

Power of Biocatalysts

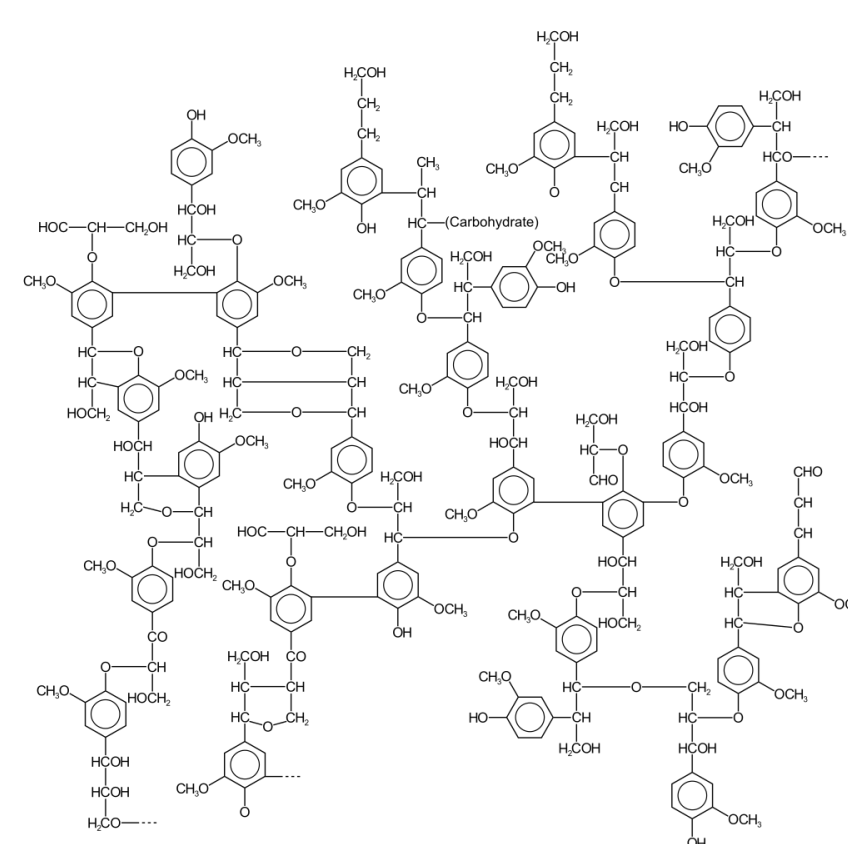


Figure 1. Idealized structure of lignin a structural polymer which composes wood.

