

# White-Rot Fungi Demonstrate First Biodegradation of Phenolic Resin

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Phenolic resins, phenol-formaldehyde polymers previously thought to be nonbiodegradable, are produced at an annual rate of 2.2 million metric tons in the United States for many industrial and commercial applications. Three independent lines of evidence established their biodegradability with the white-rot fungus *Phanerochaete chrysosporium*. Chromatic transformation of growth medium (yellow to pink) indicated initial biodegradation of the resin 3 days after inoculation. A degradation product, <sup>13</sup>C-labeled phenol, was detected with gas chromatography-mass spectroscopy. Scanning electron micrographs revealed physical evidence of degradation. This is the first demonstrated biodegradation of these phenol-formaldehyde polymers and stands as a platform for investigation into bioremediation and biorecycling of phenolic resins.

## Introduction

Phenolic resins (PRs) are complex synthetic polymers made from phenol and formaldehyde, and are classified as thermoset resins. Phenolic resins have various commercial, industrial, and manufacturing applications and are particularly important in the construction industry, where they represent the major worldwide adhesive resin for exterior-grade plywood (1), oriented strandboard, medium-density fiberboard, and other engineered wood products (2). Their resistance to attack by both fungi and termites (3–5) establishes them as a valuable construction material. Because PRs are composed of a thermoset, 3-dimensional network that in essence becomes a single molecule, they are difficult to dissolve, and cannot be melted or recast (6). These properties have generated a large market for such durable polymers, but also make them extremely challenging to degrade or recycle.

As a result of their durability, virtually all of the phenolic polymers that are produced find their way permanently into landfills after their initial use is complete. Given that the annual U.S. production is over 2.2 million metric tons and rising (6), and that PRs are not known to be degraded in the environment, the long-term accumulation prospects are vast. These factors are creating the impetus for inventive methods of recycling synthetic polymer material. One such method was developed in 1997 by Japan's Institute of Resources & Environment and Mitsui SRC Development Co. (7) but the intensive heat and use of solvents such as tetralin translate into costly operational expenses, as well as additional

chemical waste management needs. Therefore, ideas for utilizing fungi to biodegrade PRs during recycling processes could be an attractive alternative.

White-rot fungi have evolved to produce a very powerful and nonspecific bank of enzymes called ligninases that degrade lignin (8). The free-radical nature of ligninases allows such fungi to decompose a wide spectrum of persistent organic pollutants such as DDT, TNT, pyrenes, PCBs, dioxins, and many others (8, 9). The white-rot fungus *Phanerochaete chrysosporium* is one of the most notable species used for such research.

The progress of biodegradation in plastics was previously reviewed (10), but no mention of phenolic polymers was made. In two other papers, Milstein et al. (11) and Chen et al. (12) showed that the white-rot basidiomycetes *P. chrysosporium*, *Pleurotus ostreatus*, and *Trametes versicolor* were able to biodegrade lignin-styrene copolymerization products. Since the molecular structure of PR is similar to that of lignin (Figure 1), and the enzymatic arsenal of white-rot fungi is adept at deconstructing lignin, we hypothesized that phenolic resin polymers could be degraded by these fungi.

To test this hypothesis, we examined eleven strains of fungi (five species of white-rot fungi and one species of brown-rot fungus, Table S1 in the Supporting Information) for the degradability of pure PR. First, a qualitative assay was designed for the survey, followed by a spectroscopic assay involving PR production and degradation using <sup>13</sup>C-labeled phenol. This allowed for accurate detection of evidence from degradation using gas chromatography-mass spectroscopy (GC-MS). Third and finally, visual evidence of degradation was observed with scanning electron microscopy (SEM) of PR chips grown with the fungi. These three separate tiers of evidence demonstrate that *P. chrysosporium* can biodegrade phenol-formaldehyde polymer.

## Materials and Methods

**Phenolic Resin Production.** Every manufacturer has a slightly different (and proprietary) formulation for their PR. We used a typical formulation that could be representative of that found in PR-containing commercial products (13). One hundred and eight grams of phenol (90% weight/weight) was dissolved in approximately 12 mL of deionized (DI) water, mixed with 66 g of para-formaldehyde (reagents obtained from Fisher Scientific (Fairlawn, NJ)), and mixed inside a three-necked glass round-bottomed flask. A glass stir rod with a Teflon paddle was inserted in the vertical neck of the flask and connected to an overhead stirrer. A water-cooled condenser was inserted into one of the lateral necks on the flask, and an addition funnel was inserted in the remaining neck.

