentrations (7, 12).

Results from the screening of many white rot fungi by using the N-limited BIII medium (39) indicate that many white rot fungi have no detectable LiP activity (2, 9, 28, 41). Curiously, some of these LiP-negative white rot fungi do possess the LiP genes (22, 37). One such fungus, *Bjerkandera adusta*, only produces LiP in a rich glucose-peptone medium (22), whereas almost no LiP activity was observed in N-limited BIII media (9, 22, 41).

The objective of this study was to determine which component(s) of the glucose-peptone medium was responsible for inducing the production of LiP and other ligninolytic enzymes in *Bjerkandera* sp. strain BOS55. This strain, which was isolated from decaying wood buried in the litter layer of a beech forest, is an outstanding degrader of polycyclic aromatic hydrocarbons (16). Aside from LiP, the fungus produces MnP (9) and a novel manganese-independent peroxidase (MIP) (10).

MATERIALS AND METHODS

Microorganism. *Bjerkandera* sp. strain BOS55 was isolated from forest soil samples with a selective medium containing hemp (*Cannabis sativa* L.) stem wood, guaiacol, and benomyl (9). The fungus was maintained on malt extract agar plates (per liter: 15.0 g of agar, 3.5 g of malt extract, and 5.0 g of glucose) at 4°C, from which they were transferred to new malt extract agar plates and incubated at 30°C for 4 to 6 days before use in experiments in this study. In all cases, single 6-mm-diameter mycelium-agar plugs (obtained along a uniform circumference) from the plates were used as the inocula.

Media. The standard basal medium used in the experiments was N limited (2.2 mM NH_4^+-N; added as diammonium tartrate) BIII mineral medium (39), with 10 g of glucose liter^{-1} as the primary substrate and 2 mg of thiamine liter^{-1} in a 20 mM 2,2-dimethylsuccinate (pH 4.5) buffer. Different media were prepared with extra N from various sources and

* Corresponding author.

set at several pH values. The extra N sources used were a mixture of 20 L-amino acids (Ala, Arg, Asn, Asp, Cys, Gly, Glu, Gln, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val), peptone, yeast extract, soybean protein, diammonium tartrate, or a combination of these. The media with the mixture of L-amino acids contained a final concentration of 0.25 g of each amino acid liter^{-1}. The media were adjusted to pH values of 4.5, 6.0, or 7.3 by NaOH addition.

The glucose-peptone medium used in the experiments was modified from that of Kimura et al. (22) and contained (per liter) 10 g of glucose, 5 g of peptone, 2 g of yeast extract, 1 g of KH_2PO_4, and 0.5 g of $MgSO_4 \cdot 7H_2O$ (pH 4.5).

All media were filter sterilized (Schleicher & Schuell FP 030/3; pore size, 0.2 μm).

Culture conditions. Aliquots (5 ml) of media were placed in loosely capped 250-ml serum bottles. The bottles were incubated statically under an air atmosphere at 30°C in complete darkness. The 250-ml serum bottles used for CO_2 evolution measurements, on the contrary, were sealed with gas-impermeable rubber septa. The headspaces of the cultures were aseptically flushed with air at the time of inoculation and after each gas sampling.

Enzyme assays. LiP activity was assayed with veratryl alcohol as described by Tien and Kirk (39) but was corrected for veratryl alcohol oxidase activity (i.e., activity in the absence of H_2O_2). MnP activity was measured by a method modified from that of Pasczynski et al. (30). The reaction mixture contained 50 mM sodium malonate (pH 4.5), 1 mM 2,6-dimethoxyphenol, 1 mM $MnSO_4$, and up to 600 μl of culture broth in a total volume of 1 ml. The reaction was initiated by adding 0.4 mM H_2O_2, and MnP was corrected for laccase activity and for MIP activity. MIP activity was measured by a method modified from that of de Jong et al. (10). The reaction mixture contained 50 mM sodium malonate (pH 4.5), 1 mM 2,6-dimethoxyphenol, 1 mM EDTA, and up to 550 μl of culture broth in a total volume of 1 ml. The reaction was initiated by the addition of 0.4 mM H_2O_2.

