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Kraft Pulp Bleaching and Delignification by Dikaryons and Monokaryons of *Trametes versicolor*

KATHERINE ADDLEMAN AND FREDERICK ARCHIBALD*

Pulp and Paper Research Institute of Canada, 570 St. John's Boulevard, Pointe Claire, Quebec H9R 3J9, and Macdonald College, McGill University, 21111 Lakeshore Road, Sainte Anne de Bellevue, Quebec H9X 1C0, Canada*

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The ability of 10 dikaryotic and 20 monokaryotic strains of *Trametes* (*Coriolus*) *versicolor* to bleach and delignify hardwood and softwood kraft pulps was assessed. A dikaryon (52P) and two of its mating-compatible monokaryons (52J and 52D) derived via protoplasting were compared. All three regularly bleached hardwood kraft pulp more than 20 brightness points (International Standards Organization) in 5 days and softwood kraft pulp the same amount in 12 days. Delignification (kappa number reduction) by the dikaryon and the monokaryons was similar, but the growth of the monokaryons was slower. Insoluble dark pigments were commonly found in the mycelium, medium, and pulp of the dikaryon only. Laccase and manganese peroxidase (MnP) but not lignin peroxidase activities were secreted during bleaching by all three strains. Their laccase and MnP isozyme patterns were compared on native gels. No segregation of isozyme bands between the monokaryons was found. Hardwood kraft pulp appeared to adsorb several laccase isozyme bands. One MnP isozyme (pI, 3.2) was secreted in the presence of pulp by all three strains, but a second (pI, 4.9) was produced only by 52P. A lower level of soluble MnP activity in one monokaryon (52D) was associated with reduced bleaching ability and a lower level of methanol production. Since monokaryon 52J bleached pulp better than its parent dikaryon 52P, especially per unit of biomass, this genetically simpler monokaryon will be the preferred subject for further genetic manipulation and improvement of fungal pulp biological bleaching.

In response to environmental concerns and increasingly stringent emissions standards, the pulp and paper industry is looking for ways to decrease the levels of chlorinated lignin residues in its effluents, through both production process changes and improved treatment technologies. Considerable interest has focused on the white rot basidiomycete fungi, since they are the only group of organisms known to be capable of the preferential degradation of native lignins and the complete degradation of wood (1). The kraft process, at present the most common commercial chemical delignification method, produces a dark pulp because of the color of residual modified lignin residues. These are normally bleached or removed by the use of chlorine, chlorine dioxide, and caustic (NaOH) extractions. A dikaryon (strain 52P; ATCC 20869) of *Trametes* (*Coriolus*) *versicolor*, a common white rot basidiomycete species found worldwide, has been shown to bleach and delignify kraft pulp (23, 28), offering a possible alternative to chlorine. This organism produces laccases (23, 30) and two extracellular peroxidases, lignin peroxidase (LP) (10, 18) and manganese peroxidase (MnP) (17), although LP is not detected during biological bleaching (3). During hardwood and softwood kraft pulp biological bleaching, the kappa number, a measure of the residual lignin in the pulp, is reduced (23, 29) and methanol is released by laccase or MnP-mediated demethylation of the pulp (6, 24). Our incomplete understanding of the overall mechanisms of biological bleaching, however, as well as the long incubation times required have so far discouraged development on an industrial scale.

Each dikaryotic cell of *T. versicolor*, a tetrapolar heterokaryon, contains two nonidentical nuclei derived from the fusion of two different, sexually compatible primary mycelia, each bearing a single nucleus per cell. The dikaryotic mycelia of most *T. versicolor* isolates, including strain 52, become pigmented in culture, producing brown exudates and darkening some softwoods during decay (12). This organism also produces "pseudo-sclerotial plates" in response to environmental stimuli, such as invasion by another fungus or dessication (27). These structures are dense aggregations of darkly pigmented swollen hyphae (20). In addition, dikaryons in general have faster growth rates than monokaryons of the same species (31). These factors suggest that the use of a monokaryon of *T. versicolor* may be preferable to the use of a dikaryon for the development of a biological bleaching system, particularly if the monokaryon shows a reduced tendency to produce dark pigments in culture. Furthermore, two laccase isozymes from dikaryons have been shown to segregate into compatible monokaryons of *Coriolus hirsutus* (19). If a monokaryon that has reduced numbers of isozymes of secreted enzymes, such as laccase and MnP, and that can biologically bleach and delignify comparably to the dikaryon presently used (strain 52P) can be obtained, it would provide a much simpler genetic and biochemical system for the elucidation of the mechanisms of biological bleaching and delignification and for further genetic manipulation, gene isolation, and mutagenesis.

Two mating-compatible monokaryotic strains were derived from the *T. versicolor* dikaryon 52P to compare the parent strain and its progeny with regard to characteristics relevant to biological bleaching. Kappa number reduction and methanol production as measures of delignification, growth rates, final pulp viscosity, and production of isozymes of secreted enzymes suspected of association with delignification, biological bleaching, and pulp yield were monitored. Our results showed that monokaryons, in gen-

* Corresponding author.

eral, bleached hardwood kraft pulp more extensively than dikaryons and that one protoplast-derived monokaryon from strain 52P brightened pulp faster and to a greater extent than its dikaryotic parent, with significantly less biomass production and without the appearance of dark particles. Lower levels of MnP in the other monokaryon of this compatible pair were associated with delayed hardwood kraft pulp bleaching and reduced methanol production.