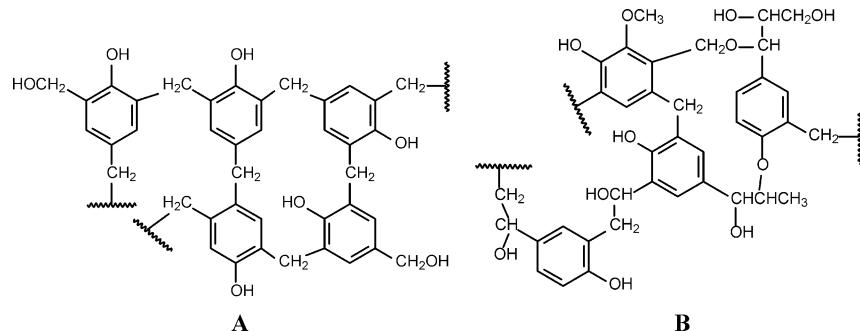
Sixteen grams of a 50% (w/v) aqueous NaOH solution was added to the flask dropwise with the separatory funnel, while stirring, and the solution was allowed to mix in a water bath at 70 °C for 1 h. The temperature was maintained using a Therm-O-Watch L6-1000SS (Instruments for Research and Industry, Terre Haute, IN). After the solution was cooled to 30 °C, 10–11 mL of 50% (w/v) NaOH and 7 mL of 30% (w/v) NH<sub>4</sub>OH were added and thoroughly stirred. This water-soluble pink solution, now called the A-stage resin, was cooled to room temperature and refrigerated until ready to cure. Resin for the <sup>13</sup>C-labeled experiments followed a similar scaled down procedure (approximately 5% of constituents), except that <sup>13</sup>C-labeled phenol (Sigma, St. Louis, MO) was substituted for phenol in the production process.

**Curing and Preparation of Resin.** A-stage resin was cured in a clean dry glass Petri dish at a depth of approximately 1

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**FIGURE 1.** Two-dimensional comparison between chemical structures of a phenol–formaldehyde resin (**A**) and a model section of lignin (**B**).

mm. All resin was cured at 98–102 °C for at least 17 h in an Isotemp Vacuum Oven (Fisher Scientific International). The brown polymer disks were allowed to cool to room temperature then placed in a paper towel and smashed with a wrench to produce approximately 5 mm × 5 mm fragments. The fragments were then autoclaved for 20 min in the glass Petri dish. Complete curing was tested by placing polymer chips in 1.5% malt agar for 3 days and observing for color change, which would indicate incomplete polymerization.

**Qualitative Assay for Phenolic Resin Degradation.** Fungal cultures (Table S1) were maintained on 1.5% malt agar slants at 2–7 °C, and transferred to 1.5% malt agar plates for experiments as needed. All cultures were received from the Forest Products Laboratory in Madison, WI. All strains were tested in the qualitative assay, but a smaller subset consisting of *P. chrysosporium* (strains BKM-F-1767 and ME-446), *Pleurotus ostreatus* (FP-90031-Sp and FP-101509-Sp), *Oligoporus placentus* (*Postia placenta*) (Mad-575), *Schizophyllum commune* (Jaquiot), and *Trichaptum biforme* (FP-86522-Sp) were used for the experiments with  $^{13}\text{C}$ -labeled phenol (chosen from qualitative assay results). One gram of polymer fragments was embedded in mature cultures grown on 1.5% malt agar plates. Maturity of a culture was characterized by hyphae growing to the edge of the plate and forming a dense layer of surface hyphae. Cultures were observed for as long as 30 days, watching for chromatic transformations or other cultural changes, followed by inspection for hyphal growth on polymer fragments using dissecting, light, and scanning electron microscopes.

**Isotopic Analysis for Degradation of Phenolic Resin into Phenol.** Phenol containing isotopically labeled carbon atoms, totaling a molecular weight (MW) of 100, was used during the production of the phenolic resin. One gram of polymer chips was embedded into each mature fungal culture in a hood. A sterilized chemistry scoop was used when weighing the polymer chips to randomize the variation in the size of chips that were added to each plate.

