Enhanced Expression and Hydrogen Peroxide Dependence of Lignin Peroxidase from *Streptomyces viridosporus* T7A

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The volumetric and specific activities of *Streptomyces viridosporus* T7A lignin peroxidase isoform ALiP-P3 were increased by formulating a novel corn-starch-based growth medium by optimizing the concentrations of starch, casein, yeast extract, $CaCO_3$, NH_4Cl, and trace metals. This medium increases cell densities by 10-fold, cell-specific ALiP-P3 activity by 6-fold, and volumetric ALiP-P3 activity by 60-fold compared to those obtained using a yeast-extract-based medium. The presence of increased concentrations of ALiP-P3 in the starch-based cultures was confirmed by Western blot analysis. In addition, the lignin peroxidase activity was found to be highly dependent upon the concentration of the necessary cofactor, hydrogen peroxide; using 2,4-dichlorophenol as the substrate, ALiP-P3 activity increased 1448-fold as the concentration of H_2O_2 was varied from 0.1 to 250 mM.

Introduction

Lignin is the second most abundant biopolymer, comprising 15% of the Earth's biomass (Hammel, 1992) and consists of an apparently random complex of phenolic and non-phenolic compounds (Crawford, 1981). The majority of bonds within a typical lignin structure consist of arylglycerol β-aryl ether (β-O-4) and diaryl propane (β-1) bonds (Crawford, 1981). Since lignin also contains molecules of different chirality, lignin-degrading enzymes must be non-stereoselective (Crawford, 1981). Because of the irregularity of the lignin structure, relatively few microorganisms are capable of degrading lignin.

The most-studied, lignin-degrading system is that of the white rot fungus, *Phanerochaete chrysosporium*. *P. chrysosporium* secretes both lignin peroxidase and manganese peroxidase for lignin degradation (Kirk and Farrell, 1981; Tien and Kirk, 1984). Lignin peroxidase from *P. chrysosporium* is a heme protein that catalyzes the H_2O_2-dependent oxidation of lignin (Tien and Kirk, 1984). Using its nonspecific peroxidases, *P. chrysosporium* is able to degrade a wide range of synthetic compounds including azo dyes, 1,1′-(2,2,2-trichloroethylidene)bis[4-chlorobenzene] (DDT), 3,4-dichloroaniline, and pentachlorophenol (Higson, 1991).

The Gram-positive bacterium, *Streptomyces viridosporus* T7A, also produces a variety of enzymes which play a role in lignin degradation. These extracellular enzymes include various peroxidases, cellulases, esterases, and endoglucanases (Adhi *et al.*, 1989; Borgmeyer and Crawford, 1985; Crawford *et al.*, 1983; Ramachandra *et al.*, 1987). *S. viridosporus* T7A produces four extracellular lignin peroxidase isoforms, and although all require hydrogen peroxide as an electron donor, each isoform has a different substrate range (Ramachandra *et al.*, 1988). The isoform with the greatest known substrate range, ALiP-P3, catalyzes the degradation of lignin model compounds by oxidative cleavage of $C_\alpha-C_\beta$ and $C_\alpha-$carbonyl bonds and is the only isoform detectable with an enzyme assay using 2,4-dichlorophenol as the substrate (Ramachandra *et al.*, 1988). In addition, either one or more of the isoforms catalyzes the oxidation of syringic acid, vanillylacetone, and vanillic acid (Spiker *et al.*, 1992). Lignin peroxidase of *S. viridosporus* T7A has been studied in the degradation of Kraft indulin lignin (Giroux *et al.*, 1988), acetovanillone (Spiker *et al.*, 1992), vanillyl alcohol (Spiker *et al.*, 1992), guaiacol (Spiker *et al.*, 1992), and humic acid model compounds (Kontchou and Blondeau, 1991). Because *S. viridosporus* T7A lignin peroxidase (ALiP) is known to degrade lignin and various phenolic compounds, *Streptomyces* may be used to degrade or mineralize recalcitrant waste compounds such as azo dyes (Paszczynski *et al.*, 1992) and pesticides (Gauger *et al.*, 1986; Gunner and Zuckman, 1968; Paszczynski *et al.*, 1992).

Unlike *P. chrysosporium*, which produces lignin peroxidase only under nitrogen, carbon, or sulfur-limiting conditions (Jeffries *et al.*, 1981; Kirk *et al.*, 1978), *S. viridosporus* T7A produces lignin peroxidase during growth-associated conditions in yeast-extract-based medium (Korus *et al.*, 1991). However, poor *S. viridosporus* T7A culture conditions can result in low lignin peroxidase yields which often require time-consuming enzyme concentration steps. Low ALiP expression also requires cultures with high cell densities to successfully screen *Streptomyces* species for lignin peroxidase activity. By enhancing ALiP expression, the need to concentrate or process *Streptomyces* cultures can be diminished. Sufficiently large amounts of enzyme will also be required in any reactor utilizing ALiP for bioremediation.

