APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1989, p. 1165–1168
0099-2240/89/051165-04$02.00/0
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Vol. 55, No. 5

Production of Major Extracellular Enzymes during Lignocellulose Degradation by Two Streptomycetes in Agitated Submerged Culture

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Received 21 November 1988/Accepted 17 February 1989

Streptomyces viridosporus T7A and _S. badius_ 252 were grown in 1 to 2% (wt/vol) slurry cultures with mineral salts solution containing 0.6% yeast extract and 100/200 mesh ground and extracted corn lignocellulose at 37°C. Enzyme activities rapidly increased in the first 3 to 4 days and then declined and remained at a relatively constant level. Concentrations of endoglucanase and xylanase produced by _S. badius_ were lower than those produced by _S. viridosporus_. However, the lignin-peroxidase peak concentration was threefold higher than with _S. viridosporus_ and was obtained at 9 to 10 days of incubation. By polyacrylamide gel analysis, it was determined that peroxidases from both species consisted of four enzymes, with only one, the lignin peroxidase, having high activity. A culture pH of 8.5 was preferable for lignocellulose degradation by _S. badius_.

When grown on corn lignocellulose, *Streptomyces viridosporus* T7A produces extracellular enzymes that extensively degrade both lignin and carbohydrate components (4, 9). The most abundant lignin degradation catabolite produced during this oxidative degradation is a polymeric water-soluble modified lignin polymer, acid-precipitable polymeric lignin (APPL) (2, 3). The same degradative mechanism also has been examined in mutant strains with enhanced abilities to produce APPL (4, 9). These APPL-overproducing strains also overproduce peroxidases and cellulases, indicating that these enzymes are involved in lignin biodegradation (9). Recently, lignin peroxidase was shown to be a major component of the ligninolytic system of *S. viridosporus* T7A (10).

Previously, we reported lignin degradation and production of APPL by *S. viridosporus* T7A in submerged, agitated cultures (1). Compared with the solid-state degradation of lignin by *S. viridosporus* T7A (3, 7), the agitated culture reduced the incubation time and gave higher APPL production. In the present study, we used agitated cultures to characterize the production of major extracellular enzymes (endoglucanase, xylanase, and lignin peroxidase) during corn lignocellulose degradation by *S. viridosporus* T7A and *S. badius* 252. Results showed that *S. badius* produces the same lignin peroxidases as *S. viridosporus* T7A but has a higher peroxidase activity.

MATERIALS AND METHODS

Microorganisms. Stock cultures of *S. viridosporus* T7A (ATCC 39115) and *S. badius* 252 (ATCC 39117) were maintained at 4°C on yeast extract-malt extract-dextrose agar and yeast extract-malt extract agar, respectively (8). Spore suspensions from 2- to 8-week-old stock slants were used to prepare the inoculum culture.

Batch experiments. (i) Inoculum cultures. A 10% total working volume of 0.6% (wt/vol) yeast extract-mineral salt medium was added to a 0.5-liter fermentor (Bioflo C32; New Brunswick Scientific Co., Inc., Edison, N.J.) and autoclaved (121°C, 1 h). The sterilized mixture was then inoculated with a spore suspension of *S. viridosporus* T7A or *S. badius* 252. Incubation was carried out at 37°C with high agitation and high aeration. After growing for 24 to 48 h, the resulting logarithmic-phase culture was transferred to the working fermentor.

(ii) First experiments. A 1% (wt/vol), completely extracted (1, 3), 100/200 mesh dried corn lignocellulose suspension in 1.35 liters of mineral salts supplemented with 0.6% yeast extract was added to a 2.0-liter fermentor (Bioflo C32; New Brunswick Scientific Co., Edison, N.J.) and autoclaved (121°C, 1 h). The sterilized suspension was then inoculated with 150 ml of inoculum culture of *S. viridosporus* T7A. Incubation was carried out at 37°C with agitation (200 rpm) and aeration (0.5 liter/min). Foaming was controlled manually with antifoam MF (Hodag Chemical Co., Skokie, Ill.). Culture pH was maintained at 8.5 by automatic addition of 2 N H_2SO_4 or 2 M NaOH. Lignocellulose degradation, enzyme activities, APPL yield, and cell mass concentration were determined as described below.

