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Synthesis and Properties of Lignin Peroxidase from *Streptomyces viridosporus* T7A

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ABSTRACT

The production of lignin peroxidase by *Streptomyces viridosporus* T7A was studied in shake flasks and under aerobic conditions in a 7.5-L batch fermentor. Lignin peroxidase synthesis was found to be strongly affected by catabolite repression. Lignin peroxidase was a non-growth-associated, secondary metabolite. The maximum lignin peroxidase activity was 0.064 U/mL at 36 h.

In order to maximize lignin peroxidase activity, optimal conditions were determined. The optimal incubation temperature, pH, and substrate (2,4-dichlorophenol) concentration for the enzyme assays were 45°C, 6, and 3 m*M*, respectively. Stability of lignin peroxidase was determined at 37, 45, and 60°C, and over the pH range 4–9.

Index Entries: Lignin peroxidase; *Streptomyces viridosporus;* enzyme synthesis

INTRODUCTION

Lignin peroxidase, a major component of the lignin-depolymerizing enzyme system of *Streptomyces viridosporus* T7A, has potential applications similar to those proposed for the fungus *Phanerochaete chrysosporium* in biopulping, biobleaching, converting lignin to useful products, and treating industrial wastes (*1*). Research on lignin peroxidase characterization, (especially enzyme synthesis and stability) and on biotechnological

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applications have been hampered by poor enzyme yield. There is a considerable interest in industrial-scale production of lignin peroxidase as well as in understanding the interactions between the various enzymes of the lignin-depolymerizing system of *Streptomyces viridosporus* T7A.

In this paper, lignin peroxidase production of *Streptomyces viridosporus* T7A from sucrose, glucose, and low-viscosity carboxymethylcellulose (CMC) were investigated. Conditions for the production of lignin peroxidase, as well as some of the characteristics of lignin peroxidase, were determined.

MATERIALS AND METHODS

Microorganism and Culture Maintenance

S. viridosporus T7A (ATCC 39115) stock cultures were stored at 4°C on yeast extract–malt extract–dextrose (YEMED) agar (2) for 2–12 wk. In all experiments, spores from stock slants were used in preparing the inoculum culture.

Culture Conditions

Shake-Flask Cultures

To determine the effect of glucose concentration on the production of peroxidase, batch cultures were carried out in 250-mL shake flasks, using 100 mL liquid. Glucose levels used were 0.5, 1, and 5 g/L. A control with no glucose was also maintained. Effect of CMC (3 g/L) and yeast extract (0.5 g/L) on peroxidase activity was also carried out in 250-mL shake flasks, using 100 mL working volume. The medium contained 15 g/L sucrose; 1 g/L each of proline, asparagine, and glutamic acid; 5.3 g/L Na_2HPO_4; 1.98 g/L KH_2PO_4; 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.2 g/L NaCl, 0.05 g/L $CaCl_2 \cdot H_2O$; and 1 mL of trace element solution (2). The agitation speed and temperature were 125 rpm and 37°C, respectively. Peroxidase activities were assayed after 2.5 d.

Inoculum Cultures

Fermenter inoculum (500 mL) containing 2.5 g/L yeast extract (Difco Laboratories, Detroit, MI); 1 g/L each of proline, asparagine, and glutamic acid; 5.3 g/L Na_2HPO_4; 1.98 g/L KH_2PO_4; 0.2 g/L Mg $SO_4 \cdot 7H_2O$; 0.2 g/L NaCl; 0.05 g/L $CaCl_2 \cdot H_2O$; and 1 mL of trace element solution (2) in a 2-L shake flask was autoclaved (121°C, 30 min), cooled, and inoculated with spores of *S. viridosporus* T7A from stock slants. Incubation was carried out at 37°C and 125 rpm. After growing for 24–32 h, the resulting logarithmic-phase culture was transferred to the fermenter.

