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Ferricyanyide-based analysis of aqueous lignin suspension revealed sequestration of water-soluble lignin moieties

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Electronic Supplementary Information (ESI)

Protocol S1: Lignin preparation

Protocol S1: Preparation of Klason lignin from switchgrass

The acid-insoluble (Klason) lignin extract was prepared following a modification of previously described protocol. Approximately 500 ml of 72% sulfuric acid was added to 150 g of Putnam switchgrass (≥ 60 mesh) in clean glass beaker containing stir bar and incubated at 30°C (400 rpm) for 60 min. The slurry was diluted with 500 mL of DI-water and incubated for additional 60 min at 30°C (200 rpm). The volume of the suspension was adjusted to 4 L with DI-water and autoclaved at 121°C (15 p.s.i) 60 min in sealed pressurized bottle. The hydrolysate was incubated at 4°C for at least 2 h to precipitate the acid-insoluble lignin extract. The sediment was re-suspended in 3 – 6 L of DI-water and filtered through Miracloth (Millipore, Billerica, MA). The retentate was washed with 1 L of DI-water and re-suspended in 300 – 350 mL of DI-water. The acid-insoluble lignin suspension was treated with adding 100 mL of 72% sulfuric acid and incubated at 30°C (200 rpm) for 60 min. The slurry was diluted with 1 L of DI-water and autoclaved at 121°C (15 p.s.i) for 60 min. The hydrolysate was precipitated at 4°C for at least 2 h and filtered through Miracloth. The retentate was washed with DI-water until the pH of filtrate was ~7.0. The washed acid-insoluble (Klason) lignin extract was snap-froze in liquid nitrogen and lyophilized.

Protocol S2: Preparation of aqueous lignin suspensions from switchgrass and *Eucalyptus*

The Klason lignin from switchgrass and ionic-liquid derived lignin from *Eucalyptus* were washed and size fractionated to generate uniform suspension and remove soluble lignin moieties. Approximately 1.0 – 5.0 g of each lignin extract was moistened with DI-water and ground into fine paste with mortar and pestle. The ground lignin extracts were re-suspended in 50 – 100 mL of DI-water and sequentially filtered through 60, 40 and 20 µm Steri-flip nylon membrane filters (Millipore, MA). The 20 µm filtrates were filtered through a 0.2 µm Steri-flip membrane filter (Millipore, MA) to remove soluble lignin and low molecular weight moieties. The 0.2 µm retentate from each lignin extract was washed by re-suspending in DI-water and sequentially filtered through 20 and 0.2 µm membrane filters to further remove large aggregates and soluble low molecular weight moieties. The washing step was repeated until each 0.2 µm filtrate was colorless. Finally, the 0.2 µm retentate from each lignin extracts was re-suspended in DI-water and filtered through 20 µm Steri-flip filter. Each resulting uniform lignin suspension containing particles (≤ 20 µm particles) was autoclaved at 121°C (15 psi) for 30 - 35 min and incubated overnight at 30°C to induce germination of heat resistant spores and spore-forming microbes. The sterilization-incubation cycle was repeated two more times to ensure complete sterilization of the lignin suspensions.

Supplementary Figures:

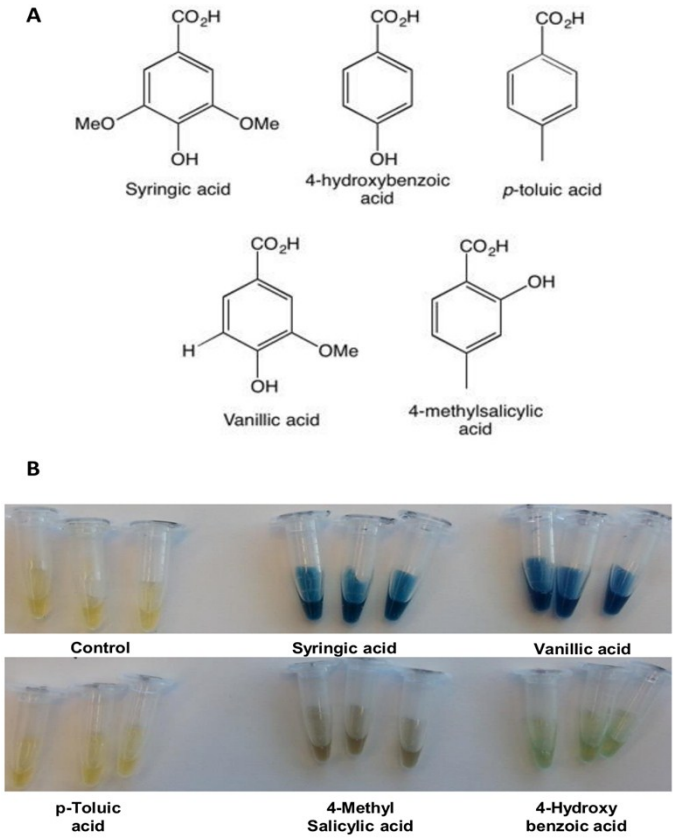

Figure S1: Phenolic and non-phenolic benzoic acid monomers. (A) Molecular structures and (B) Prussian blue production from ferricyanide reaction

