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Lignocellulose Decomposition by Selected *Streptomyces* Strains†

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From 30 actinomycete cultures isolated by enrichment technique on agar media containing newsprint as a primary carbon and energy source, three *Streptomyces* strains were selected for characterization of their lignocellulose-decomposing abilities. All three streptomycetes were capable of oxidizing specifically ^{14}C-labeled lignocelluloses to $^{14}CO_2$. These *Streptomyces* were shown to attack primarily the cellulosic (glucan) components, of which between 25 to 40% evolved as $^{14}CO_2$ during 1,025 h of incubation depending upon the culture used. Lignin-labeled lignocelluloses were also attacked, but to a lesser degree, with up to about 3.5% being oxidized to $^{14}CO_2$ depending upon the culture used. Additionally, it was shown that purified ^{14}C-labeled milled-wood lignin was attacked, with recoveries of up to 17.7% of the label as $^{14}CO_2$. This is the first conclusive evidence to show that streptomycetes can decompose lignin.

For years it has been known that certain fungi are capable of decomposing lignocellulose (11). The white rot fungi have been of particular interest because of their ability to completely oxidize both the cellulosic and lignin components of wood to CO_2 and water. The roles that nonfungal microbial groups play in lignocellulose decomposition, however, remain to be defined. It is known that certain thermophilic actinomycete species attack lignocellulose, depleting primarily carbohydrate components (3), whereas other species may also deplete lignin (1). Other workers have shown that some gram-negative eubacteria are capable of decomposing purified lignins (12, 13). Recently the first conclusive evidence was published to show that a *Nocardia* species can degrade lignin (14). The euactinomycetes have not been studied in detail with respect to lignin decomposition. In the work presented here, three *Streptomyces* strains were examined for their abilities to attack lignocellulose. Their abilities to oxidize both the cellulosic and lignin components were examined by using ^{14}C methodology (4, 5).

MATERIALS AND METHODS

Preparation of labeled lignocelluloses. Natural lignocelluloses from Douglas fir (*Pseudotsuga menziesii*) containing ^{14}C specifically in their lignin components or cellulosic (glucan) components were prepared by feeding twigs L-[*U*-^{14}C]phenylalanine or D-[*U*-^{14}C]glucose, respectively, through their cut stems, by methods previously described (4, 5), or in their lignin components by feeding twigs of red maple (*Acer rubrum*) [2′-^{14}C(side-chain)]ferulic acid (4-hydroxy-3-methoxycinnamic acid), which is known to be incorporated preferentially into lignin components of plants (8, 9). After grinding and an extensive extraction procedure (see references 4, 5, and 8 for methodology), the residual lignocellulose preparations were [^{14}C]lignin-labeled, [^{14}C]glucan-labeled, and [2′-^{14}C(side-chain)]lignin-labeled lignocelluloses, respectively. In the case of side-chain-labeled material, ^{14}C was present in the C2 of the phenylpropane side-chain units within the lignin polymer. Additionally, Douglas fir twigs were allowed to take up L-[*U*-^{14}C]histidine in order to assess incorporation of label into plant protein. This ^{14}C-protein-labeled lignocellulose was used to help assess the specificity of our labeling techniques and to determine the rates of turnover of wood protein by cultures.

Chemical analyses showed the lignocelluloses to consist of about 25% lignin and 65% carbohydrate, which was mostly cellulose along with small amounts of hemicellulose glucans. Also, other hemicelluloses, primarily xylans and mannans, were present. Crude protein content of the lignocelluloses averaged 5.0% on a Kjeldahl nitrogen basis. Previous work has shown these extractive-free ^{14}C-lignocelluloses to be good substrates for microbial degradation studies and to be essentially free of readily decomposable contaminating materials (4, 5, 8, 9, 14).

For one decomposition experiment, ^{14}C-labeled milled-wood lignin (^{14}C-MWL) was prepared from [^{14}C]lignin-labeled lignocellulose. The ^{14}C-MWL was prepared by jar-milling ^{14}C-maple in air at 2°C for 2 weeks. The milled wood was extracted for 24 h with 90% dioxane in water. Dioxane-water-soluble lignin was purified by precipitation from dichloroethane-ethanol (1:1) into ether, then into hexane. As a final

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purification, the lignin was precipitated from 90% acetic acid into water. Insolubles were collected by centrifugation, dissolved in 90% dioxane-water, and freeze dried to an off-white powder. This is a Björkman lignin (2) and is one of the best kinds of extractive lignins for microbial degradation studies (11).

