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Paper Mill Effluent Decolorization by Fifty Streptomyces Strains

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Fifty actinomycete strains isolated from lignocellulosic substrates were examined for the ability to remove the color from a paper mill effluent obtained after semichemical alkaline pulping of wheat straw. *Streptomyces* sp. strains UAH 15, UAH 23, UAH 30, and UAH 51 were selected for their ability to decolorize the effluent in a liquid medium containing 1% (wt/vol) glycerol, 0.2% (wt/vol) ammonium sulfate, and 80% (vol/vol) effluent. The highest levels of decolorization achieved after the strains grew were 60 to 65%. Strains UAH 30 and UAH 51 were selected for further study because of their different patterns of effluent decolorization during growth. Fractionation of the decolorized effluent by gel permeation chromatography demonstrated that there were reductions in the levels of absorbance of the high- and medium-molecular-weight compounds. These fractions were mainly responsible for the color of the effluent, while the last fractions, the low-molecular-weight compounds, could have been responsible for the residual color of the decolorized effluent. Thin-layer chromatography revealed significant differences among the patterns of bands corresponding to the acidified supernatants obtained after precipitation of alkali-lignin from the effluent samples decolorized by different *Streptomyces* strains.

Pulp and paper mill effluents have been recognized as environmental hazards for many years. The chemical composition of such effluents depends on the nature of the feedstocks, as well as the treatment procedure. The dark brown color of these effluents is mainly due to their high contents of oxidized and partially degraded lignin (10). Reducing this color before the effluents are discharged into natural waters is an important goal.

Several microorganisms (mainly white rot fungi) have been used to decolorize effluents from different sources because of their ability to degrade lignin under laboratory conditions (2, 3, 13, 18).

It is also known that actinomycetes play an important role in the transformation of lignocellulose (5, 11, 14, 17, 21). These microorganisms are able to degrade cellulose and hemicellulose as they oxidize and solubilize the lignin component. Several actinomycete strains have been proven to modify effluents which are produced during the chemical bleaching of pulps (20, 22). Although there is considerable potential for treating these effluents by biological methods, the mechanism of color removal is poorly understood.

In this study we determined the optimal conditions for decolorization of a paper mill effluent by several *Streptomyces* strains. Furthermore, we partially characterized the decolorized effluent.

MATERIALS AND METHODS

Microorganisms and culture maintenance. Fifty actinomycete strains were isolated from different lignocellulosic substrates by using two types of media, Bacto Actinomycete Isolation Agar (Difco) and inorganic salt-starch agar (16). Most of these strains were identified as *Streptomyces* strains on the basis of the information in *Bergey's Manual of Systematic Bacteriology* (19). Spore suspensions were obtained by growing

the strains at 28 or 37°C (depending on the strain) on G.A.E. agar containing (per liter) 10 g of glucose, 1 g of L-asparagine, 0.5 g of yeast extract, 0.5 g of K_2HPO_4 , 0.5 g of $MgSO_4 \cdot H_2O$, and 0.01 g of $FeSO_4 \cdot H_2O$. The suspensions were kept at -20°C in 20% (wt/vol) glycerol. For the experiments, stock cultures were inoculated onto the same medium, and the spores were harvested with distilled water. Standard spore suspensions (10^7 CFU ml^{-1}) were used as the initial inocula in all assays.

Effluent origin, color measurement, and pH determination. Paper mill effluents (pH 8.5) were obtained from a Spanish plant after semichemical alkaline pulping of wheat straw (soda cook liquor) and anaerobic and aerobic treatments. These effluents were collected and stored at 4°C.

The pHs of the effluents were adjusted to different values (range, pH 7.0 to 3.0) by adding 12 M HCl, and then the effluents were centrifuged at $12,000 \times g$ for 15 min and filtered through Whatman no. 1 filter paper before they were used. Color intensity was measured at 465 nm after the pH was adjusted to 7.6 with 12 M HCl. The effluent absorbance was determined spectrophotometrically and was related to the absorbance of a Pt-Co standard solution at the same wavelength (12). The initial color intensity of each effluent was 16,000 color units. The adsorption of color by the mycelia was estimated in a supernatant obtained after the mycelia were washed with 1 N NaOH.