Attempts to identify an inducer for *S. viridosporus* T7A lignin peroxidase have not been successful since neither lignocellulose (Pasti *et al.*, 1991) nor (carboxymethyl)-cellulose (Lodha *et al.*, 1991) induces synthesis of the enzyme. Various carbon sources such as sucrose, lactose, and glucose have been used in *S. viridosporus* T7A growth media, but none resulted in high peroxidase yielding cultures (Korus *et al.*, 1991). The highest specific ALiP-P3 activity was obtained using a yeast-extract-based medium which lacked a true carbon source (Korus *et al.*, 1991).

This work reports the development of a culture medium which enhances both *S. viridosporus* T7A cell density and ALiP-P3 production. Using this starch-based medium, volumetric and cell-specific ALiP activities were

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increased by 60- and 6-fold, respectively. In addition, the effect of H_2O_2 concentrations on ALiP specific activity is reported.

Materials and Methods

Organism and Culture Conditions. *S. viridosporus* T7A (ATCC 39115) was kindly supplied by Prof. D. L. Crawford (University of Idaho, Moscow). *S. viridosporus* T7A spore suspensions (5 mL) were made from R2YE (Hopwood *et al.*, 1985) slants which had been incubated at 37 °C for 1−3 weeks (Hopwood *et al.*, 1985). The spore suspensions from each slant were divided and stored as either long-term stock cultures (stored at −85 °C) or working spore suspensions (stored at −20 °C).

The shake flask cultures were conducted in 250 mL Erlenmeyer flasks, inoculated with 50 μL of the working spore suspensions, and shaken at 250 rpm in a 37 °C Series 25 orbital air bath (New Brunswick Scientific Co. Inc., Edison, NJ).

Media Optimization. To determine the effect of phosphate, carbonate, and Tris buffer on ALiP-P3 production, *S. viridosporus* T7A was cultured in duplicate flasks of 100 mL basal medium consisting of (wt/vol) 0.5% corn starch (Kingsford, Coventry, CT), 0.5% yeast extract (Difco Laboratories, Detroit, MI), 0.3% malt extract (Difco), 0.1% NH_4NO_3 (Fisher Scientific, Tustin, CA), and 0.1% (vol/vol) R2YE trace metal stock solution (Hopwood *et al.*, 1985). This basal medium was supplemented with either phosphates (0.53% Na_2HPO_4 and 0.198% KH_2PO_4, Fisher) (Korus *et al.*, 1991), 0.5% $CaCO_3$ (as precipitated chalk, Fisher), or 25 mM Tris-HCl (pH 8).

Using $CaCO_3$, the optimum concentrations of yeast extract, corn starch, casein, NH_4Cl, and trace metals on ALiP-P3 production were studied in duplicate 50 mL cultures by varying one medium component and keeping the other component concentrations constant. The yeast extract was varied in an initial basal medium consisting of (wt/vol) 1.5% corn starch, 0.7% $CaCO_3$, 0.3% casein (Aldrich Chemical Co., Inc., Milwaukee, WI), 0.1% NH_4Cl (Fisher), and 0.1% vol/vol R2YE trace metal stock solution. The yeast extract concentration which resulted in the highest ALiP-P3 activity was then fixed in the basal medium with 1.5% casein, and the NH_4Cl concentration was varied. The basal medium was next modified by using the NH_4Cl concentration that resulted in the highest ALiP-P3 activity and varying the concentration of R2YE trace metals. The concentration of trace metals that resulted in the highest ALiP-P3 activity was then fixed, and this modified basal medium was used for both corn starch and casein studies. The corn starch and casein concentrations that resulted in the highest ALiP-P3 activities (6−8 days) were used in the final basal medium to give an ALiP-P3-optimized medium.

The optimized starch-based medium (DJMM) used to determine the maximum ALiP-P3 activity, and the dry cell mass of *S. viridosporus* T7A consisted of (wt/vol) 4.0% corn starch, 2.0% casein, 0.7% $CaCO_3$, 0.3% yeast extract, 0.1% NH_4Cl, and 0.1% (vol/vol) R2YE trace metals stock solution. Triplicate 100 mL cultures were sampled daily for ALiP-P3 activity and dry cell mass.

Cultures grown in the starch-based medium were compared to triplicate, *S. viridosporus* T7A cultures in 50 mL of yeast-extract-based medium, developed by Korus *et al.* (1991) (wt/vol): 0.53% Na_2HPO_4, 0.198% KH_2PO_4, 0.25% yeast extract, 0.1% glutamic acid (Fisher), 0.1% proline (Fisher), 0.1% asparagine (Fisher), 0.02% $MgSO_4 \cdot 7H_2O$ (Fisher), 0.005% $CaCl_2 \cdot 2H_2O$ (Fisher), 0.02% NaCl (Fisher), and 0.1% (vol/vol) R2YE trace metals solution. After 3 days, the maximum ALiP-P3 activity and the dry cell mass of the yeast-extract-based medium cultures were obtained. All media were sterilized by autoclaving for 20 min at 121 °C.