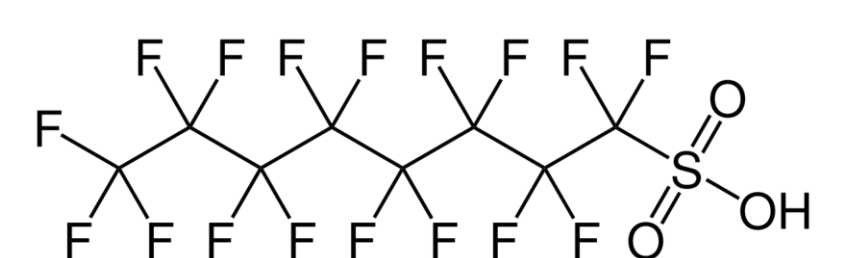


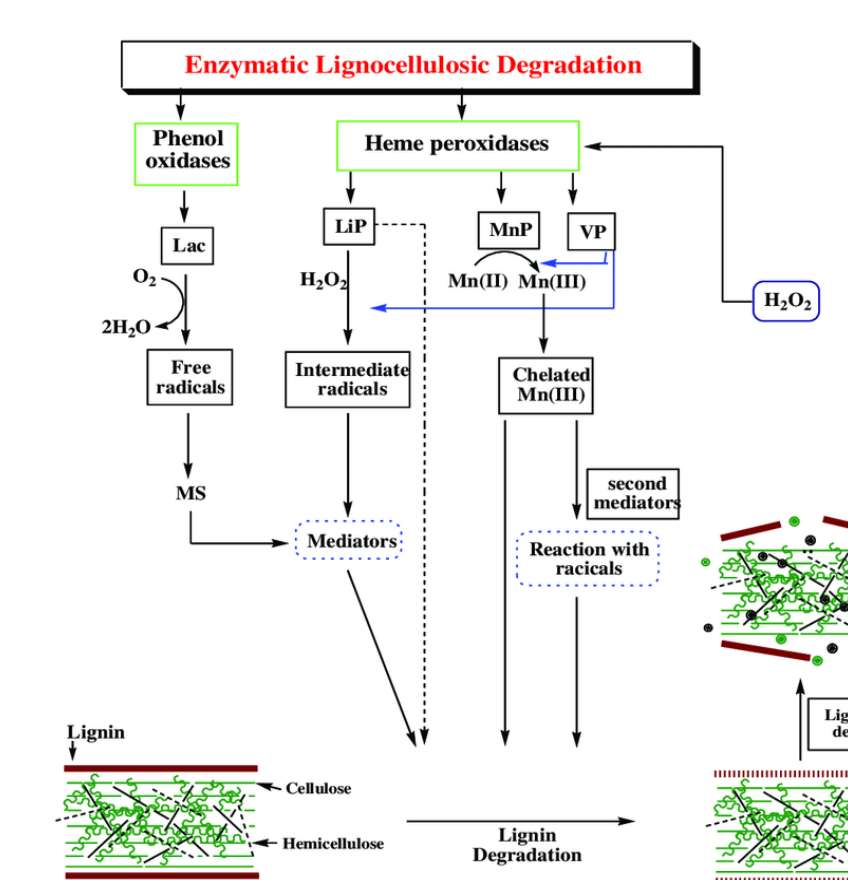
Figure 2. Perfluorooctanesulfonic acid molecule; a "forever chemical" pollutant.

Organisms have optimized through evolution, powerful tools to aid in the degradation of resilient compounds. Chitin, lignin, and cellulose are examples of natural polymers which can maintain stability through harsh conditions. Nature has also evolved counterparts, many times enzymes, to degrade them.

Synthetic polymers and "forever chemicals" such as polyethylene terephthalate (PET) and polyfluoroalkyl substances (PFAS) have been detrimental to the environment due to their longevity and resilience. Their similarity to natural polymers makes them ideal candidates for degradation by powerful natural enzymes¹.

Lignolytic Enzymes

Lignin is a crucial structural polymer comprising wood. Some organisms degrade wood for food or shelter and use lignolytic enzymes to do so.



Scheme 1. Multiple reactions in a fungal organism involved in the breakdown of lignin. This information needs to be considered when the assay for laccase is selected due to possible overlap.²