MATERIALS AND METHODS

Fungal strains. *T. versicolor* 52P (ATCC 20869) mycelium was originally isolated from a rotted *Ulmus americana* stump in Pointe Claire, Quebec, Canada. The hyphae bore clamp connections but no fruiting bodies or conidia, and the organism was tentatively identified on the basis of morphological characteristics by J. H. Ginns of Agriculture Canada. Eight other dikaryotic strains of *T. versicolor*, derived from Agriculture Canada cultures, and one isolate from Czechoslovakia were used in the preliminary bleaching screening: Paprican 464 (CCFC 22794), 466 (CCFC 94076), 467 (CCFC 175418), 468 (CCFC 197256), 469 (CCFC 197257), 470 (CCFC 197258), 471 (CCFC 197259), and 472 (CCFC 197260) and 534, the Czechoslovakian strain. Five strains had been isolated from hardwood sources, and five had been isolated from softwood sources. Twenty monokaryotic strains, kindly provided by A. D. M. Rayner (University of Bath, Bath, England), were taken from germinated single-basidiospore cultures from two fruiting bodies of *T. versicolor* representing a sampling of all four mating types. Cultures were incubated at 27°C. Plugs (1.2-cm diameter) were punched from the peripheries of 5-day-old mycological broth (MB [glucose, 40 g/liter; Soytone, 10 g/liter; pH 5.0] supplemented with trace metals [21]) agar plate colonies, lightly macerated, and stored at −80°C in 50% MB and 25% glycerol.

A defined medium, TDM (*Trametes* defined medium), was also used for biomass production. TDM contained 33.3 mM glucose, 15 mM glutamine, 5 mM NaCl, 5 mM KH_2PO_4, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 10 mM 2,2-dimethylsuccinate, and 0.8 mg of thiamine per liter; the pH was set to 5.0. The following trace metals (as a 1,000× acidic concentrate) were added to TDM: 20 μM $FeSO_4$, 2 μM $CuSO_4$, 5 μM $ZnCl_2$, 20 μM $MnSO_4$, 5 μM $CoCl_2$, 0.1 μM $NiCl_2$, and 0.5 μM $(NH_4)_6Mo_7O_{24}$. With lower glucose (8 mM), glutamine (3 mM), and manganese (5 μM in the above-described trace metal cocktail) concentrations, the latter medium was used for LP production (LP-TDM). Another defined medium, SDM-2 (27a), used for softwood pulp bleaching, contained 0.5 g of Tween 80, 10.0 g of glucose, 0.26 g of asparagine, 0.68 g of KH_2PO_4, 0.25 g of $MgSO_4 \cdot 7H_2O$, 15 mg of $CaCl_2 \cdot 2H_2O$, and 1 mg of thiamine per liter and a trace metal 1,000× acidic concentrate of 2 μM $FeSO_4$, 1 μM $CuSO_4$, 10 μM $MnSO_4$, 5 μM $ZnSO_4$, 5 μM $CoCl_2$, and 0.5 μM $(NH_4)_6Mo_7O_{24}$; the pH was set to 5.5.

Inoculum preparation. For protoplasting and pulp bleaching, five 1.2-cm-diameter plugs were taken from the growing edge of an MB agar colony and shaken on a rotary shaker (radius = 9 mm; 200 rpm) in a 500-ml Nalgene foam-plugged flask containing two glass marbles (1.0-cm diameter) and 100 ml of MB for 5 days. The resulting slurry was washed by filtration (Whatman no. 4 filter) for protoplasting or used unfiltered to inoculate bleaching flasks. In some cases, the inoculum for bleaching trials also included 0.25% (wt/vol) pulp.

Protoplast production and isolation of compatible monokaryons. Approximately 200 mg of filtered inoculum was shaken (75 rpm) for 1 to 3 h in a sterile 20-ml scintillation vial containing 2 ml of 0.1 M Na phosphate buffer (pH 5.6) with 0.1 M $MgSO_4$ and 0.4 M D-mannitol for osmotic stability and 20 mg of NovoZym 234 (Novo) as the lytic agent (10 mg/100 mg of biomass). NovoZym 234 is a preparation from *Trichoderma harzianum* containing glucanase, laminarinase, xylanase, chitinase, and protease activities. After the appearance of protoplasts, a series of dilutions were made with osmotically stabilized buffer, and the protoplasts were counted with a hemocytometer and plated onto MB agar plates containing 0.4 M D-mannitol. Regenerating colonies were examined by phase-contrast microscopy for clamp connections. Colonies lacking clamps were subcultured and inoculated in pairs on MB agar plates so that their mycelia would fuse. Compatible monokaryons were identified by the renewed production of clamp connections, the reappearance of colony characteristics specific for dikaryons, and nuclear staining with 4′,6-diamidino-2-phenylindole to confirm the presence of paired nuclei in the cells. This protoplasting procedure was carried out with dikaryotic strains 52P, 467, and 472.

