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Lignin-Solubilizing Ability of Actinomycetes Isolated from Termite (Termitidae) Gut†

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The lignocellulose-degrading abilities of 11 novel actinomycete strains isolated from termite gut were determined and compared with that of the well-characterized actinomycete, *Streptomyces viridosporus* T7A. Lignocellulose bioconversion was followed by (i) monitoring the degradation of [^{14}C]lignin- and [^{14}C]cellulose-labeled phloem of *Abies concolor* to $^{14}CO_2$ and ^{14}C-labeled water-soluble products, (ii) determining lignocellulose, lignin, and carbohydrate losses resulting from growth on a lignocellulose substrate prepared from corn stalks (*Zea mays*), and (iii) quantifying production of a water-soluble lignin degradation intermediate (acid-precipitable polymeric lignin). The actinomycetes were all *Streptomyces* strains and could be placed into three groups, including a group of five strains that appear superior to *S. viridosporus* T7A in lignocellulose-degrading ability, three strains of approximately equal ability, and three strains of lesser ability. Strain A2 was clearly the superior and most effective lignocellulose decomposer of those tested. Of the assays used, total lignocellulose weight loss was most useful in determining overall bioconversion ability but not in identifying the best lignin-solubilizing strains. A screening procedure based on $^{14}CO_2$ evolution from [^{14}C-lignin]lignocellulose combined with measurement of acid-precipitable polymeric lignin yield was the most effective in identifying lignin-solubilizing strains. For the termite gut strains, the pH of the medium showed no increase after 3 weeks of growth on lignocellulose. This is markedly different from the pattern observed with *S. viridosporus* T7A, which raises the medium pH considerably. Production of extracellular peroxidases by the 11 strains and *S. viridosporus* T7A was followed for 5 days in liquid cultures. On the basis of an increase of specific peroxidase activity in the presence of lignocellulose in the medium, the actinomycetes could be placed into the same three groups.

The lignin-solubilizing *Streptomyces* species examined to date have been shown to oxidatively depolymerize lignin as they degrade the cellulose and hemicellulose components of plant residues (8). The depolymerization reactions produce a modified water-soluble, acid-precipitable polymeric lignin (APPL) as the principal lignin degradation product (13). Extracellular lignin peroxidases are thought to participate in lignin depolymerization by fungi (22, 23), and recently, an extracellular lignin peroxidase (actinomycete lignin peroxidase P3, ALiP-P3), was discovered in *Streptomyces viridosporus* T7A (34, 35). The gene coding for ALiP-P3 has recently been cloned (37).

Several other lignin-depolymerizing actinomycetes have been described previously (41). *Thermonospora mesophila* degrades lignocellulose and produces APPL (25, 27). *Streptomyces cyaneus*, an APPL-producing actinomycete, grows on ball-milled straw and excretes an inducible extracellular protein involved in lignin solubilization (26). *Streptomyces badius* 252 (31) produces a chemically distinct APPL (5) and recently has been found to excrete peroxidases similar to those of *S. viridosporus* (1). *S. badius* 252 and *S. viridosporus* T7A have also been shown to solubilize Kraft lignins (16). Recently, Ball et al. (3) found that, of 20 actinomycetes screened by using direct and indirect assays for ligninolytic activity, *S. badius* 252, *T. mesophila*, and *S. cyaneus* were the most active lignin solubilizers.

The range of actinomycete species capable of metabolizing lignin is unknown, since research to date has been done with only a few strains. All strains examined thus far solubilize lignin to an APPL-like product. It has not been established whether species that are capable of more complete degradation of lignin (i.e., complete mineralization) exist. In addition, it is not known whether all lignin-solubilizing actinomycetes convert lignin by a similar mechanism involving extracellular lignin peroxidases. Thus, examining a larger group of actinomycetes, isolated from a diversity of natural habitats, could lead to a better understanding of the enzymology of their lignin-degrading ability, as well as to a deeper insight into the role these bacteria play in recycling lignin in nature. In this study, we report on the lignin-solubilizing abilities of lignocellulose-decomposing actinomycetes previously isolated from termite gut (30). Their degradative traits were determined and compared with those of *S. viridosporus* T7A. Results show that certain termite gut strains are superior to *S. viridosporus* T7A in lignocellulose-degrading ability. There are also differences in enzymology between these strains that may shed some light on the mechanism of lignocellulose degradation.

