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# Effect of substrate nitrogen on lignin degradation by *Pleurotus ostreatus*

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**Abstract.** In order to determine the effect of substrate nitrogen (N) on ligninolytic activity, *Pleurotus ostreatus* was grown in solid media containing either growth-limiting (1 mM) or excess (10 mM) $NH_4Cl$. After 25 days, $^{14}C$-$CO_2$ production from $^{14}C$-cornstover lignin in low-N medium was 3 times that in nitrogen (N)-rich medium. Supplementation of low-N medium with glucose (0.3%) further enhanced ligninolytic activity. Decolorization of an aromatic, polymeric dye, Poly R-481, in solid media was also greatest under N-limiting conditions.

**Key words:** *Pleurotus ostreatus* – Poly R-481 – Cinnamic acid – Biodegradation – Secondary Metabolism – Nitrogen Catabolite Repression – White Rot – Cornstover – Bioconversion – Lignin

Second only to cellulose, lignin is the most abundant biological compound found in nature (Crawford 1981), yet it is degraded by only a small number of microorganisms; primarily the "white rot" basidiomycetous fungi. Of obvious ecological significance, lignin biodegradation by these fungi also has promising commercial applications.

Decomposition of lignocellulosic crop residues by white rot fungi results in improved digestibility by ruminants. Zadražil and Brunnert (1982) observed a 33% increase in wheat straw digestibility after colonization by *Phanerochaete chrysosporium* (= *Sporotrichum pulverulentum*). In southern Chile, wood decomposed by a consortium of fungi (primarily *Ganoderma applanatum* and *Armillaria* spp.) is used as a highly digestible feed known as "Palo Podrido" (Zadražil et al. 1982). The edible "oyster mushroom", *Pleurotus* spp., shows particular promise as a bioconversion organism. Its colonization of beechwood sawdust and rape and sunflower straws (Zadražil 1980), wheat straw (Zadražil 1977; Zadražil and Brunnert 1980; Lindenfelser et al. 1977), and cotton straw (Platt et al. 1983) significantly increases the digestibility of these materials.

Cultural conditions that promote ligninolytic activity most likely result in the greatest improvements in substrate digestibility, as lignin is a major digestibility-limiting component of plant fiber (Crawford 1981). As an aspect of secondary metabolism in fungi (Gold et al. 1982; Fenn and Kirk 1981), ligninolytic activity is stimulated by nutrient limitation; nitrogen (N)-limitation being the most effective means of promoting longterm lignin degradation by *Phanerochaete chrysosporium* (Reid 1983a, b, 1979; Buswell et al. 1982; Kirk et al. 1978; Fenn et al. 1981).

In this study we examined the effect of growth-limiting and excess substrate N (as $NH_4Cl$) on ligninolytic activity in *Pleurotus ostreatus*. We also developed a novel method for the preparation of $^{14}C$-cornstover lignin by (U-$^{14}C$)-cinnamic acid uptake via adventitious roots.

## Materials and methods

### *Cultures*

*Pleurotus ostreatus* was obtained from CM Mushroom Co., Hayward, CA, USA. Cultures were maintained on solid media described below and on malt extract agar slants.

### *Media*

Basal medium contained the following (per l): $KH_2PO_4$, 5.0 g; $CaCl_2 \cdot 2H_2O$, 0.2 g; $MgSO_4$, 0.5 g; trace minerals, 20 ml; vitamins, 20 ml. The pH was adjusted to 5.0 with KOH. Trace minerals contained (per l): $CuSO_4 \cdot 5H_2O$, 0.2 g; $ZnSO_4 \cdot 7H_2O$, 0.1 g; $MnCl_2 \cdot H_2O$, 0.1 g; Na molybdate, 0.05 g; Na EDTA, 0.6 g; and $FeSO_4 \cdot 7H_2O$, 1.0 g. The vitamin solution (Scott and Dehority 1965) contained (per 100 ml): pyridoxine HCl, 10 mg; riboflavin, 10 mg; thiamine·HCl, 10 mg; nicotinamide, 10 mg; calcium-D-pantothenate, 10 mg; para-amino benzoic acid (PABA), 0.5 mg; folic acid, 0.25 mg; biotin, 0.26 mg; $B_{12}$, 0.025 mg.

