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Overproduction of Lignin-Degrading Enzymes by an Isolate of *Phanerochaete chrysosporium*

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Phanerochaete chrysosporium **is a white rot fungus which secretes a family of lignin-degrading enzymes under nutrient limitation. PSBL-1 is a mutant of this organism that generates the ligninolytic system under nonlimiting conditions during primary metabolism. Lignin peroxidase, manganese peroxidase, and glyoxal oxidase activities for PSBL-1 under nonlimiting conditions were 4- to 10-fold higher than those of the wild type (WT) under nitrogen-limiting conditions. PSBL-1 was still in the log phase of growth while secreting the enzymes, whereas the WT had ceased to grow by this time. As in the WT, manganese(II) increased manganese peroxidase activity in the mutant. However, manganese also caused an increase in lignin peroxidase and glyoxal oxidase activities in PSBL-1. Addition of veratryl alcohol to the culture medium stimulated lignin peroxidase activity, inhibited glyoxal oxidase activity, and had little effect on manganese peroxidase activity in PSBL-1, as in the WT. Fast protein liquid chromatography (FPLC) analysis shows production of larger amounts of isozyme H2 in PSBL-1 than in the WT. These properties make PSBL-1 very useful for isolation of large amounts of all ligninolytic enzymes for biochemical study, and they open the possibility of scale-up production for practical use.**

Lignin degradation is considered the rate-limiting step in carbon cycling on Earth. Only a few organisms are capable of degrading this aromatic polymer. Lignin is made up of phenylpropanoid units which are linked by a variety of carbon-carbon and carbon-oxygen bonds; this makes lignin very difficult to degrade. This property offers protection to plant tissues from pathogenic attack by microorganisms. The predominant degraders of lignin are filamentous fungi. Lignin peroxidases (ligninases) were first discovered in one of these, the white rot basidiomycete *Phanerochaete chrysosporium* (12, 32), and have since been found in other fungi (1, 27). To degrade the lignin polymer, *P. chrysosporium* secretes a family of H_2O_2-utilizing heme proteins (13, 20, 26). These are divided into two catalytically distinct classes, the manganese peroxidases (11, 28) and the lignin peroxidases. Recently, another enzyme which is part of the lignin-degrading system, glyoxal oxidase, was characterized and found to produce extracellular H_2O_2 (17). Besides lignin, ligninases can also degrade a number of organic pollutants, such as chlorinated aromatics, DDT [1,1′-(2,2,2-trichloroethylidene) bis(4-chlorobenzene)], and benzo[*a*]pyrene (5, 8). With this enzymatic capability, these proteins may prove useful for cleanup of toxic wastes that are otherwise resistant to degradation. Other potential applications include the use of ligninase in the pulp and paper industry (30).

However, since these fungi produce ligninases in only relatively small amounts, this limits their use commercially. The ligninolytic system is synthesized only in response to nitrogen (18), carbon, or sulfur (14) deprivation, which triggers secondary metabolism. Thus far, mutants have been described which are able to synthesize part of the ligninolytic system under nonlimiting conditions (3, 6, 22), but in quantities that are smaller than that produced by the wild type (WT) under nutrient limitation.

Recently, a procedure was described in which lignin model compounds were covalently attached to lysine to form an adduct that would act as a ligninase-dependent amino acid source (31). A mutant of *P. chrysosporium* was selected from a lysine auxotroph under nutrient-rich conditions with this adduct as the sole lysine source (35). The ligninases of the lysine auxotroph must oxidize the adduct to yield free lysine to complement the auxotrophy. This procedure allowed isolation of a mutant which produces lignin peroxidases under nutrient sufficiency (35). Previous reports demonstrate changes only in ligninase activity in deregulated mutants. In this report, we describe the characteristics of the entire ligninolytic system of this isolate under limiting and nonlimiting conditions. We compare the mutant and the WT with respect to growth, glyoxal oxidase activity, and manganese peroxidase activity, as well as lignin peroxidase activity.