MATERIALS AND METHODS

Microorganisms and culture maintenance. Eleven wild-type actinomycetes were selected from 20 strains isolated from higher termites in Kenya (30). All strains have been identified as *Streptomyces* strains, primarily *Streptomyces chromofuscus* and *Streptomyces rochei* (M. B. Pasti, M. Basaglia, G. Concheri, S. Cardinali, D. L. Crawford, and M. P. Nati, Proceedings of the European Actinomycetes Group 4th Conference, 21–22 April, Udine, Italy, in press)

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TABLE 1. Ratio of extracellular peroxidase specific activity for each *Streptomyces* strain grown in liquid medium in the presence versus the absence of lignocellulose

Actinomycete	Strain	Ratio[a]
S. chromofuscus	A2	1.18
S. diastaticus	A3	1.04
S. rochei	A4	1.02
S. chromofuscus	A6	0.99
S. cyaneus	A7	1.09
S. chromofuscus	A8	1.26
S. rochei	A10	0.86
S. chromofuscus	A11	0.92
S. diastaticus	A12	0.85
S. rochei	A14	0.44
S. chromofuscus	A20	0.62
S. viridosporus	T7A	0.99

[a] Enzyme activity was measured by spectrophotometric assay of A_{510} as described in Materials and Methods, and the ratio was expressed as activity units per minute per milligram of intracellular protein in media with lignocellulose versus activity units per minute per milligram of intracellular protein in media without lignocellulose.

on the basis of the key of Williams et al. (38). Table 1 summarizes their identities to genus and species. Wild-type *S. viridosporus* T7A (ATCC 39115) was isolated from Idaho soil by D. L. Sinden (M.S. thesis, University of Idaho, Moscow, Idaho, 1972). Stock cultures of the Kenyan isolates were maintained at 4°C after growth and sporulation at 37°C on the following medium (in grams per liter of deionized water): NH_4NO_3, 1; KH_2PO_4, 0.4; yeast nitrogen base (Difco Laboratories, Detroit, Mich.), 0.67; yeast extract (Difco), 0.2; lactose, 15; agar, 18. *S. viridosporus* T7A was maintained at 4°C after growth and sporulation at 37°C on yeast extract-malt extract glucose agar (33). Stock cultures were subcultured every 2 to 10 weeks. In all experiments, unless otherwise reported, a spore suspension was used as the initial inoculum. The suspension was obtained by suspending the spores from an agar slant culture in 10 ml of sterile distilled water containing 0.1% (wt/vol) Tween 80. Spore suspensions were sometimes stored for 2 to 10 weeks at 4°C prior to being used.

