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Lignin Degradation and Production of Microbially Modified Lignin Polymers by *Streptomyces viridosporus* in Slurry Reactors

Scientific Note

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Index Entries: Lignin; APPL; *Streptomyces*; slurry culture.

INTRODUCTION

In nature, lignin is found covalently linked to cellulose, the most abundant compound in the biosphere, as lignocellulose (*1*). Lignin, a polyphenolic polymer, is degraded by some fungi and filamentous bacteria (*1–3*). Bacteria of the genus *Streptomyces* are known to attack lignocellulose by degrading both the lignin and polysaccharide components (*2–6*). Quantitatively, the most important lignin degradation catabolite produced during degradation of lignin by these filamentous bacteria is a polyphenolic water soluble modified lignin polymer, acid precipitable polymeric lignin (APPL), which precipitates from solution upon acidification (*6–8*). Among five selected *Streptomyces* grown on ground and extracted corn stover lignocellulose in solid-state fermentations, *Streptomyces viridosporus* T7A showed the highest yield of APPL (*9*). Currently,

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lignin degradation and APPL production by streptomycetes have been demonstrated only in bubbler tube cultures (*10*) and in solid-state fermentations (*7,11*). Typically, lignocellulose degradation and APPL production by *S. viridosporus* in solid-state fermentation of ground and extracted corn lignocellulose requires 6–8 wk incubation at 37°C to produce an average lignocellulose weight loss of 36% and an APPL yield of 98 mg/g of initial lignocellulose (*7,8,10,11*).

APPLs have some unique commercially valuable properties if they could be produced inexpensively on a large scale. For example, APPLs have both antioxidant (*11*) and immunoadjuvant properties (*12*), and they have other possible uses as a phenolic chemical feedstock (*9*).

A proposed solid state fermentation process for industrial production of APPLs has been developed (*9*). Solid-state fermentation has the advantage of low agitation, high surface contact with culture mycelia, high oxygen transfer, and it closely resembles the cultural conditions of lignocellulose degradation in nature. It has been hypothesized that some extracellular enzymes involved in lignin degradation are cell associated (*13–15*), which requires intimate hyphal-lignocellulose contact. Stable mutants and protoplast fusion recombinants have been developed that produce APPL at a faster rate and in higher yields than the wild type strain (*15,16*). Commercial production of APPL using solid-state fermentation has the disadvantages of scaleup difficulties, long incubation times (6–8 wk), and space limitations caused by large surface area requirements.

In this paper we report on lignocellulose degradation and APPL production by *S. viridosporus* in slurry culture operated at 3 and 5% (w/v) lignocellulose concentrations. Shake flasks and stirred reactors were employed. The effect of pH on lignocellulose degradation and APPL production was also determined, with pH 8.5–8.7 being optimal. After only a 2-wk incubation lignocellulose degradation and APPL yields in pH 8.5 to 8.7 cultures were similar if not better than 6–8 wk solid-state fermentations.

MATERIALS AND METHODS

Microorganism

Stock cultures of *Streptomyces viridosporus* T7A (ATCC 39115) were maintained at 4°C on yeast extract-malt extract-dextrose agar (YEMED) (*17*). Spore suspensions from 2–8 wk-old stock slants were used as the initial inoculum in all experiments.

Slurry Culture in Stirred Reactors

All stirred slurry cultures were run in 2 L fermentor vessels equipped with agitation, temperature, and pH control (Bioflo C32, New Brunswick Scientific Co., Edison,NJ). Corn stalks (*Zea mays*) were dried,