In addition to comparison of these enzymes in the WT and PSBL-1, we determined the effect of manganese and veratryl alcohol on ligninolytic enzymes of PSBL-1. Current literature is conflicting on the role of these two components in affecting the level of enzyme activities (2, 4, 9, 23, 36). Using this mutant of *P. chrysosporium*, we were able to clearly demonstrate the necessity of manganese for all ligninolytic enzymes and the enhancement of lignin peroxidase activity by veratryl alcohol.

MATERIALS AND METHODS

Chemicals. Veratryl alcohol was purchased from the Aldrich Chemical Company, Milwaukee, Wis., and was vacuum distilled. All other chemicals were of reagent grade and were used without further purification.

Growth of *P. chrysosporium*. The WT strain BKM-F-1767 (ATCC 24725) and the lysine auxotroph PSBL-1 were maintained on agar medium containing 1% malt, 0.2% Bacto-Peptone, 1% yeast extract, 0.1% asparagine, 0.2% KH_2PO_4, and 0.1% $MgSO_4 \cdot 7H_2O$.

P. chrysosporium was grown in nitrogen-limited medium

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as previously described (34). Nonlimited cultures contained the same amounts of all other components, but 10 times the concentration of ammonium tartrate (11 mM). Carbon-limited cultures contained 1/10 the concentration of glucose (0.1%) and the same concentration of ammonium tartrate as the nonlimited cultures. The fungus was grown in shallow stationary cultures (10 ml in 125-ml flasks) containing 10^5 spores per ml. The flasks were flushed on day 3 with water-saturated 100% O_2. PSBL-1 cultures were supplemented with 0.3 mM lysine.

Veratryl alcohol was added on day 3 during O_2 flushing. The $MnSO_4$ concentration was varied within the Basal III medium component. For these experiments, veratryl alcohol and additional trace elements other than those in Basal III medium were deleted (34). For dry-weight determinations, the mycelial mat from the above cultures was dried at 80°C for 3 h in tared weighing pans.

Enzyme assays. All enzyme assays were performed with the extracellular fluid, which was collected by filtration through Whatman no. 4 filter paper. Three culture flasks were collected per datum point, and results are given as the mean of three datum points. Vertical bars on each figure represent the standard error of the mean.

Lignin peroxidase activity was measured by monitoring the oxidation of veratryl alcohol to veratraldehyde as indicated by an increase in A_{310} (33). The assay mixture contained 25 mM sodium tartrate (pH 2.5), 2 mM veratryl alcohol, 0.4 mM H_2O_2, and 50 to 275 μl of extracellular fluid in a total volume of 0.5 ml. The reaction was initiated by addition of H_2O_2.

Glyoxal oxidase activity was monitored by measuring the amount of H_2O_2 evolved, by using a peroxidase-coupled assay (29) as modified by Kersten and Kirk (17). The reaction mixture consisted of 50 mM sodium 2,2-dimethylsuccinate (pH 6.0), 10 mM methylglyoxal as the substrate, 0.01% phenol red, 10 μg of horseradish peroxidase type II (Sigma Chemical Co., St. Louis, Mo.) per ml, and up to 300 μl of extracellular fluid per ml of reaction mix. A 5-ml mixture was prepared, and the reaction was initiated by the addition of methylglyoxal. Oxidation of phenol red was determined by monitoring the change in A_{610}. The reaction was carried out at room temperature. The mixture was sampled every 30 s for 4 min by removing 1 ml and adding it to 50 μl of 2 N sodium hydroxide.

Manganese peroxidase activity was determined by monitoring the oxidation of phenol red spectrophotometrically at 610 nm (11). The reaction mix contained 50 mM sodium succinate (pH 4.5), 50 mM sodium lactate (pH 4.5), 0.1 mM $MnSO_4$, 3 mg of gelatin per ml, 50 μM H_2O_2, and 0.1 mM phenol red. The reaction was initiated with H_2O_2 and was conducted at 30°C. The mixture was sampled by removing 1 ml of the 5-ml total amount and adding it to 40 μl of 5 N sodium hydroxide every minute for 4 min.

One unit of enzyme activity is equivalent to 1 μmol of product formed per min.