Culture conditions. (i) Degradation of [^{14}C-lignin]- and [^{14}C-cellulose]lignocelluloses. The microorganisms were grown on lignocellulose in liquid medium in test tube bubbler cultures as previously described (10). The ^{14}C-lignocellulose substrates used for each microorganism were as follows: (i) white fir phloem [^{14}C-cellulose]lignocellulose (*Abies concolor*), 830 dpm/mg (Klason analysis: ^{14}C was 28.2% insoluble and 65% soluble); (ii) white fir phloem [^{14}C-lignin]lignocellulose (*A. concolor*); 990 dpm/mg, (Klason analysis: ^{14}C was 82.3% insoluble and 12.4% soluble) (11, 14). Fifty milligrams of air-dried ^{14}C-lignocellulose was sterilized in a metal-capped culture tube by autoclaving at 121°C for 1 h. After being cooled, 10 ml of sterile nitrogen-free mineral salts solution (12) supplemented with 0.6% (wt/vol) yeast extract (Difco) was added to each tube. Each culture tube was then inoculated with 0.2 ml of actinomycete spore suspension. For each strain and ^{14}C-lignocellulose substrate, five replicate culture tubes were used, along with a similar number of uninoculated, incubated controls. After inoculation, each culture tube was fitted with a bubbler tube, which provides an air inlet tube and an air outlet tube, and sterile CO_2-free air was continuously passed through each culture tube as previously described (6, 7). $^{14}CO_2$ produced as a result of ^{14}C-lignocellulose degradation was continuously trapped in a 2 M NaOH solution. Cultures were incubated at 37°C for 21 days. The percentage of initial ^{14}C evolved after 21 days as $^{14}CO_2$, as well as that recovered as ^{14}C-labeled water-soluble products, was then determined by liquid scintillation counting as previously described (15).

(ii) Solid-state fermentation assay. Cultures were grown on ground (40 mesh) and extracted lignocellulose prepared from corn stalks (*Zea mays*) as previously described (7). Five grams of air-dried lignocellulose per flask was added to 1-liter Corning no. 1460 reagent bottles, which were then autoclaved unplugged at 121°C for 1 h and again for 20 min after foam plugs were inserted.

To prepare actinomycete inocula, each of the 12 strains (5 ml of spore suspension) was inoculated into 2-liter flasks, each containing 1 liter of nitrogen-free mineral salts solution (12) supplemented with 0.6% (wt/vol) yeast extract. Cultures were incubated with shaking (200 rpm) at 37°C for 3 days and were then aseptically transferred to a sterile 1-liter graduated separatory funnel (Kimble no. 29040f). The cells were allowed to settle to the cylinder bottom, and then 50-ml samples of the gravity-concentrated cell suspension (0.1 ± 0.02 g, dry weight) were added to each reagent bottle containing the 5 g of sterile corn lignocellulose. Each bottle was then rolled on its side to distribute the lignocellulose and cells over its inside surface (32). Five replicates for each strain and for the uninoculated sterile control were incubated in a horizontal position for 4 weeks at 37°C. To harvest the lignocellulose, 250 ml of deionized H_2O was added to each bottle and the lignocellulose was scraped from the walls of the bottle. The bottle was next placed in a steamer for 1 h and then stored overnight at 4°C. The weight of residual lignocellulose plus biomass was then determined gravimetrically by filtration onto preweighed filter paper (9). The filtrate was acidified to a pH of 2.0 with concentrated HCl, and the precipitated APPL was collected by centrifugation, washed, dried, and weighed (2, 13).

Chemical analyses. The lignin, carbohydrate, and organic nitrogen contents of residues were determined by the Klason lignin procedure (28), the Somogyi-Nelson carbohydrate assay (2), and the micro-Kjeldahl method (24), respectively.

Production of extracellular peroxidase in liquid culture. Each *Streptomyces* strain was grown in a cotton-plugged 250-ml flask containing 0.25% (wt/vol) yeast extract (Difco), 0.1% (wt/vol) L-proline, 0.1% (wt/vol) asparagine, and 0.1% (wt/vol) glutamic acid in 50 ml of nitrogen-free mineral salts solution (12). Each strain was grown in this medium in the presence and absence of lignocellulose. The lignocellulose substrate was ground (200 mesh) extracted corn (*Z. mays*) stover lignocellulose and was used at a final concentration of 0.05% (wt/vol). Inoculated cultures and inoculated controls were incubated at 37°C and 200 rpm for up to 5 days. Every 12 h, samples were taken and the supernatant peroxidase activity was determined.