ground in a Wiley mill to pass a 20 mesh screen, and then sequentially extracted with hot water, benzene-ethanol (1:1), ethanol, and hot water, after which the residue was air dried (*7*). Dried 30 or 50 g samples of lignocellulose were added to 2 L Bioflo vessels with 1 L of mineral salts medium (5.3 g of Na_2HPO_4, 1.98 g of KH_2PO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.2 g of NaCl, 0.05 g of $CaCl_2 \cdot 2H_2O$, and 1 mL of trace elements solution (*17*) in 1 L of deionized water; pH 7.1) also containing 0.6% (w/v) yeast extract (Difco Lab., Detroit, Michigan). Lignocellulose and medium were autoclaved (121°C, 1 h) together. Each sterilized slurry was inoculated with a 10 mL spore suspension prepared from one stock slant of *S. viridosporus*. Cultural parameters were temperature (37°C), agitation (200 rpm), and aeration (0.5 L/min). Filter sterilized, humidified, CO_2 free air was supplied to all stirred slurry cultures. Foaming was controlled manually with antifoam MF (Hodag Chemical Co., Skokie, IL). For 3 and 5% (w/v) stirred slurry cultures the initial pH was maintained at 7.1–7.4 for the first 3 d and then was aseptically changed to pH 8.5–8.7 for the remainder of the 2 wk incubation. For one 5% (w/v) slurry culture the pH was maintained at pH 7.0–7.2 for the entire 2 wk incubation. Uninoculated sterile controls were incubated under the same conditions as described above for each slurry culture. Lignocellulose degradation and APPL yield were determined for each reactor as described below.

Slurry Culture in Shake Flasks

Streptomyces viridosporus T7A was grown in 3% and 5% (w/v) shaken slurry cultures with 100 mL of mineral salts solution containing 0.6% yeast extract and 40 mesh ground and extracted corn lignocellulose in 500-mL cotton-plugged Erlenmeyer flasks. Slurry culture pH was adjusted to pH 8.0 prior to autoclaving (15 min), which resulted in a final pH of 7.1–7.2. Sterilized slurry cultures were inoculated with spores and incubated at 37°C and 200 rpm. Inoculated (two flasks) and uninoculated (one flask) samples were harvested every 3–4 d for a total incubation of 6 wk. The final pH for each harvested flask was also determined. Lignocellulose degradation and APPL yield were determined as described below.

Chemical Characterization of Lignocellulose Residues and APPLs

For harvest of each slurry culture (stirred reactor and shaken flask) an equal volume (1 L or 100 mL) of deionized water was added. The vessel or flask was placed in a steamer (100°C) for 1 h, and the residue was collected by suction filtration onto preweighed filter paper (Whatman No. 1) (*7*). To determine lignocellulose weight loss, insoluble residues on the filters were air dried at 70°C, allowed to equilibrate to room temperature, and the filters were reweighed. APPLs were recovered from the aqueous filtrate by acidification to pH 1.5–2.0 with 12 *M* HCl. The precipitate that formed was collected by centrifugation, washed once with deionized water, air dried (70°C), allowed to equilibrate to room tempera-

ture, and then weighed to determine the APPL yield for each slurry culture (*7*).

Both insoluble residues and APPLs were subjected to the following chemical assays. The Klason lignin contents were determined by a modified Klason procedure (*18*). Total carbohydrate contents in the Klason filtrates were determined by measurements of reducing sugars using a modified Somogy-Nelson procedure (*19*). The percent nitrogen and crude protein were determined by micro-Kjeldahl analysis (*18,20*). The percent cell mass in the harvested residue was determined by assuming the total protein in the culture residue minus the protein in the uninoculated sterile control was from the cell mass. *S. viridosporus* has a protein content of 57% as determined by micro-Kjeldahl. Ash content for each APPL was determined by combustion of about 100 mg samples at 500°C in an environment of pure oxygen (*7*).

RESULTS AND DISCUSSION

Shake flask experiments were used for obtaining preliminary data on lignocellulose degradation and APPL production by *S. viridosporus* strain T7A in slurry reactors. Time dependence of lignocellulose degradation (expressed in terms of weight, lignin, and total carbohydrate loss), APPL yield, cell growth, and pH change were determined. The results for 3 and 5% shaken slurry cultures are shown in Figs. 1–5. Smooth lines have been drawn in each figure but do not represent any fitted or theoretical predictions. The maximum degradation of lignocellulose (weight attributed to cell mass has been subtracted) in both 3% and 5% shaken slurry cultures was achieved after about 2 wk incubation (53 and 60%, respectively). There was little additional weight loss for the remainder of the 6 wk (Figs. 1 and 2). After a 6 wk incubation the 3% and 5% uninoculated controls showed 7.3 and 9.2% lignocellulose weight losses, respectively. The losses of lignin and carbohydrate were expressed as percent losses of initial content. Lignin and carbohydrate initial contents were 27 and 45%, respectively. Carbohydrate loss was rapid over the first wk and slow for the following 5 wk. Lignin loss was approximately linear over the initial 2 wk, and there was very little lignin loss over the following 4 wk. For 3 and 5% uninoculated controls carbohydrate losses of 2.18 and 1.26%, respectively, were observed after 6 wk of incubation. No lignin losses were observed.