FPLC analysis. The extracellular enzyme profile was determined by fast protein liquid chromatography (FPLC) with an anion-exchange Mono Q column (Pharmacia LKB Biotechnologies, Piscataway, N.J.) (19). Extracellular enzymes were concentrated 20-fold and eluted from the column with a linear gradient from 10 mM to 1 M sodium acetate (pH 6.0) over 40 min at 2 ml/min. Only the first 25 min of the profile is shown (Fig. 7), since no other peaks elute after this time. The elution profile was monitored at A_{280}.

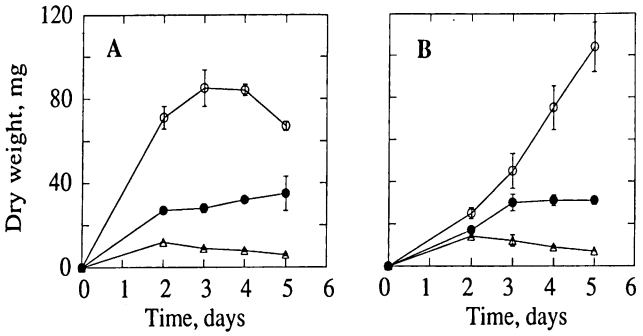

FIG. 1. Growth of *P. chrysosporium* WT (A) and PSBL-1 (B) under various nutrient culture conditions: nonlimiting (○), nitrogen limiting (●), and carbon limiting (△). Growth was measured by dry-weight determinations.

RESULTS

Effect of nutrient conditions on growth and ligninolytic-enzyme activity. Growth of the *P. chrysosporium* mutant PSBL-1 gave contrasting results from growth of the WT, depending on the nutrient conditions (Fig. 1). Under nitrogen-limiting conditions, the WT and the mutant showed similar growth rates, as measured by dry weight. Growth peaked on day 2 or 3, after which it leveled off. In carbon-limited cultures, growth reached a maximum on day 2 for both strains and then gradually declined. Thus, the WT and PSBL-1 behaved similarly under nutrient limitation in their growth patterns as determined by mycelial dry weight. PSBL-1, however, showed a high rate of growth under nonlimiting conditions throughout the experiment. These were also the culture conditions which promoted the highest level of ligninase activity. Therefore, PSBL-1 was still actively growing after the onset of ligninolytic-enzyme production, as indicated by these dry-weight determinations. This is in contrast to the WT, which produced ligninases only after cessation of growth on day 2.

Lignin peroxidase activity for the WT was as shown previously (19); under nitrogen limitation it peaked on day 5 at 90 U/liter of extracellular fluid (15 U/mg of protein) (Fig. 2), and under carbon limitation it peaked on day 3 at 33 U/liter (Fig. 2). No activity was present without nutrient deprivation. In contrast, PSBL-1 showed an eightfold-higher lignin peroxidase activity in nutrient-rich medium (729 U/li-

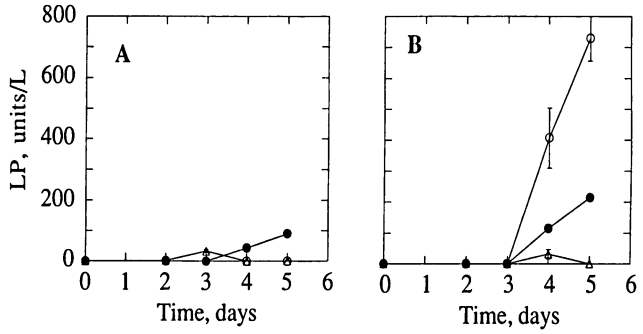

FIG. 2. Extracellular lignin peroxidase (LP) activity of the *P. chrysosporium* WT (A) and PSBL-1 (B) cultures under various nutrient conditions: nonlimiting (○), nitrogen limiting (●), and carbon limiting (△). Lignin peroxidase activity was measured by veratryl alcohol oxidation as described in Materials and Methods.