Enzyme assay. Peroxidase activity was routinely assayed with 2,4-dichlorophenol (2,4-DCP) (Sigma) as the substrate (34). A final volume of 1.0 ml of reaction mixture contained 50 mM potassium phosphate buffer (pH 7.00), 3 mM 2,4-DCP, 0.164 mM 4-aminoantipyrine (Sigma Chemical Co., St. Louis, Mo.), 4.0 mM hydrogen peroxide, and 0.1 ml of centrifuged culture supernatant. The reaction was initiated by the addition of hydrogen peroxide, and the increase in A_{510} was monitored for 2 min at 37°C, starting 15 s after the addition of hydrogen peroxide. One unit of enzyme activity was expressed as the amount of enzyme required for an increase in absorbance of 1.0 AU/min.

Intracellular protein was determined by boiling harvested culture pellets for 20 min in 1 M NaOH prior to protein

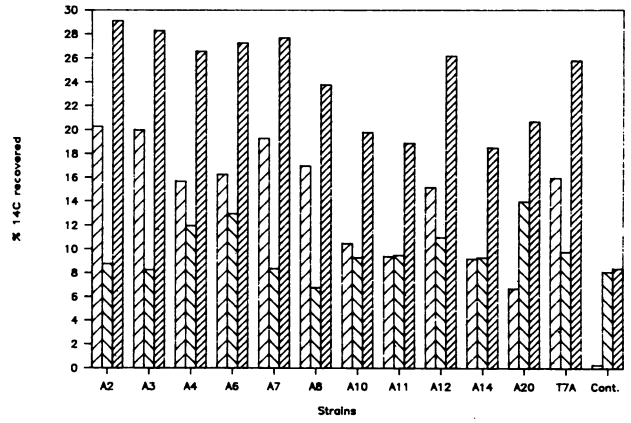

FIG. 1. Degradation of [^{14}C-cellulose]lignocellulose to $^{14}CO_2$ and ^{14}C-labeled water-soluble products by 12 strains after 3 weeks of incubation. Symbols: ▨. percentage of $^{14}CO_2$ recovered; ▭. percentage of ^{14}C-labeled water-soluble products recovered; ▨. percentage of total ^{14}C recovered.

estimation by colorimetric procedure no. TPRO-562 of Sigma. Intracellular protein was used as the index of cellular growth. Extracellular protein was also determined by the same method (Sigma). Peroxidase specific activity was expressed as activity units per minute per milligram of intracellular protein.

RESULTS AND DISCUSSION

Degradation of [^{14}C-cellulose]- and [^{14}C-lignin]lignocelluloses. Degradations of the two labeled lignocelluloses to $^{14}CO_2$ and ^{14}C-labeled water-soluble products by each actinomycete are shown in Fig. 1 and 2. After 3 weeks of incubation, the mineralization of the [^{14}C-cellulose]lignocellulose to $^{14}CO_2$ averaged 18 to 20% for strains A2, A3, A7, A8, about 14 to 16% for strains A4, A6, *S. viridosporus* T7A, and A12, and 6 to 10% for strains A10, A11, A20, and A14. Sterile controls produced a negligible 0.3%. The accumulation of ^{14}C-labeled water-soluble products as a result of cellulose metabolism by the strains is also shown in Fig. 1. The uninoculated controls showed an average 8.3% solubilization. The strains that best mineralized [^{14}C-cellulose] lignocellulose to $^{14}CO_2$ generally produced less ^{14}C-labeled water-soluble products than did the less efficient lignocellulose degraders. Consequentially, the total amount of ^{14}C recovered ($^{14}CO_2$ and ^{14}C-labeled water-soluble products) averaged 24 to 29% for almost all strains; lower values (18 to 20%) were observed in strains A10, A11, A14, and A20. In all strains except A20, cellulose mineralization predominated over the accumulation of ^{14}C-labeled water-soluble products, suggesting that with A20, end product inhibition of cellulose degradation may be higher than that for the other strains. The fact that almost all of the strains showed a good ability to attack the cellulosic fraction in the lignocellulose substrate confirms previously reported data that were based on nonradiorespirometric techniques (30).