APPL yields in 3% shaken slurry cultures reached a maximum of 40 mg/g of initial lignocellulose, and APPL accumulation followed a similar pattern to lignin weight loss (Fig. 3). Despite some inconsistencies in data for 5% shaken slurry cultures, the pattern had the same trend, reaching a maximum of 28 mg APPL per g of initial lignocellulose (Fig. 3). For 3 and 5% uninoculated controls, APPL yields were 5.1 mg and 4.8 mg/g of initial lignocellulose, respectively. Both lignin loss and APPL yield gradually increased until the maximum APPL yield was achieved at

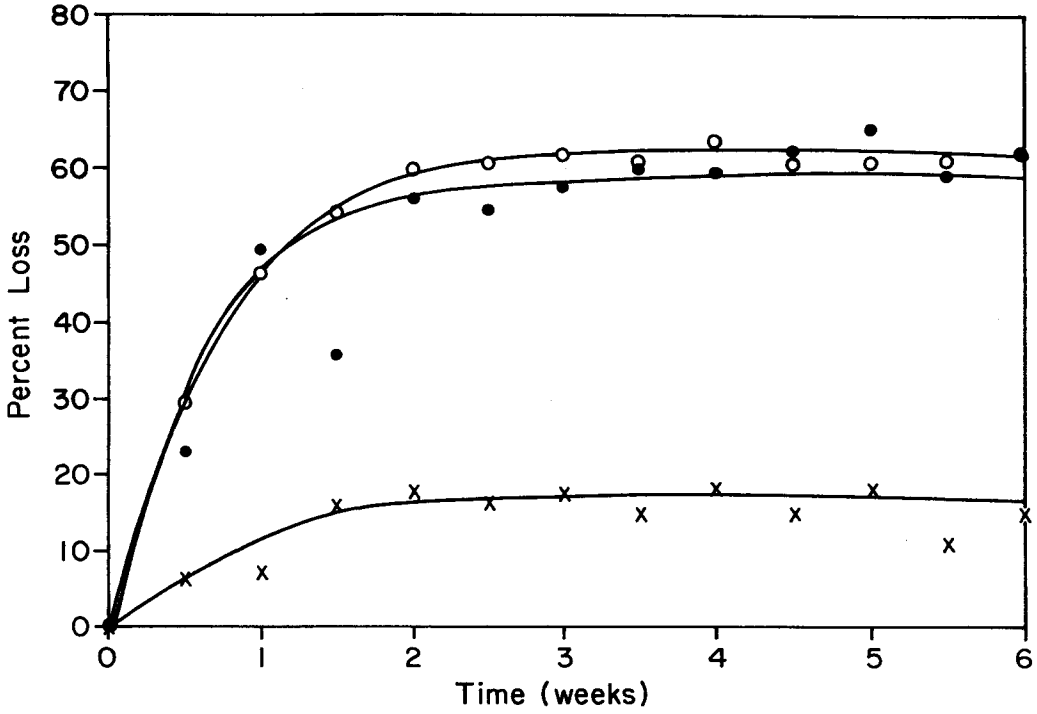

Fig. 1. Corn lignocellulose degradation by *S. viridosporus* T7A in 3% shaken slurry cultures incubated at 37°C for 6 wk reported as total lignocellulose (○), carbohydrate (●), and lignin weight loss (X).

approximately 2 wk, showing a strong correlation between the loss of insoluble lignin and the accumulation of soluble APPL. Time course data obtained with 8 wk dampened (solid-state) cultures showed both APPL yield and lignin loss continuous throughout the incubation (*8*). The shaken slurry cultures at 2 wk incubation gave a similar APPL yield (28 mg/g initial lignocellulose) and lignin loss (12–15%) as compared to the solid-state system at 6 wk (8).