ALiP-P3 Enzyme Assay. *S. viridosporus* T7A lignin peroxidase activity of the culture supernatant was assayed using a modified 2,4-dichlorophenol assay (Ishida *et al.*, 1987; Ramachandra *et al.*, 1988; Spiker *et al.*, 1992) which is based on the reaction of ALiP-P3-oxidized 2,4-dichlorophenol with 4-aminoantipyrene to form a colored antipyrylquinonimine (Litvinchuk *et al.*, 1991) that strongly absorbs at 510 nm. The ALiP-P3 activity was analyzed daily by centrifuging a 1.0 mL sample from each shake-flask culture for 5 min at 12 000 rpm (model Z229; Hermle, Gosheim, Germany). The final concentrations of the 1.0 mL aqueous assay mixture consisted of 100 mM Tris buffer (pH 8.0), 5.0 mM 2,4-dichlorophenol (Eastman Kodak Co., Rochester, NY), 16.4 mM 4-aminoantipyrene (Fisher), 4.0 mM H_2O_2 (Fisher), and 1−100 μL of culture supernatant. After the addition of H_2O_2, the absorbance change at 510 nm was monitored for 5 min at 25 °C with a Cary 1 spectrophotometer (Varian Instrument Group, Palo Alto, CA). The initial rate of DCP degradation was calculated using an molar absorptivity of 18 500 M^{-1} cm^{-1}, which was determined as described by Metelitza *et al.* (1991).

The effect of H_2O_2 on ALiP-P3 activity was determined using triplicate ALiP-P3 assay measurements for 0.1−500 mM hydrogen peroxide concentrations with ALiP-P3 samples obtained from a 100 mL optimized starch-based culture of *S. viridosporus* T7A. After maximum ALiP-P3 production was achieved (6 days), 5.0 mL of the culture was removed and centrifuged at 19 000 rpm for 10 min at 4 °C using a model J2-21 centrifuge (Beckman Instruments, Inc., Fullerton, CA). The hydrogen peroxide dependence was then examined at two different ALiP-P3 concentrations: undiluted supernatant and a 1/10× dilution of the supernatant in distilled, deionized water diluent.

To verify that the ALiP-P3 enzyme assay measured ALiP-P3 activity linearly over the working range of 0.0−100.0 nmol/(mL·min), the ALiP-P3 activity of supernatant from a *S. viridosporus* T7A culture grown in optimized starch medium (DJMM) was determined and compared to five samples formed by diluting the supernatant with distilled, deionized water (all activities measured in triplicate). A linear dependence was determined over the entire working range ($R^2 = 0.997$); hence, the ALiP-P3 activity measured with the assay was directly proportional to the enzyme concentration.

Dry Cell Mass. The dry cell mass of each culture was obtained by filtering either 5.0 mL of the starch-based culture or the entire 50 mL yeast-extract-based culture through preweighed, type A/E glass fiber filter paper (Gelman Sciences, Ann Arbor, MI). The collected cells were dried at 80 °C overnight in an oven and then weighed using a model PM200 balance (Mettler-Toledo AG, Greifensee, Switzerland).

Western Blot Analysis. To verify the presence of ALiP-P3, a 12% resolving SDS-PAGE gel (Laemmli, 1970) was run (Mini-Protean II, Bio-Rad Laboratories, Hercules, CA) at constant voltage (150 V) for 1 h using supernatant samples obtained from 100 mL shake-flask cultures (1.0 mL samples were centrifuged for 5 min at 12 000 rpm, and the supernatant was assayed for ALiP-P3 activity). Gels were loaded such that each sample contained the volume of supernatant required for an ALiP-P3 activity of 0.0338 nmol/min. The proteins of the SDS-PAGE gels were then transferred (Mini Trans-Blot, Bio-Rad) to a nitrocellulose membrane (Bio-Rad) according to the manufacturer's transfer protocol. The ALiP-