Radioactivity counting. ^{14}C was quantified by liquid scintillation techniques previously described (4, 5).

Source of lignocellulosic substrates. The wood sources for work presented here were twigs from Douglas fir (*P. menziesii*) or red maple (*A. rubrum*). Both labeled and unlabeled substrates were prepared from sapwood or the outer new growth of the wood lying above the heartwood.

Isolation and selection of lignocellulose-decomposing actinomycetes. Isolations were made at room temperature from natural samples including forest soils, garden soils, and decomposing plant materials. Cultures were isolated by enrichment technique. Serially diluted natural samples were plated onto a mineral salts agar medium containing 40-mesh ground newsprint (0.5%) as a carbon and energy source and vitamin-free Casamino Acids (0.025%) as a supplemental nitrogen source. From original isolation plates incubated at room temperature, 30 mesophilic actinomycete strains were purified by restreaking onto the same medium. All of these isolates cleared newsprint agar to varying degrees within 7 to 14 days. Each isolate was screened for its ability to decompose lignocellulose in a 14-day growth experiment where the isolate was inoculated into 10 ml of lignocellulose medium in a test tube. The medium consisted of mineral salts solution containing 0.10% KH_2PO_4, 0.4% $Na_2HPO_4 \cdot 7H_2O$, 0.02% NaCl, 0.02% $MgSO_4 \cdot 7H_2O$, and 0.005% $CaCl_2 \cdot 2H_2O$ plus 0.025% vitamin-free Casamino Acids (Difco) and a 0.5% level of ground, extracted fir lignocellulose. Lignocellulose and mineral salts plus Casamino Acids solutions were autoclaved separately, then mixed before inoculation. Inoculated cultures were incubated for 14 days at room temperature, under constant aeration. Sterile, humidified air entered each test tube through a glass-tube bubbler which penetrated nearly to the culture tube bottom.

From the 30 isolates, 3 organisms were selected for further study based upon their abilities to cause substantial weight loss of lignocellulose. All three, cultures 28, 87A, and 177, were identified as strains of *Streptomyces* as determined from direct microscopic examinations of sporulating colonies on agar media. Culture 28 produces long openly spiraled spore chains on a grey aerial mycelium. No melanoid pigment is produced on rich organic media. Culture 87A is similar, but produces a lightly yellow pigment on such media. Culture 177 produces long, straight spore chains in a grey aerial mycelium and a brown-to-black melanoid pigment on rich organic media.

Characterization of lignin- and cellulose-degrading abilities. *Streptomyces* strains 28, 87A, and 177 were examined, utilizing ^{14}C methodology, for their abilities to oxidize lignin and cellulose within the lignocellulose complex. Fifteen-milliliter cultures were run in duplicate, each containing 13.5 ml of mineral salts solution, 1.5 ml of an amino acid solution (containing equal amounts of the 20 common amino acids to a final concentration of 0.25% in mineral salts), a known amount of ^{14}C-lignocellulose plus sufficient unlabeled lignocellulose to bring the final concentration to 0.5%. Sources and specific activities of all ^{14}C-lignocelluloses are reported in the Results section.

Tubes were inoculated and incubated with constant aeration at room temperature. Degradation of ^{14}C-labeled substrate was monitored by following $^{14}CO_2$ evolution from actively growing cultures over time. $^{14}CO_2$ was trapped by passage of all exit gases through 10 ml of aqueous 8% NaOH. Traps were changed periodically, and $^{14}CO_2$ was quantified.

RESULTS

All 30 isolates screened by the weight-loss test caused weight losses of lignocellulose in excess of that for uninoculated controls. After 14 days growth, the cultures were harvested and all residual insolubles were collected by centrifugation, with two distilled water washes. Average losses of insolubles ranged between 7.2 and 24.8% as compared with 5.0% for controls. The three best cultures in this respect were *Streptomyces* strains 28, 87A, and 177, which caused weight losses of 20.2, 13.3, and 24.8%, respectively (average of two determinations). These weight-loss values did not account for bioconversions of lignocellulose into cell mass; therefore, they were considered to be minimal decomposition values. Kjeldahl nitrogen analyses of residues, however, showed that those strains which caused considerable weight loss of substrate also produced significant amounts of cell mass. In the case of the three streptomycete strains, residues, after 14 days, averaged 10 to 12% crude protein.