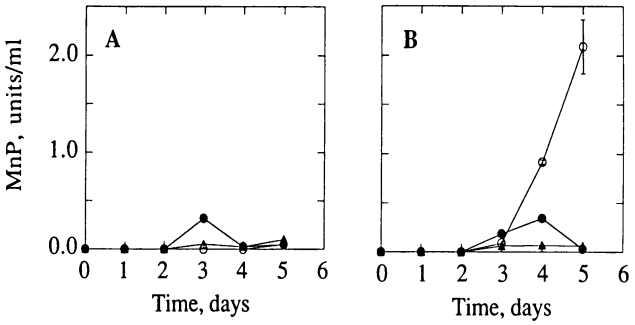

FIG. 3. Extracellular manganese peroxidase (MnP) activity of the *P. chrysosporium* WT (A) and PSBL-1 (B) cultures under various nutrient conditions: nonlimiting (○), nitrogen limiting (●), and carbon limiting (△). Manganese peroxidase activity was measured by oxidation of phenol red as described in Materials and Methods.

ter, 25 U/mg of protein) than the highest WT activity under nitrogen-limiting conditions. The activity for PSBL-1 under nitrogen-limiting conditions (214 U/liter) was also higher than that in the WT, whereas in carbon-limiting medium the two were similar (17 U/liter for PSBL-1).

Manganese peroxidase activity was similar in both strains under nutrient deprivation (Fig. 3). Under nitrogen limitation, activity peaked on day 3 in the WT at 318 U/liter (7 U/mg of protein) and day 4 in PSBL-1 at 342 U/liter, with the two strains attaining the same level of activity. Under carbon limitation, the isolates showed the same low level of activity (100 U/liter for the WT and 70 U/liter for PSBL-1), which did not decrease over time. As with lignin peroxidase, no manganese peroxidase activity was observed under nutrient-rich conditions in the WT. However, under nutrient-rich conditions, PSBL-1 had nearly an order of magnitude higher activity than did the nitrogen-starved WT. PSBL-1 manganese peroxidase activity peaked at almost 2,600 U/liter of extracellular fluid (38 U/mg of protein) by day 6 (data not shown).

The response of the glyoxal oxidase activity to different nutrient conditions in PSBL-1 was similar to that of lignin peroxidase and manganese peroxidase (Fig. 4). Nearly four-fold-higher activity was found with the mutant under nonlimiting conditions (38 U/liter) than with the nitrogen-starved

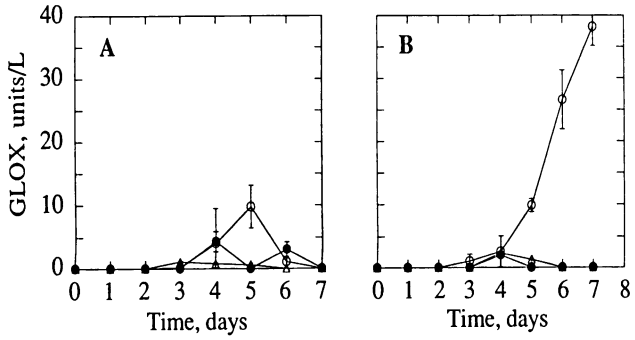

FIG. 4. Extracellular glyoxal oxidase (GLOX) activity of the *P. chrysosporium* WT (A) and PSBL-1 (B) cultures under various nutrient conditions: nonlimiting (○), nitrogen limiting (●), and carbon limiting (△). Glyoxal oxidase activity was measured by monitoring the evolution of H_2O_2 in a coupled assay with horseradish peroxidase as described in Materials and Methods.