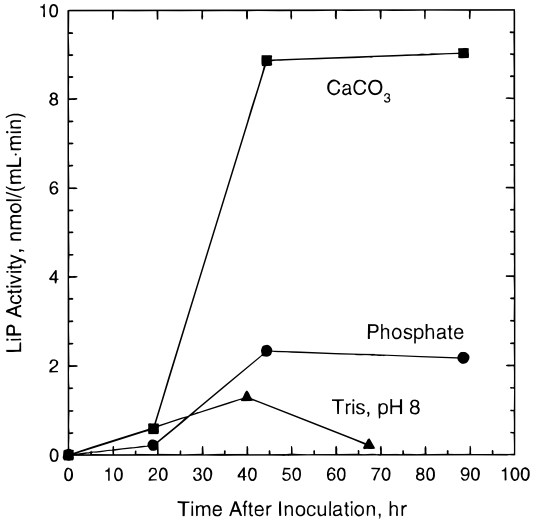

Figure 1. Phosphate, $CaCO_3$, and Tris buffer and ALiP-P3 production. Data represent the average of duplicate 100 mL media cultures consisting of the following basal medium (wt/vol): 0.5% corn starch, 0.5% yeast extract, 0.3% malt extract, 0.1% NH_4NO_3, and 0.1% (vol/vol) R2YE trace metal stock solution. In addition to the basal medium, the phosphate-containing medium contained 0.53% Na_2HPO_4 and 0.198% KH_2PO_4, the calcium-containing medium used 0.5% wt/vol $CaCO_3$, and the Tris-containing medium used 25 mM Tris (pH 8). Symbols: ●, phosphate-containing medium; ■, $CaCO_3$-containing medium; ▲, Tris-containing medium.

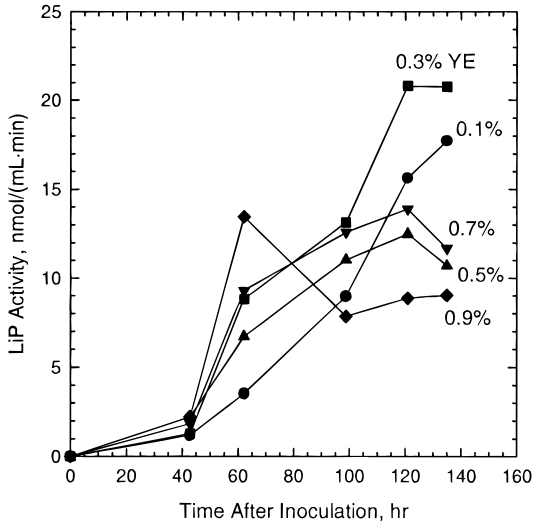

Figure 2. Yeast extract and ALiP-P3 production. Data represent the average of duplicate 50 mL cultures containing basal medium (wt/vol): 1.5% corn starch, 0.7% $CaCO_3$, 0.3% casein, 0.1% NH_4Cl, and 0.1% vol/vol R2YE trace metal stock solution, with varying concentrations of yeast extract (YE).

P3 bands were detected using the ProtoBlot Western blot AP system (Promega Corp., Madison, WI) by following the manufacturer's protocol. Rabbit anti-ALiP-P3 primary antibody (Magnuson *et al.*, 1991), generously provided by Prof. D. L. Crawford, was used at 1:2000 dilution along with rabbit anti-IgG alkaline phosphatase conjugate (Promega).

Results

Buffer and ALiP-P3 Production. The effect of both phosphate and $CaCO_3$ on *S. viridosporus* T7A ALiP-P3 production is shown in Figure 1. The maximum ALiP-P3 production in the calcium carbonate-containing media was 8.9 nmol/(mL of culture supernatant·min), compared to 2.3 nmol/(mL of culture supernatant·min) obtained in the phosphate-containing medium. A 25 mM Tris buffer (pH 8) was also tested and resulted in a maximum ALiP-P3 activity of 1.7 nmol/(mL of culture supernatant·min). Phosphates have been shown to repress antibiotic production by *Streptomyces* (Demain and Piret, 1991) and may have a similar effect on ALiP-P3 production. In addition, calcium carbonate is insoluble and is only consumed when needed. On the basis of these determinations, $CaCO_3$ was used as the buffering agent in all further media evaluations.

Yeast Extract and ALiP-P3 Production. Figure 2 shows the ALiP-P3 activity resulting from altering the yeast extract concentration in a basal medium from 0.1 to 0.9%. The highest ALiP-P3 activity was obtained using 0.3% (wt/vol) yeast extract, whereas higher concentrations inhibited ALiP-P3 production by as much as 57% (Figure 2, 0.9% yeast extract). Similarly, Korus *et al.* (1991) reported the same 0.3% (wt/vol) optimum yeast extract concentration in yeast-extract-based medium and that higher concentrations (0.8% wt/vol yeast extract) inhibited ALiP-P3 production by as much as 82%.

Ammonium Chloride and ALiP-P3 Production. Ammonium chloride, used as an inorganic nitrogen source, was varied from 0 to 0.9% in a medium consisting of (wt/vol) 1.5% corn starch, 0.7% $CaCO_3$, 1.5% casein, 0.3% yeast extract, and 0.1% (vol/vol) R2YE trace metal stock solution. Figure 3 shows that NH_4Cl concentrations greater than 0.1% (wt/vol) were found to limit ALiP-P3 production. Consequently, 0.1% (wt/vol) NH_4Cl was used in the following media formulations.