Due to their differences in growth rates, the amount of time each strain was grown before  $^{13}\text{C}$ -labeled polymer was embedded varied, as did the incubation period before the plates were extracted (Table S1). Triplicates of each fungal culture were embedded with polymer for each day that was to be extracted and observed for any change. Three entire plates were then extracted separately on each corresponding day (Table S1). Three plates for each day were necessary because the entire culture in each plate was extracted, preventing further sampling of that culture on the subsequent days.

Polymer chips were removed from the agar and stored in a glass Petri dish with sterilized, moistened paper towel for microscopic inspection. The medium was weighed by subtraction from the Petri dish, and placed in a clean stainless steel blender. Using a plastic syringe, 10 mL of DI water was added to the blender, and the medium was blended to an

even slurry. The slurry was transferred into a 50 mL plastic centrifuge tube, along with a series of three 10 mL DI water washes of the blender. The slurry was centrifuged in an International Equipment Company clinical centrifuge at 3000 rpm for 10 min, and the supernatant fraction was poured into a clean 60 mL glass extraction vial, along with two centrifuged 10 mL DI washes of the slurry. The water extract was transferred into a 125 mL separatory funnel with one 5 mL DI rinse of the vial.

The water extract in the separatory funnel was extracted through a series of four 5 mL additions of methylene chloride ( $\text{CH}_2\text{Cl}_2$ ), catching the  $\text{CH}_2\text{Cl}_2$  in a 20 mL glass scintillation vial and discarding the water extract. The  $\text{CH}_2\text{Cl}_2$  in the scintillation vial was evaporated to approximately 5 mL under gaseous nitrogen stream. Completely evaporated samples were resuspended in 5 mL of  $\text{CH}_2\text{Cl}_2$ . Samples were chemically dried by adding 0.25 g of sodium sulfate ( $\text{Na}_2\text{SO}_4$ ) drying agent for at least 5 min, and then filtered through a glass wool column in a Pasteur pipet into a clean 10 mL glass vial. The 20 mL scintillation vial was rinsed twice with ~1 mL of  $\text{CH}_2\text{Cl}_2$ , which was then pipetted through the glass wool column into the 10 mL glass vial. This sample was evaporated completely under gaseous nitrogen stream, and stored at room temperature until ready to run on the gas chromatograph–mass spectrometer (GC–MS).

The samples were resuspended in 2 mL of  $\text{CH}_2\text{Cl}_2$  immediately before they were run on a Varian Saturn 2100D GC–MS with a CP-Sil 8 CB low bleed/MS Chrompack capillary column and Saturn WS software. The settings used consisted of a run time of 7.2 min, temperature zones between 110 and 210 °C, pressure of 11.1 pounds per square inch (psi), column flow of 1.0 mL/min, linear velocity of 36.6 cm/s, total flow of 24.1 mL/min, the split state on, and a split ratio of 20:1. A 1  $\mu\text{L}$  sample of the 2 mL suspension was injected using a Hamilton Co. no. 701 (Reno, NV) 10  $\mu\text{L}$  glass syringe. A sharp peak at approximately 6.2 min with a mass spectrum showing a molecular weight of 100 was indicative of  $^{13}\text{C}$ -labeled phenol present in the sample. A positive control of pure  $^{13}\text{C}$ -labeled phenol was run along with negative controls of polymer chips embedded in agar without fungi, and 1.5% liquid malt extract with and without fungi. Standard errors were calculated of peak heights and used for comparisons between treatments and controls.