Growth and biomass production. Dikaryotic strain 52P and the protoplast-derived monokaryotic strains, 52J and 52D, were plated on MB agar or TDM agar. Inoculum plugs were from the same medium composition as that of the final medium. After 5 days of growth at 27°C, each mycelium was placed in boiling water to liquify the associated agar, rinsed several times with boiling water, placed on a preweighed Whatman no. 4 filter, and oven dried (50°C) to a constant weight. Ergosterol was measured by the method of Seitz et al. (33).

Biological bleaching. A 2% suspension of water-washed kraft pulp in a 500-ml glass flask was inoculated with a 15% (vol/vol) inoculum to yield a 200-ml final volume. For the preliminary screening of dikaryons and monokaryons, this system was miniaturized to 10% of the above-described volume in a 50-ml flask. The suspension was shaken at 200 rpm and 27°C. Hardwood pulp A (washed brownstock from a central Canadian kraft mill) contained 8.3% softwood and six hardwoods, with maple being predominant (44.1%). The initial brightness was 30.5% (ISO [International Standards Organization]), the kappa number was 14.8, and the viscosity was 24.3 $mPa \cdot s^{-1}$. A second hardwood pulp brownstock, B, from the same mill, contained 55.3% maple and 7.9% softwood and had an initial brightness of 30.9% (ISO), a kappa number of 12.5, and a viscosity of 12.3 $mPa \cdot s^{-1}$. The manganese level in this pulp was 42.5 ppm. The softwood kraft pulp was a pure furnish of black spruce from the Paprican pilot plant and had an initial brightness of 34.5% (ISO), a kappa number of 25.0, and 38.8 ppm of manganese. Small handsheets (2 by 4 cm) were prepared by filtering 20 ml of the pulp suspension, blotting it, and air drying it for 24 h. Brightness was measured (457 nm) by use of a Perkin-Elmer λ3B spectrophotometer equipped with a reflectance sphere. Daily handsheets were prepared during hardwood pulp bleaching; the final harvesting of the pulp and supernatant was done after 5 days. Handsheets were prepared from the softwood pulp every 3 days, and final harvesting was done on the 12th day. Final brightness, kappa number, and viscosity values were obtained from standard handsheets produced from the pulp obtained at harvest. All bleaching experiments were performed in triplicate. Dark particles that were produced during dikaryon incubation with pulp and that appeared in the handsheets were quantified by use of a standard dirt count procedure (standard T437 om-85 of the

TABLE 1. Morphological and microscopic characteristics of *T. versicolor* dikaryon 52P and monokaryons 52J and 52D grown on MB agar plates for 1 week at 27°C

Strain	No. of nuclei/cell	Clamp connections	Arthrospores[a]	Colony texture	Colony color	Exudate
52P	2	+	−	Dense, flat	White-brown	Clear, brown
52J	1	−	+	Loose, high	White	Absent
52D	1	−	+++	Loose, high	White	Absent

[a] Observations were made microscopically: −, no arthrospores; +, a few arthrospores; +++, very numerous arthrospores.

Technical Association of the Pulp and Paper Industry [TAPPI], Atlanta, Ga.) and electronic quantitation.

Measurement of lignin degradation. Delignification in the biological bleaching system was measured as the change in the handsheet kappa number (standard T236 cm-85 of TAPPI) and by measuring methanol production in the supernatant by gas chromatography (6, 23).

Enzyme production and assays. Laccase activity was measured daily in the bleaching supernatants by monitoring the oxidation of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) at 420 nm. One unit of activity equalled 1 μM ABTS oxidized per min at 25°C and pH 5.0. LP was produced in LP-TDM (described above). The inoculum was prepared in 100 ml of this medium with 20 mycelium agar plugs and shaking for 24 h at 200 rpm in a 500-ml Nalgene flask containing two 1-cm glass marbles. Shaking was continued for another 24 h with the marbles removed. This inoculum was transferred to 900 ml of fresh LP-TDM in a 2.8-liter Fernbach flask (60 rpm; radius = 0.9 cm). Veratryl alcohol (1 mM) was added at inoculation and again after 7 days, when a daily flushing with 2.0 liters of O_2 was begun. LP activity was measured daily after day 7 by monitoring the oxidation of veratryl alcohol (2 mM) to veratraldehyde at 310 nm with 0.1 mM H_2O_2 in 100 mM tartrate buffer (pH 2.5) (molar extinction coefficient, 9,300 M^{-1} cm^{-1}). One unit of activity equalled 1 μM veratryl alcohol oxidized per min at 25°C (36). MnP activity was measured by monitoring the change in the optical density produced by the formation of a malonate-Mn^{3+} complex at 270 nm with 50 mM Na malonate (pH 4.5), 0.2 mM $MnSO_4$, and 0.1 mM H_2O_2 as substrates (15). Xylanase activity was determined by inoculating 1.5% agar plates containing 1.0% birchwood xylan (Sigma) and trace metals with 3-mm-diameter mycelium plugs, flooding the plates with 0.1% aqueous Congo red once colonies reached 5 mm in diameter, shaking the plates at 50 rpm for 15 min, and then rinsing the plates with 1 N NaCl for 15 min. The cleared zone around each colony indicated the relative amount of xylanase produced. Cellulase activity was also measured with Congo red as described above but on 1.0% carboxymethyl cellulose (CMC) agar plates containing trace metals. Cellulase production in the biological bleaching system was also assessed by measuring handsheet pulp viscosity with a capillary viscosimeter (standard T230 om-82 of TAPPI).