Decolorization screening under different conditions. Primary screening was performed on G.A.E. agar plates containing 30% (vol/vol) total effluent. Each plate was inoculated with 20 μl of a standard spore suspension, and the plates were incubated at 28 or 37°C for 7 days.

In liquid medium, 200- μl spore suspensions were inoculated into 100-ml flasks containing 20 ml of 80% (vol/vol) total effluent and mineral salt medium (4) supplemented with a carbon source (glycerol, mannitol, glucose, or, starch) at a concentration of 1% (wt/vol) and a nitrogen source (0.6% [wt/vol] yeast extract or 0.2% [wt/vol] ammonium sulfate). Each carbon source was tested with each nitrogen source.

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Consequently, eight different media were assayed. The initial pH of each medium was adjusted to 7.0.

After the optimal medium for decolorization was selected, different inocula (0.1, 0.5, 1.0, 1.5, and 2.5 ml of standard spore solution per 100 ml of medium) were also assayed. Cultures were incubated for 7 days at 28 or 37°C with shaking at 200 rpm.

Cell growth and color loss. The time course of growth was determined in 100-ml flasks containing 20 ml of mineral salt medium supplemented with 80% (vol/vol) total effluent, 1% (wt/vol) glycerol, and 0.2% (wt/vol) ammonium sulfate. The cultures were incubated under the conditions described above.

Cell growth was estimated by determining the dry weight of the mycelia. After the mycelia were separated by centrifugation at $3,000 \times g$, for 10 min, the pH, color loss, and molecular distribution of the effluent components were determined daily for the culture supernatants.

Gel permeation chromatography. The pH values of untreated and decolorized effluents were adjusted to 7.6. The molecular size distribution in each effluent was determined by loading 2 ml of the effluent onto a Sephadex G-100 column (40 by 2.5 cm; Pharmacia) in 0.1 M Tris-HCl buffer (pH 7.5). The flow rate was 1.5 ml min^{-1} . Sample elution was monitored by measuring the A_{280} with a Hi-Load system apparatus (Pharmacia) and collecting 3-ml fractions. Color absorbance was measured at 465 nm with a Beckman model DU-50 spectrophotometer.

Alkali-lignin preparation. Untreated and decolorized effluents were acidified with 12 M HCl to pH 1 to 2 and then centrifuged at $12,000 \times g$ for 10 min. Alkali-lignin was obtained after each precipitate was washed with deionized water. The pH of the acidified supernatant was adjusted to 7.6, and this supernatant was chromatographed as described above.

Thin-layer chromatography. Acidified supernatants that were obtained after alkali-lignin precipitation were extracted with 1 volume of diethyl ether, and the extracts were evaporated to one-tenth of the initial volume. Ether-soluble concentrated fractions were spotted onto silica gel aluminum sheets (Scharlau Aluchrom SI F₂₅₄) and developed in toluene-acetic acid (7:3) (15). Bands were visualized under short-wave UV light and photographed by using type 55 ASA/DIN 50/18° Polaroid black and white film.

RESULTS AND DISCUSSION

On the basis of the results of the primary screening, *Streptomyces* sp. strains UAH 15, UAH 23, UAH 29, UAH 30, UAH 51, and UAH 59 were selected because of their ability to remove the color from solid medium and produce clear halo around mycelial growth after 7 days of incubation.

The optimal conditions for effluent decolorization were determined by using liquid medium. Different degrees of decolorization were observed in the eight media tested, depending on the carbon and nitrogen sources added. The highest degrees of decolorization were observed when we used media containing glucose and ammonium sulfate, glycerol and yeast extract, and glycerol and ammonium sulfate (Fig. 1). The strains produced significant growth in all media, and glycerol and ammonium sulfate were the carbon and nitrogen sources which resulted in the highest degrees of decolorization in all of the strains tested (65, 60, 63, and 65% color loss for strains UAH 15, UAH 23, UAH 30, and UAH 51, respectively, after 7 days of growth). It should be emphasized that in the presence of yeast extract, all of the strains produced a great deal of pigment, which could have masked decolorization; therefore, yeast extract is not suitable for decolorization studies.