Fermenter Culture Conditions

Fermentation was performed in a 7.5-L fermenter (Microferm, New Brunswick Scientific Co., Edison, NJ). A volume of 4.5 L containing 2.5

g/L yeast extract supplemented with 1 g/L each of proline, asparagine, and glutamic acid in mineral salts was added to the fermenter and autoclaved (121°C, 1 h). The sterile medium was inoculated with the 500-mL inoculum culture of *S. viridosporus* T7A to give a final volume of 5 L. Incubation was carried out at 37°C with agitation of 100 rpm and aeration of 1 L/min. Foaming was controlled manually with antifoaming agent MF (Hodag Chemicals Co., Skokie, IL). The culture pH was not controlled.

Crude Enzyme Solution

After 2 d of growth, culture supernatant solutions were harvested by filtration through No. 1 filter paper (Whatman, Inc., Clifton, NJ). Proteins in the filtrate were ultrafiltered through a 10,000 MW cut-off membrane (Amicon PM10) to concentrate the enzymes six-fold. This concentrated crude enzyme solution was used for all experiments.

Enzyme Assay

Peroxidase activity was assayed with 2,4-dichlorophenol (Sigma Chemicals Co., St. Louis, MO) as the substrate (*3*). Activity of 2,4-dichlorophenol is associated with the dominant lignin peroxidase produced by *S. viridosporus* (*4*). A final volume of 1.0 mL of reaction mixture contained 100 m*M* sodium succinate buffer (pH 5.5), 82 m*M* 4-aminoantipyrine, 1.0 m*M* 2,4-dichlorophenol, 4.0 m*M* hydrogen peroxide, and 100 μL of the enzyme preparation. The reaction was initiated by the addition of hydrogen peroxide, and the increase in A_{510} using a Hewlett-Packard UV/visible diode array spectrophotometer Model 8452A was monitored for 2 min at 37°C. One unit of enzyme activity was expressed as the amount of enzyme required for an increase of 1.0 U of absorbance/min. Specific activity was expressed as units of activity per milligram cell dry weight.

Cell Dry Weight

Cells were filtered onto preweighed filter paper (Whatman No. 1) dried at 70°C for 36 h and weighed.

Enzyme Denaturation

Crude enzyme solution (2 mL) was placed in a tube and kept in a water bath at 37°C for 24 h. Enzyme activity was measured every 3 h. A similar procedure was used to observe the enzyme denaturation at 45 and 60°C.

RESULTS AND DISCUSSION

Peroxidase Repression by Glucose

As shown in Fig. 1, increasing concentrations of glucose progressively reduced the production of lignin peroxidase. A reduction of approx 70%

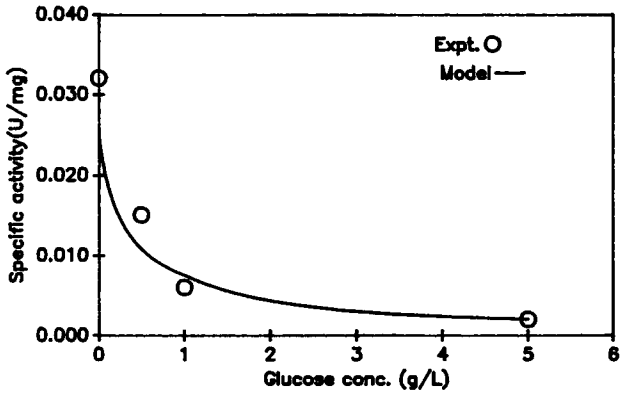

Fig. 1. Effect of glucose concentration on the production of lignin peroxidase by *S. viridosporus* T7A.

in peroxidase specific activity occurred at a 0.5 g/L glucose concentration, indicating strong peroxidase repression by glucose.

The above behavior can be satisfactorily explained by the following relationship:

$$A = K_1 / (K_2 + C)$$

or

$$(1 / A) = (K_2 / K_1) + (C / K_1)$$

where A = specific enzyme activity (U/mg dry cell), C = glucose concentration (g/L), and K_1,K_2 = constants. The R^2, K_1, and K_2 values calculated for the linear fit using SAS PROC REG analysis (SAS Institute, Cary, NC) were 0.99, 0.011 (U·g/mg cells·L) and 0.43 g/L, respectively.