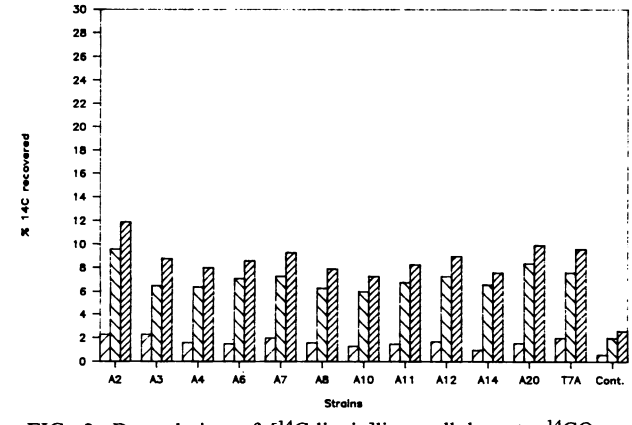

FIG. 2. Degradation of [^{14}C-lignin]lignocellulose to $^{14}CO_2$ and ^{14}C-labeled water-soluble products by 12 strains after 3 weeks of incubation. Symbols: ▨. percentage of $^{14}CO_2$ recovered: ▭. percentage of ^{14}C-labeled water-soluble products recovered: ▨. percentage of total ^{14}C recovered.

TABLE 2. Percentage of lignocellulose weight loss in solid-state fermentation after 4 weeks of incubation at 37°C

Strain	% Loss
A2	49.64
A8	48.76
A3	44.32
A7	43.75
A4	40.11
T7A	36.04
A6	35.02
A10	34.89
A11	33.10
A20	29.27
A14	26.46
A12	25.83

Figure 2 shows the pattern observed for [^{14}C-lignin]lignocellulose degradation. The total ^{14}C recovered ($^{14}CO_2$ and ^{14}C-labeled water-soluble products) varied little between the different cultures, averaging 1 to 2% for $^{14}CO_2$ evolution and 6 to 10% for ^{14}C-labeled water-soluble products, while the uninoculated control values were 0.6 and 2%, respectively. The values observed for the nontermite strain *S. viridosporus* T7A were slightly lower than those previously reported (32). Previous data, however, were based on the use of a different labeled substrate. Previous work (11, 31) has shown that different amounts of $^{14}CO_2$ and ^{14}C-labeled water-soluble products can be expected from different labeled lignocelluloses when a particular strain is used. Our data indicate that the ^{14}C-lignin component of *A. concolor* was not as good a substrate for bioconversion by the control strain *S. viridosporus* T7A as compared with other lignocelluloses. Therefore, when directly comparing new strains of lignin-decomposing bacteria with previously proven strains like *S. viridosporus* T7A, one should always consider the influence of the particular substrates utilized. Consequently, as a result of the lack of standardization of the substrates chosen, a comparison of new strains with other bacteria already tested on labeled lignocellulosic materials (4, 17–21, 26, 29, 36, 39) does not seem appropriate.

In this study, mineralization of ^{14}C predominated in the [^{14}C-cellulose]lignocellulose assay whereas, with [^{14}C-lignin]lignocellulose as a substrate, ^{14}C-lignin solubilization predominated.

Lignocellulose degradation and APPL production in the solid-state fermentation assay. After 4 weeks of incubation, the corn stover lignocellulose was decomposed to substantial weight losses of 40 to 50% by strains A2, A3, A4, A7, and A8, 30 to 40% by *S. viridosporus* T7A, A6, A10, and A11, and 20 to 30% by strains A12, A20, and A14 (Table 2). The