Carbon dioxide analysis. Carbon dioxide evolution was determined in the headspace by gas chromatography with a Packard 427 (Palo Alto, Calif.) apparatus fitted with a thermal conductivity detector (140°C). The column (Hayesep Q; Chrompack, Middelburg, The Netherlands) was maintained at 110°C, and helium was used as a carrier gas (30 ml min^{-1}). The injection port was maintained at 110°C. The injection volume was 100 μl.

Chemicals. Mycological peptone (9.5% total nitrogen), yeast extract (11.0% total nitrogen), and soybean protein (14.7% total nitrogen) were obtained, respectively, from Oxoid Ltd. (Basingstoke, Hampshire, England), Life Technologies Ltd. (Paisley, United Kingdom), and Purina Protein Europe (Brussels, Belgium). The L-amino acids were obtained from either Merck (Darmstadt, Germany) or Janssen Chimica (Geel, Belgium). All other chemicals were commercially available and were used without any further purification.

TABLE 1. Effects of various N supplements on the ligninolytic enzyme activity and the CO_2 production at pH 4.5

Day and medium[a]	Cumulative CO_2 (μmol)	Activity (nmol min^{-1} ml^{-1})		
		MnP	MIP	LiP
Day 5				
B-g	120 ± 8	113 ± 34	11 ± 1	0
B-G	116 ± 22	74 ± 27	12 ± 2	0
B-g-N	143 ± 12	220 ± 27	25 ± 5	0
B-g-S	647 ± 77	849 ± 42	49 ± 5	11 ± 3
B-g-Y	395 ± 48	542 ± 76	25 ± 6	6 ± 2
B-g-P	706 ± 43	1,239 ± 184	57 ± 4	9 ± 2
B-g-P-Y	698 ± 71	1,486 ± 200	127 ± 40	12 ± 2
g-Y	311 ± 27	40 ± 12	23 ± 6	13 ± 2
g-P-Y	813 ± 74	461 ± 54	222 ± 1	69 ± 11
Day 7				
B-g	203 ± 15	240 ± 37	16 ± 6	0
B-G	209 ± 32	149 ± 31	16 ± 1	0
B-g-N	335 ± 64	ND[b]	ND	0
B-g-S	1,369 ± 30	998 ± 152	36 ± 2	26 ± 2
B-g-Y	815 ± 19	448 ± 88	13 ± 4	0
B-g-P	1,235 ± 56	630 ± 154	65 ± 6	41 ± 4
B-g-P-Y	1,371 ± 42	882 ± 182	141 ± 13	68 ± 31
g-Y	608 ± 59	8 ± 7	15 ± 7	0
g-P-Y	1,390 ± 69	217 ± 21	150 ± 22	67 ± 10
Day 12				
B-g	400 ± 8	192 ± 46	11 ± 3	0
B-G	417 ± 57	254 ± 18	13 ± 3	0
B-g-N	1,098 ± 57	ND	ND	0
B-g-S	1,870 ± 46	306 ± 131	98 ± 17	63 ± 4
B-g-Y	1,409 ± 35	70 ± 10	6 ± 5	0
B-g-P	1,745 ± 76	223 ± 79	53 ± 1	29 ± 6
B-g-P-Y	1,923 ± 92	480 ± 104	108 ± 38	61 ± 20
g-Y	1,084 ± 62	1 ± 1	4 ± 4	0
g-P-Y	1,739 ± 120	63 ± 30	41 ± 10	14 ± 4

[a] B, BIII plus 2.2 mM NH_4^+-N; g, 10 g of glucose per liter; G, 20 g of glucose per liter; N, 34.0 mM NH_4^+-N; S, 5 g of soybean protein (52.5 mM N) per liter; P, 5 g of peptone (34.0 mM N) per liter; Y, 2 g of yeast extract (15.7 mM N) per liter.

[b] ND, not determined.