**Scanning Electron Microscopy of Samples.** Phenolic polymer chips no larger than 3 mm × 5 mm embedded in 10-day-old cultures of *P. chrysosporium* (*Pc*) were incubated at room temperature for 28 days. Polymer samples were removed and fixed for 24 h with a 3% glutaraldehyde solution in 0.1 M cacodylate buffer. Ten chips were washed in 100% ethanol with a bristle brush to remove surface hyphae before being fixed with glutaraldehyde. Fixing buffer was discarded, followed immediately by five washes, 10 min each, with 0.1 M cacodylate buffer. Chips were secondarily fixed with 1% osmium tetroxide for 1 h. Osmium solution was discarded



**FIGURE 2.** Pink chromatic transformation in cultures of *Phanerochaete chrysosporium* (*Pc1*) grown with phenolic resin (PR). (A) *Pc1* grown alone on 1.5% malt agar for 13 days (control). (B) 13-day old culture of *Pc1* grown on 1.5% with PR embedded for 3 days. (C) PR embedded in 1.5% malt agar alone for 13 days (control).

and the samples were washed three times, 15 min each, with 0.1 M cacodylate buffer. The samples were dehydrated using a grade series of Fisher ethanol involving five minutes in 30% ethanol, 10 min in 50% ethanol, 10 min in 70% ethanol, 15 min in 95% ethanol, and two 15 min washes in 100% ethanol. Samples were stored in 100% ethanol for at least 1 h before proceeding to critical point drying in a Samdri-PVT-3B from Tousimis Research Corporation. A Denton Vacuum Desk II was used for gold coating, and Cambridge Instruments Stereocam 90 scanning electron microscopes (SEMs) at the University of Wisconsin—La Crosse and the Forest Products Laboratory, United States Forest Service in Madison were used to inspect them.

## Results and Discussion

After manufacturing our own phenolic resin from phenol and formaldehyde, we performed a preliminary qualitative assay in which we found that growing fungi on 1.5% malt agar and embedding chunks of PR into the culture produced positive results. A chromatic transformation with pink coloration (Figure 2B) was observed in cultures of *P. chrysosporium* grown with the polymer, whereas controls of PR embedded in 1.5% malt agar without the fungus (Figure 2C) and fungus grown without the polymer (Figure 2A) showed no color change. These data implicate a fungal role in the transformation.

We hypothesized that these results indicated polymer degradation because the PR, before being cured, is a pink water soluble substance called the A-stage resin, which consists of monomers of phenol molecules connected by formaldehyde. During the curing process these A-stage monomers are then cross-linked into a larger polymeric composite. If these cured PR composites were degraded, we would expect that A-stage monomers would be an initial degradatory product, resulting in a pink chromatic transformation of the cultures. The A-stage resin may then be further degraded into its initial constituents of phenol and formaldehyde.

The white-rot fungus, *P. chrysosporium*, exhibited this color shift in the surface hyphae (Figure 2B) as early as 2 days after polymer introduction. The other four white rot species surveyed (*Schizophyllum commune*, *Trichaptum biforme*, *Pleurotus ostreatus*, and *Pleurocybella porrigens*) and the brown-rot fungus (*Oligoporous* [=Postia] *placentus*) all demonstrated no change in culture morphology when incubated with the polymer.

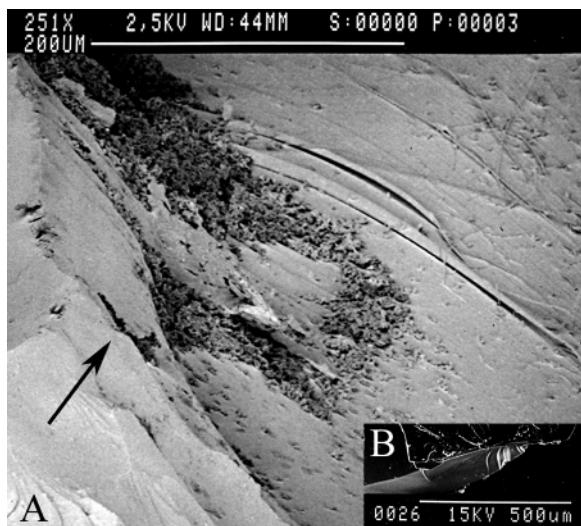
The validity of this qualitative method for detection was verified spectroscopically utilizing isotopically  $^{13}\text{C}$ -labeled phenol and GC-MS. Two strains of *P. chrysosporium* (*Pc1* and *Pc2*), and one strain each of *S. commute*, *P. ostreatus*, and *O. placentus* (fungi not positive in the first assay) were tested. Extracts made from *P. chrysosporium* (*Pc1* and *Pc2*)