(iii) Second experiments. The system for the second experiments was similar to that of the first experiment, with *S. viridosporus* T7A and *S. badius* 252 and a total working volume of 3.5 liters with 2% lignocellulose. Culture parameters were as follows: temperature, 37°C; agitation, 300 rpm; aeration, 1.0 liter/min; and pH, 8.5. The pH of the *S. badius* 252 culture was initially 6.75 and then was monitored at suitable time intervals. Enzyme activities and APPL yield were determined as described below.

Lignocellulose loss and cell mass determination. Residues from each sample of the first experiments were collected by suction filtration onto preweighed glass filter paper (GF/A; Whatman, Inc., Clifton, N.J.). To determine lignocellulose weight loss, insoluble residues on the filters were air dried at 100°C and allowed to equilibrate to room temperature, and the filters were reweighed. The percent nitrogen and crude protein of the insoluble residues were estimated by micro-Kjeldahl analysis (5, 6). The percent cell mass was determined by taking the total protein in the culture residue and subtracting the protein in the uninoculated sterile control. *S. viridosporus* has a protein content of 57% as determined by micro-Kjeldahl analysis (1).

Estimation of APPL. Concentrations of water-soluble APPL present in the culture filtrates were estimated by using a turbidimetric assay (3). Culture filtrates (1.5 ml) were acidified with 0.15 ml of 12 M HCl. The A_{600}s of acidified samples were recorded. APPL contents were calculated

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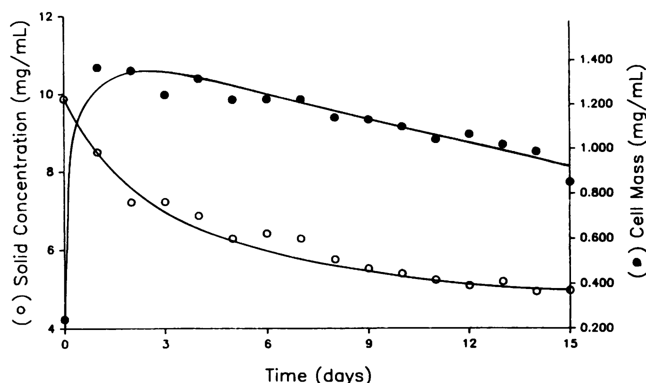

FIG. 1. Lignocellulose (○) and cell (●) concentrations during degradation of 1% (wt/vol) corn lignocellulose by *S. viridosporus* T7A.

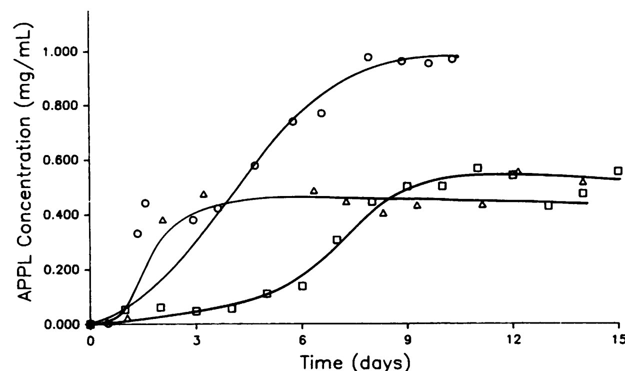

FIG. 2. Production of water-soluble APPL by *S. viridosporus* T7A with 1% corn lignocellulose (□) and 2% corn lignocellulose (○) and by *S. badius* 252 with 2% corn lignocellulose (△).

from a standard curve prepared by using a gravimetrically predetermined APPL content of 2-week-old culture filtrates of *S. viridosporus* T7A.

Enzyme assays (9). (i) Peroxidase. Peroxidase activity was assayed by monitoring the oxidation of 2,4-dichlorophenol (Sigma Chemical Co., St. Louis, Mo.) in the presence of hydrogen peroxide. A final volume of 1.0 ml of reaction mixture contained a 100 mM sodium succinate buffer (pH 5.5), 82 mM 4-aminoantipyrine (Sigma), 1.0 mM 2,4-dichlorophenol, 4.0 mM hydrogen peroxide, and 200 μl of 10-fold-concentrated enzyme preparation. The reaction was initiated by the addition of hydrogen peroxide, and the increase in A_{510} was monitored for 1 min at 37°C. One unit of enzyme activity was expressed as the amount of enzyme required for an increase of 1.0 absorbance unit per min.