Both the 3% and 5% shaken slurry cultures produced similar patterns for change in pH during the course of the incubation (Fig. 4). The trend was to a higher pH of 8.0 and 8.4, and nearly all the change in pH occurred early, during the time of greatest lignocellulose degradation indicating the production of alkaline products during growth of *S. viridosporus*. The source of alkaline products may be yeast extract. This pattern has also been detected in solid-state fermentation when the medium contained 0.6% yeast extract (*10*). The smaller pH change in the 5%, compared to the 3%, shaken slurry culture was probably a result of the higher lignocellulose concentration in the 5% slurries, which probably resulted in a higher organic acid and CO_2 production because of lignocellulose degradation (*10*). This smaller increase in pH could also explain the lower APPL yield for the 5% shaken slurry culture because optimum

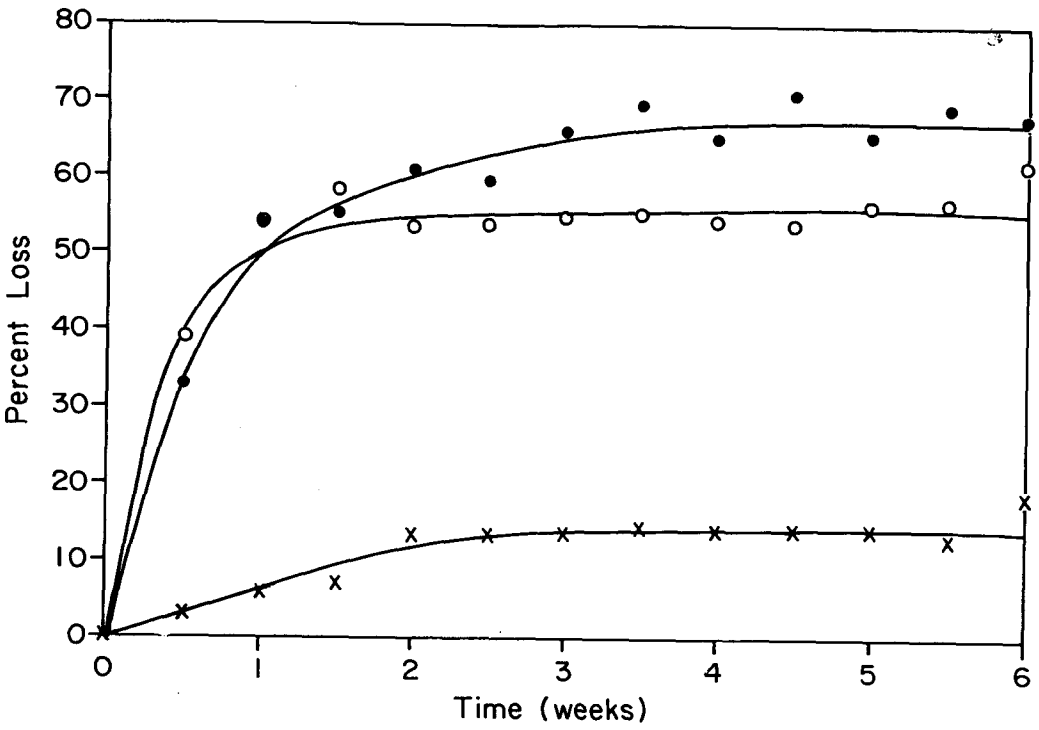

Fig. 2. Corn lignocellulose degradation by *S. viridosporus* T7A in 5% shaken slurry cultures incubated at 37°C for 6 wk reported as total lignocellulose (○), carbohydrate (●), and lignin weight loss (X).

APPL production occurs at pH 8.5–8.8 (*10*). In uninoculated controls the pH gradually decreased from 7.2–6.9 over the 6 wk incubation. For both the 3% and 5% shaken slurry cultures rapid growth occurred during the first 5 d followed by relatively stationary growth for the rest of the incubation period (Fig. 5).

Results similar to the flask cultures were observed in 3% and 5% slurry cultures of *S. viridosporus* T7A in stirred reactors incubated for 2 wk at 37°C (Table 1). The dominant factor affecting lignocellulose degradation and APPL production was pH. The effect of pH on weight loss and APPL production in 5% stirred slurry reactors can be seen in Table 1. The percent lignocellulose weight losses for pH 7.0 and 8.5 were 39.6 and 46.8%, respectively, while APPL production was 16.3 mg and 98.7 mg/g of initial lignocellulose, respectively. This 83% reduction in APPL production because of pH was also reflected in the much lower lignin loss at pH 7.0 compared to pH 8.5. At the suboptimal pH 7.0, APPL showed a higher protein concentration, lower Klason lignin content, and higher carbohydrate component. These findings confirm and extend previous work of Pometto and Crawford (*10*), which demonstrated maximum APPL production at pH 8.5–8.8.