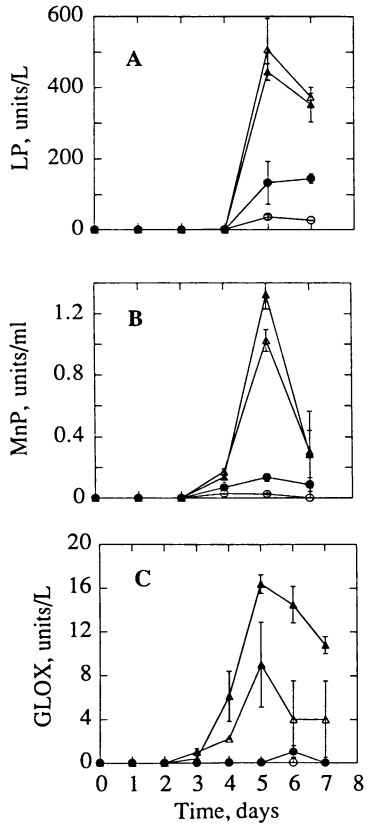

FIG. 5. Enzyme activity in extracellular fluid of *P. chrysosporium* PSBL-1 cultures with different concentrations of manganese sulfate. Lignin peroxidase (LP) (A), manganese peroxidase (MnP) (B), and glyoxal oxidase (GLOX) (C) activities are shown. Enzyme activities were measured as described in Materials and Methods. Manganese sulfate concentrations: ○, 6 μM; ●, 30 μM; △, 118 μM; ▲, 237 μM.

WT (10 U/liter). Again, activity with this enzyme was similar in the two strains under carbon and nitrogen limitation. Surprisingly, glyoxal oxidase activity in the WT was highest under nonlimiting conditions.

Effect of manganese on ligninolytic enzymes of PSBL-1. In previous studies, the manganese concentration in the medium was shown to have an effect on manganese peroxidase activity, but not on lignin peroxidase or glyoxal oxidase activity (2, 4). In our studies, manganese clearly stimulated manganese peroxidase activity at concentrations of up to 237 μM in PSBL-1 (Fig. 5B), as was the case in the WT. However, manganese concentrations of up to 119 μM also stimulated lignin peroxidase activity, which peaked on day 4 at 507 U/liter (Fig. 5A). A higher concentration did not stimulate lignin peroxidase activity further. Glyoxal oxidase activity was entirely dependent on manganese (Fig. 5C); as the manganese concentration increased, the glyoxal oxidase activity increased. In fact, this enzyme activity could not be detected in the medium containing only low concentrations (6 μM) of manganese.

Effect of veratryl alcohol on ligninolytic enzymes of PSBL-1. As in the WT (9, 23, 36), veratryl alcohol caused an increase in extracellular lignin peroxidase activity in a concentration-dependent fashion in PSBL-1 (Fig. 6A). Concentrations of 0.4 and 2.0 mM stimulated activity almost threefold and over

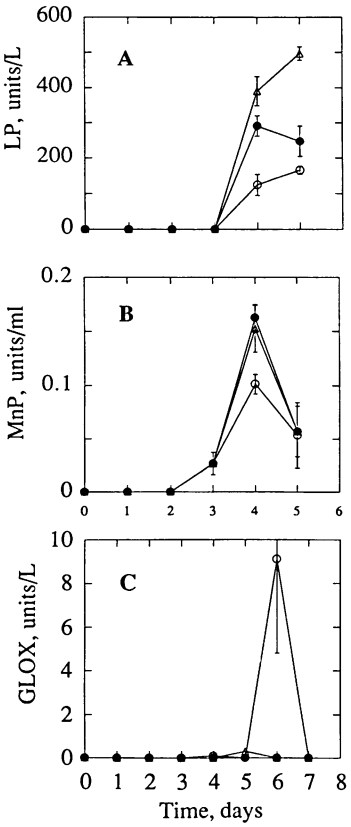

FIG. 6. Enzyme activity in extracellular fluid of *P. chrysosporium* PSBL-1 cultures with different concentrations of veratryl alcohol. Lignin peroxidase (LP) (A), manganese peroxidase (MnP) (B), and glyoxal oxidase (GLOX) (C) activities are shown. Enzyme activities were measured as described in Materials and Methods. Veratryl alcohol concentrations: ○, 0 mM; ●, 0.4 mM; △, 2.0 mM.