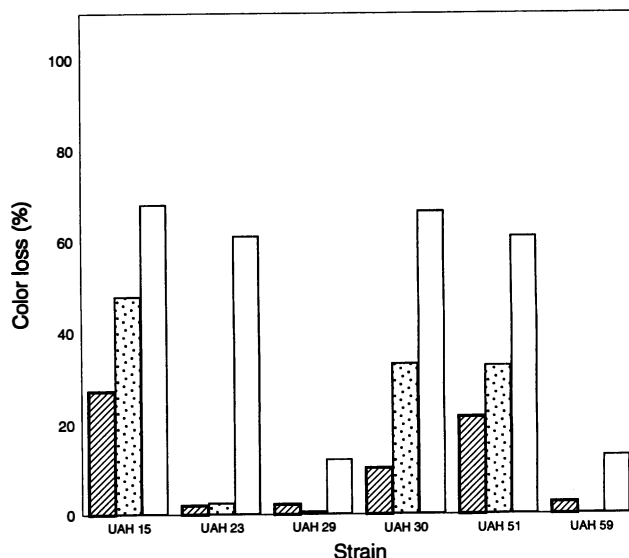


FIG. 1. Percentages of decolorization by different *Streptomyces* strains obtained with glucose and ammonium sulfate (cross-hatched bars), glycerol and yeast extract (stippled bars), and glycerol and ammonium sulfate (open bars).

On the basis of these results, strains UAH 15, UAH 23, UAH 30, and UAH 51 were selected for further study. The time course of growth of these strains and the pattern of color loss that occurred with growth are shown in Fig. 2. The most significant color loss in the effluent was observed during the exponential phase of growth in all of the strains studied. However, with some of the strains (UAH 15 and UAH 51) color loss was also significant during the stationary phase of growth.

In the strains tested, the pH values of the decolorized effluents were not lower than 5 during incubation. The final pH

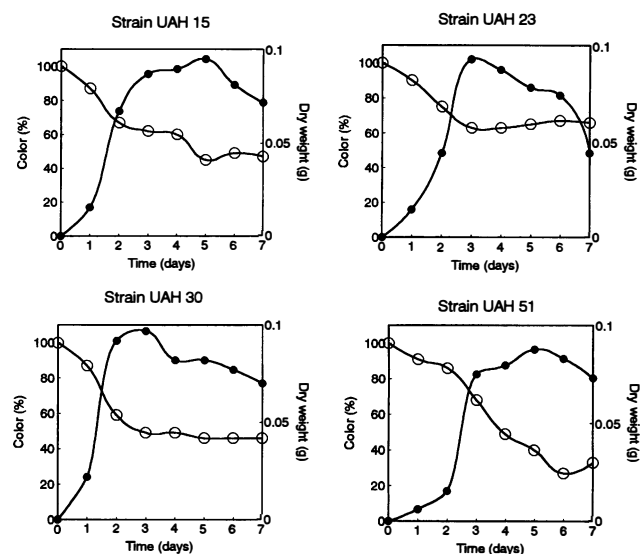


FIG. 2. Relationship between time course of growth (●) and color loss (○) of the effluent in mineral salt medium supplemented with 80% (vol/vol) effluent, glycerol, and ammonium sulfate.