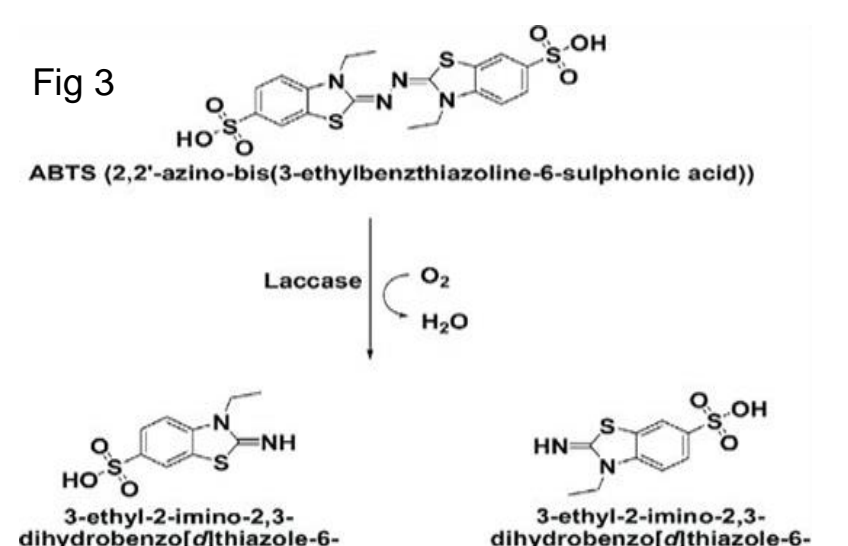
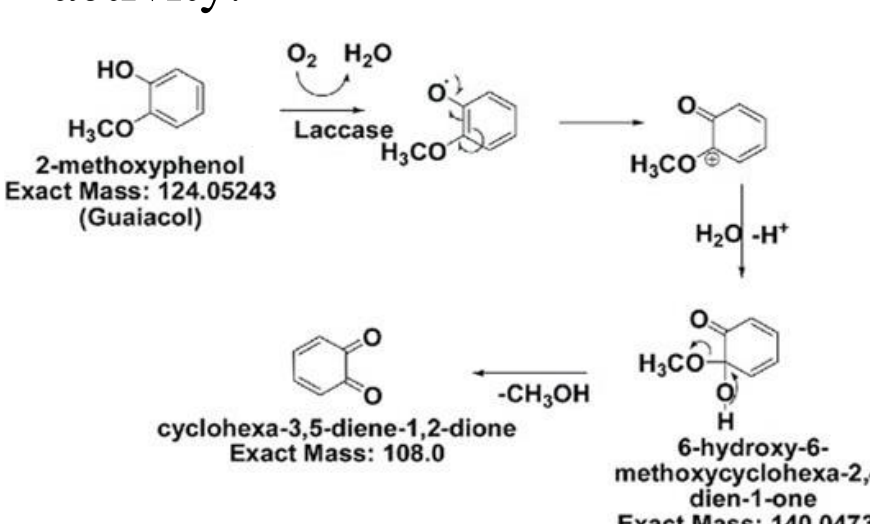


Fig 3: The mechanism for the laccase-catalyzed degradation of ABTS and guaiacol into colored compounds.³ The colored products allow for visualization of laccase activity.



Laccase

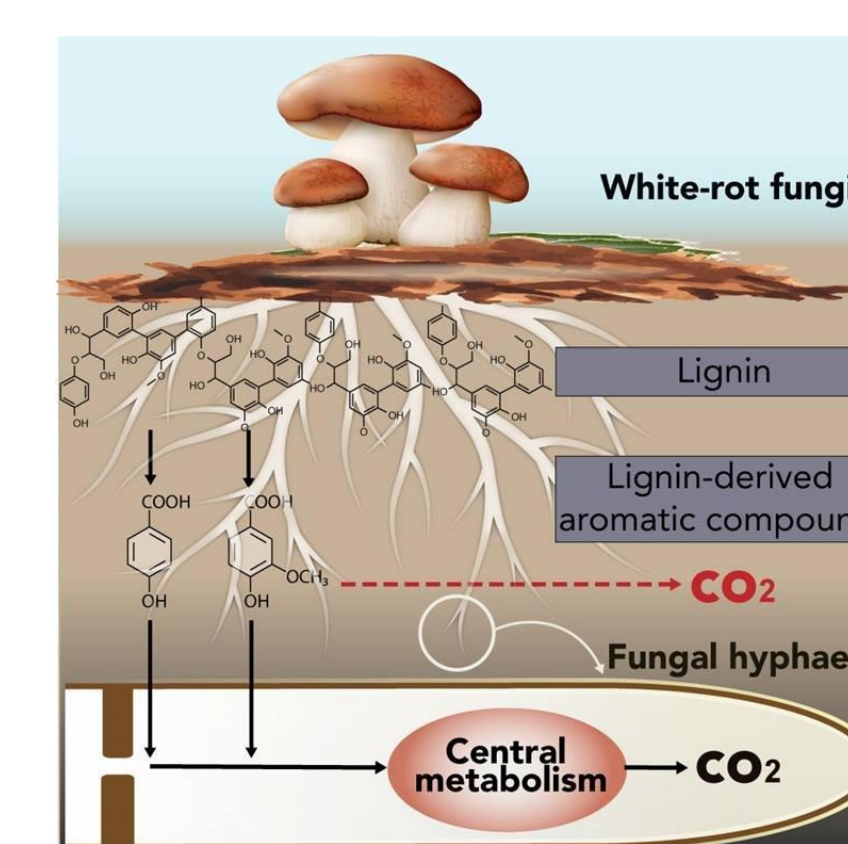


Figure 3. Laccase is a powerful enzyme used by fungi to decompose lignin into compounds which are incorporated into their metabolism.³