Gel electrophoresis. Isozyme patterns of laccases and peroxidases of concentrated and dialyzed supernatant samples (10,000-molecular-weight cutoff) were displayed by use of native 8 to 25% acrylamide gradient gels. Laccase bands were visualized by soaking the gels in 1 mM 3,3′-diaminobenzidine (DAB) in 0.2 M Na acetate buffer (pH 4.5). For peroxidase bands, 0.5 mM H_2O_2 was added to the DAB solution. Isoelectric focusing on pH 3 to 9 and pH 4 to 6.5 gels of partially purified peroxidase isozymes by fast protein liquid chromatography with a Mono Q anion-exchange column was used to identify the isoelectric points of the peroxidase isozymes.

RESULTS AND DISCUSSION

Isolation of compatible monokaryons from regenerated protoplasts. NovoZym 234 provided faster and more complete cell wall lysis than various combinations of lytic enzymes, such as cellulase and chitinase. Also, mannitol provided more osmotic stability than equal concentrations of sucrose. This optimized system yielded approximately 10^5 protoplasts per ml from approximately 100 mg of wet mycelium, and these protoplasts were stable for several days at 4°C. Only occasional hyphal filaments remained after 3 h of incubation. About 20% of the colonies regenerated from them lacked clamp connections. The presence of clamps was accepted as an indicator of the dikaryotic state, since fluorescence microscopy confirmed that only hyphae bearing clamp connections contained two nuclei per cell, as previously reported for this species (39). Two colonies were identified as the mating-compatible monokaryons (named 52J and 52D) when crossing them resulted in the reappearance of clamp connections throughout the colony as well as the characteristic color, texture, and exudate seen only in the dikaryon (Table 1). Crossing either 52J or 52D with four monokaryotic colonies from other strains of *T. versicolor* (Paprican 467 and 472) resulted in the appearance of clamp connections in every case, confirming the identity of Paprican 52P as *T. versicolor*.

Comparison of monokaryons and dikaryons. (i) Growth rate. Significantly more biomass was produced in 5 days by the parent dikaryon, 52P (0.14 g [dry weight] per plate), than by monokaryon 52J or 52D (0.09 or 0.07 g [dry weight] per plate, respectively) on MB agar. Monokaryon 52J produced less ergosterol (14.0 μg/ml) than 52P (50.0 μg/ml) after incubation in 1% hardwood kraft pulp for 5 days. Ergosterol is a steroid that is found in fungal cell membranes and that has been used to measure biomass production (33). Growth was restricted on the defined medium (TDM), and all three strains produced similar yields.

(ii) Biological bleaching and other changes in pulp properties. Both monokaryotic and dikaryotic strains of *T. versicolor* were able to biologically bleach, delignify, and demethylate kraft pulps, with some variability. In many different trials with dikaryotic strains, final hardwood pulp brightness increases after 5 days ranged from −1.4 to +18.1%, with an average of +7.8 points (standard deviation = 1.0; Table 2). Brightness increases were significantly higher ($P < 0.01$) with the monokaryotic strains (Table 3) and ranged from +6.5 to +25.6, with an average of +16.5 points (standard deviation = 1.8). There was no difference in bleaching by the monokaryotic basidiospore cultures from either fruiting body X or fruiting body Y. Softwood pulp was only effectively bleached by strain 52P (data not shown) and darkened

TABLE 2. Ability of wild dikaryons of *T. versicolor* to bleach hardwood kraft pulp, demethylate lignin, and secrete relevant enzymes[a]

Strain	Source	% Final brightness (ISO)	pH	Laccase (U/ml)	MnP (ΔOD[b]/min/ml)	Methanol (mg/liter)	Xylan[c]	CMC[c]
52P	Elm	51.9 ± 1.2	4.9	0.05 ± 0.01	0.35 ± 0.09	8.88 ± 1.2	++	+++
464	Beech	35.8 ± 1.7	3.4	0.01 ± 0.00	<0.01	2.34 ± 0.7	+	++
466	Poplar	46.1 ± 1.1	4.9	0.27 ± 0.03	0.53 ± 0.27	6.20 ± 1.0	+	++
467	Hickory	44.0 ± 2.1	4.7	0.15 ± 0.01	0.18 ± 0.09	5.88 ± 1.4	++	+++
468	Cedar	45.2 ± 1.1	4.5	0.54 ± 0.10	0.80 ± 0.09	6.18 ± 0.2	++	+
469	Fir	49.6 ± 0.2	4.5	0.17 ± 0.02	0.18 ± 0.00	6.42 ± 0.3	+++	+++
470	Fir	55.3 ± 1.9	4.9	0.58 ± 0.17	0.35 ± 0.00	5.34 ± 1.2	+++	+++
471	Cedar	40.0 ± 1.0	3.9	0.02 ± 0.00	<0.01	2.00 ± 0.5	+	+++
472	Fir	40.9 ± 1.1	4.8	0.15 ± 0.02	0.44 ± 0.09	4.70 ± 0.5	++	++
534	Oak	41.3 ± 1.4	4.4	0.01 ± 0.00	<0.01	6.66 ± 1.7	++	++
Control	HWKP[d]	37.2 ± 0.1	6.6	<0.01	<0.01	0.00	−	−