grown with  $^{13}\text{C}$ -labeled polymer produced chromatograms with distinct peaks at 6.2 min (Figure S1E in the Supporting Information), with the mass spectrum of that peak having a molecular weight of 100 (Figure S1F). These data matched the peak of the  $^{13}\text{C}$ -labeled phenol standard, shared nearly identical fragmentation patterns with the  $^{13}\text{C}$ -labeled phenol standard, and were easily distinguishable from possible contaminant nonlabeled phenol (MW = 94) that would have come from the fungi or other external sources (Figure S1A and B). The controls, with pure  $^{13}\text{C}$ -labeled polymer embedded in 1.5% malt agar alone, with *P. chrysosporium* grown in 1.5% malt agar alone, or with a fungus not exhibiting a chromatic transformation in the qualitative experiment, all exhibited no peaks when run on the GC-MS (data not shown). This constituted our second line of evidence for biodegradation of phenol-formaldehyde polymer by fungi.

Further investigations for visual evidence of PR degradation using SEM and *Pc1* and *Pc2* also proved to be fruitful. Polymer chips that had been embedded in plates with *Pc1* and *Pc2* were compared to polymer chips where the hyphae had been washed away in 100% ethanol, and a control of pure PR that had been embedded in medium without fungi. The presence of hyphae growing on all the unwashed polymer chips was quite obvious, but these hyphae obscured observation of possible hyphal penetration of the polymer surface. Some hyphae still remained on polymer pieces that were washed, but the physical degradation was much more evident by areas of pockmarked surface and jagged-edged holes in the polymer (Figure 3A), in contrast to the “skating rink” smoothness of control polymer chips (Figure 3B) embedded in medium without fungi.

Since 2.2 million metric tons of phenolic resins are annually produced, recycling has become a major concern (6). The implications of this discovery of PR degradation by fungi for the PR manufacturing, recycling, and waste management industries, as well as for the construction industry, have one major repercussion: The ability of white-rot fungi to create a water-soluble byproduct from cured PR in a short period of time could be incorporated into a large-scale PR recycling process. The current process for recycling PR requires a heating stage in nitrogen at 5–6 bar (7), whereas supplementing this stage with fungal degradation might offset some energy and chemical requirements. Furthermore, the byproducts of PR degradation by *P. chrysosporium* are water soluble, which might make it possible to recover PR production constituents by washing cultures with a polar solvent, possibly water.

The phenomenon that PRs can be biodegraded also could change previous attitudes toward the preservative benefit that these compounds provide for wood-polymer composites. The biological resistance, from both fungi and termites of wood treated with phenol-formaldehyde resin was well



**FIGURE 3.** Scanning electron micrograph of the surface of a phenolic polymer chip (A) embedded in malt agar with *Phanerochaete chrysosporium* (*Pc1*) for 28 days and washed with alcohol before fixation. Jagged edged holes (A, see arrow) are present, along with smaller pock marks in the smooth polymer surface. The major area of degradation (A, center) is mottled in appearance, with the heaviest degradation occurring at the top of the micrograph. (B) Control polymer chip embedded in malt agar without fungi. Note the glasslike appearance when the chip had been broken. No degradation was seen in B. (A, bar = 200  $\mu$ m; B bar = 500  $\mu$ m).

established and tested in the 1980s and early 1990s (3–5), and has made it a major worldwide adhesive resin for exterior-grade plywood (1), oriented strandboard, medium-density fiberboard, and other engineered wood products (2). Further investigations into the effect of wood content on *P. chrysosporium*'s ability to degrade PRs, and the effects this degradation has on dimensional stability of wood-composite materials could be of interest.

The previously mentioned three separate tiers of evidence demonstrate that *P. chrysosporium* can biodegrade phenol-formaldehyde polymer. This research represents the first demonstrated fungal biodegradation of phenol-formaldehyde polymer. The white-rot fungus, *P. chrysosporium*, produced a chromatic transformation in culture with PR, isotopically labeled PR verified the pink color change was caused by the degradation of PR, and SEM visually confirmed the PR degradation. The aspects surrounding exactly how degradation is accomplished, what factors affect this process, and how this process might be utilized may be of interest to individuals in the phenolic resin, recycling, construction materials, and waste management industries.

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### Supporting Information Available

Fungal cultures used, length of culture growth before and after resin extraction, GC–MS chromatograms. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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