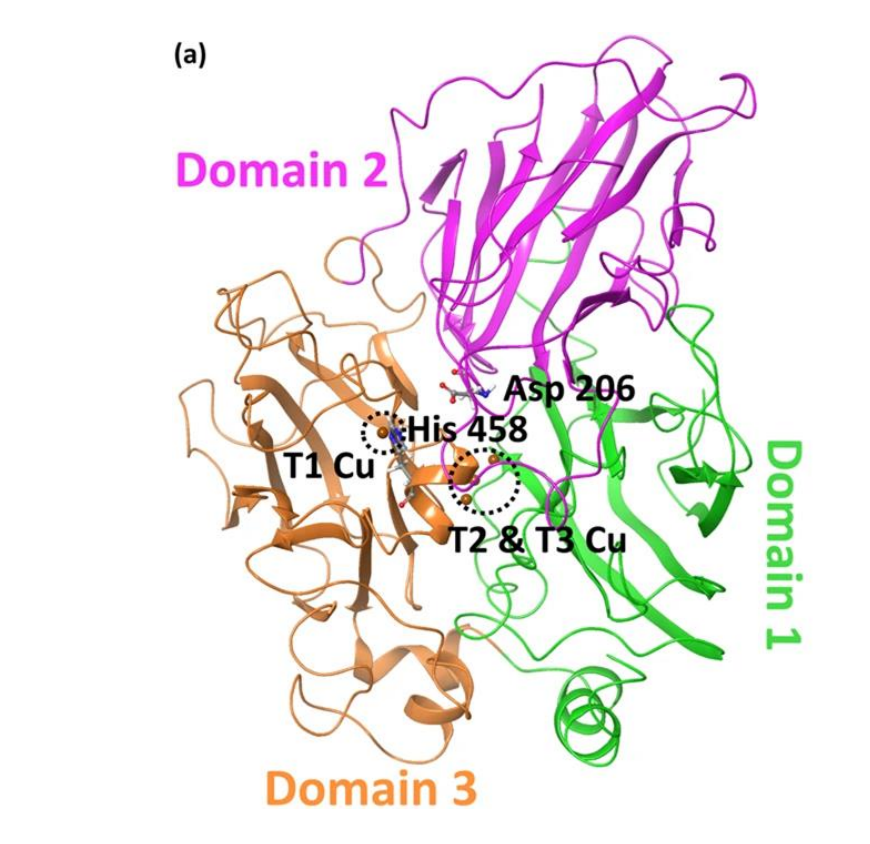


Figure 4. Crystal structure of a laccase enzyme of *Trametes versicolor* with T1, T2 and T3 copper sites^{6,2}.

Laccases are copper dependent multicopper oxidases that catalyze the oxidation of a variety of phenolic substrates. In wood-rot fungi laccase is used in the degradation of lignin from wood. The enzyme is also present in many different organisms. The enzyme converts phenol groups to phenoxy radicals which can lead to degradation of the compound or cross-linking with other compounds.

Experimental Methods

Different wood-rot fungi species were collected from a wooded property local to the Mahwah, New Jersey area. Each collected fungal species was inoculated to separate plates containing malt extract agar (MEA). In order to prevent contamination, each fungus specimen was cleaned with 4% bleach prior to plating. After incubation at 30°C for 5 to 10 days, each plate contained what appeared, under qualitative measures, to be a singular species of fungi.