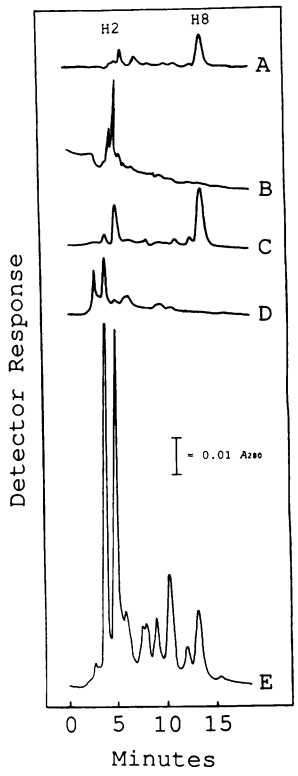

FIG. 7. FPLC Mono-Q anion-exchange column elution profile of extracellular proteins isolated from day 5 stationary cultures. (A) WT, nitrogen limited; (B) WT, carbon limited; (C) PSBL-1, nitrogen limited; (D) PSBL-1, carbon limited; (E) PSBL-1, nonlimited. The extracellular fraction was concentrated 20-fold. The profile shows A_{280}. Mono-Q chromatography is described in Materials and Methods.

fourfold, respectively, by day 4. Veratryl alcohol also caused a slight increase in the manganese peroxidase activity, with a 30% increase in activity in the presence of 0.4 or 2.0 mM veratryl alcohol (Fig. 6B). Surprisingly, even at low concentrations (0.4 mM), veratryl alcohol caused a decrease in extracellular glyoxal oxidase activity (Fig. 6C). In fact, enzyme activity was apparent only in cultures which did not contain supplementary veratryl alcohol.

Extracellular protein analysis. The FPLC analysis of the WT and PSBL-1 after 5 days of growth is given in Fig. 7. The two isolates produced similar proteins under nutrient limitation. For both strains, a protein which has a retention time corresponding to that of isozyme H8 was predominant under nitrogen limitation, whereas H2 was predominant under carbon limitation. However, PSBL-1 produced large amounts of H2 when grown without nutrient limitation (Fig. 7E), as well as relatively large amounts of the other isozymes, including the manganese peroxidases. The difference in isozyme production between the WT and PSBL-1 may account for the differences found in specific activity for the lignin peroxidases and manganese peroxidases.

DISCUSSION

We previously described the isolation of a mutant, PSBL-1, which produces ligninases under nutrient-rich conditions (35). In the present study, we further characterize this mutant and demonstrate that it also produces the manganese peroxidases and glyoxal oxidase under nutrient-rich conditions. The extracellular enzymes are produced in much larger amounts than in the WT. Thus, this mutant should prove extremely useful for biochemical and regulatory studies. We have demonstrated that the ligninolytic enzymes are produced during log-phase growth, indicating that PSBL-1 is able to produce the entire ligninolytic system under nonlimiting conditions during primary metabolism.

Studies by Liwicki et al. (24) and Kelley (15) showed that idiophasic events can be uncoupled, indicating different control mechanisms for various secondary metabolic functions. In the study by Liwicki et al. (24), loss of lignin-degrading ability did not result in loss of other idiophasic functions, such as sporulation and secondary metabolite production. Kelley (15) isolated mutants which were unable to degrade lignin and were deficient in glucose oxidase activity but could still perform such secondary metabolic functions as formation of conidiospores and synthesis of veratryl alcohol. In this work, we have shown that growth can continue while lignin peroxidases, manganese peroxidases, and glyoxal oxidase are produced, demonstrating that secondary metabolic events can be controlled at different levels. Specifically, an idiophasic event is now occurring during primary metabolism.

Other researchers have isolated several mutants that are deregulated in that they produce lignin peroxidases under nonlimiting nutrient conditions. Buswell et al. (6) reported

an isolate (INA-12) that produces ligninase when in the presence of ammonia nitrogen but not glutamate. Kuwahara and Asada (22) isolated a strain that could also produce ligninase under high-nitrogen conditions. Boominathan et al. (3) isolated two nitrogen-deregulated mutants, *der*8-2 and *der*8-5. This group found manganese peroxidase, lignin peroxidase, and glucose oxidase activity in nitrogen-containing medium, but the activity was lower than in the WT under limiting conditions. A strain which was isolated by Galliano et al. (10) was enhanced in cellulase activity and also showed higher xylanase, protease, and ligninase activity as compared with the WT. These results, together with our data, indicate that a wide-domain regulatory mechanism may be at work that controls the ligninolytic system or possibly all extracellular enzyme activities. Such a system is in operation in other fungi, for example *Aspergillus nidulans*. The *areA* gene product is the regulator in nitrogen control (7). It acts as a positive regulator in repression of a number of enzymes by nitrogen metabolites (for a review, see reference 25). This regulatory system is subject to pathway-specific elements as well. Since our work, as well as that by others described above, indicates a general change in the entire ligninolytic system, such a wide-domain regulatory protein may be involved. This is likely to be the site of the mutation in PSBL-1.