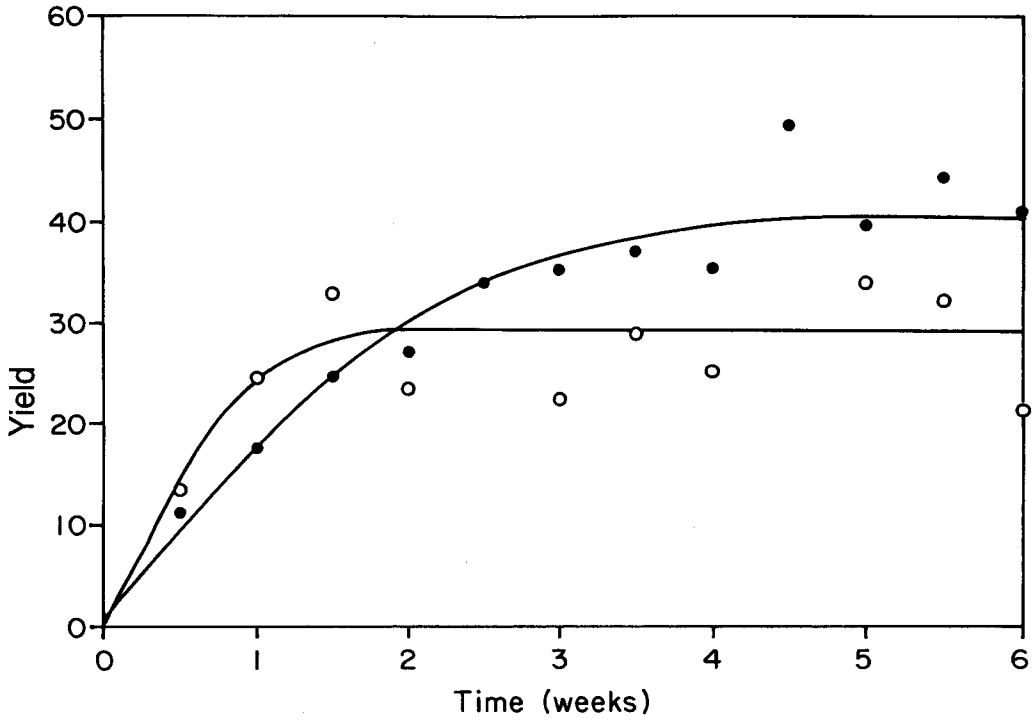

Fig. 3. APPL production from corn lignocellulose by *S. viridosporus* T7A in 3% (●) and 5% (○) shaken slurry cultures incubated at 37°C for 6 wk. Values represent APPL yields expressed as milligrams of APPL recovered per gram of initial lignocellulose.

Some differences in lignocellulose degradation and APPL production were also observed between the 3 and 5% stirred slurry cultures maintained at pH 8.5 (Table 1). At harvest of the 3 and 5% cultures APPL yields were 72.7 mg and 98.7 mg/g of initial lignocellulose, respectively. For the uninoculated controls the corresponding values were 9.4 mg and 6.6 mg/g. Percent lignin loss was considerably greater for the 5% slurry. Lignin losses for the uninoculated controls were 1.9 and 3.6% for the 3 and 5% slurries, respectively. The greater total lignin loss relative to APPL accumulation in the 5% slurry may be the result of greater mineralization of lignin to CO_2 and production of low molecular weight lignin products in the 5% stirred slurry culture. Cell mass was actually higher in the 5% slurry on a culture volume basis (3.8 g/L for the 5% slurry and 3.4 g/L for the 3% slurry). Actual lignocellulose weight loss, not including cell mass, and carbohydrate loss were similar in both cultures. For the uninoculated 3 and 5% controls, weight losses were 9.1 and 10.7%, respectively. The only component of APPL that varied significantly from the 3 and 5% stirred slurry cultures was the ash content. This difference may be due to a higher phosphate level in the APPL from 5% slurry cul-