RESULTS

The effect of various N supplements on the production of ligninolytic enzymes (LiP, MnP, and MIP) and the metabolic activity (as indicated by the CO_2 production) after 5, 7, and 12 days are shown in Table 1. All of the N supplements caused higher CO_2 production. Extra glucose, instead of extra N, did not stimulate the metabolic activity. The activity of MnP and MIP was stimulated by organic forms of N. The BIII, present in the basal medium, greatly stimulated the production of MnP, whereas the production of LiP was only delayed by BIII. The LiP activity was induced by all of the organic N sources (peptone, yeast extract, and soybean protein). No LiP activity was detected in the culture fluids of the basal medium and the basal media supplemented with either extra diammonium tartrate (NH_4^+-N) or extra glucose (Table 1).

Peptone, yeast extract, and soybean protein are undefined sources of organic N. Twenty L-amino acids were screened for inducing LiP activity in the basal medium (pH 4.5), in order to find a defined organic nitrogen source which can induce LiP. Only L-asparagine induced LiP activity at pH 4.5 (results not shown). L-Histidine also induced LiP activity, but only when the initial pH of the medium was uncorrected (pH 6.0) and not when set at pH 4.5 (results not shown). Apparently, both the type of N source and the pH effects were very important.

The role of N supplements at different pH values on the CO_2 production and LiP activity was evaluated. The CO_2 production was almost not influenced by the initial pH and the kind of N nutrient that was supplemented. The amount of N, on the contrary, was of great importance for the CO_2 production. The N-sufficient media supported distinctly higher metabolic rates compared with those in the low-

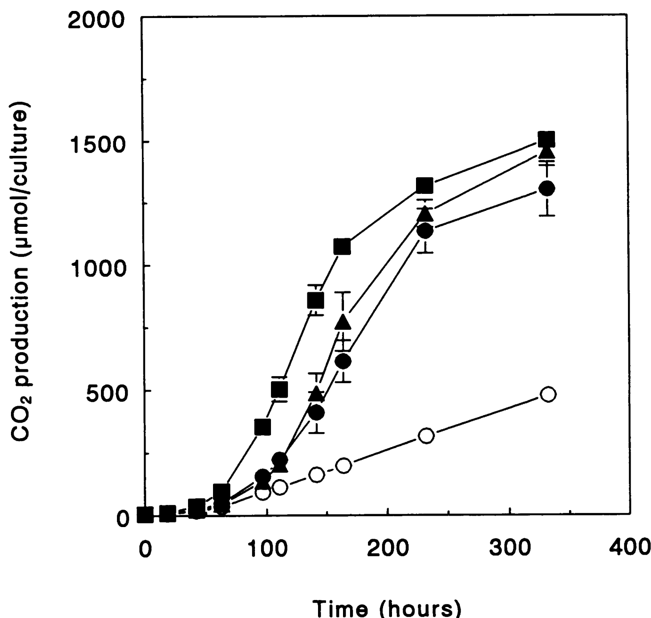

FIG. 1. Effects of various N sources at pH 6 supplemented to the basal medium (2.2 mM NH_4^+-N) on the CO_2 production: ○, no supplement; ●, 34.0 mM NH_4^+-N; ■, 5 g of peptone (34.0 mM N) per liter; and ▲, 5 g of L-amino acid mixture (57.1 mM N) per liter.

ammonium N basal medium, as shown in Fig. 1 for pH 6, confirming that the basal medium was N limited. Figure 2A and B show that in the NH_4^+-N-containing media, LiP was only detected in those with an initial pH of 7.3. The peak LiP activity in the medium supplemented with 34.0 mM NH_4^+-N occurred at day 5, while a lower peak occurred at day 8 in the basal medium containing 2.2 mM NH_4^+-N. In the media supplemented with peptone, the first and also highest LiP activity peak was also observed in the pH 7.3 medium and occurred at day 5. However, high levels of LiP activity were also produced in the peptone media at lower pH values. In the media supplemented with the mixture of twenty L-amino acids, high LiP activity was only found in the medium with an initial pH of 6.0. The LiP activity peak in this medium occurred at day 5. The pH during the experiment is given in Fig. 3. The pH, initially set at pH 6.0 and 7.3, gradually declined, so that by day 10 the pH values in all treatments were in the range of 4.5 to 5.5. However, on day 5, when LiP activity first appeared, the pH had only declined by about 0.5 and 1.0 U in the media with starting pH values of 6.0 and 7.3, respectively.