The larger amounts of enzyme production in PSBL-1 than in the WT may be attributed to the rich nutrient conditions in mutant cultures. Extracellular enzyme production is more likely to be maintained at maximal levels when carbohydrates and nitrogen are present in unrestricted quantities. This is supported by the fact that nutrient limitation results in equivalent amounts of enzyme production in PSBL-1 and the WT.

Other researchers have shown that addition of manganese and veratryl alcohol to the medium alters enzyme production (2, 4, 9, 36). If this is true, these effects should be apparent with this mutant as well. Also, since this mutant already produces large amounts of these enzymes, it would be useful to determine how to optimize the production of each component of the ligninolytic system to facilitate further studies of the individual enzymes. In our studies, increasing the manganese concentration increased lignin peroxidase, manganese peroxidase, and glyoxal oxidase activities. Brown et al. (4) saw similar results with the WT and suggested that manganese is involved in transcriptional regulation of the manganese peroxidase gene. They went on to say that manganese levels may activate a secondary messenger, such as cyclic AMP, from which a global response would be expected. On the basis of our findings, this is possible and merits further study. In contrast, Kern (16) found an increase only in activities of lignin peroxidases, not in activities of manganese peroxidases or glyoxal oxidase, when manganese(IV) oxide was added to the cultures. He postulated that manganese(IV) oxide may protect ligninase from inactivation by H_2O_2. This difference in results may be due to the different oxidation states of the manganese, since we add it as $MnSO_4$. Bonnarme and Jeffries (2) also saw an increase only with manganese peroxidases, not lignin peroxidases, after the addition of manganese. However, this work was done in shaking culture, not stationary culture as was used in the present study. Culture conditions can account for vast differences in the response of the organism, as we have seen in our studies with PSBL-1 (unpublished results). Thus, the effects of manganese are quite variable, depending on the culture conditions and the form of manganese added.

The presence of additional veratryl alcohol in the culture medium is known to enhance the activity of lignin peroxidases. Our studies with this mutant strain support this, since lignin peroxidase activity is much higher in cultures grown in the presence of veratryl alcohol and since manganese peroxidase activity is not greatly affected. Evidence thus far suggests that veratryl alcohol has a protective effect by preventing inactivation of the lignin peroxidases by H_2O_2 (36), although some suggest that veratryl alcohol actually increases the synthesis of lignin peroxidase proteins (9). It is surprising that glyoxal oxidase is completely inactive in cultures grown in the presence of this component, since veratryl alcohol seems to be necessary for optimal lignin peroxidase activity. This suggests, on a practical level, that in optimizing for production of glyoxal oxidase, the culture medium should not contain veratryl alcohol.

Other variations in culture conditions when using WT *P. chrysosporium* have improved lignin peroxidase production, although not to the extent of the activity produced by PSBL-1. For example, growth in shaking culture under low-manganese conditions gave 400 U/liter (2), whereas low levels of carbon and medial nitrogen with the addition of 0.1% Tween 80 gave activities as high as 356 U/liter (21).

We have shown that the PSBL-1 mutant of *P. chrysosporium* can overproduce the ligninolytic system under nutrient-rich conditions and can give enzyme yields of up to an order of magnitude higher than the WT. This makes PSBL-1 very useful for isolation of large amounts of the ligninolytic enzymes for biochemical study and opens the possibility of scale-up production for practical use. The nature of the mutation indicates that a global regulatory change may be responsible for the overproduction. More studies are necessary to elucidate the precise nature of the mutation in PSBL-1 and to further characterize the regulation of the ligninolytic enzymes by various factors.

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