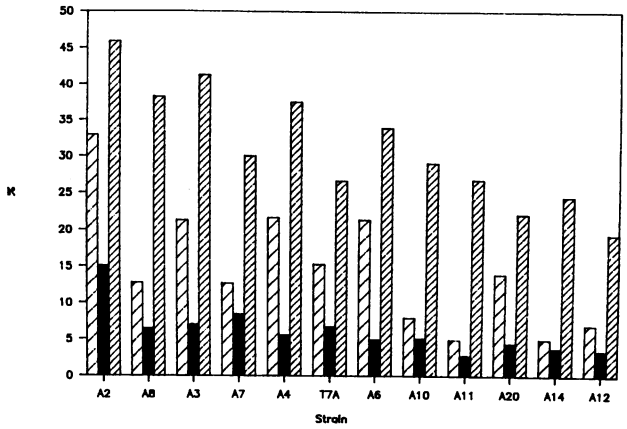

FIG. 3. Degradation of *Z. mays* lignocellulose by 11 Kenyan isolates and *S. viridosporus* T7A in solid-state fermentation after 4 weeks of incubation. Symbols: ▨, percentage of Klason lignin loss; ■, percentage of APPL yield (percentage of initial substrate lignin); ▨, percentage of carbohydrate loss.

strain showing the highest Klason lignin loss (33%) was strain A2, followed by strains A3, A4, and A6 (22%), strains A7, A8, *S. viridosporus* T7A, and A20 (15%), and strains A10, A11, A12, and A14 (less than 10%) (Fig. 3). APPL production over the 4-week incubation period averaged 5 to 8% of the initial lignin in the substrate (25% Klason lignin basis), except for strain A2, whose value was 15%, and strains A11 and A14, whose values were 2 to 3% (Fig. 3). On the basis of carbohydrate loss, the strains could be listed in three major groups: strains A2, A3, A4, and A8 with carbohydrate losses of 38 to 48%, strains A7, A6, *S. viridosporus* T7A, A10, and A11 with losses of 20 to 30%, and strains A20, A12, and A14 with losses below 20% (Fig. 3). All values reported above are averages of five replicates. In addition, the values are reported after the subtraction of the values for the corresponding controls. The initial Klason lignin and carbohydrate contents of the lignocellulose were 25 and 56%, respectively.

The values of *S. viridosporus* T7A for weight, carbohydrate, and Klason losses were similar to those previously reported, but the APPL yield was slightly lower (5). The APPL harvesting method could be partially responsible for the difference. As reported by Giroux et al. (16), the time of harvesting after starting the APPL precipitations can considerably influence the recovery of APPL.

The strains showing greater weight losses also showed greater Klason lignin losses and relatively high APPL productions. Exceptions were strains A4, A6, and A20, where the low values of weight loss and APPL production seemed to indicate a low bioconversion, despite relatively a great Klason lignin loss. This might be explained considering that, in grass lignocelluloses, lignin is closely associated with carbohydrates and that strains A4, A6, and A20 are known to be good cellulolytic microorganisms (30). The cellulase complex may have removed carbohydrates together with some attached lignin. Once this lignin was removed, A4, A6, and A20 might not have an efficient system with which to attack the remaining lignin and/or to effect bioconversion of lignin to APPL. Moreover, if we consider the ^{14}C-labeled water-soluble products from [^{14}C-cellulose]lignocellulose degradation as shown in Fig. 1, A4, A6, and A20 showed the highest values. On the other hand, in strains A2, A3, A4, A7, and A8, the ^{14}C-labeled water-soluble products recovered were very low, indicating that the lignin-carbohydrate complexes undergo extensive further degradation. In fact, in these strains, APPL production is higher than in the other strains. Strains A10, A11, A12, and A14 seemed to show that weight loss was due more to carbohydrate loss than to lignin loss, a conclusion that is also supported by the low APPL yields.

Strains A6 and T7A raised the pH of the medium from the original value of 7.1 up to 7.5 to 8.0 after 4 weeks of growth. All of the other strains showed a final pH of around 6.6 to 6.7. The increasing alkalinity of the medium in *S. viridosporus* T7A culture was previously reported by Pometto and Crawford (32). In comparison, cultures of the Kenyan isolates showed a final slightly acidic pH. These strains, having been isolated from a different habitat from that of *S. viridosporus*, may also differ in their metabolism of organic compounds, so that fewer basic metabolites are released into the growth medium.