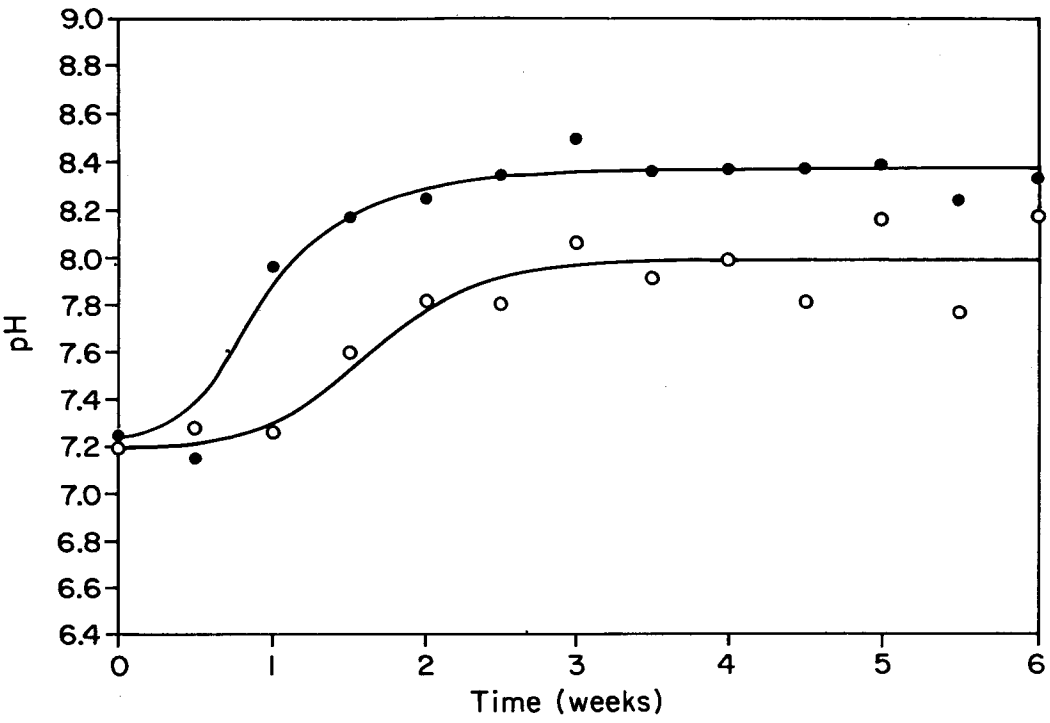

Fig. 4. pH during degradation of corn lignocellulose and APPL production by *S. viridosporus* T7A in 3% (●) and 5% (○) shaken slurry cultures incubated at 37°C for 6 wk.

tures. Previously, APPLs high in ash and phosphate were observed in APPLs produced in shake flask cultures with *Streptomyces badius* 252 (*8*).

In comparisons of the stirred slurry cultures and solid-state fermentations (Table 1), no significant differences were observed in APPL composition except for the ash content of the 5% stirred slurry culture. In most cases weight, lignin, and carbohydrate losses were less in solid-state fermentations than in the slurry reactor because of greater mineralization and solubilization of the lignocellulose in the stirred slurry cultures than in solid-state cultures. Similar yields of APPL were observed for solid-state fermentations and stirred slurry cultures system at pH 8.5. The greatest difference between slurry cultures and solid-state fermentations was the length of incubation time required for maximum degradation. The solid-state fermentations required 6–8 wk incubation while stirred slurry culture systems required only a 2 wk incubation to achieve equal or better results. Furthermore, our results confirm the previous work of Pometto and Crawford (*10*) that pH 8.5 to 8.8 produces the highest APPL yield.