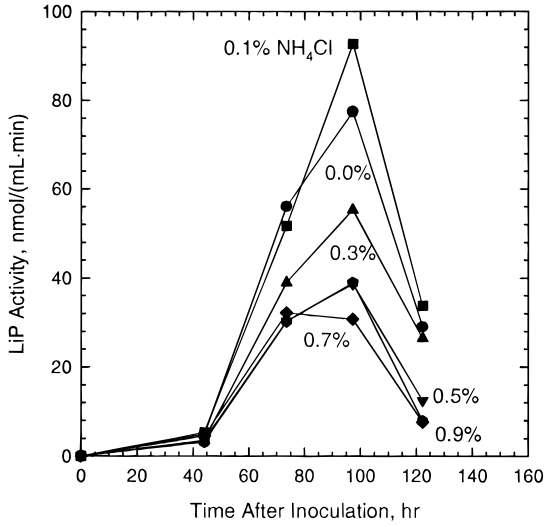

Figure 3. Ammonium chloride and ALiP-P3 production. Data represent the average of duplicate 50 mL cultures of basal medium (Figure 2 legend with 0.3% yeast extract and 1.5% casein) with varying concentrations of NH_4Cl.

Trace Metals and ALiP-P3 Production. Other researchers have speculated that the activity of enzymes with metal cofactors may be impacted deleteriously by the depletion of trace metals at high cell density (Murdock *et al.*, 1993). To examine whether varying the trace metal concentration affects ALiP-P3 expression and activity, *S. viridosporus* T7A was cultivated in a basal medium consisting of (wt/vol) 1.5% corn starch, 1.5% casein, 0.7% $CaCO_3$, 0.3% yeast extract, and 0.1% NH_4Cl. No significant difference in ALiP-P3 production was observed over a 50× concentration range (0.1−5.0% vol/vol) of R2YE trace metal stock solution (data not shown).

Corn Starch and ALiP-P3 Production. Figure 4 shows that the maximum ALiP-P3 activity was obtained using a corn starch wt/vol concentration of 4.0%. The basal medium used in the corn starch experiments consisted of 1.5% casein, 0.7% $CaCO_3$, 0.3% yeast extract,

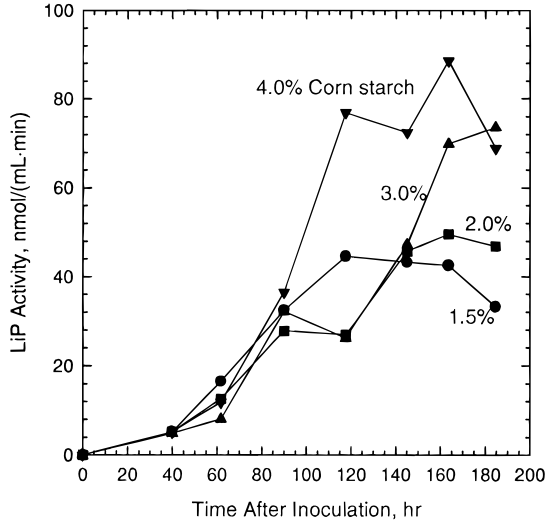

Figure 4. Corn starch and ALiP-P3 production. Data represent the average of duplicate 50 mL cultures of basal medium (Figure 2 legend with 0.3% yeast extract and 1.5% casein) with varying concentrations of corn starch.

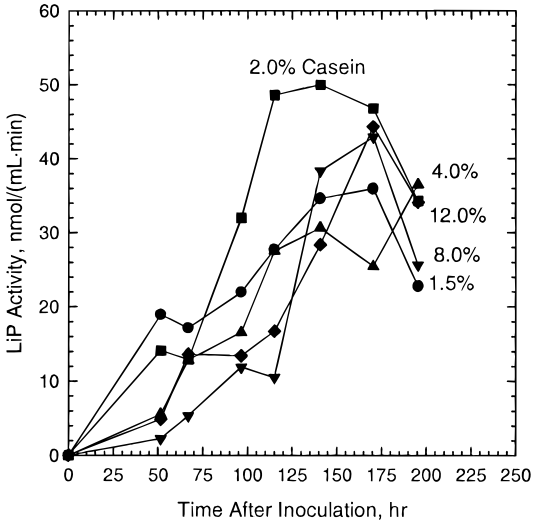

Figure 5. Casein and ALiP-P3 production. Data represent the average of duplicate 50 mL cultures of basal medium (Figure 2 legend with 0.3% yeast extract) with varying concentrations of casein.

0.1% NH_4Cl, and 0.1% trace metal stock solution. The data also indicate that corn starch concentrations greater than 4.0% might result in even higher levels of ALiP-P3 activity. Above 4.0% starch concentrations, however, the autoclaved medium becomes very viscous and is difficult to agitate.