Effect of CMC and Yeast Extract on Lignin Peroxidase Production

The effects of CMC and yeast extract (YE) on peroxidase production and cell mass were examined (Fig. 2). When YE (0.5 g/L) was used without a supplemental carbon source, high peroxidase activity (0.04 U/mL) and good growth (0.8 g/L) were obtained. When CMC (3 g/L) was used instead of YE, there was an 83% reduction in peroxidase activity relative to activity with YE. Previous studies established 3 g/L CMC as the optimum concentration for lignin peroxidase activity (*5*). When CMC (3 g/L) and YE (0.5 g/L) were used togather, there was a 25% reduction in peroxidase activity. Thus, YE without a supplemental carbon source was preferred for the production of lignin peroxidase.

Growth and Product-Formation Curves

Figure 3 shows that product formation was not growth-associated. This is similar to the ligninolytic system in *Phanerochaete chrysosporium*, in which lignin peroxidase is a secondary metabolite (*6,7*). Product formation

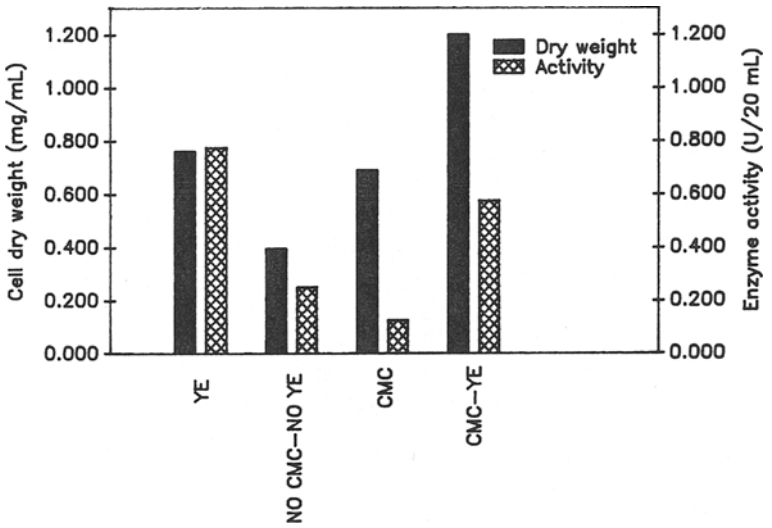

Fig. 2. Growth and production of lignin peroxidase by *S. viridosporus* T7A with CMC (3 g/L) and YE (0.5 g/L) in shake flasks. These carbon sources were added to a medium containing 15 g/L sucrose and 1 g/L each of proline, asparagine, and glutamic acid in a mineral salt solution.

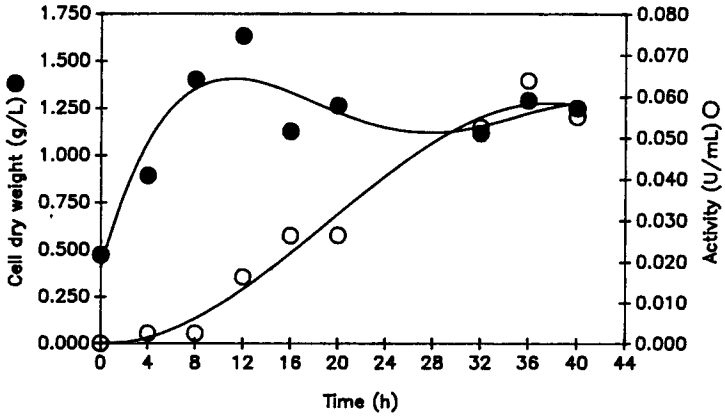

Fig. 3. Cell mass and lignin peroxidase activity during batch cultivation of *S. viridosporus* T7A in 7.5-L fermenter.

began after 8 h, and maximum activity was reached at 36 h. The maximum enzyme concentration was 0.064 U/mL at 36 h. Growth was exponential for approx 8 h, and cell mass was relatively constant after 15 h. The specific growth rate and doubling time for the initial 8 h were 0.14/h and 5.1 h, respectively.