For media with variable amounts of $NH_4Cl$, basal medium was prepared at 125% stock concentration. Appropriate volumes of 0.4 M $NH_4Cl$ and distilled water were added to achieve 100% stock concentration. Media were autoclaved 25 min, at 125° C. To each flask, 1 ml 15% sterile glucose was added (0.3% final conc.).

Solid media contained, per liter basal medium: agar, 15 g (1.5% final conc.); either 54 mg $NH_4Cl$ (1 mM final conc.), or 535 mg $NH_4Cl$ (10 mM final conc.); either 3.0 g glucose, or 0.15 g Klasson-extracted unlabeled cornstover autoclaved separately in 15 ml tubes and sprinkled atop each hardened agar plate. For dye decolorization experiments, 200 mg Poly R-481 (Sigma) polymeric dye (Dawson 1981) was added per liter (0.02% final conc.).

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*Nitrogen limited growth*

In 125 ml conical flasks, 50 ml cultures were shaken at 150 rpm, 28° C. At 0, 4, 6, 8, 10, 14, and 18 day intervals, 0.1 ml culture aliquots were taken for $NH_4^+$ determination by the phenol colorimetric method of Chaney and Marbach (1962). Entire flask contents were filtered when no $NH_4^+$ was detected, or when there was no change from the previous assay. Cultures were filtered through pre-dried (1 h, 105° C), tared, 0.65 µm nitrocellulose filters (Millipore), and the mycelia were rinsed with 100 ml buffered saline (0.9% NaCl in 0.1 M $KH_2PO_4$ adjusted to pH 7.2 with KOH). Filters and mycelia were dried 2 h at 105° C prior to weighing.

Inoculum was prepared by adding a plug from a plate colony, made with a no. 3 corkborer, to 50 ml medium (0.3% glucose, 1.2 mM $NH_4Cl$) in a 125 ml conical flask. After flasks were shaken 2 days at 28° C, the cultures were homogenized 1 min (Omni-Mixer, Sorvall). The homogenate was transferred back to flasks and incubated another 6 days. Mycelial pellets were homogenized again, and 2 ml of homogenate was inoculated into each experimental flask. The homogenate (0.1 ml) was also spread on nutrient agar plates to test for contamination.

*($U_{14}C$)-trans-cinnamate preparation*

This radiolabeled lignin precursor was prepared by the enzymatic deamination of phenylalanine (Pometto and Crawford, 1981), using phenylalanine ammonia-lyase (PAL; EC 4.3.1.5). The reaction mixture contained the following: (U-$^{14}$C)-L-phenylalanine (0.56 µmol, ICN), 250 µCi; Tris buffer (0.05 M, pH 8.75), 13 ml; L-phenylalanine (0.1 M in Tris buffer), 20 µl; PAL, 50 µl (3.2 IU/ml; Sigma). The mixture was incubated 4 h at 30° C, and yielded 49% conversion. After reaction with additional enzyme (25 µl, 5.5 h), 75% of the substrate had been converted to cinnamate. Cinnamate formation was monitored by U.V. absorbance (268 nm).

Unreacted phenylalanine was removed from the reaction mixture using ion-exchange chromatography. The mixture, containing an additional 20 µl 0.1 M phenylalanine, was loaded into a 5 ml disposable column containing 3 ml DEAE cellulose (pre-swollen, Whatman) in 0.05 M pH 8.75 Tris buffer. Additional phenylalanine was added to facilitate eluent detection by U.V. absorbance (254 nm). Phenylalanine eluted immediately, and cinnamate followed after buffer was changed to 0.01 M pH 6.5 phosphate. The latter 19.6 ml fraction contained 88.5 µM cinnamate at $2.4 \times 10^7$ DPM/ml.

Purity of fractions was evaluated by thin-layer chromatography. Five microliters of each fraction were spotted on cellulose TLC strips (Baker) and run in the organic phase of benzene:acetic acid:$H_2O$ (2:2:1). Strips were analyzed on a radiochromatogram scanner (Packard, model 7201). A single peak corresponding to either cinnamate or phenylalanine was detected on each strip.

*In vivo labeling*

Potted 7-week-old field corn absorbed label through the severed ends of adventitious roots, a variation of an earlier method involving stalk penetration by hypodermic needles (Haider et al. 1977). Label in 0.01 M pH 6.5 phosphate buffer, was contained in 2 shortened 5 ml pyrex tubes, each sealed around the root with a septum stopper. A 1 ml syringe barrel with a 25-gauge needle penetrated each septum, through which label solution could be added as required. Solution, once absorbed was replaced with water which was consumed until harvest. Lateral roots eventually grew from adventitious roots inside the vials.