[a] An initial screening with reduced volumes (10%) of the standard biological bleaching mixture.
[b] Change in the optical density units at 270 nm.
[c] Clearing of CMC-Congo red and birchwood xylan-Congo red agar plates: −, no clearing around colony; +++, extensive clearing; + and ++, intermediate clearing.
[d] HWKP, 2% hardwood kraft pulp alone (sterile).

substantially after incubation with some of the other dikaryotic strains. The great variability found among dikaryotic strains in their ability to bleach kraft brownstock may explain the conflicting reports on the efficacy of various *T. versicolor* strains in biologically bleaching and degrading lignin (14, 23, 38). Since strain 52P bleached both pulps well, this dikaryon and its derived compatible monokaryons were selected for further investigation. In a total of 20 bleaching trials under various conditions and with different pulps, monokaryon 52J showed the fastest rate of brightening and the largest total mean brightness increase, followed by 52P and 52D, although the differences were not significant because of the variability produced by the dissimilar systems (Fig. 1). However, in four trials with the same hardwood pulp in a 2% suspension, 52J brightened the pulp significantly more than 52P ($P < 0.05$; Table 4).

TABLE 3. Screening of hardwood kraft pulp bleaching by monokaryons derived from basidiospores from two fruiting bodies (X and Y) of *T. versicolor* and from protoplasts of two dikaryotic strains

Strain	% Brightness (ISO)	Laccase (U/ml)	pH
X1	45.2 ± 1.7	<0.01	4.4
X2	49.0 ± 6.5	<0.01	4.1
X3	46.7 ± 1.2	0.03 ± 0.02	3.9
X4	44.0 ± 1.5	<0.01	3.0
X5	43.3 ± 0.6	<0.01	4.7
X6	55.8 ± 2.6	<0.01	4.1
X7	52.4 ± 0.8	0.05 ± 0.03	4.1
X8	52.2 ± 0.9	0.12 ± 0.02	4.0
X9	43.9 ± 0.7	0.03 ± 0.01	3.8
X10	55.3 ± 0.8	0.07 ± 0.03	3.9
Y1	59.2 ± 1.3	<0.01	3.9
Y2	44.3 ± 1.1	0.13 ± 0.09	4.1
Y3	51.1 ± 0.5	0.04 ± 0.01	4.0
Y4	56.7 ± 0.8	<0.01	4.1
Y5	40.1 ± 1.0	<0.01	3.7
Y6	53.6 ± 1.4	<0.01	4.0
Y7	42.9 ± 0.9	<0.01	4.0
Y8	55.5 ± 1.6	<0.01	4.0
Y9	54.1 ± 1.0	0.01 ± 0.01	4.0
Y10	53.7 ± 0.4	0.03 ± 0.01	3.8
52J	54.5 ± 1.7	0.09 ± 0.03	3.9
52D	52.7 ± 0.9	0.01 ± 0.01	3.9
467S	44.8 ± 1.1	0.06 ± 0.02	4.0
467T	51.1 ± 1.2	0.12 ± 0.01	3.7
Control	33.6 ± 0.1	<0.01	5.0

Dark particles occurred in the 52P-bleached pulp and with several other screened dikaryons but never with monokaryotic cultures. Phase-contrast microscopy of these particles revealed swollen and pigmented hyphae entwined in the pulp fibers, reminiscent of the hyphal morphologies described in antagonism lines at the borders between different *T. versicolor* dikaryons in nature (20, 39).

(iii) Delignification and demethylation. Although delignification, as measured by kappa number reduction, was the same for the parent dikaryon, 52P, and its daughter monokaryons, significantly more methanol from hardwood pulp was produced by 52J, the most efficient bleacher, than by 52D, which bleached the least (Table 4). However, methanol production, which precedes delignification (6), was independent of biological bleaching in the preliminary dikaryotic and monokaryotic screening (Tables 2 and 3). Methanol was found only in low levels at harvest in the reduced-volume system, possibly reflecting a decrease over time, which has been noted (6). The ability of monokaryons to degrade wood more while producing less biomass than dikaryons has been reported for the white rot fungi *Poria vaillantii* and *Serpula lacrimans* (9, 13), although a similar study of *C. hirsutus* showed that neither daughter monokaryon had ligninolytic ability exceeding that of the parent dikaryon (40).