With the substrates and cultural conditions used, and by the assays employed, several of the Kenyan actinomycetes appear capable of greater lignocellulose degradation than does *S. viridosporus* T7A. Strain A2 was a particularly interesting culture, showing especially good lignin-converting capacity.

Weight loss tests were useful as a measure of overall lignocellulose bioconversion ability, but were not useful in the identification of good lignin-solubilizing strains. The screening procedure based on $^{14}CO_2$ evolution was more definitive in measuring lignin-solubilizing ability than was the weight loss procedure, unless this latter procedure was carried out together with the measurement of APPL yield. The correlation might be even stronger if APPL were measured by the procedure of Giroux et al. (16).

Conditions used in the present research were optimized for *S. viridosporus* T7A. Since there are distinct differences between the Kenyan strains and *S. viridosporus* T7A, differences can also be expected for optimal lignin metabolism. The APPLs produced by the Kenyan bacterial strains also need to be chemically characterized in order to better understand the metabolic capabilities of these strains and to elucidate how they attack the lignin polymer. Finally, cellulolytic enzymes probably play a role, in addition to cellulose hydrolysis, in the degradation of lignocellulose, increasing the accessibility of ligninolytic enzymes to the lignin in the plant tissue.

Extracellular peroxidase production in liquid culture. All 11 of the Kenyan strains showed extracellular peroxidase activity against 2,4-DCP in media with and without lignocellulose. With each strain, the peak of peroxidase activity (activity units per minute per milligram) in both media occurred at the same time, shortly after growth peaked. Maximal activity was reached after 2 days of growth with all strains, except for A4, A20, and T7A, which reached their peaks at 3.5, 1, and 3 days, respectively. The peak in peroxidase activity occurred 12 to 24 h after the growth peak for all of the strains, except for strain A20, where the two maximal values occurred simultaneously. The peroxidase activities of the strains could be compared by expressing activity as a ratio of the peak of enzyme specific activity from media with versus without lignocellulose. These rates were determined prior to death phase to avoid extraneous factors, such as proteolysis, that would affect peroxidase activity. Such a ratio is useful for comparing strains for peroxidase enhancement in activity in the presence of lignocellulose. On the basis of ratio values, the actinomycetes fell into the same three major lignocellulose-degrading ability