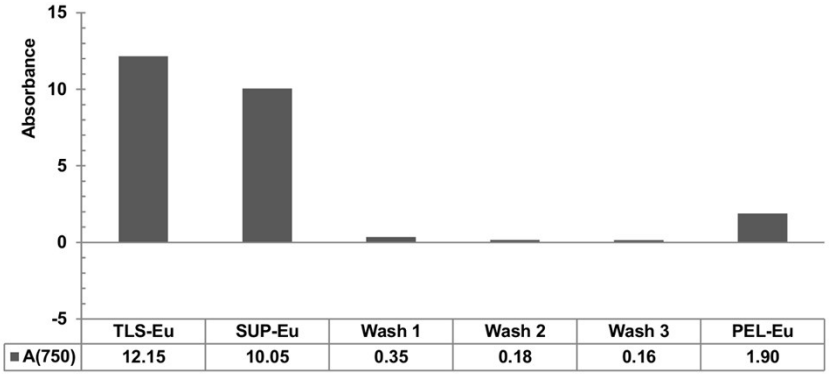

Figure S2. Ferricyanide analysis of lignin extracts from *Eucalyptus* (Eu). Ferricyanide activity of total lignin suspension (TLS), supernatant (SUP) containing soluble lignin moieties, pellet (PEL) from Eucalyptus. The supernatant was filtered through 0.45 um filter, while the pellet was washed three times with DI-water (Wash 1 – 3). The ferricyanide activity of each samples was determined at 750 nm wavelength.

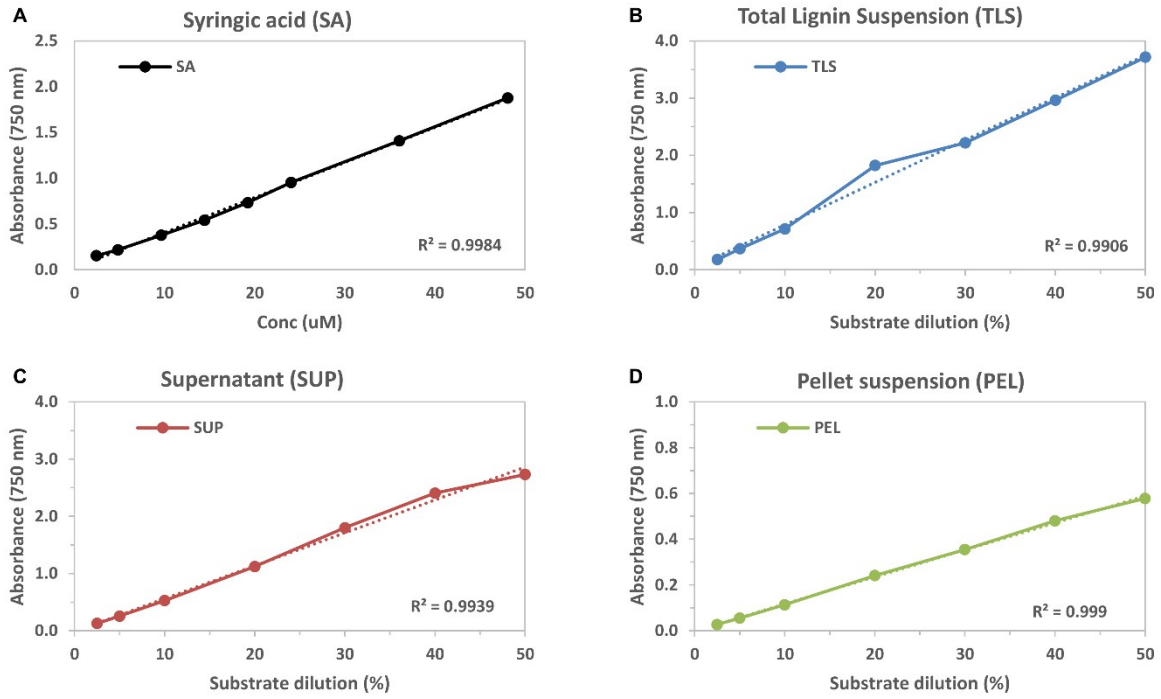

Figure S3: 96-well plate ferricyanide assay analysis of syringic acid and lignin extract from *Eucalyptus*: Plot of ferricyanide activity of different concentrations of syringic acid (A). Plot of ferricyanide activity of different dilutions of total lignin suspension (B), 0.2 µm filtered supernatant (C) and pellet (D) from *Eucalyptus* lignin extract. Dotted lines represent trend-line (R^2 values inserted). The supernatant and pellet were obtained by centrifuging the TLS at 20,000 × *g* for 30 min. The reaction was carried out in 96-well plate and the ferricyanide activity of each reaction was determined at 750 nm wavelength. The pellet was washed three times with DI-water.

Supplementary Table S1: Minimal Salt Medium (MSM)

Nutrient	**Amount (g/L)**
K_2HPO_4	1.6
KH_2PO_4	0.4
$MgSO_4 \cdot 7H_2O$	0.2
NaCl	0.1
$FeSO_4 \cdot 7H_2O$	0.003
$CaCl_2 \cdot 2H_2O$	0.025
NH_4NO_3	0.5

Dissolve salts in 1 L of DI-water (pH = 7.2 – 7.5) and filter sterilize using 0.2 µm membrane filter. Medium can also be autoclaved without the $CaCl_2 \cdot 2H_2O$.

(MSM broth was adapted from Berki and Khan, 1986)

Reference:

1. R. M. Behki and S. U. Khan, *Journal of Agricultural and Food Chemistry*, 1986, 34, 746-749.