(iv) Secreted enzymes. LP activity was never detected in the bleaching system, although low levels were found after 12 days in pulp-free LP-TDM with the dikaryon (6 U/liter) and the monokaryons (52J: 2 U/liter; 52D: 0.5 U/liter). The absence of LP activity in the *T. versicolor* bleaching system, as measured by veratryl alcohol and Azure B oxidation assays (2), may have been the result of protein aggregation, inhibitors, pulp adsorption, or protease-mediated degradation occurring in the bleaching supernatant (3, 11). However, earlier work with 52P and the metavanadate ion as an LP inhibitor suggested that this enzyme is not involved in pulp biological bleaching and is secreted by *T. versicolor* only under high-oxygen, 12-day, nitrogen-limited growth (secondary metabolism) conditions (3). In the present work, the carryover of nitrogenous nutrients from the MB inoculum

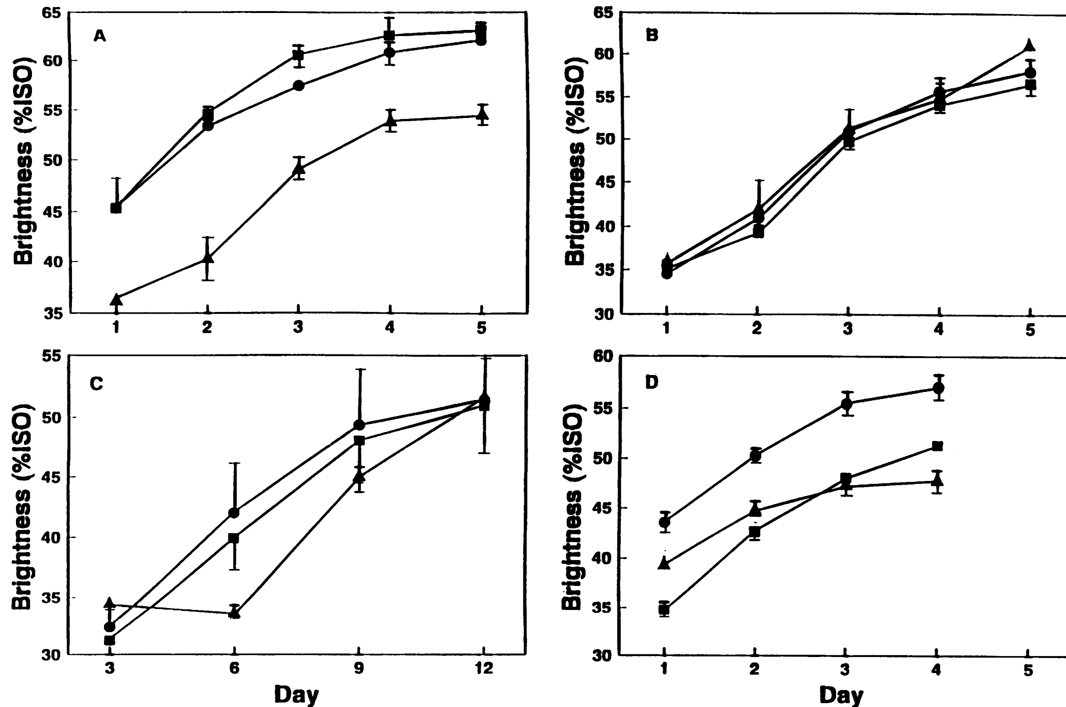

FIG. 1. Biological bleaching by a parent dikaryon (52P) (■) and two mating-compatible monokaryons (52J [●] and 52D [▲]) derived from it. (A) Tween 80 (0.05%) was added to the 2% hardwood pulp suspension. (B) An SDM-2 culture was used for the inoculum and included in the 2% hardwood pulp suspension. (C) Softwood kraft pulp (black spruce) (2%) in SDM-2 (12 days). (D) Hardwood pulp suspension (1%). Error bars represent $P < 0.05$.

into the pulp suspension, the short incubation, and the agitation used in the bleaching system (200 rpm) may have suppressed both the production and the activity of LP, as in *Phanerochaete chrysosporium* (37). However, the addition to the pulp flasks of Tween 20, a detergent known to protect LP in agitated media and possibly to moderate adsorption effects (16), did not result in the production of detectable LP.

MnP produced by 52P during biological bleaching has been found to demethylate and delignify kraft pulp in vitro (24). Secreted MnP activity in culture supernatants was substantial during 52P and 52J biological bleaching. MnP was also produced constitutively in the pulp-free inoculum (52P: 0.5 U/ml; 52J: 0.45 U/ml; molar extinction coefficient, 8,850 M^{-1} cm^{-1}). There was a lag period in the secretion of MnP for strain 52D, the level being 0.02 U/ml in the inoculum. A strong positive correlation ($P < 0.01$) (34) between the level of MnP and brightening was found (Fig. 2). This result corroborates results found in the accompanying paper, in which MnP was present at maximum levels when the rate of bleaching was at its highest (24). However, when the 52D inoculum was induced to produce high levels of MnP prior to the bleaching trials by the addition of 0.25% HWKP, 0.2 mM $MnSO_4$, and a chelator (4, 7, 8), there was no improvement in the rate or extent of biological bleaching by this strain (results not shown), a result that suggests that other unknown secreted enzymes or compounds are also involved in bleaching. Reduced MnP levels in 52D were also associated with low methanol production ($P < 0.01$; Table 4). This result suggests that MnP is responsible for hardwood pulp demethylation (24). In the preliminary screening of dikaryons and monokaryons (Tables 2 and 3), enzyme activity levels were only measured at harvest (day 5), although most pulp bleaching occurred between days 1 and 3. This result may explain why no relationship was found between MnP activity and pulp bleaching in this screening.