When the streptomycetes were grown on ^{14}C-lignocelluloses, incubations were extended to 1,025 h (about 43 days). As a result, overall substrate decomposition was greater than that observed after only 14 days of growth. Cultures 28, 87A, and 177 caused weight losses of 35.8, 28.8, and 36.0%, respectively. This level of decomposition was as great as that obtained with fungi such as *Trichoderma viride* grown on substrates containing less lignin than the fir lignocellulose (10). All three cultures evolved $^{14}CO_2$ from all of the ^{14}C-lignocellulose substrates, with the cellulosic components being attacked most readily. As shown in Fig. 1, a substantial portion of the total ^{14}C localized in [^{14}C]glucan-labeled lignocellulose was oxidized to $^{14}CO_2$. *Streptomyces* 177 gave the highest conversions of substrate to $^{14}CO_2$. All three also evolved $^{14}CO_2$ from [^{14}C]lignin-labeled lignocellulose, although to a more limited degree as compared with the labeled cellulosic components. Typical [^{14}C]lignin oxidation curves are shown in Fig. 2 (for [^{14}C]lignin-labeled lignocellulose labeled by the phenylalanine incorporation method) and Fig. 3

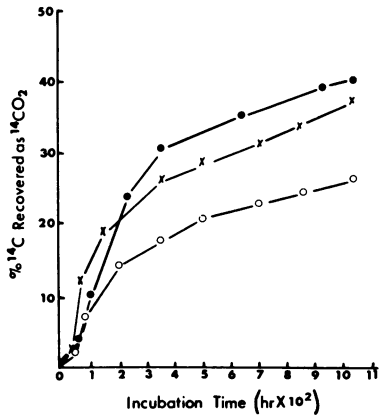

FIG. 1. *Oxidation of [^{14}C]glucan-labeled fir lignocellulose by selected Streptomyces strains. Symbols: ●, culture 177; ×, culture 28; ○, culture 87A. Total of 5.0 × 10^4 dpm per tube. Specific activity of the ^{14}C-lignocellulose was 1.1 × 10^5 dpm/mg.*

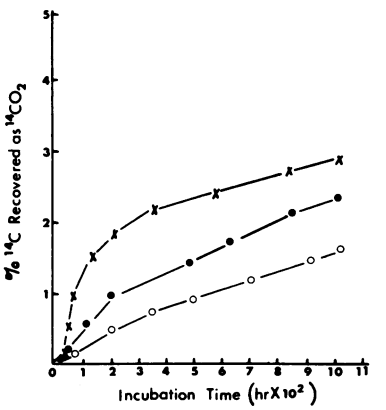

FIG. 2. *Oxidation of [^{14}C]lignin-labeled fir lignocellulose by selected Streptomyces strains. Symbols: ●, culture 177; ×, culture 28; ○, culture 87A. Total of 7.5 × 10^4 dpm per tube. Specific activity of the ^{14}C-lignocellulose was 7.3 × 10^3 dpm/mg.*

(for [^{14}C]lignin-labeled lignocellulose labeled by the ferulic acid incorporation method). Evolution of $^{14}CO_2$ over time followed a predictable respiration pattern for both glucan- and lignin-labeled substrates. After 48- to 72-h lags, cultures rapidly reached a maximal rate of metabolic activity which after about 200 h began slowly decreasing through the rest of the incubation period. Often near 1,000 h, a temporary increase, or pulse, in $^{14}CO_2$ evolution occurred, probably as cellular lysis began and some biomass was oxidized via cryptic growth and endogenous metabolism.