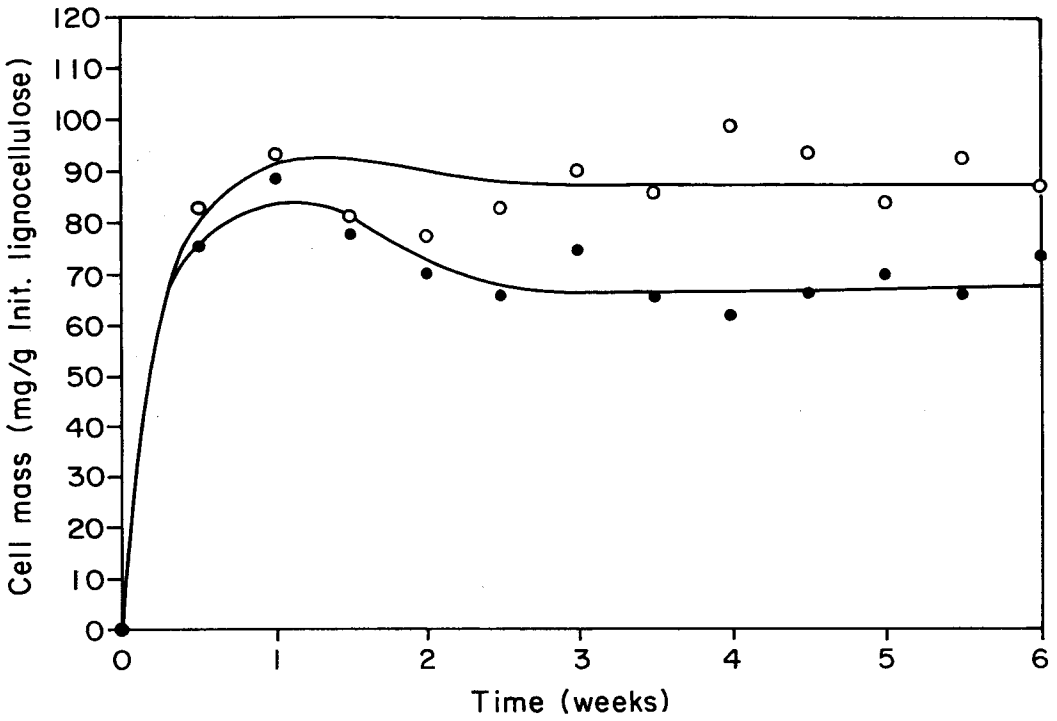

Fig. 5. Cell mass production (dry wt) by *S. viridosporus* T7A during degradation of corn lignocellulose in 3% (•) and 5% (○) shaken slurry cultures incubated at 37°C for 6 wk.

This is the first report of lignin degradation by *S. viridosporus* T7A in submerged, agitated cultures. The use of such cultures is very important for future commercialization of APPL production since incubation time is reduced, APPL yield is high, and scaleup is more easily achieved than with solid-state fermentations. Additional research will be needed to examine the performance of the slurry culture system for APPL production with enhanced APPL producing strains such as UV mutant *S. viridosporus* T7A-81 (*7*) and protoplast fusion recombinants of *Streptomyces* (*16*). In addition, other lignin degrading *Streptomyces* such as *S. badius* 252 (*5*), *S. flavovirens* 28 (*8*), and *S. setonii* 75Vi2 (*10*) need to be examined in a slurry culture.

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Table 1
Corn Stover Lignocellulose Degradation and APPL Production by *S. viridosporus* T7A in Stirred Slurry Cultures and Solid-state (Dampened) Fermentations

	Slurry culture			Dampened Culture		
pH:	8.5	8.5	7.0	7.0[10]	8.4[10]	Uncontrolled[7] 7.1 –8.5
Slurry conc'n (%-w/v):	3%	5%	5%	—	—	—
Lignocellulose loss (%)	46.7	46.8	39.6	20.0	26.5	36.2
Lignin loss (%)	35.7	60.3	15.5	34.0	33.0	19.7
Carbohydrate loss (%)	47.1	43.3	41.0	38.0	46.0	44.4
APPL yield (mg/g initial lignocellulose)	72.7	98.7	16.3	52.0	74.4	98.0
APPL component						
lignin (%)	65.1	61.0	41.8	67.9	65.5	55.2
protein (%)	17.9	14.5	32.2	15.0	11.9	—
carbohydrate (%)	5.1	5.0	10.1	2.0	2.2	10.6
ash (%)	5.2	15.6	9.1	5.3	3.4	4.0
Harvest time (wk)	2	2	2	6	6	8
Cell mass (cell dry wt g/L)	3.4	3.8	—	—	—	—

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