Effect of Temperature on Enzyme Activity

The effect of temperature on the enzyme reaction is shown in Fig. 4a. The reaction at each temperature was carried out at pH 5.5 for 2 min.

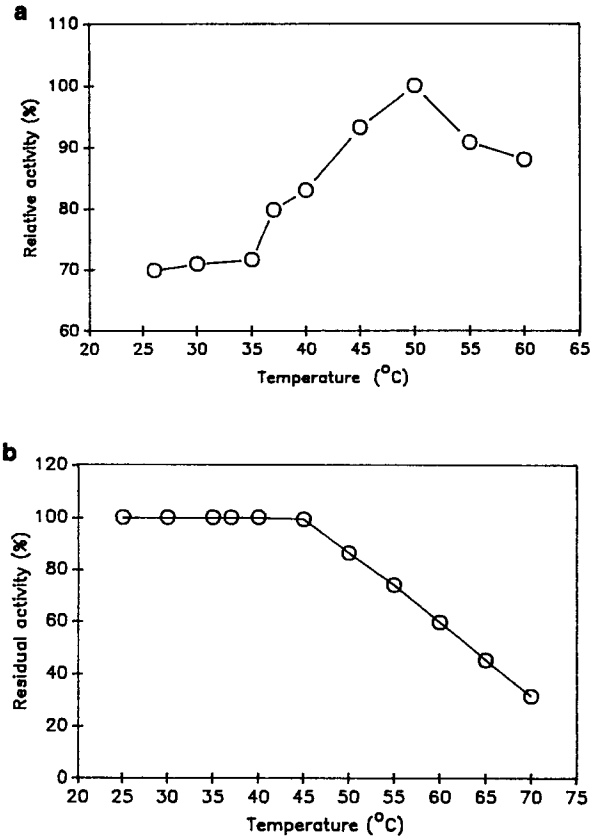

Fig. 4. (a) Influence of temperature on the activity of lignin peroxidase produced by *S. viridosporus* T7A. Enzyme assays were carried out at each temperature at pH 5.5. (b). Influence of temperature on the stability of lignin peroxidase produced by *S. viridosporus* T7A. Crude enzyme solutions were held at each temperature for 30 min prior to the enzyme assays at 37°C and pH 5.5.

Apparent enzyme activity was highest at 50°C. Since the enzyme might be inactivated at this high temperature, the temperature stability of the enzyme was examined (Fig. 4b). Crude enzyme solutions were maintained in a water bath at each temperature for 30 min. Enzyme activity was measured at each temperature using a Hewlett-Packard UV/visible diode array spectrophotometer with temperature-control system attached. The enzyme was inactivated above 45°C. On the basis of these data, a temperature of 45°C was used for the measurement of enzyme activity.

Effect of pH on Enzyme Activity

The effect of pH on the enzyme reaction is shown in Fig. 5a. Sodium succinate buffer (0.5*M*) in the range of pH 4.5–6 and potassium phosphate buffer (0.5*M*) in the range of pH 6.5–8.5 were used. The optimum pH of lignin peroxidase lies near pH 6. Since the enzyme might not be stable at this pH, the pH stability of the enzyme was examined (Fig. 5b). Crude