Laccase activity was always present during bleaching by

TABLE 4. Changes in handsheet properties and methanol production during biological kraft pulp bleaching with dikaryon 52P and derived monokaryons 52J and 52D of *T. versicolor*[a]

Strain	% Brightness (ISO)[b]		Kappa number		Viscosity ($mPa \cdot s^{-1}$), HW	Particle count (ppm)[c], HW	Methanol (mg/liter), HW
	HW	SW	HW	SW			
52P	51.3 ± 0.4	51.0 ± 5.3	8.8 ± 1.1	8.2 ± 2.0	7.8 ± 0.5	3,197 ± 300	18.0 ± 3.5
52J	57.0 ± 0.8	51.4 ± 3.9	8.7 ± 0.7	8.4 ± 0.6	8.3 ± 0.3	236 ± 51	21.4 ± 1.3
52D	47.8 ± 1.0	51.6 ± 0.3	9.1 ± 0.4	8.6 ± 0.1	9.0 ± 0.7	ND	11.9 ± 4.1
Control	36.4 ± 0.1	34.6 ± 0.1	12.3 ± 1.3	24.9 ± 0.6	10.5 ± 0.1	ND	1.9 ± 0.1

[a] Hardwood (HW) and softwood (SW) pulp suspensions (2%) were incubated at 200 rpm and 27°C for 5 days (HW) and 12 days (SW). ND, not determined.
[b] Final measurement after 5 days of pulp incubation.
[c] Enumeration by use of a computer-based scanner of darkly pigmented particles in handsheets.

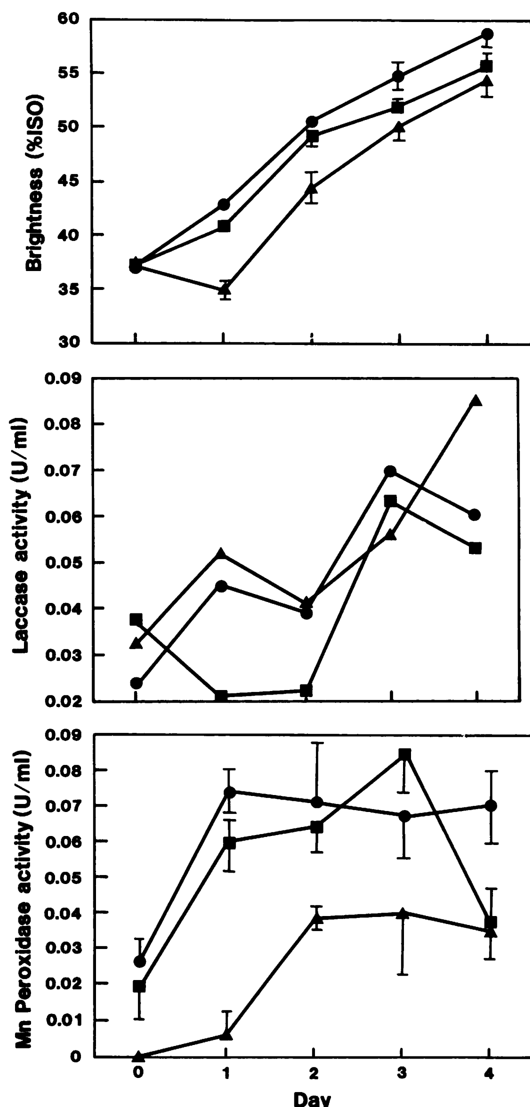

FIG. 2. Brightness and laccase and MnP activity levels measured daily during a 4-day bleaching trial of a 2% hardwood kraft pulp suspension. A molar extinction coefficient of 8,850 M^{-1} cm^{-1} was used for the unit transformation of MnP activity. Error bars indicate $P < 0.05$. The laccase graph lacks error bars, indicating no significant differences between strains. Symbols are as defined in the legend to Fig. 1.

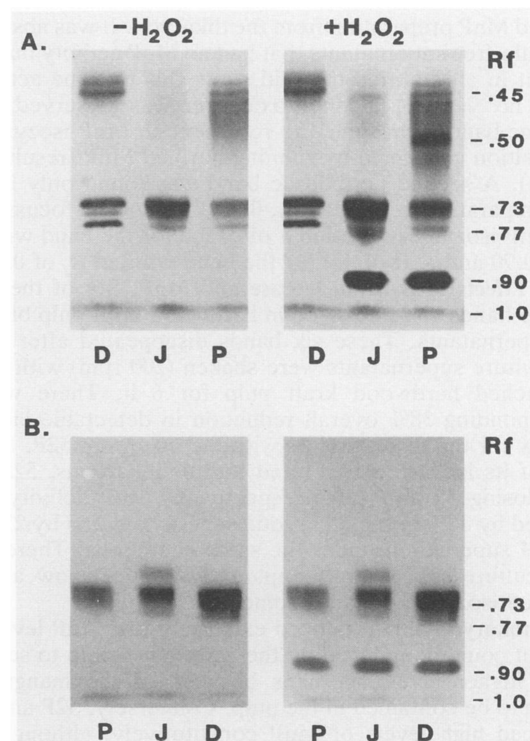

FIG. 3. Laccase and peroxidase isozyme bands produced by the parent dikaryon, 52P (P), and its monokaryotic progeny, 52J (J) and 52D (D), in the presence and absence of hardwood kraft pulp in native polyacrylamide gel electrophoresis. Concentrated, dialyzed supernatants from 5-day-old inoculum flasks without pulp (A) and from 5-day-old hardwood pulp bleaching flasks (B) were used. Laccase bands were visualized by soaking the gel in acetate buffer containing DAB. H_2O_2 (0.1 mM) was subsequently added to the solution to visualize peroxidase activity.