During the pH experiment, the production of MnP and MIP was also monitored (results not shown). Again, it was found that organic nitrogen sources stimulate the MnP and MIP production. Very high stimulation of MnP production occurred under those conditions that induced LiP production, provided that the N sources were organic.

DISCUSSION

According to the present-day paradigm, white rot fungi produce only ligninolytic enzymes in response to nutrient limitation. The most-studied system is the triggering of LiP production by N depletion (5, 23). However, Kimura et al. (22) found that wild-type *B. adusta* produced LiP only in an organic N-rich medium. In accordance with their results, organic N-rich media induced LiP production in *Bjerkandera* sp. strain BOS55. The LiP production could also be induced by soybean protein and yeast extract, but not with high NH_4^+-N at pH 4.5. From these results it can be concluded that the production of LiP was induced by peptides and proteins and not by the vitamins or by the minerals in the glucose-peptone medium. These components also stimulated the production of MnP and MIP, although some production of MnP and MIP was observed in the basal medium. Extra glucose did not stimulate CO_2 production, whereas all of the nutrient nitrogen supplements did; the growth therefore in the basal medium was truly N limited. Under the N-limited conditions of this study and other studies (9, 22, 41), no LiP activity was found in *Bjerkandera* spp., indicating a distinct regulation of LiP production in this genus compared with that in *Phanerochaete chrysosporium* with respect to the depletion of nitrogen nutrients.

In wild-type strains of *P. chrysosporium*, high levels of complex N nutrients such as peptone, yeast extract, and proteins do not cause drastic repressions in the ligninolytic activity ([^{14}C]lignin to $^{14}CO_2$) (33, 34). In some cases, the complex N nutrients stimulate the initial rate of lignin mineralization compared with that in N-limited basal medium (33, 34). Assuming that peroxidases were required for the observed ligninolysis (24), *P. chrysosporium* probably produces ligninolytic peroxidases in the presence of complex organic N nutrients, as we and Kimura et al. (22) have found for *Bjerkandera* spp. Dosoretz et al. (12) observed that unsubmerged cultures of *P. chrysosporium* overproduced LiP (800 nmol liter^{-1} min^{-1}) in medium with very high NH_4^+-N (45 mM) when the carbon became limiting, corresponding to the moment when the fungus' own endogenous nitrogen was being degraded. The complex organic nitrogen supplements could have a role in imitating the conditions that the fungus encounters during C limitation. To test this hypothesis, future investigations should determine whether amino acids and peptides released from the autolysis of cell constituents can induce LiP production.

The production of LiP in *Bjerkandera* sp. strain BOS55 was pH dependent. At pH 4.5, asparagine was the only L-amino acid that induced the production of LiP. Histidine induced LiP production at pH 6.0 but not at pH 4.5. The pH greatly influenced the LiP production in media supplemented with NH_4^+-N, a mixture of amino acids, or peptone. In contrast, the pH had no effect on the metabolic activity of the fungus as evidenced from the CO_2 production data. Roche et al. (36) conducted research on the effect of pH and 13 mM L-asparagine on the LiP production by the mutant strain *P. chrysosporium* INA-12. The highest LiP activity was detected at pH 5.4. These findings suggest that LiP activity induction in N-deregulated mutants of *P. chrysosporium* is also pH dependent.

The BIII mineral nutrient solution, present in the basal medium, greatly stimulated the production of MnP. BIII contains Mn(II), and thus increased concentrations of manganese probably led to increased MnP production, as was found previously with other white rot fungi (2, 4, 31). Some researchers reported that small additions of manganese also stimulate LiP production (4, 23); however, others report that high manganese concentrations inhibited LiP production (2, 31). In this study it was observed that the production of LiP was only delayed by the presence of BIII.