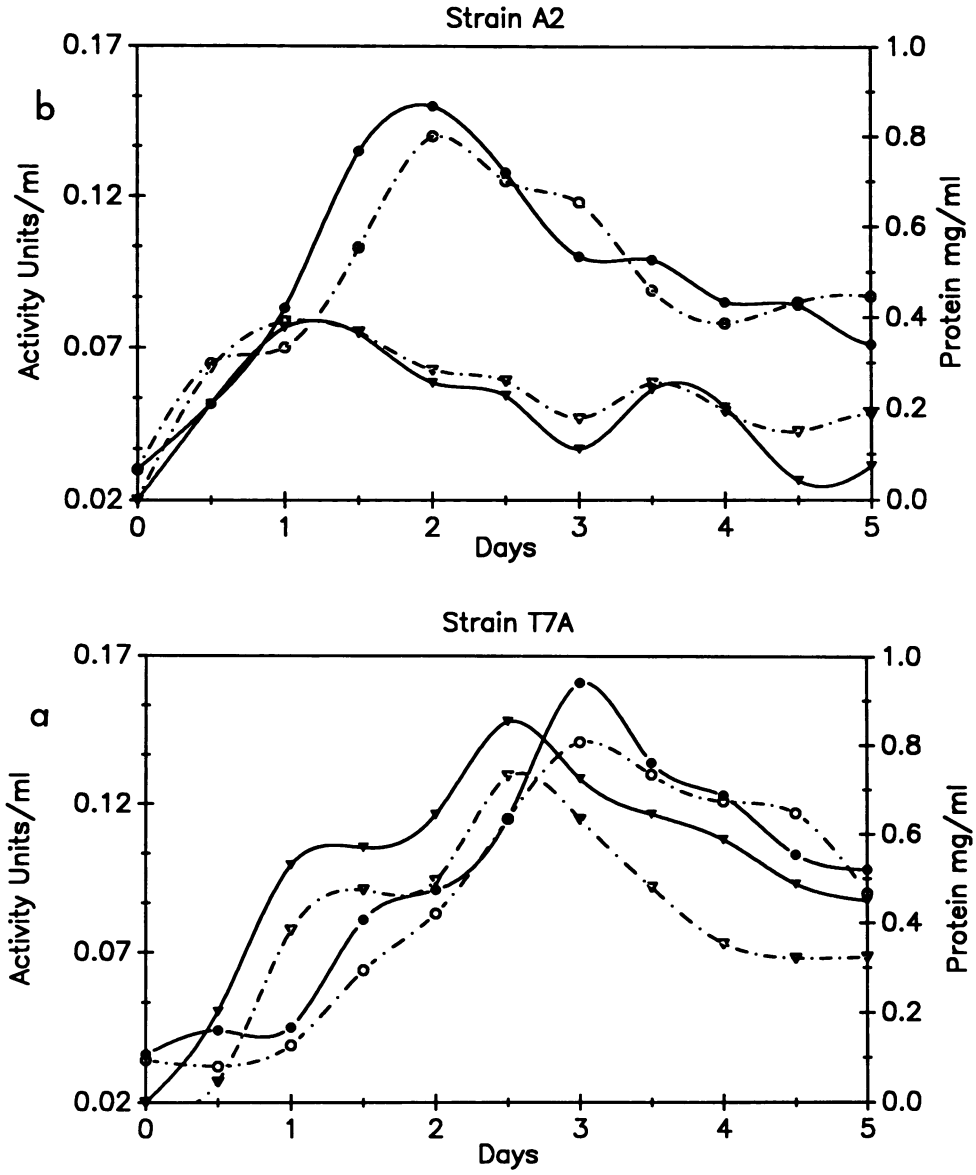

FIG. 4. Relationship between peroxidase activity (● and ○) and culture growth (▼ and ▽) for *S. chromofuscus* A2 (a) and *S. viridosporus* T7A (b). Cultures were grown in medium without (open symbols) or with (filled symbols) lignocellulose (0.05% wt/vol) as described in Materials and Methods. Enzyme assays were performed with nonconcentrated culture supernatants.

groups as they did when the other assays were used (Table 1). The best lignocellulose degraders (the first group was strains A8, A2, A7, and A3) increased their synthesis of peroxidase more than did the other strains when grown in medium containing lignocellulose. The intermediate group (strains A4, T7A, A6, and A11) showed a slightly detectable or not detectable increase, and, with the poorly lignocellulolytic group (strains A10, A12, A20, and A14), the ratio actually fell to less than one. An example of the relationship between peroxidase activity and culture growth in the presence or absence of lignocellulose for *S. chromofuscus* A2 versus *S. viridosporus* T7A is shown Fig. 4.

Overall, these data indicate that about 20 to 25% enhancement of production of 2,4-DCP-oxidizing peroxidases is typically observed in the most highly lignocellulolytic strains on the addition of lignocellulose to the growth medium. Previously, it has been shown that 2,4-DCP is a good substrate for assaying actinomycete lignin peroxidase (35, 37). Thus, the peroxidases of the Kenyan *Streptomyces* strains, such as *S. chromofuscus* A2, may be induced to higher levels and/or may be superior to the peroxidases of *S. viridosporus*. However, further research is needed to determine if this is the case, or if the superior lignocellulolytic activity is due to the activities of other enzymes acting in concert with the peroxidases.

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