Casein and ALiP-P3 Production. The optimum casein concentration for ALiP-P3 production was determined by varying casein from 1.5 to 12% in a basal medium of (wt/vol) 1.5% corn starch, 0.7% $CaCO_3$, 0.3% yeast extract, 0.1% NH_4Cl, and 0.1% trace metal stock solution. Figure 5 shows that the maximum ALiP-P3 activity was obtained using a 2.0% casein concentration and higher casein concentrations both depressed ALiP-P3 production and delayed maximum volumetric activity by 1 day.

ALiP-P3 Production in Optimized Media. To maximize the expression of ALiP-P3, the optimal wt/vol concentrations of the key medium components (assuming each component may be optimized independently of the others) were used to formulate the novel starch-based medium, named DJMM (Deokjin Maximal Medium). Figure 6 shows the dry cell mass and ALiP-P3 activity of 100 mL cultures of *S. viridosporus* T7A in DJMM. The average maximum *volumetric* ALiP-P3 activity in cultures using this optimized medium was 184 nmol/mL·min (Figure 6, 220 h), which was 60-fold higher than that synthesized by cultures in yeast-extract-based medium (3 nmol/mL·min, 65 h). The average maximum *specific* ALiP-P3 activity of *S. viridosporus* T7A grown in DJMM was 10 865 nmol/(g of cell·min) (Figure 6, 220 h); in contrast, the average maximum specific ALiP-P3 activity obtained from yeast-extract-based medium was 1805 nmol/(g of cell·min) (after 72 h). Hence, this novel medium yields a 600% increase in specific ALiP-P3 activity compared to that obtained using yeast-extract-based media. This also represents a 280% improvement over the maximum specific activity reported by Korus *et al.* (1991), 3892 nmol/(g of cell·min), obtained using the same strain in yeast extract (0.05% wt/vol) medium. However, the ALiP-P3 activity reported is not directly comparable since Korus *et al.* (1991) used a higher assay temperature (37 °C) and different substrate concentrations. Using an optimized 0.30% wt/vol yeast-extract-based medium, Korus *et al.* (1991) also reported a 1.8 mg/mL cell density at maximum ALiP-P3 production (5

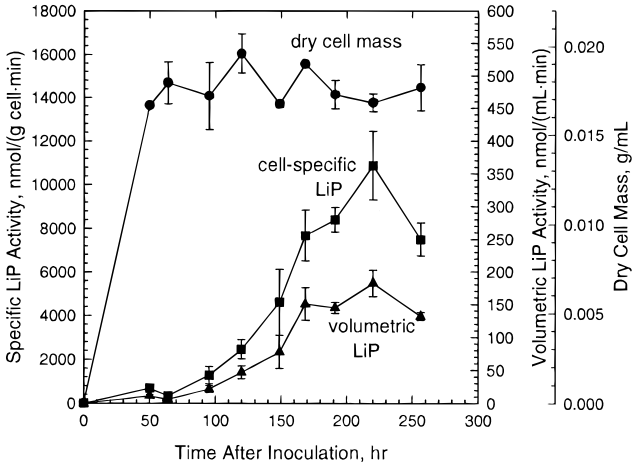

Figure 6. Cell-specific and volumetric ALiP-P3 activity of *S. viridosporus* T7A in final, optimized starch medium. Data represent averages of triplicate 100 mL cultures of the final, optimized, high-ALiP-P3-producing medium (DJMM). Error bars indicate standard deviation. Symbols: ■, cell-specific ALiP-P3 activity; ●, dry cell mass; ▲, volumetric ALiP-P3 activity.

nmol/mL·min, after 3 days) which occurred in the stationary growth phase (although ALiP-P3 production began during the growth phase). In DJMM cultures, maximum ALiP-P3 production similarly occurred well into the stationary growth phase (Figure 6), although the average cell density in the starch-based cultures at maximum ALiP-P3 production (17.4 mg/mL) was 10-fold higher than that of yeast-extract-based cultures of this study (1.7 mg/mL), which agrees well with the value of Korus *et al.* (1991) for the same medium.

The most important components of DJMM for high ALiP-P3 production were corn starch, casein, and $CaCO_3$. The use of corn starch resulted in high cell densities, whereas casein and $CaCO_3$ enhanced the cell-specific ALiP-P3 activity.