MnP production in *Bjerkandera* sp. strain BOS55 was greatly stimulated in media containing peptone or the amino acid mixture under those conditions that caused high pro-

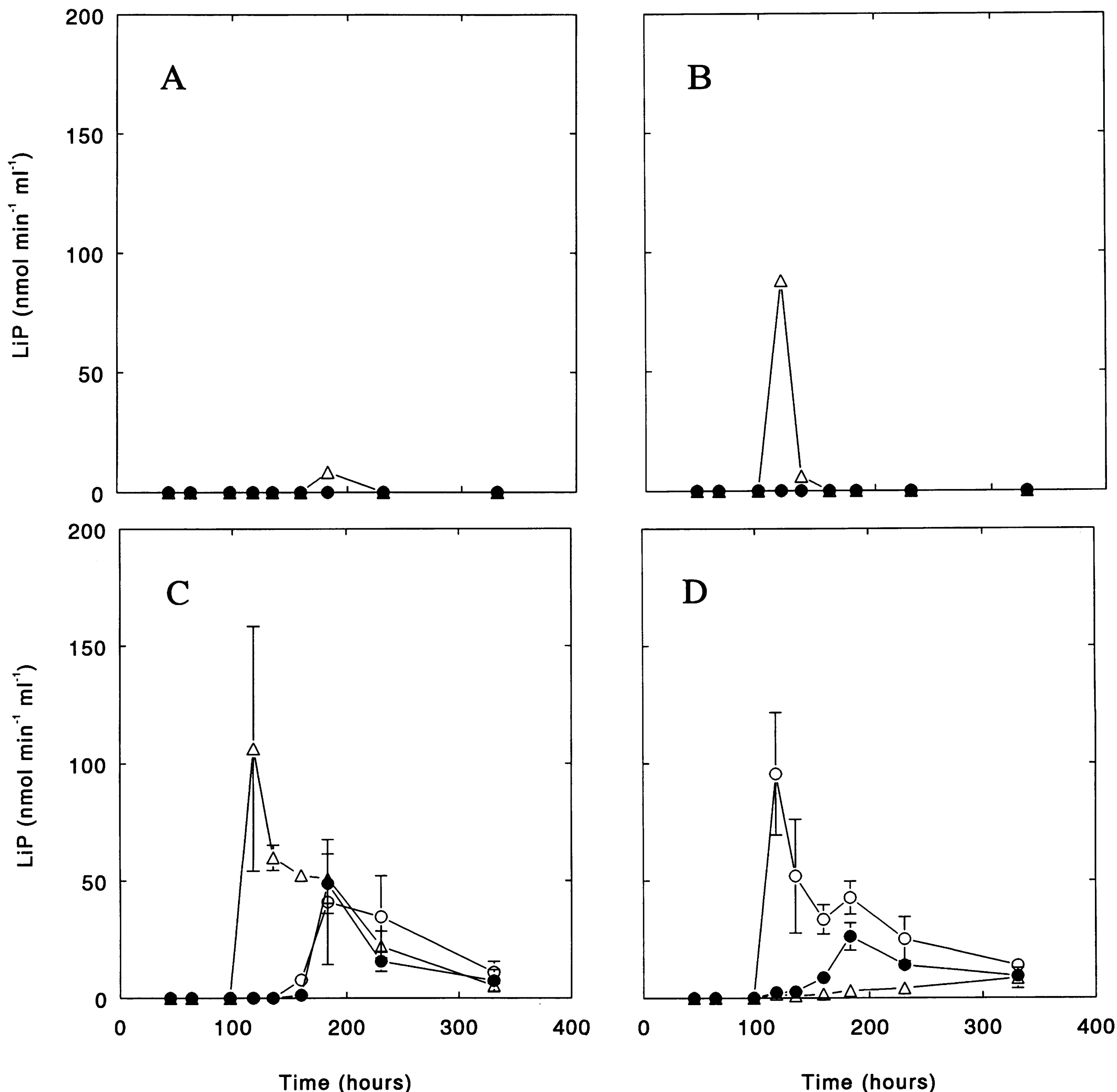

FIG. 2. Effects of various N sources (none [A], 34.0 mM NH_4^+-N [B], 5 g of peptone [34.0 mM N] per liter [C], and 5 g of L-amino acid mixture [57.1 mM N] per liter [D]) supplemented to the basal medium (2.2 mM NH_4^+-N) on LiP production. The supplements were tested with three different initial pH values of the media; pH 4.5 (●), pH 6.0 (○), and pH 7.3 (△).

ductions of LiP as well. A possible explanation is a parallel regulation of the LiP and MnP production in response to organic N. However, it is possible that the extra MnP activity is only an artifact, since, LiP, together with veratryl alcohol, is able to oxidize Mn(II) to Mn(III) in vitro (3, 32). Veratryl alcohol is produced by *Bjerkandera* sp. strain BOS55 (11).