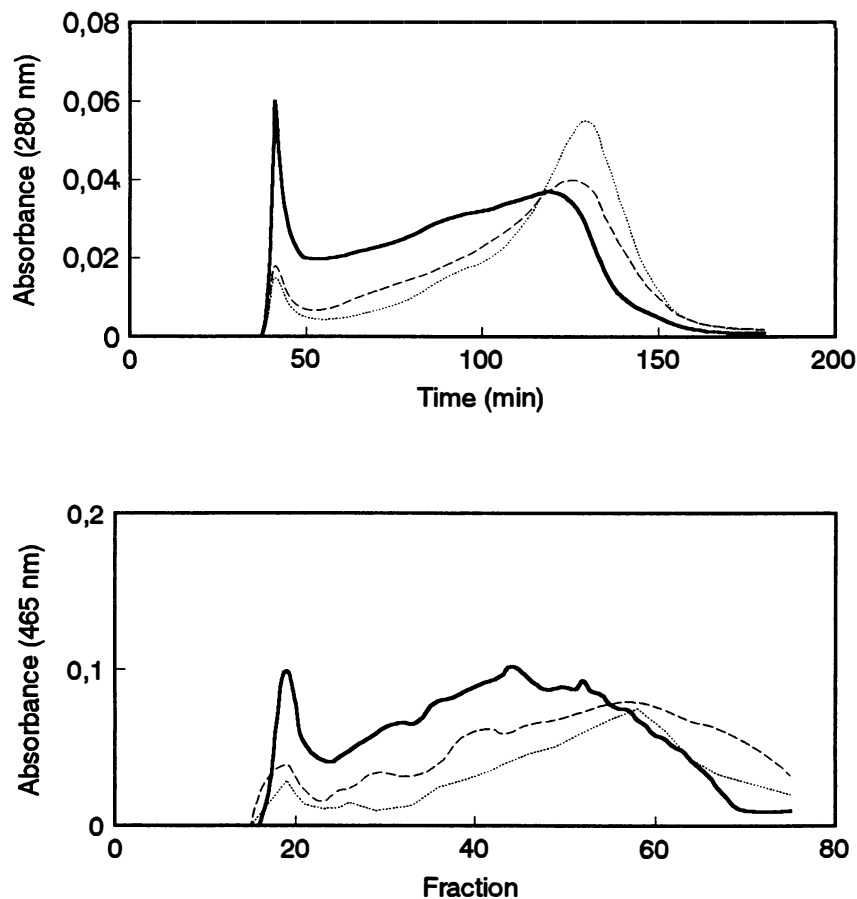


FIG. 3. Sephadex G-100 elution patterns at 280 and 465 nm obtained with untreated effluent (—) and decolorized effluent after 2 days (---) and 6 days (···) of growth of strain UAH 30.

values reached with strains UAH 15, UAH 23, UAH 30, and UAH 51 were 6.57, 6.38, 5.90, and 5.0, respectively. In blank experiments, the degrees of color loss in untreated effluent were 4% at pH 7.0, 8% at pH 6.5, 12% at pH 6.0, 16% at pH 5.5, and 17% at pH 4.5. Therefore, we believe that the decolorization process is not just a consequence of acidification of the medium, but biological transformation should be considered.

On the basis of the different patterns of decolorization, strains UAH 30 and UAH 51 were selected for further study. To find out whether the inoculum size had an effect on the degree of decolorization observed in the effluent, different concentrations of spores were assayed. We observed decolorization of the effluent only when the inoculum size was more than 10^6 CFU ml⁻¹.

Studies of molecular size distribution in decolorized effluents are useful for determining the modifications that occur in the chromophoric groups responsible for the color (8, 9). The patterns of elution from Sephadex G-100 columns at 280 and 465 nm obtained with both untreated effluents and effluents decolorized by strains UAH 30 and UAH 51 are shown in Fig. 3 and 4, respectively. At both wavelengths, the patterns of elution from the effluents decolorized by the strains exhibited decreases in absorbance of the high- and medium-molecular-weight compounds that were correlated with incubation time. Effluent decolorized by strain UAH 51 exhibited gradual decreases in absorbance of the high- and medium-molecular-

weight compounds during growth (Fig. 4), while strain UAH 30 produced only slight changes after 2 days of growth (Fig. 3). An increase in the A_{280} of the low-molecular-weight compounds was observed in the effluent decolorized by strain UAH 30. However, in the effluent decolorized by strain UAH 51 a decrease in the absorbance of these compounds at the same wavelength was detected.

At 465 nm, the elution patterns of the high- and medium-molecular-weight compounds in the effluents decolorized by both strains were similar to the patterns obtained at 280 nm. In contrast, the low-molecular-weight absorbance in effluent decolorized by strain UAH 51 at 465 nm was different from the absorbance in effluent decolorized by strain UAH 30. The differences could be attributed to different transformation mechanisms of the chromophores in the two strains.