Mature plants were severed from their roots, rinsed, divided by length into thirds, split lengthwise, and dried overnight at 50° C. Sections were milled separately in a blender. Triplicate 0.5 g samples of control and radiolabeled stover from each section underwent Van Soest acid detergent lignin (ADL) determination (Goering and Van Soest 1970). Specific activities (DPM/mg dry stover) of both acid-digested fractions and unextracted samples were determined by combustion (Packard Tricarb, model 2450), the $^{14}C$-$CO_2$ was trapped in an organic amine reagent (Carbasorb, Packard) and automatically mixed with liquid scintillation counting (LSC) fluor.

Klason extraction of labeled and unlabeled stover was done according to the method of Crawford and Crawford (1978). Stover from the bottom third of the plant was extracted twice in water (stirred, 4 h, 65° C; 33 g in a 1 of distilled water) and rinsed through 2 layers of cheesecloth until the filtrate was clear. A 22 h Soxhlet extraction in benzene/ethanol (2:1) followed. After a 7 h Soxhlet extraction in 95% ethanol, stover was dried 15 h at 75° C. During extraction, 22.8% of the initial radioactivity was lost.

According to Crawford et al. (1983) ferulic and coumaric acids esterified to the periphery of the lignin polymer may be degraded using nonligninolytic enzymes and should, therefore, be eliminated by base hydrolysis. To accomplish this, Klason-extracted stover (5 g) was stirred in 200 ml 0.1 N NaOH (or distilled water in the control) 4 h at room temperature. To terminate the reaction, 1 ml 36 N $H_2SO_4$ was added. Stover was rinsed through cheesecloth with deionized water until no counts were detected in the filtrate. Hydrolysis caused a 16.8% loss of initial counts; only 0.4% were lost in the control (by water extraction only). Dried stover was passed through a Wiley-mill with a 20-mesh screen, then stored frozen.

*Growth on radiolabeled cornstover*

Experimental flasks each contained 10 ml solid media with 1 mM or 10 mM $NH_4Cl$, or 1 mM $NH_4Cl$ + 0.3% glucose. All flask media were covered with 150 mg sterile milled stover (2:1, extracted, unlabeled:NaOH-treated, labeled; 262 DPM/mg). To permit periodic flushing of accumulated $CO_2$, flasks were sealed with wired down stoppers that were penetrated by glass tubing filled with glass wool.

Flasks were inoculated with 2 no. 3 corkborer plugs from colonies grown 4 days on low-N (1 mM) or high-N (10 mM) media containing glucose or stover. Cultures were transferred 3 times on their respective media prior to inoculation. Control flasks were uninoculated.

Flasks were placed in a 28° C incubator, the tubing attached to a manifold to allow fifteen flasks to be flushed at a time. After all inoculated flasks were completely colonized, they were flushed 20 min with $CO_2$-free air (bubbled through 0.1 N KOH, 2.8 ml/s) 10, 17, and 25 days after inoculation. To trap $CO_2$, the flushed gas was bubbled through 4 ml organic amine (Oxisorb-$CO_2$, NEN) in 5 ml septum vials sealed with teflon-coated septa (Supelco), penetrated by 25-gauge needles. Flasks were flushed for 10 min with moist air (bubbled through water; 2.8 ml/s) and resealed.

**Table 1.** $^{14}$C-trans cinnamate absorption and incorporation in cornstover

| Plant Section | DPM/mg lignin $\pm \sigma$ | DPM/mg stover $\pm \sigma$ | % Incorporation[a] $\pm \sigma$ |
|---|---|---|---|
| Bottom | 4,080 ± 90 | 1,290 ± 200 | 14.0 ± 1.7 |
| Middle | 225 ± 133 | 74 ± 23 | 3.7 ± 1.9 |
| Top | 53 ± 23 | 32 ± 2 | 8.8 ± 3.8 |

[a] $\text{Incorporation} = \frac{\text{DPM/mg lignin}}{\text{DPM/mg stover}} \cdot \%\ \text{lignin [dry basis]}$

*$CO_2$ assay*

Carbon dioxide was determined according to Chung and Trlica (1978). A 160 µl aliquot of vial contents was added to 3 ml solvent (100% ethanol/dioxane, 1:1) and 6 drops thymol blue (0.2% in dioxane). The mixture was titrated with 0.05 N Na-methylate in dry benzene/methanol (9:1), under a stream of $N_2$. The assay was standardized by $CO_2$ evolving from preweighed $NaHCO_3$ reacted with 2 ml 4 N $H_2SO_4$. The remaining vial contents were mixed with 7.5 ml LSC fluor (Oxiprep II, NEN) and counted.