Bjerkandera spp. are wild-type strains that produce LiP in response to N-sufficient conditions. Since the growth is also stimulated by N, upon further optimization it may be possible to obtain commercially interesting yields of the ligninolytic enzymes. However, the importance of N stimulation in nature is not clear, because the N content of most wood is very low (14). Certain conditions in which the fungi could have access to high levels of N could occur naturally. Some N_2-fixing bacteria are associated with the major decay fungi in wood (1, 26). Cowling and Merrill (8) suggested that nitrogen from in situ N_2 fixation might supplement the existing and meager N resources in wood available to the fungi. Furthermore, lots of wood being decayed by white rot fungi is buried in the nitrogen-rich litter layer of forest soils.

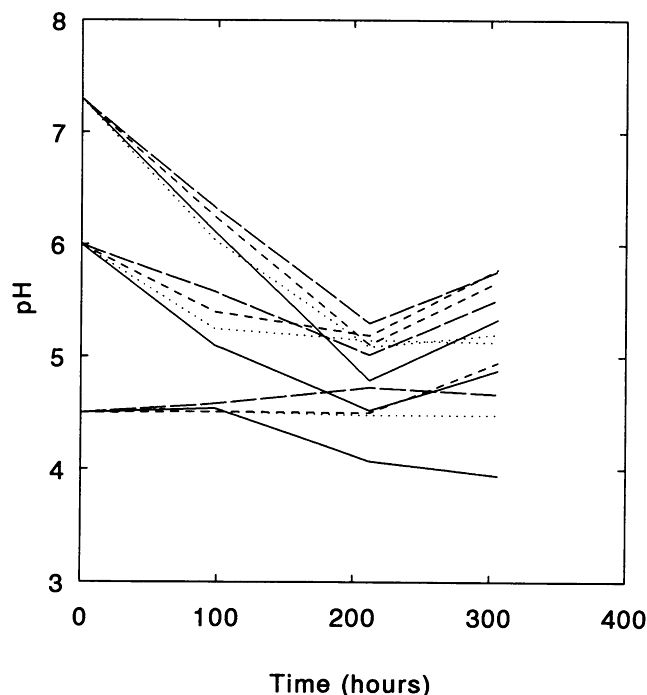

FIG. 3. The pH of the basal media supplemented with various nutrient nitrogen sources that were initially set at 4.5, 6.0, or 7.3. The supplements were none (······), 34.0 mM NH_4^+-N (——), 5 g of peptone per liter (— — —), and 5 g of L-amino acid mixture per liter (- - -).

ACKNOWLEDGMENT

The work presented in this report was funded in part by the Royal Netherlands Academy of Arts and Sciences.