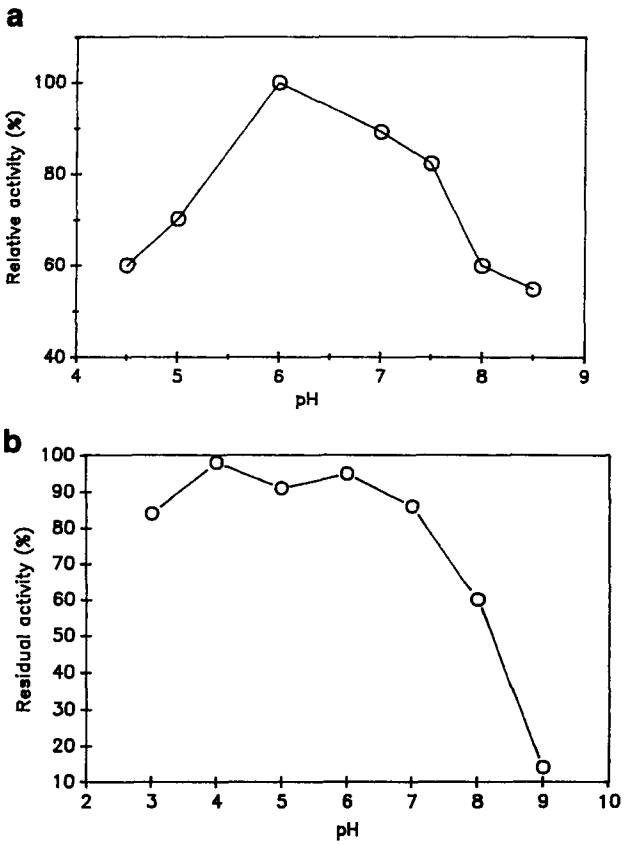

Fig. 5. (a) Influence of pH on the activity of lignin peroxidase produced by *S. viridosporus* T7A. Sodium succinate butter (0.5*M*) in the range of pH 4.5–6 and potassium phosphate buffer (0.5*M*) in the range of pH 6.5–8.5 were used. (b) Influence of pH on the stability of lignin peroxidase produced by *S. viridosporus* T7A. Crude enzyme solutions were held at each pH for 10 h at 4°C prior to enzyme assays at 37°C and pH 5.5.

enzyme solution (100 μL) was added to six tubes, each containing 300 μL buffer in the range of pH 4–9. Thus, crude enzyme solutions were maintained at each pH for 10 h at 4°C. The enzyme was found to be most stable (more than 90% stability) in the range of pH 4–6.

Effect of Substrate Concentration on Enzyme Reaction

The relationship between enzyme activity, E, and 2,4-dichlorophenol concentration, S, can be represented by a hyperbolic saturation curve (Fig. 6) and described by the following Michaelis-Menten-type equation:

$$E = E_{max} (S / K_m + S)$$

or

$$1 / E = (K_m / E_{max}) [(1 / S) + (1 / E_{max})]$$

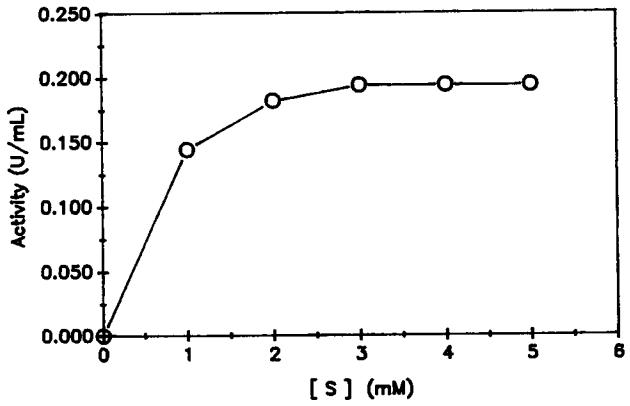

Fig. 6. Effect of substrate concentration (2,4-dichlorophenol) on lignin peroxidase activity.

where E = enzyme activity (U/mL), E_{max} = maximum enzyme activity (U/mL), S = final substrate concentration (mM) in the reaction mixture; and K_m = the saturation constant representing the substrate concentration at which $E = (1/2)\ E_{max}$.