To analyze the ability of the different species to oxidise phenolic compounds two qualitative assays were performed: One assay to study oxidation of the compound ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) and a second studying oxidation of guaiacol (2-methoxy phenol). Both of these are phenol containing compounds whose oxidation by Lignolytic enzymes can be observed as a color change-Green in case of ABTS and a reddish brown in the case of guaiacol. To perform the assay-we used potato dextrose agar (PDA) plates. PDA media was prepared as per instructions and autoclaved at 250°F for 30 min) with the following setup: A series of 3 plates per condition (ABTS and guaiacol) per species was set up: PDA, PDA with ABTS and PDA with guaiacol. with and without 0.15mM CuSO₄. Condition (1) contained 0.2mM ABTS, condition (2) contained 0.2mM ABTS and 0.15mM CuSO₄, condition (3) contained 4mM guaiacol, condition (4) contained 4mM guaiacol and 0.15mM CuSO₄, and condition (5) acted as a negative control with only PDA. Each plate was then inoculated with the respective fungal species. Results were gathered through visual observation of color change.

For identification purposes, the fungal species were cultured in PDA Broth. The broth was prepared per instructions and autoclaved for 30 minutes at 250°F (121°C) along with mason jars that were used as the culturing vessels. Liquid cultures facilitated DNA extraction from the fungal mycelium. The Norgen Biotech Corp Fungi/Yeast Genomic DNA Isolation Kit was used to extract DNA and the procedure was followed with some modifications. To assist in the lysing of the cells the contents of the bead tube were incubated with 200 units of Lyticase and Proteinase K, shaken at maximum speed for 10 minutes on a vortex with the Ambion Vortex Adapter attached and sonicated in a 50/50 water-ice mixture for 20 minutes. The final modification was made to help concentrate the DNA. The lysate was separated into two separate spin columns. One of the columns was eluted and the flowthrough from that column was used to elute the second spin column. PCR was run on the isolated DNA. A total of three PCR reactions were run per species. All reactions contained the same recipe: (1) RNase Free Water (13µl) (2) 5x SuperFi Buffer (3) dNTPs (0.5 µl) (4) Template DNA (3µl) (5) Polymerase SuperFi (0.25µl) (6) 10 µM Forward Primer (1.25 µl) (7) 10 µM Reverse Primer (1.25 µl). The distinction comes with what primers were used in each reaction. PCR: (1) contained previously published primers⁴ for ITS1 and ITS 4 (2) contained ITS 2 and ITS 4 (3) contained ITS 1 and ITS 3. The thermal cycle conditions used were 95°C for denaturation, 64.7°C for annealing, and 72°C for extension.