REFERENCES

1. **Aho, P. E., R. J. Seidler, H. J. Evans, and P. N. Raju.** 1974. Distribution, enumeration, and identification of nitrogen-fixing bacteria associated with decay in living white fir trees. Phytopathology **64:**1413–1420.
2. **Bonnarme, P., and T. W. Jeffries.** 1990. Mn(II) regulation of lignin peroxidases and manganese-dependent peroxidases from lignin-degrading white rot fungi. Appl. Environ. Microbiol. **56:**210–217.
3. **Bono, J., P. Goulas, J. Boe, N. Portet, and J. Seris.** 1990. Effect of Mn(II) on reactions catalyzed by lignin peroxidase from *Phanerochaete chrysosporium*. Eur. J. Biochem. **192:**189–193.
4. **Brow, J. A., J. K. Glenn, and M. H. Gold.** 1990. Manganese regulates expression of manganese peroxidase by *Phanerochaete chrysosporium*. J. Bacteriol. **172:**3125–3130.
5. **Buswell, J. A.** 1992. Fungal degradation of lignin, p. 425–480. *In* D. K. Arora, B. Rai, K. G. Mukerji, and G. R. Knudsen (ed.), Handbook of applied mycology, vol. 1. Soil and plants. Marcel Dekker, Inc., New York.
6. **Buswell, J. A., B. Mollet, and E. Odier.** 1984. Ligninolytic enzyme production by *Phanerochaete chrysosporium* under nitrogen suffiency. FEMS Microbiol. Lett. **25:**17–22.
7. **Chen, A. H. C., C. G. Dosoretz, and H. E. Grethlein.** 1991. Ligninase production by immobilized cultures of *Phanerochaete chrysosporium* grown under nitrogen-sufficient conditions. Enzyme Microb. Technol. **13:**404–407.
8. **Cowling, E. B., and W. Merrill.** 1966. Nitrogen in wood and its role in wood deterioration. Can. J. Bot. **44:**1533–1544.
9. **de Jong, E., F. P. de Vries, J. A. Field, R. P. van der Zwan, and J. A. M. de Bont.** 1992. Isolation and screening of basidiomycetes with high peroxidative activity. Mycol. Res. **96:**1098–1104.
10. **de Jong, E., J. A. Field, and J. A. M. de Bont.** 1992. Evidence for a new extracellular peroxidase: manganese-inhibited peroxidase from the white rot fungus *Bjerkandera* sp. BOS55. FEBS Lett. **299:**107–110.
11. **de Jong, E., J. A. Field, J. A. F. M. Dings, J. B. P. A. Wijnberg, and J. A. M. de Bont.** 1992. *De-novo* biosynthesis of chlorinated aromatics by the white rot fungus *Bjerkandera* sp. BOS55: formation of 3-chloro-anisaldehyde from glucose. FEBS Lett. **305:**220–224.
12. **Dosoretz, C. G., N. Rothschild, and Y. Hadar.** 1993. Overproduction of lignin peroxidase by *Phanerochaete chrysosporium* (BKM-F) under nonlimiting nutrient conditions. Appl. Environ. Microbiol. **59:**1919–1926.
13. **Faison, B. D., and T. K. Kirk.** 1985. Factors involved in the regulation of a ligninase activity in *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **49:**299–304.
14. **Fengel, D., and G. Wegener (ed.).** 1989. Wood: chemistry, ultrastructure, reactions. Walter de Gruyter & Co., Berlin.
15. **Fenn, P., and T. K. Kirk.** 1981. Relationship of nitrogen to the onset and suppression of ligninolytic activity and secondary metabolism in *Phanerochaete chrysosporium*. Arch. Microbiol. **130:**59–65.
16. **Field, J. A., E. de Jong, G. F. Costa, and J. A. M. de Bont.** 1992. Biodegradation of polycyclic aromatic hydrocarbons by new isolates of white rot fungi. Appl. Environ. Microbiol. **58:**2219–2226.
17. **Field, J. A., E. de Jong, G. F. Costa, and J. A. M. de Bont.** 1993. Screening for ligninolytic fungi applicable to the biodegradation of xenobiotics. Tibtech. **11:**44–49.
18. **Freer, S. N., and R. W. Detroy.** 1982. Biological delignification of ^{14}C-labeled lignocelluloses by basidiomycetes: degradation and solubilization of the lignin and cellulose components. Mycologia **74:**943–951.
19. **Gold, M. H., M. Kuwahara, A. A. Chiu, and J. K. Glenn.** 1984. Purification and characterization of an extracellular H_2O_2-requiring diarylpropane oxygenase from the white rot basidiomycete, *Phanerochaete chrysosporium*. Arch. Biochem. Biophys. **234:**353–362.
20. **Jeffries, T. W., S. Choi, and T. K. Kirk.** 1981. Nutritional regulation of lignin degradation by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **42:**290–296.
21. **Keyser, P., T. K. Kirk, and J. G. Zeikus.** 1978. Ligninolytic enzyme system of *Phanerochaete chrysosporium*: synthesized in the absence of lignin in response to nitrogen starvation. J. Bacteriol. **135:**790–797.
22. **Kimura, Y., Y. Asada, and M. Kuwahara.** 1990. Screening of basidiomycetes for lignin peroxidase genes using a DNA probe. Appl. Microbiol. Biotechnol. **32:**436–442.
23. **Kirk, T. K., and R. L. Farrell.** 1987. Enzymatic "combustion", the microbial degradation of lignin. Annu. Rev. Microbiol. **41:**465–505.
24. **Kirk, T. K., and K. E. Hammel.** 1992. What is the primary agent of lignin degradation in white-rot fungi?, p. 535–540. *In* M. Kuwahara and M. Shimada (ed.), Biotechnology in pulp and paper industry. Uni Publishing Co., Ltd., Tokyo.
25. **Kirk, T. K., E. Schultz, W. J. Connors, L. F. Lorenz, and J. G. Zeikus.** 1978. Influence of culture parameters on lignin metabolism by *Phanerochaete chrysosporium*. Arch. Microbiol. **117:**277–285.
26. **Larsen, M. J., M. F. Jurgensen, and A. E. Harvey.** 1978. N_2 fixation associated with wood decayed by some common fungi in western Montana. Can. J. For. Res. **8:**341–345.
27. **Leatham, G. F., and T. K. Kirk.** 1983. Regulation of ligninolytic activity by nutrient nitrogen in white-rot basidiomycetes. FEMS Microbiol. Lett. **16:**65–67.
28. **Nerud, F., Z. Zouchová, and Z. Mišurcová.** 1991. Ligninolytic properties of different white-rot fungi. Biotechnol. Lett. **13:**657–660.
29. **Orth, A. B., M. Denny, and M. Tien.** 1991. Overproduction of lignin-degrading enzymes by an isolate of *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **57:**2591–2596.
30. **Pasczynski, A., R. L. Crawford, and V. B. Huynh.** 1988. Manganese peroxidase of *Phanerochaete chrysosporium*: puri-