(ii) Endoglucanase. Endoglucanase was assayed with low-viscosity carboxymethyl cellulose (Sigma) as the substrate. One ml of 1% (wt/vol) carboxymethyl cellulose in 0.5 M phosphate buffer (pH 6.0) was added to a test tube containing 0.5 ml of unconcentrated culture supernatant. The test tubes were incubated at 45°C for 10 min. The reaction was stopped by adding 3.0 ml of 3,5-dinitrosalicylate (Sigma) reagent, and the tube was then boiled for 15 min. A 1.0-ml volume of 40% (wt/vol) sodium potassium tartarate was added to the tube to help maintain color stability. After cooling the tube at room temperature, the A_{575} was recorded. The amount of reducing sugar released was estimated from a glucose standard curve. One unit of enzyme activity was expressed as the amount of enzyme which produced 1 μmol of reducing sugar per min.

(iii) Xylanase. Xylanase was assayed as described above for endoglucanase, except that carboxymethyl cellulose was replaced with 1.0 ml of 0.5% (wt/vol) oat spelt xylan (Sigma) in 0.5 M phosphate buffer.

(iv) Peroxidase activity staining on gels. Native polyacrylamide gels were run for each 10-fold concentrate. The gels were then treated for 5 min at 37°C. Peroxidase assay was performed by using 197.2 mg of L-dopa–0.1 g of 4-aminoantipyrine–4 ml of 0.3% hydrogen peroxide in 100 ml of 0.1 M phosphate buffer. Peroxidase bands stained red.

RESULTS AND DISCUSSION

The first experiment was carried out to explore the general pattern of lignocellulose degradation and APPL production by *S. viridosporus* T7A in agitated, submerged cultures. Time dependence of lignocellulose degradation (expressed in terms of remaining total weight concentration), cell growth, and APPL yield were determined. The total lignocellulose loss and cell growth (Fig. 1) were similar to results obtained in shaken-flask experiments (1). The maximum degradation of lignocellulose (50 to 53% with weight attributed to cell mass subtracted) in both experiments was achieved after approximately 2 weeks. Experiments with 2% initial lignocellulose gave large variations in total lignocellulose weight loss determinations (data not shown).

APPL concentrations by *S. viridosporus* T7A for both first (1% initial lignocellulose concentration) and second (2% initial lignocellulose concentration) experiments in this study reached maximum values of 0.52 mg/ml and 1.0 mg/ml, respectively, after 9 to 10 days (Fig. 2). Shaken-flask cultures at 2 weeks of incubation gave maximum yields of 1.2 mg/ml and 1.4 mg/ml, respectively, for 3 and 5% initial lignocellulose concentrations (1).

Unlike *S. viridosporus* T7A, *S. badius* 252 grows poorly in the solid-state lignocellulose system (2). Therefore, the time course of APPL production was previously followed in liquid shaken cultures after establishment of growth on dampened lignocellulose (2). In the present study, APPL production by *S. badius* 252 in agitated, submerged culture followed a similar pattern, reaching a maximum rapidly and then remaining constant for the duration of the experiment (Fig. 2). However, maximum APPL concentration could be attained faster (3 days compared with 8 days) and much higher (0.45 mg/ml compared with 0.06 mg/ml) relative to

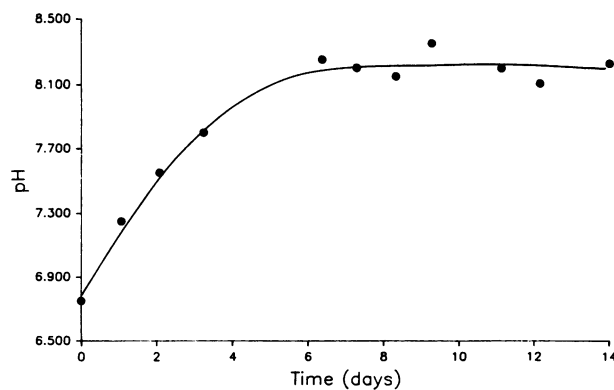

FIG. 3. pH values during *S. badius* culture with 2% corn lignocellulose.