The optimum substrate concentration in the reaction mixture was 3 mM. The E_{max} and K_m values for the nonlinear fit using SAS PROC NLIN analysis (SAS Institute, Cary, NC) were 0.22 U/mL and 0.4 mM, respectively.

Enzyme-Deactivation Model

The following Arrhenius model was used to describe the deactivation of lignin peroxidase with time:

$$E = E_0 * \exp [(-k_d)\ (t)]$$

or

$$1n\ (E_0/E) = k_d\ (t)$$

where E_0 = initial enzyme activity, E = enzyme activity at time t, and k_d = deactivation constant (h^{-1}).

The experimental data for peroxidase deactivation at 37, 45, and 60°C were fitted using the above model (Fig. 7). Results obtained using SAS PROC REG linear analysis (SAS Institute) are listed in Table 1. Deactivation at 45°C is 1.7 times faster than deactivation at 37°C, whereas deactivation at 60°C is 9.4 times faster than deactivation at 37°C.

Energy of Activation

Data from Table 1 were used to determine energy of activation using the Arrhenius equation:

$$k_d = A\ \{\exp [-E_a/(RT)]\}$$

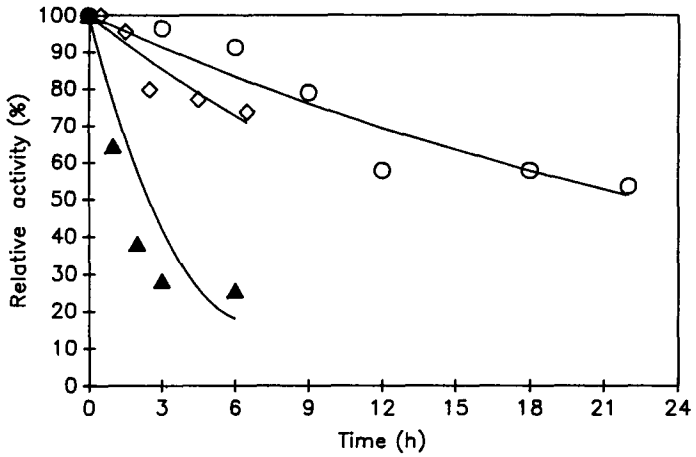

Fig. 7. Deactivation of lignin peroxidase at 37 (○), 45 (◇), and 60°C (▲).

Table 1
The Model Parameter and R^2 Value
for the Relationship Between Peroxidase Activity and Time

Temperature(°C)	k_d (h^{-1})	R^2
37	0.0306	0.955
45	0.0533	0.940
60	0.2869	0.900

or

$$\ln k_d = \ln A - E_a/(RT)$$

where E_a = activation energy, R = gas law constant, A = frequency factor, and T = temperature (K). The R^2, E_a, and A calculated for the linear fit using SAS PROC REG analysis were 0.94, 1813.2 J/mol, and 9.39/h, respectively.

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REFERENCES

1. Buswell, J. A. and Odier, E. (1987), Lignin biodegradation. *CRC Crit. Rev.* **6,** 1–60.
2. Pridham, T. G., and Gottlieb, D. (1948), *J. Microbiol.* **56,** 107–114.

3. Pasti, M. B., Pometto, A. L. III, Nuti, M. P., and Crawford, D. L. (1990), *Appl. Environ. Microbiol.* **56,** 2213–2118.
4. Wang,. Z., Blakeley, B. H., Crawford, D. L., Hertel, G., and Rafii, F. (1990), *J. Biotechnol.* **13,** 131–144.
5. Ramachandra, M., Adhi, T. P., and Crawford, D. L., The influence of culture parameters on the production of lignin peroxidase by *Streptomyces viridosporous* T7A. Poster presented at ASM Annual Meeting, New Orleans, May (1989).
6. Linko, S. (1988), *Enzyme Microbiol. Technol.* **10,** 410–417.
7. Faison, B. D., and Kirk, T. K. (1985), *Appl. Environ. Microbiol.* **49,** 299–304.