Results

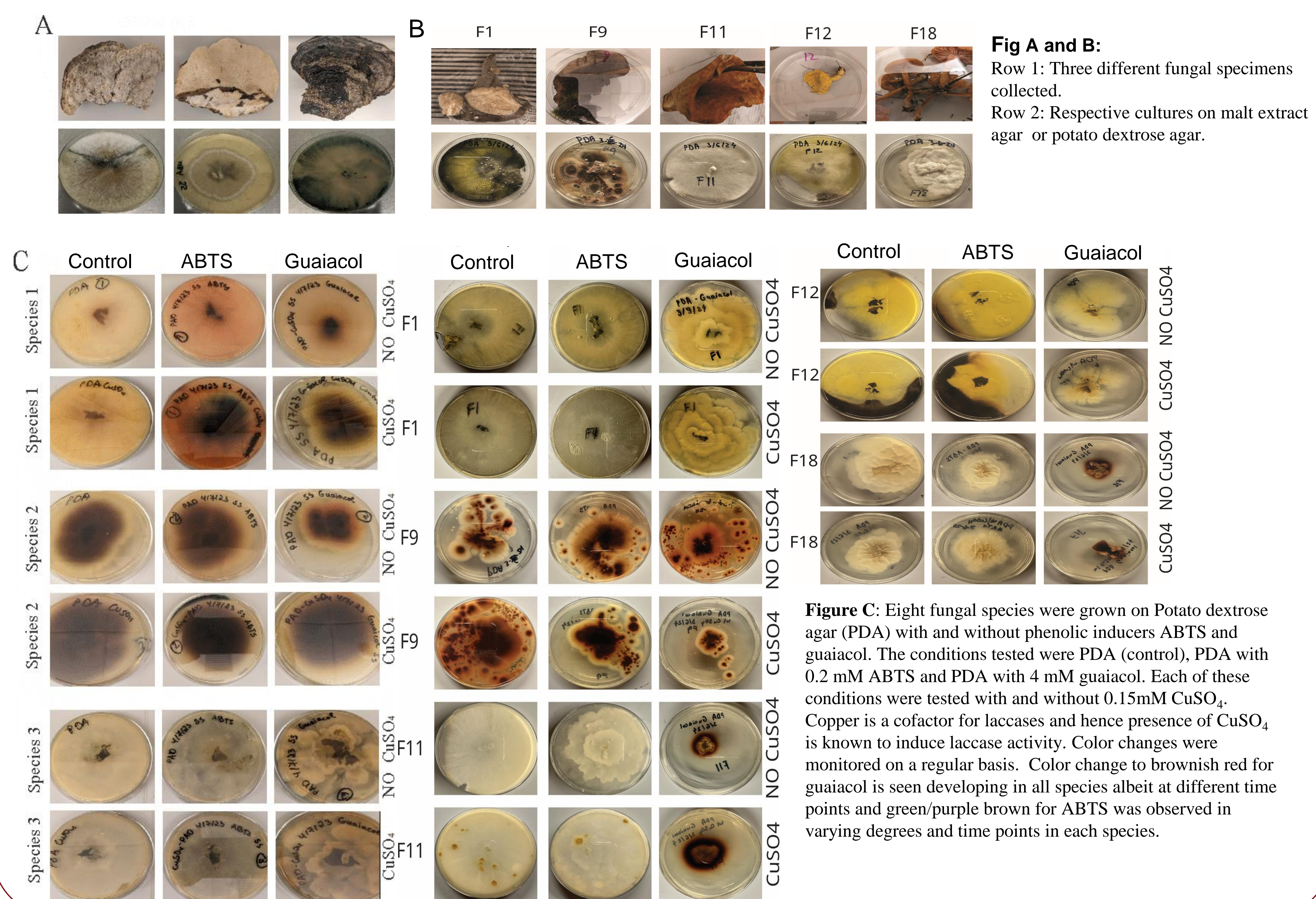
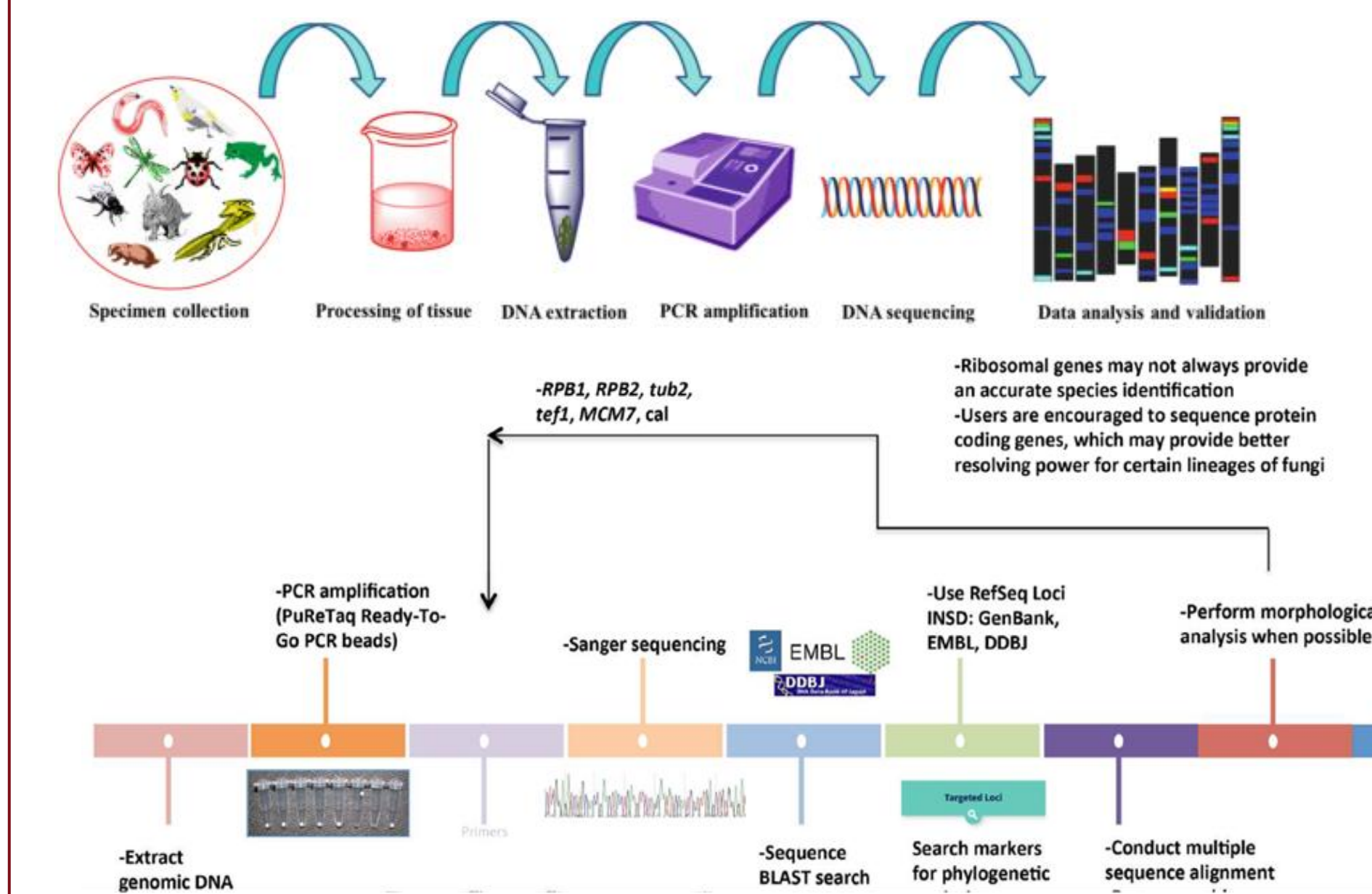