dikaryotic strains and was usually secreted by the monokaryotic strains as well. The levels varied and were independent of pulp bleaching and methanol production (Tables 2, 3, and 4 and Fig. 2). Differences between phenol oxidase (laccase) production and peroxidase production by various strains of this species have been noted previously (35). Mn(II), which is present in kraft pulps and in the media, has been reported to regulate the production of both laccase and MnP (25). However, there was no correlation between MnP and laccase levels in the bleaching flasks. No regulation of laccase by manganese was found in another white rot fungus, *Dichomitus squalens* (26).

Birchwood xylan and CMC indicator plates showed similar patterns of solubilization by the three strains, indicating that all produced active xylanases and cellulases in similar concentrations (results not shown).

(v) Isozymes of laccase and MnP. As in many other fungi, secreted enzymes of *T. versicolor* are characterized by having multiple isozymes, making their purification and functional interpretation very difficult. Several laccase isozymes have been reported for *T. versicolor* (5, 30, 32); a total of nine different peroxide-independent DAB-oxidizing bands were produced by 52P, 52J, and 52D in MB, five protein bands being common to all the strains, in different concentrations (Fig. 3). Discrete segregation of the isozyme bands between the two monokaryons, which when mated combine to produce the dikaryon band pattern, as has been reported for *C. hirsutus* (19), did not occur. The major laccase bands, at R_fs of 0.71, 0.73, and 0.74, were found in all three strains, although they were difficult to separate in 52P and 52J. The multiple more slowly migrating isozymes found in 52P appeared to be a composite of those seen in the monokaryotic supernatants, although the most slowly migrating monokaryotic isozymes were not seen on the dikaryotic gel. Such multiple bands possibly indicate incremental glycosylations of a single gene product (19) or other post-translational processing, such as aggregation, acetylation, or protease cleavage.

Two isozymes of MnP from *T. versicolor* were first reported in 1987 (17). A peroxidase band (R_f, 0.90) was found in all the bleaching cultures as well as in the partially

purified MnP preparation from the dikaryon. It was absent in 52D pulp-free supernatants that had no MnP activity but was present in all cultures that did show this enzyme activity. Since no veratryl alcohol oxidation was observed, this isozyme band is presumed to represent an MnP isozyme, a supposition confirmed by running purified MnP (results not shown). A second peroxidase band was found only in the MB preparation from 52P (R_f, 0.50). Isoelectric focusing of the MnP isozymes revealed a pI of 3.2 for the band with an R_f of 0.90 and a pI of 4.9 for the band with an R_f of 0.50.

(vi) Effects of pulp on laccase and MnP. Six of the nine laccase bands were not seen in hardwood kraft pulp bleaching supernatants. These six bands disappeared after pulp-free culture supernatants were shaken (200 rpm) with fresh unbleached hardwood kraft pulp for 6 h. There was a corresponding 38% overall reduction in detectable laccase activity for the three strains, with the dikaryon, 52P, losing 12% of its laccase activity and the monokaryons, 52J and 52D, losing 43 and 60%, respectively. MnP activity was reduced by 38% in the dikaryotic supernatant and by 27% in the 52J supernatant under the same conditions. Therefore, pulp culture supernatants apparently do not show all the secreted isozymes because some bind to pulp.

Monokaryon 52D produced extremely low MnP levels in MB but could be induced by the addition of pulp to secrete much higher levels, perhaps because of the manganese known to be contained in the pulp. Conversely, 52P and 52J produced high levels of MnP constitutively, although the presence of pulp was also stimulatory, but to a lesser degree.

Conclusions. The characteristics of (i) reduced biomass and slower growth rate, (ii) no dark pigment production, (iii) superior biological bleaching ability, and (iv) a simpler system for genetic manipulation and biochemical analysis justify replacing the parent dikaryon with monokaryon 52J in future work on biobleaching and biological delignification.

The involvement of MnP, but not LP, in pulp bleaching, delignification, and demethylation is strongly suggested by (i) the significant positive correlation between MnP activity and pulp bleaching and demethylation and (ii) the absence of LP in the bleaching system. Also, although laccase was present during bleaching, the lack of correlation among laccase levels, bleaching, and methanol production suggests that it is not involved. These conclusions corroborate results found in the accompanying paper (24).

Since pulp adsorbs some laccase and MnP isozymes strongly and others only weakly, assaying these enzymes in pulp bleaching supernatants may not be an accurate assessment of the total enzyme activity secreted into or functioning in these systems. Also, laccase and MnP isozymes from the parent dikaryon were not segregated discretely into the mating-compatible monokaryotic nuclei; i.e., each nucleus coded for enzyme forms that did bind and enzyme forms that did not bind to pulp fibers.

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