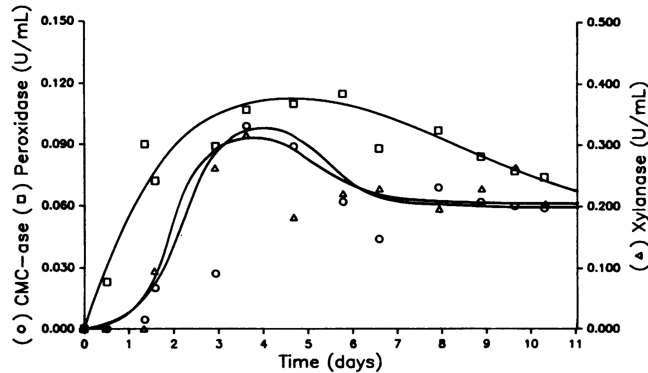

FIG. 4. Extracellular enzyme activities during 1% corn lignocellulose degradation by *S. viridosporus* T7A. ○, Endoglucanase; ●, xylanase; △, lignin-peroxidase.

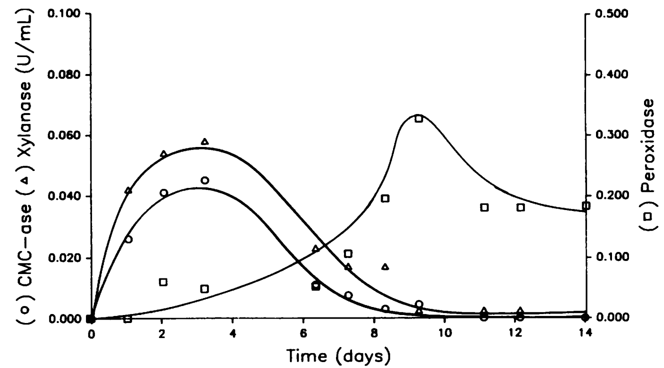

FIG. 6. Extracellular enzyme activities during 2% corn lignocellulose degradation by *S. badius* 252. ○, Endoglucanase; △, xylanase; □, lignin-peroxidase.

shaken cultures (2). These differences probably result from better aeration and agitation in the agitated, submerged culture.

Culture pH was previously reported as a dominant factor in APPL production by *S. viridosporus* T7A in solid-state fermentation (7) as well as in agitated, submerged culture (1). The present study with *S. badius* 252 showed a similar pH pattern during the course of incubation (Fig. 3). The trend was to a higher pH of 8.3, and nearly all APPL production occurred during the time of rapid increase in pH. This indicated that a higher maximum production of APPL by *S. badius* 252 could also be obtained by maintaining culture pH at values between 8.0 and 8.5. By changing pH from 7.0 to 8.5 in 5% agitated slurry cultures with *S. viridosporus* T7A, it was shown that the APPL yield was increased approximately sixfold from 0.815 to 4.935 mg/ml (1).

The first experiments of this study were also intended to explore enzyme production in agitated, submerged cultures. Extracellular enzyme production by *S. viridosporus* during 1% corn lignocellulose degradation in agitated, submerged cultures is shown in Fig. 4. Each enzyme reached its peak activity early, and then activity dropped and finally reached a constant level. These similar trends in activities indicate a close interaction among these key enzymes which act synergistically in degrading lignocellulose.

With *S. viridosporus* T7A, maximum peroxidase activity was observed early in the stationary-growth phase (Fig. 1 and 4). This also has been observed with *S. viridosporus* T7A in liquid culture supplemented with 0.05% (wt/vol) lignocellulose (7). Lignin peroxidase, endoglucanase, and xylanase all reached maximum activities simultaneously with *S. viridosporus* (Fig. 4 and 5).