It has been reported that small amounts of incorporated [^{14}C]phenylalanine (3 to 5%) may end up in plant protein as a result of uptake by

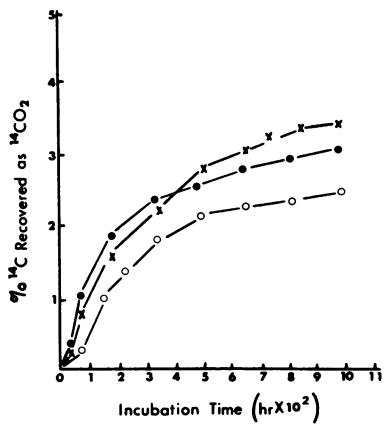

FIG. 3. *Oxidation of [2'-^{14}C(side chain)]lignin-labeled maple lignocellulose by selected Streptomyces strains. Symbols: ●, culture 177; ×, culture 28; ○, culture 87A. Total of 1.08 × 10^4 dpm per tube. Specific activity of the ^{14}C-lignocellulose was 1.44 × 10^2 dpm/mg.*

twigs (7). Because the fir lignocellulose used here contained about 5.0% protein, it is possible that microbial oxidation of [^{14}C]phenylalanine-contaminated protein may have accounted for the low levels of lignin degradation observed for the phenylalanine-labeled substrate (although not for the ferulic acid-labeled one). To examine this possibility, lignin-labeled fir lignocellulose was analyzed for phenylalanine-labeled protein by thin-layer chromatography. In addition, specifically protein-labeled fir lignocellulose (labeled with [^{14}C]histidine) was used as a substrate to help assess the contribution of ^{14}C-protein to the $^{14}CO_2$ liberated during growth of the cultures on [^{14}C]lignin lignocellulose. Also, cultures 28 and 177 were grown in media containing ^{14}C-MWL, and the ability of both to oxidize this purified lignin preparation was determined.

For thin-layer chromatography separation, 50-mg samples of lignin-labeled fir lignocellulose (3.64 × 10^5 dpm) in 2.5 ml of 6 N HCl were hydrolyzed in sealed ampoules at 121°C for 18 to 24 h to release wood protein as free amino acids. Typically, 37.5% of the total ^{14}C was solubilized. This, however, included acid-soluble [^{14}C]lignin components as well as any [^{14}C]phenylalanine released from protein. To distinguish between these components, acid hydrolysates were chromatographed by thin-layer chromatography. One hundred-microliter samples (5.5 × 10^3 dpm) were spotted onto plates containing Adsorbosil-5 (Applied Scientific, State College, Pa.) as sorbent and developed in a butanol-ethanol-water (7:2:3) solvent system. Phenylalanine and tyrosine standards were run simultaneously. After development, air-dried plates

were sprayed with ninhydrin or potassium ferricyanide-ferric chloride solutions to detect amino acids or phenolic aromatics, respectively. The solvent system used did not separate phenylalanine from tyrosine; therefore, any plant conversions of [^{14}C]phenylalanine to [^{14}C]tyrosine were not considered. This solvent did separate most phenolic-staining spots from phenyalalanine, which traveled at the slower R_f. Greater than 95% of the ^{14}C applied was recovered between the origin and solvent front. Numerous ninhydrin-reactive spots, including one corresponding to phenylalanine, were detected. However, only 7.9% of the total ^{14}C applied traveled with phenylalanine. This averaged 2.96% of the total ^{14}C present in the lignocellulose (7.9 of 37.5%). Most ^{14}C traveled faster than phenylalanine at R_f's corresponding to phenolic aromatics (acid-solubilized lignin fragments). It can, therefore, be concluded that less than 3% of the total ^{14}C in this lignocellulose was localized in phenylalanine within plant protein, since the separation of phenylalanine from contaminating phenolics was not complete. Due to the inherent variability between different plants, we recommend that this analysis be routinely run on all new [^{14}C]lignin lignocelluloses labeled by the phenylalanine method. As shown in the present case, ^{14}C-contaminated protein does not appear to be a problem.

To assess the potential contribution of $^{14}CO_2$ from microbially oxidized protein contaminants in the [^{14}C]lignin lignocellulose, one experiment was run where cultures were grown in the presence of [^{14}C]histidine-labeled fir lignocellulose. Acid hydrolysis of this preparation, followed by chromatography, showed that 70% of the ^{14}C was acid hydrolyzable and essentially all was localized with [^{14}C]histidine spots on the thin-layer chromatography plates. In 1,025 h of incubation, none of the streptomycetes evolved more than 1.9% of the [^{14}C]histidine-labeled protein as $^{14}CO_2$. The potential interference with the lignin oxidation assay would at a maximum be a negligible 0.06% of the $^{14}CO_2$ evolved (1.9% of 2.96%).