To investigate the molecular weight patterns in the effluents tested, gel permeation chromatography of the effluent was performed after chemical removal of the alkali-lignin fraction. The resulting chemically treated effluent had a residual yellow color. The profile obtained with Sephadex G-100 at 280 nm indicated that low-molecular-weight compounds were present, and high- and medium-molecular-weight compounds were not detected (Fig. 5). These results suggest that the high- and medium-molecular-weight compounds are responsible for most of the color in the effluent, while the residual color of the decolorized effluent could be due to low-molecular-weight compounds.

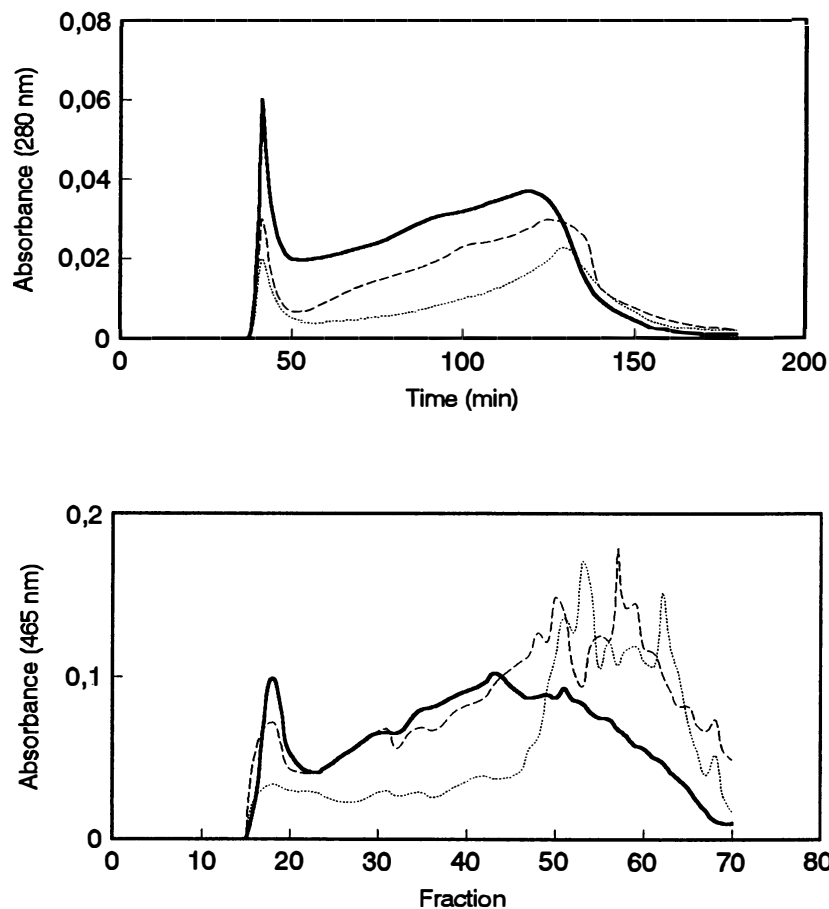


FIG. 4. Sephadex G-100 elution patterns at 280 and 465 nm obtained with untreated effluent (—) and decolorized effluent after 2 days (---) and 6 days (···) of growth of strain UAH 51.

The results of thin-layer chromatography of the compounds extracted from acidified supernatants obtained from both untreated effluents and effluents decolorized by strains UAH 51 and UAH 30 are shown in Fig. 6. Because aromatic compounds can be visualized under short-wave UV light (15), the bands detected could be attributed to lignin-related compounds. A constant pattern of bands was obtained with the effluent decolorized by strain UAH 30 after 4 or more days of

incubation (Fig. 6A), while in the effluent decolorized by strain UAH 51 patterns of bands having different R_f values were obtained at different times during the decolorization process (Fig. 6B). These results suggest that strains UAH 30 and UAH 51 may have different mechanisms for decolorizing the effluent.

Although the percentage of adsorption of the color to the mycelia was estimated to be 20% of the initial color after 4 to 5 days of incubation, it was almost impossible to recover all of the color from the mycelia because of its structure under the conditions which we used (solid pellets).