*Dye decolorization on solid media*

Half of each low and high-N dye plate, containing 0.3% glucose, was covered with unlabeled, extracted cornstover. Each were inoculated with 2 no. 3 corkborer mycelial plugs; one from a low-N culture, the other from a high-N culture. Plates were incubated at 28° C.

## Results

*Radiolabel incorporation into cornstover*

The bottom third of the corn plant absorbed the most label, having 1,290 DPM/mg dry stover (Table 1). Counts incorporated into lignin were also highest in this section (4,080 DPM/mg lignin), corresponding to 14% of absorbed ($^{14}$C)-cinnamate (Table 1).

*Growth-limiting $NH_4Cl$ concentration*

Six, 7, and 10 days after inoculation, ammonium chloride was no longer detectable in cultures containing 0.25, 0.50, and 1.00 mM $NH_4Cl$, respectively. Cells were harvested at those times. Eighteen-day 5.00 mM cultures were also harvested when there was no significant depletion of substrate $NH_4Cl$ after 14 days. Cultures grown in 1.00 mM $NH_4Cl$ yielded dry cell weights half that of cultures grown in 5.00 mM $NH_4^+$ (0.442 mg/ml vs. 0.88 mg/ml; Fig. 1). For this reason, 1.00 mM $NH_4^+$ was considered the growth limiting nitrogen concentration, and was used in subsequent experimental media.

*$^{14}CO_2$ production as affected by nitrogen limitation*

Taking into account variations in total $CO_2$ output between samples, nitrogen suppression of ligninolytic activity in *Pleurotus ostreatus* is evident (Fig. 2). Nitrogen-limited cultures yielded significantly higher amounts of $^{14}$C-$CO_2$ (DPM)/mmol $CO_2$ 10, 17, and 25 days after inoculation; counts from nitrogen-rich cultures were consistantly lower on these days.

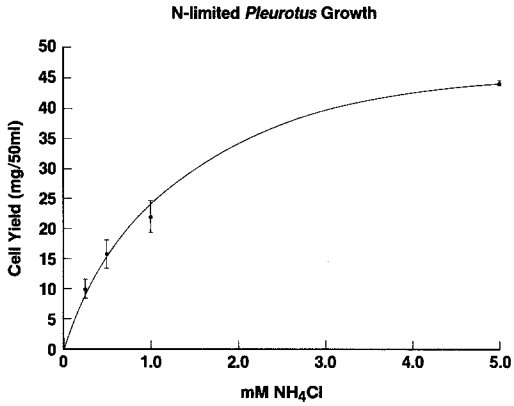


**Fig. 1.** *Pleurotus* dry cell yields with respect to substrate $NH_4Cl$ levels

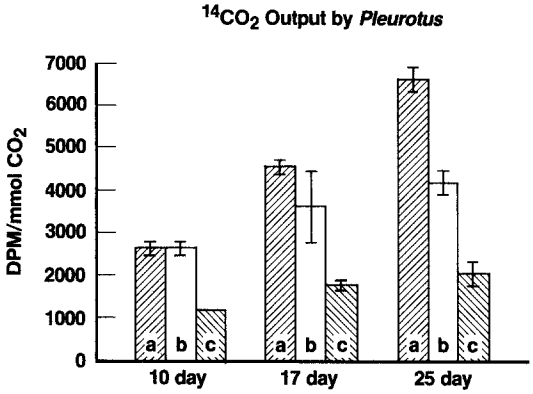


**Fig. 2.** $^{14}$C-cornstover lignin decomposition by *Pleurotus ostreatus* in solid media containing: *a* 1,0 mM $NH_4Cl$, glucose supplemented; *b* 1,0 mM $NH_4Cl$; *c* 10 mM $NH_4Cl$