Western Blot Verification of ALiP-P3 Production. To confirm that the increased peroxidase activity results from enhanced production of the *S. viridosporus* T7A lignin peroxidase isoform ALiP-P3, a Western immunoblot was conducted using a polyclonal antibody against ALiP-P3 of *S. viridosporus* T7A (Magnuson *et al.*, 1991). Samples were taken from the culture of *S. viridosporus* T7A grown in both yeast-extract-based medium (a similar

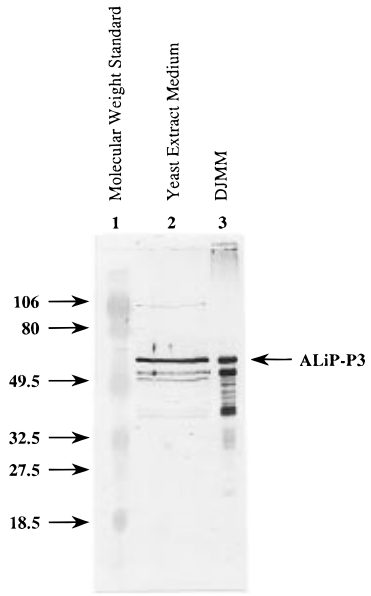

Figure 7. Western blot analysis of ALiP-P3 in both yeast extract and starch-based medium using rabbit ALiP-P3 antibody with culture supernatant samples: lane 1, MW standard; lane 2, *S. viridosporus* T7A in yeast-extract-based medium; lane 3, *S. viridosporus* T7A in final starch-based medium. Molecular weight standards (prestained; in kDa): phosphorylase *b* (106), bovine serum albumin (80), ovalbumin (49.5), carbonic anhydrase (32.5), soybean trypsin inhibitor (27.5), and lysozyme (18.5).

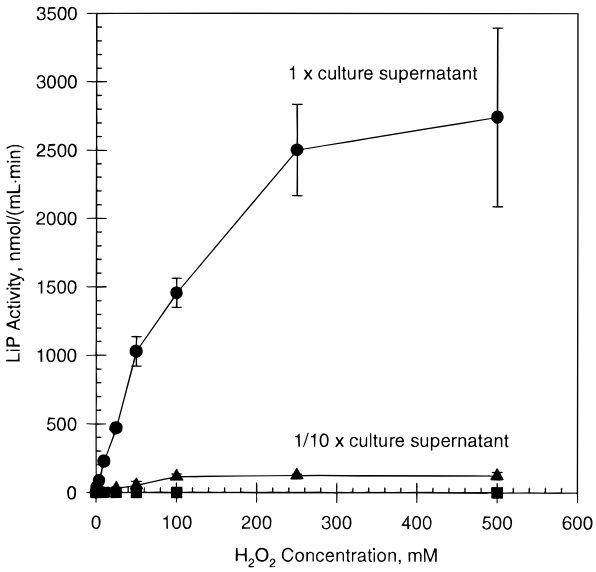

Figure 8. Hydrogen peroxide dependence of ALiP-P3 activity. Data represent triplicate enzyme samples obtained from a 100 mL culture of the final, optimized, starch-based medium (error bars indicate standard deviation). Symbols: ■, no enzyme control; ●, 1× culture supernatant; ▲, 1/10× culture supernatant.

medium from which the ALiP-P3 antibodies were isolated) and *S. viridosporus* T7A grown in the new starch-based medium. The size of proteins detected on the Western blot was calculated from a linear plot of ln(size of molecular weight markers) vs migration distance (R^2 = 0.994). As shown in Figure 7, ALiP-P3 from *S. viridosporus* T7A grown in both media gave a strong 63 kD ALiP-P3 band (lanes 2 and 3). This indicates the enhanced ALiP-P3 activity is due to increased ALiP-P3 production by the *S. viridosporus* T7A cultures grown in the starch-based medium. This banding pattern is similar to previously reported western blots of affinity-purified ALiP-P3 (Magnuson and Crawford, 1992). Although Magnuson and Crawford do not report a molecular weight for the affinity-purified ALiP-P3, their results show that only one protein band reacted strongly with the ALiP-P3 antibody. In addition to the strong 63 kD ALiP-P3 band, an intense 58 kD band arose in the starch-based sample (lane 3) that was much weaker in the yeast-extract-based culture. This second band may be a degradation product of the 63 kD ALiP-P3 band or an unidentified isoform with particularly high affinity to the polyclonal antibody. For the starch cultures, the 63 and 58 kD bands were clearly visible by SDS-PAGE (gel not shown).

Hydrogen Peroxide and ALiP-P3 Activity. As expected for a peroxidase, Figure 8 shows that ALiP-P3 activity was highly dependent on the hydrogen peroxide concentration present in the assay. Between a concentration range of 0.1 mM and 500 mM H_2O_2, peroxidase activity increased 1448-fold for the undiluted, concentrated ALiP-P3 and 264-fold for the 1/10× ALiP-P3. The H_2O_2 concentration dependence of undiluted ALiP-P3 was linear between 0.1 and 50 mM H_2O_2 ($y = 0.0996 + 0.3726x$; $R^2 = 0.998$). This large increase in ALiP-P3 activity was not due to oxidation of 2,4-dichlorophenol by H_2O_2; as shown in Figure 8, the addition of H_2O_2 without ALiP-P3 does not result in increased ALiP-P3 activity. The increase of ALiP-P3 activity at 1/10× dilution shows that enzyme activity at the concentration produced by *S. viridosporus* T7A in the yeast-extract medium was also highly dependent of H_2O_2.