Fig A and B: Row 1: Three different fungal specimens collected. Row 2: Respective cultures on malt extract agar or potato dextrose agar.

Figure C: Eight fungal species were grown on Potato dextrose agar (PDA) with and without phenolic inducers ABTS and guaiacol. The conditions tested were PDA (control), PDA with 0.2 mM ABTS and PDA with 4 mM guaiacol. Each of these conditions were tested with and without 0.15mM CuSO₄. Copper is a cofactor for laccases and hence presence of CuSO₄ is known to induce laccase activity. Color changes were monitored on a regular basis. Color change to brownish red for guaiacol is seen developing in all species albeit at different time points and green/purple brown for ABTS was observed in varying degrees and time points in each species.

Species Identification



Identification of fungal species:

- Based on morphological features such as gills, pores, warts, caps etc⁴.
- Using DNA barcoding. 75% of fungal species can be identified by amplifying and sequencing the ITS region in the fungal genome.⁴ ITS-internal transcribed spacer is a 600 bp segment of DNA that is found flanking the 5.8s rRNA between 18s and 28s subunit genes. The region is easily amplified with universal primers for this region. The DNA sequencing information is used to identify species by using Basic Local Alignment Search Tool (BLAST) in Genbank to verify the identity of the specific species.
- Double barcoding for the remaining 25% of species that cannot be easily identified using ITS sequence. This involves determining the sequence of genes such as Tef-1 (translocation elongation factor) and calmodulin (cal)⁴.

Next Steps

- Optimize PCR and carry out Identification of fungal species using DNA Barcoding (described above)
- Determining the types and specific isoforms of lignolytic enzymes that are produced in each of these species.
- Studying mRNA and protein expression patterns in response to a variety of phenolic compounds.

References

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