fication. Methods Enzymol. **161**:264–270.

31. **Perez, J., and T. W. Jeffries.** 1992. Roles of manganese and organic acid chelators in regulating lignin degradation and biosynthesis of peroxidases by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **58**:2402–2409.
32. **Popp, J. L., B. Kalyanaraman, and T. K. Kirk.** 1990. Lignin peroxidase oxidation of Mn^{2+} in the presence of veratryl alcohol, malonic or oxalic acid, and oxygen. Biochemistry **29**:10475–10480.
33. **Reid, I. D.** 1983. Effects of nitrogen supplements on degradation of aspen wood lignin and carbohydrate components by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **45**:830–837.
34. **Reid, I. D.** 1983. Effects of nitrogen sources on cellulose and synthetic lignin degradation by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **45**:838–842.
35. **Reid, I. D.** 1991. Nutritional regulation of synthetic lignin (DHP) degradation by *Phlebia* (*Merulius*) *tremellosa*: effects of nitrogen. Can. J. Bot. **69**:156–160.
36. **Roche, P., J. A. Buswell, R. B. Cain, and E. Odier.** 1989. Lignin peroxidase production by strains of *Phanerochaete chrysosporium* grown on glycerol. Appl. Microbiol. Biotechnol. **31**:587–591.
37. **Rüttimann, C., E. Schwember, L. Salas, D. Cullen, and R. Vicuña.** 1992. Ligninolytic enzymes of the white rot basidiomycetes *Phlebia brevispora* and *Ceriporiopsis subvermispora*. Biotechnol. Appl. Biochem. **16**:64–76.
38. **Tien, M., and T. K. Kirk.** 1984. Lignin-degrading enzyme from *Phanerochaete chrysosporium*: purification, characterization, and catalytic properties of a unique H_2O_2-requiring oxygenase. Proc. Natl. Acad. Sci. USA **81**:2280–2284.
39. **Tien, M., and T. K. Kirk.** 1988. Lignin peroxidase of *Phanerochaete chrysosporium*. Methods Enzymol. **161**:238–248.
40. **Tien, M., and S. B. Myer.** 1990. Selection and characterization of mutants of *Phanerochaete chrysosporium* exhibiting ligninolytic activity under nutrient-rich conditions. Appl. Microbiol. Biotechnol. **56**:2540–2544.
41. **Waldner, R., M. S. A. Leisola, and A. Fiechter.** 1988. Comparison of ligninolytic activities of selected white-rot fungi. Appl. Microbiol. Biotechnol. **29**:400–407.