Discussion

S. viridosporus T7A secretes four isoforms of lignin peroxidase (Ramachandra *et al.*, 1987). Of the four, isoform ALiP-P3 has the widest known substrate range (Ramachandra *et al.*, 1987). *S. viridosporus* T7A lignin peroxidase was previously obtained from a yeast-extract-based medium, and concentrating LiP from this medium using ultrafiltration (Lodha *et al.*, 1991) is relatively easy because all of the medium components are soluble in water. However, relatively high cell densities were not obtained and the peroxidase assay results were often inconsistent because of the relatively low amount of enzyme present in the unconcentrated culture (Lodha *et al.*, 1991). Using yeast-extract-based medium, background levels observed during the peroxidase assays (roughly 0.3 nmol/mL·min, obtained by using distilled deionized water rather than enzyme from supernatant) were often up to 10% of the maximum peroxide activities measured (data not shown). Consequently, the peroxidase present in the culture had to be concentrated for further analysis (e.g., SDS-PAGE). In addition, degradation and mineralization experiments may be hampered by the limited amount of enzyme in these *Streptomyces* cultures.

By identifying suitable carbon and nitrogen sources that both enhanced growth of *S. viridosporus* T7A and induced ALiP-P3 expression, and by optimizing the major component concentrations, higher volumetric and cell-specific activities were achieved. Corn starch was supplied as a carbon source, and two sources of nitrogen were provided. The organic, primary nitrogen source was casein, and the inorganic, supplementary nitrogen source used was ammonium chloride. Using these components and a calcium carbonate buffer, a 10-fold enhancement in *S. viridosporus* T7A cell density, a 60-fold increase in volumetric activity, and a 6-fold increase in cell-specific activity were observed.

Corn starch and casein, like lignin, are polymeric compounds which may require extracellular enzymes

that are not normally required in simple glucose and ammonium chloride media. By providing a carbon and nitrogen source which must be broken down by the cell to be assimilated, lignin peroxidase may be fortuitously produced. Higher total lignin peroxidase yields may also be obtained because of the rich supply of carbon and nitrogen which is available once starch and casein have been degraded into their unit components.

Because the lignin peroxidase assay is specific to the ALiP-P3 isoform (Ramachandra *et al*., 1988), the ALiP-P3 assay results indicate that the increase in peroxidase activity is due to increased production of ALiP-P3. This was corroborated by the Western blot analysis which indicated the ALiP-P3 obtained from the yeast-extract-based medium was identical to that of the starch-based cultures. Using the optimized starch-based medium, background levels of peroxidase activity were reduced to 0.3% of the measured activity and concentration of the culture supernatant was no longer required for the ALiP-P3 band to be visible with SDS-PAGE (data not shown).

The specific activity of ALiP-P3 was also shown to be very sensitive to hydrogen peroxide concentrations. Because there was no appreciable ALiP-P3 activity measured in the assay negative controls (culture supernatant containing ALiP-P3 but unsupplemented with H_2O_2), the peroxide in the supernatant synthesized by *S. viridosporus* T7A was negligible compared to the H_2O_2 added during the ALiP-P3 assay. Using H_2O_2 concentrations greater than that produced by *S. viridosporus* T7A, specific ALiP-P3 activity was increased by more than 1448-fold (0.1−250 mM H_2O_2). Since the H_2O_2 dependency study was conducted using the 2,4-dichlorophenol assay, either one of the two other substrates, 2,4-dichlorophenol and 4-aminoantipyrene, may be limiting the peroxidase reaction at H_2O_2 concentrations greater than 250 mM. In any case, the results of this study are applicable to the development of a reactor using *S. viridosporus* T7A lignin peroxidase. Because the maximum ALiP-P3 activity was achieved with peroxide concentrations toxic to bacteria, the degradation of DCP would best be accomplished in a separate reactor or compartment distinct from cell growth and ALiP-P3 production. Such a system would require a stable enzyme at room temperature for maximum efficiency. Although crude ALiP-P3 preparations were quite stable stored at 4 °C (67% of the initial activity remained after 2 years) and retained as much as 40% of their initial activity after autoclaving for 20 min at 121 °C (unpublished results), Lodha *et al*. (1991) have reported ALiP-P3 inactivation at moderate temperatures (30% residual activity after 30 min at 60 °C). While these considerations must be still be addressed, larger amounts of ALiP-P3 lignin peroxidase can be easily obtained with DJMM without time-consuming concentration steps, and in any reaction catalyzed by ALiP-P3, the benefit of adding additional hydrogen peroxide should be considered to increase the reactor efficiency.

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