**Table 2.** Average cumulative $^{14}$C output 25 days after inoculation

| Medium from which inoculum originated | Experimental medium | Average cumulative 25 days $^{14}$C-$CO_2$ output (% input DPM $\pm \sigma$) |
|---|---|---|
| 10 mM $NH_4Cl$/stover | 10 mM $NH_4Cl$/stover | 4.6 ± 1.3 |
| 1 mM $NH_4Cl$/stover | 10 mM $NH_4Cl$/stover | 3.7 ± 0.0 |
| 1 mM $NH_4Cl$/stover | 1 mM $NH_4Cl$/stover | 11.3 ± 0.3 |
| 1 mM $NH_4Cl$/glucose | 1 mM $NH_4Cl$/stover | 12.6 ± 1.0 |
| 1 mM $NH_4Cl$/stover | 1 mM $NH_4Cl$/stover + glucose | 15.8 ± 0.7 |

On a cumulative basis, nitrogen-limited (1 mM) cultures produced 3 times the amount of $^{14}$C-$CO_2$ as the nitrogen-rich cultures (10 mM; Table 2). The former cultures completely oxidized 11.3% of labeled substrate lignin to $^{14}$C-$CO_2$, whereas the latter cultures degraded only 3.7%. No counts were detected in uninoculated controls. The suppressive effect of excess nitrogen was independent of the amount of nitrogen in the inoculm media (Table 2).

Glucose supplementation of nitrogen-limited cultures resulted in the highest ligninolytic activity (Fig. 2), causing 15.8% of labeled substrate lignin to be completely oxidized to $^{14}C$-$CO_2$ (Table 2).

*Dye decolorization on solid media*

Nitrogen-limited colonies decolorized dye more rapidly and completely than those grown with excess nitrogen. After 6 days, colonies in nitrogen-rich media formed a decolorized zone 2/3 the width of zones surrounding the colonies in nitrogen-limited media; although colony diameters were the same. Decolorized zones spread outwards from the colonys' center. At 14 days, the nitrogen-limited plate was completely decolorized while the nitrogen-rich plate had changed little. The presence of stover on the plates did not affect decolorization, nor did the medium from which inoculum originated (1 mM or 10 mM $NH_4$ Cl).

## Discussion

The method described here, involving incorporation of a radiolabeled lignin precursor via adventitious roots, is a significant improvement over previous methods involving label uptake by severed plant sections (Crawford 1981). The latter methods use a short (1 – 7 days) period for label absorption, thereby allowing only peripheral lignin to be labeled. Use of such radioactive lignin may lead to overestimation of ligninolytic activity (Reid and Seifert 1982). Labeling via adventitious root uptake during plant growth and maturation may lead to a more even distribution of radiolabeled lignin throughout the cell wall. This method also yields larger amounts of radiolabeled plant material using relatively small amounts of the $^{14}C$-lignin precursor. Since cinnamic acid is a specific precursor, its incorporation does not necessitate the proteolytic extraction step required when $^{14}C$-phenylalanine is employed (Reid 1983a).

While $^{14}C$-$CO_2$ output adequately reflects ligninolytic activity, it does not represent all degraded lignin. Reid and Seifert (1982) noted that a significant (46%) amount of lignin degraded by *Phanerochaete chrysosporium*, remained as a soluble fraction not oxidized to $CO_2$. Kirk et al. (1978) arrived at similar results. $^{14}C$-$CO_2$ produced does, however, reflect overall ligninolytic activity to sufficiently demonstrate its suppression by excess substrate nitrogen and stimulation by $O_2$ (Freer and Detroy 1982; Reid and Seifert 1982).

The results presented here indicate that ligninolytic activity in *Pleurotus ostreatus* is indeed suppressed by excess substrate nitrogen, and that growth-limiting nitrogen levels (1 mM) stimulate the extent of lignin degradation. This effect is already established in *Phanerochaete chrysosporium* (Keyser et al. 1978). Addition of $NH_4$ prior to the expected onset of ligninolytic activity delays the appearance of this activity, and excess nitrogen depresses total, long-term activity (Buswell et al. 1982; Kirk et al. 1978; Fenn et al. 1981; Reid 1979, 1983a, b). These suppressive effects occur regardless of the nitrogen source (Buswell et al. 1982; Fenn and Kirk 1981; Reid 1983b), although the degree of suppression varies, with glutamate and glutamine being the most inhibitory. Degradation of dimeric model compounds by *Phanerochaete chrysosporium* is also suppressed by excess nitrogen in the medium (Ander et al. 1983; Shimada and Gold 1983; Glenn et al. 1983).