On the basis of the results obtained by gel permeation chromatography and thin-layer chromatography, we suggest that not only is decolorization of the effluent a consequence of mycelial adsorption of the color but also transformation of the major chromophoric groups occurs in the effluent. Transformation was observed only in strains in which previous adsorption occurred, and decolorization of the effluent by both strains required initial adsorption of the color to the mycelia. Characterization of alkali-lignin and lignin-related compounds will be necessary in order to confirm these results.

It has been found that actinomycetes produce a ligninolytic system during growth on lignocellulosic materials (1, 14). However, even though ligninolytic enzymes, such as phenol oxidases, are involved in the decolorization of paper mill effluents in white rot fungi (6, 7), no data concerning the role

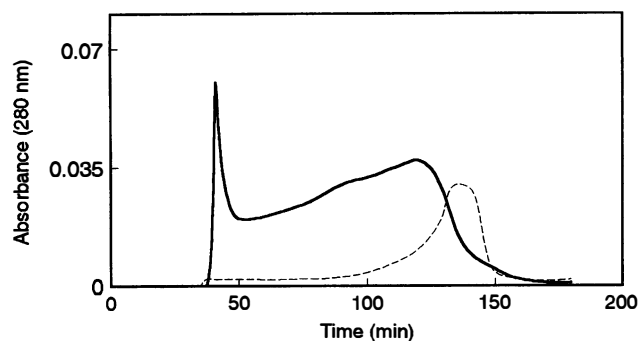


FIG. 5. Sephadex G-100 elution patterns obtained with untreated effluent (—) and effluent after chemical removal of the alkali-lignin fraction (---).

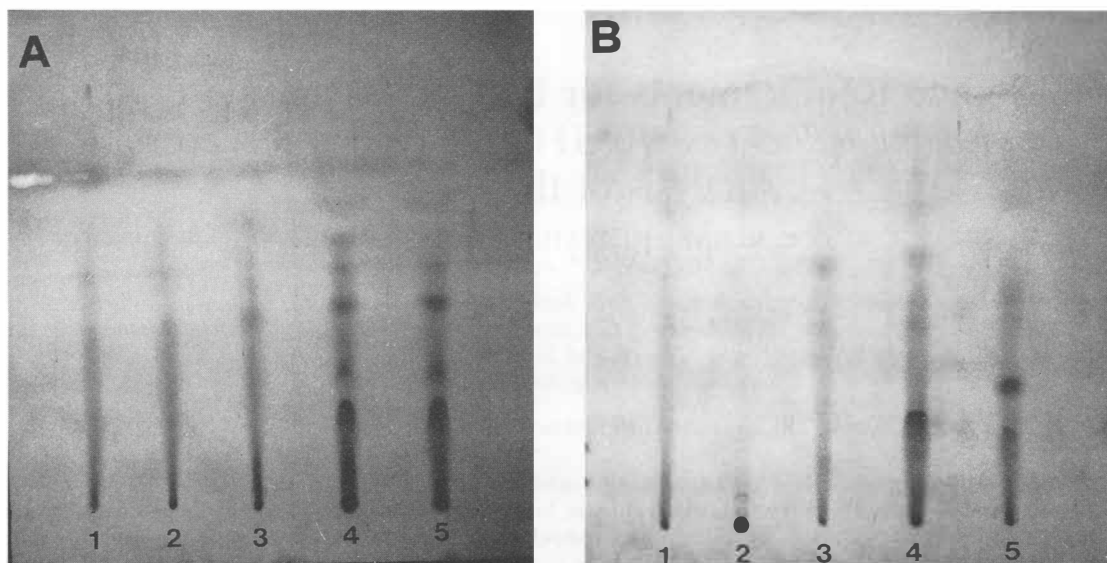


FIG. 6. Thin-layer chromatograms, visualized by UV light, showing low-molecular-weight compounds extracted from untreated effluent and effluents decolorized by strains UAH 30 (A) and UAH 51 (B). Lanes 1, untreated effluent; lanes 2 through 5, decolorized effluent after 2, 3, 4, and 6 days of growth, respectively.

of these enzymes in actinomycetes have been reported. Additional studies should be performed with selected *Streptomyces* strains to determine the possible involvement of ligninolytic enzymes in decolorization of effluents.

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