The patterns of extracellular enzyme activities produced by *S. viridosporus* T7A and *S. badius* 252 were similar, with the exception of peroxidase activities. The peak activities for both endoglucanase and xylanase were approximately twice as large for *S. viridosporus* T7A relative to *S. badius* 252 (Fig. 5 and 6). In contrast, the peak peroxidase activity for *S. badius* 252 was approximately three times larger than that for *S. viridosporus* T7A. However, *S. viridosporus* T7A culture showed maximum peroxidase activity approximately 5 days earlier than did *S. badius* 252.

Recently, a major component of the ligninolytic system of *S. viridosporus* T7A has been identified as an extracellular lignin-inducible peroxidase, which is one of four distinct peroxidases (7, 9). In the present study, polyacrylamide gel analysis showed that *S. badius* produced a similar peroxidase system with four enzymes (Fig. 7). This is the first report showing that a wild type of a *Streptomyces* species other than *S. viridosporus* is capable of producing similar ligninolytic enzymes.

During corn lignocellulose degradation in slurry cultures of *S. viridosporus* T7A, the depletion of lignin occurred in parallel with the depletion of carbohydrate (1). There was also a strong correlation between lignin depletion and APPL accumulation (1). Interestingly, the present study showed that the drop in the activities of peroxidase, endoglucanase, and xylanase was followed by production of APPL. Maximum APPL concentration was observed when the activities

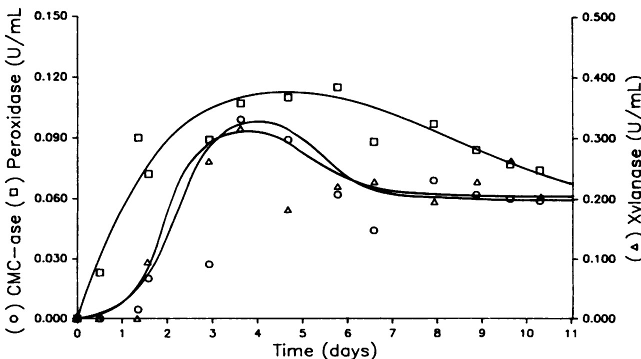

FIG. 5. Extracellular enzyme activities during 2% corn lignocellulose degradation by *S. viridosporus* T7A. ○, Endoglucanase; △, xylanase; □, lignin-peroxidase.

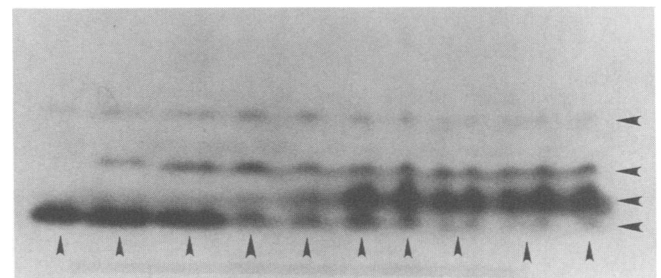

FIG. 7. Polyacrylamide gel electrophoresis showing four isoforms of lignin peroxidase (arrowheads at right) produced by *S. badius* 252 at the times corresponding to those in Fig. 6 (arrowheads at bottom; from left to right, 1.1, 2.1, 3.2, 6.4, 7.3, 8.3, 9.3, 11.1, 12.1, and 14.0 d).

of extracellular enzymes were relatively constant. Since lignocellulose degradation is a heterogenous enzymatic process, the first step is adsorption of key enzymes onto the surface of lignocellulose particles. The drop in extracellular enzyme activities could be partially attributed to this adsorption process. Adsorption was indicated by a decrease in peak activity of peroxidase with increasing initial lignocellulose concentration: 0.240 U/ml in 0.05% lignocellulose (7), 0.18 U/ml in 1% lignocellulose (Fig. 4), and 0.11 U/ml in 2% lignocellulose (Fig. 5). Controlled studies of lignocellulose degradation would be best accomplished in a cell-free system. Key extracellular enzymes have been identified, and our future work will examine the interactions of these key enzymes during lignocellulose degradation.

ACKNOWLEDGMENTS

This work was supported by a grant from the National Science Foundation (CBT-8713198), by Department of Energy grant DE-FG786ER13586, and by MUCIA-Indonesia XVIIth WB Education Project.

We thank Muralidhara Ramachandra and Anthony L. Pometto III for technical advice and assistance.

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