Nitrogen suppression in *Pleurotus* and white rot fungi other than *Phanerochaete* has not been so well-established. Buswell et al. (1982) mention unpublished results demonstrating nitrogen suppression in *Pleurotus ostreatus* and 4 other white rot fungi using synthetic $^{14}C$-lignin. With *Pleurotus ostreatus*, Freer and Detroy (1982) observed a 15% reduction in ligninolytic in the presence of excess (20 mM) $(NH_4)_2$ $SO_4$. Zadražil and Brunnert (1980) could not demonstrate nitrogen suppression in *Pleurotus ostreatus* "florida" or *Ganoderma applanatum*, probably because 30 mM $NH_4NO_3$ was the lowest level of substrate nitrogen. Leatham and Kirk (1983) found increased ligninolytic activity with nitrogen supplementation (34 mM glutamate) in *Lentinus edodes*, and activity in *Pleurotus ostreatus* was not affected. They did not establish, however, that the 2.6 mM $NH_4$-tartrate they used was at a growth-limiting level.

The nature of the native substrate used by ligninolytic fungi may influence whether the organism is susceptible to nitrogen suppression. A ligninolytic basidiomycete (NRRL 6464) isolated from a nitrogen-rich substrate (cattle dung) did not exhibit N-suppression under conditions suppressive to both *Phanerochaete chrysosporium* and *Pleurotus ostreatus* (Freer and Detroy 1982).

Our experimental results also demonstrate enhancement of ligninolytic activity by glucose supplementation of nitrogen-limited cultures. This stimulatory effect of carbohydrate has also been found in *Phanerochaete chrysosporium*. Although ligninolytic activity under nitrogen-rich conditions is promoted when carbohydrate is depleted (Jeffries et al. 1981; Kirk et al. 1978), long-term depression of lignin degradation occurs when carbon is limited (Kirk et al. 1978). Excess carbohydrate promotes long-term ligninolytic activity in *Phanerochaete chrysosporium* (and *Coriolus versicolor*; Kirk et al. 1976); and high substrate C:N ratios yield optimal activity (Reid 1979).

The fact that excess substrate nitrogen suppressed decolorization of dye plates suggests that the aromatic, polymeric dye is modified during secondary metabolism by the ligninolytic enzyme system. Glenn and Gold (1983) demonstrated that culture parameters affecting ligninolytic activity in *P. chrysosporium* affect dye decolorization in a similar way. Oxygen stimulated decolorization in static cultures, whereas excess nitrogen (12 mM $NH_4$-tartrate) was suppressive; decolorizing activity appeared at the time during growth when ligninolytic activity should also appear. A pleiotropic *Phanerochaete* mutant lacking ligninolytic and other secondary metabolic functions failed to decolorize the polymeric dye.

An extracellular peroxidase has recently been found to decolorize Poly R-481, phenol red and *o*-dianisidine in ligninolytic *P. chrysosporium* cultures (Kuwahara et al. 1984). This enzyme may also be responsible for dye decolorization observed in our *Pleurotus* cultures. The peroxidase is similar to the extracellular diarylpropane monoxygenase isolated by Tien and Kirk (1983), as its activity is $H_2O_2$-dependent and it is a heme protein of similar size ($\sim 46$ $K_d$ vs $\sim 41$ $K_d$). This enzyme requires, however, $Mn^{2+}$ and lactate for activity and is unable to significantly oxidize 2-keto-4-thiomethylbutyric acid (KTBA).

There is no evidence that dye decolorization corresponds to structural decomposition (e.g. ring cleavage) per se. It is possible, therefore, that the extracellular dye-decolorizing

peroxidase has no direct lignin-degrading activity. Its role in the ligninolytic system may, in fact, be indirect, such as inactivating toxic aromatic degradation products and/or regulating synthesis of lignin- and polysaccharide-degrading enzymes (Ander and Eriksson 1976).

The fact that ligninolytic activity is suppressed by excess substrate nitrogen must be taken into account when designing experiments concerned with the bioconversion of crop residues by white rot fungi. Decolorization of the aromatic, polymeric dye, poly R-481, may serve as a simple means to anticipate N-suppression and other conditions affecting ligninolytic activity. As a screening method for lignin-degrading organisms, dye decolorization also shows promise.

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