Finally, cultures 28 and 177 were grown in 10 ml of the mineral salts-amino acid solution supplemented with 45 mg of cold fir lignocellulose and 5.0 mg of ^{14}C-MWL (Fig. 4). Both strains oxidized a significant portion to $^{14}CO_2$. Percent recoveries after 1,025 h were, in fact, considerably higher for ^{14}C-MWL than for natural lignins. MWL was, therefore, less resistant to attack by these cultures than were natural lignins complexed with carbohydrate. With $^{14}CO_2$ recoveries of 17.7 and 15.0%, respectively, for cultures 28 and 177, these data confirm the lignin-degrading abilities of these organisms and support the

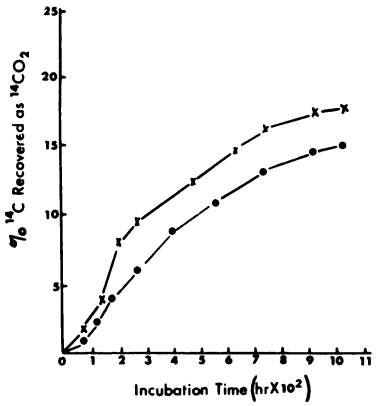

FIG. 4. *Oxidation of ^{14}C-MWL by selected Streptomyces strains. Symbols: ●, culture 177; ×, culture 28. Total of 6.0 × 10^3 dpm per tube. Specific activity of the ^{14}C-lignocellulose was 1.2 × 10^3 dpm/mg.*

data obtained when cultures were grown on [^{14}C]lignin-labeled lignocelluloses. Recently, other workers have reported similar decomposition of unlabeled MWL by eubacteria such as *Pseudomonas*, *Flavobacterium*, and *Aeromonas* species (12).

As a result of growth on these ^{14}C-lignocelluloses, not only was ^{14}C oxidized to $^{14}CO_2$, but additional label was solubilized as well. By examining culture supernatants for radioactivity at the time of harvest, it was possible to determine how much of the insoluble lignin or cellulosic substrate had become water soluble as a result of growth by each culture. Soluble ^{14}C was quantified by taking five replicate 1.0-ml samples of each supernatant, placing them in scintillation vials, evaporating the samples to dryness, adding scintillation fluid (10 ml), and then counting each vial with all data being compared with uninoculated controls. Typically, cultures growing at the expense of [^{14}C]glucan lignocelluloses solubilized as much as 7 to 14% of the total ^{14}C in excess of what the cultures oxidized to CO_2. Likewise, additional solubilization of [^{14}C]lignin components typically occurred, ranging between 4 and 8% of the total ^{14}C. Since solubilization is a form of partial degradation, these *Streptomyces* strains actually decomposed more of the substrate than $^{14}CO_2$ data alone accounted for. When all of the ^{14}C data are used to calculate theoretical weight loss of substrate for specific experiments, the calculated values match actual weight losses very well when estimates of cell mass present are considered.

DISCUSSION

For the *Streptomyces* strains examined, it was shown that all were good lignocellulose degraders, with abilities seemingly similar to the few

soft-rot fungi that have been studied (6); that is, they carry out a limited attack on lignin, but primarily deplete carbohydrates. The validity of considering as significant the limited degradation of [^{14}C]lignin was substantiated by growth of the cultures on several lignin preparations. The combined data from all of the lignin-labeled substrates, as a whole, show conclusively that these cultures do attack the lignin polymer. Of particular interest was the observation that the streptomycetes decomposed MWL more readily than natural lignins. Other workers have isolated eubacteria that decompose such purified lignins (12, 13). It is possible that their organisms may not have attacked natural lignins so readily had they been examined in that respect.

We have recently determined that all three streptomycetes are capable of utilizing single-ring aromatics such as parahydroxybenzoate as sources of carbon and energy (unpublished data). This too would support the finding that the cultures attack lignin. Trojanowski et al. (14) found that their lignin-degrading *Nocardia* was capable of attacking various single-ring aromatics. This is the first conclusive evidence to show that lignin degradation is carried out by strains of *Streptomyces*. Although with the strains examined here, total lignin degradation was not great, it seems likely that more active cultures remain to be isolated.

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