Assessing lignin types to screen novel biomass-degrading microbial strains: Synthetic lignin as useful carbon source.

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Materials and methods.- Oxalic acid, horseradish peroxidase, coniferyl alcohol, hydrogen peroxide, dimethyl carbonate and 2-methyltetrahydrofuran (2-MeTHF) were obtained from Sigma-Aldrich and used without further purification. Beech wood was obtained from a local supplier, the particle size was reduced with a cutting mill with a 10 mm sieve and dried at 50 °C until constant weight (ca. 24 h).

Exemplary procedure of the OrganoCat process and isolation of lignin.- In a 80 mL glass-made high pressure reactor 180.3 mg (0.1 mol L^{-1} referring to the aqueous phase) oxalic acid was dissolved in 20 mL distilled water and 20 mL 2-methyltetrahydrofuran (2-MeTHF) were added. 2000.4 mg (100 g L^{-1} referring to the aqueous phase) beech wood chips (10 mm, dried at 50 °C until constant weight) were added and the reactor was closed tight. For 3 h the reaction mixture was stirred at 140 °C. After cooling down to room temperature the reactor was opened, the cellulose enriched pulp was filtered off and the organic phase was isolated by centrifugation and decantation. 2-MeTHF was removed *via* distillation and 251.6 mg (12.6 wt% of initial biomass) solid, brown lignin powder was obtained, which was then used for subsequent processing steps. A complete characterization of the lignin has been published elsewhere.[1]

Synthetic lignin production (DHP – dehydrogenative polymers).- DHP was produced according to the *Zutropf* method.[2] Two solutions were prepared: in solution A coniferyl alcohol (0.25 – 1.00 mM) and HRP (3 mg) were dissolved in water. For solution B, a phosphate buffer (60 mL; 50 mM, pH 6.5) containing 0.019 % H_2O_2 was used. Both solutions were gradually added together to a phosphate buffer (15 mL; 50 mM, pH 6.5) over 2 h at room temperature and stirred overnight. The resulting precipitate was centrifuged (6,000 rpm, 30 min), washed with distilled water, and dried under vacuum to afford the DHP (synthetic lignin). The characterization of the resulting lignin based on NMR and ESI-MS has been published elsewhere.[3]

Catalyst-free lignin dearomatization.- 10 g L^{-1} OrganoCat lignin was dissolved in DMC and the reaction was shaken (up to 5 mL) in a Thermomixer comfort from Eppendorf (15 mL) or stirred at 80 °C. H_2O_2 was added to start the reaction. During the reaction, samples (typically 100 – 200 µL) were taken, and dried at 100 – 110 °C. Samples were analyzed by ^{1}H-NMR to determine the degree of dearomatization. The analytic characterization of the two dearomatized derivatives can be found in the literature.[1]

Isolation and identification of lignin degrading bacteria.- A mineral salts medium (MM), pH 7, composed of 2.0 g L^{-1} $(NH_4)_2SO_4$, 0.85 g L^{-1} $NaH_2PO_4 \cdot x$ 2 H_2O, 1.55 g L^{-1} K_2HPO_4 and 0.1 g L^{-1} $MgCl_2 \cdot x$ 6 H_20, was additionally supplemented with 0.1 g L^{-1} yeast extract and with a mixture of trace elements, prepared as a 1,000x stock solution containing 0.6 mg L^{-1} ferric sulfate, 0.2 mg L^{-1} zinc sulfate, 0.4 mg L^{-1} copper sulfate, 0.2 mg L^{-1} manganese sulfate, and 0.2 mg L^{-1} cobalt sulfate. To this media, a defined lignin-related substrate was added as carbon source for enrichment of lignin-degrading bacteria. The different carbon sources used for this study were (i) natural lignin obtained from hardwood; (ii) synthetic lignin; (iii) 50 % dearomatized natural lignin and (iv) 100 % dearomatized natural lignin. Solubilization of carbon sources (ii) and (iii) in minimal medium resulted in a decrease of pH to about 4. Hence, additional cultures were performed adjusting the pH to 7 with the addition of sodium hydroxide after solubilization of the carbon source and before inoculation with the soil sample.

As inoculum material, soil collected from decayed material of a fallen tree in Aachen (Germany) was used. The inoculum was prepared by suspending 10 g of soil in 100 mL of sterile 0.9 % (w/v) NaCl. After incubation for 30 min at 30 °C with shaking at 200 rpm the mixture was settled down for another 30 min and 1 mL aliquots of the supernatant were used to inoculate four 250 mL Erlenmeyer flasks containing 50 mL of MM. The cultures were grown at 30 °C in a shaking incubator and after 48 h, 1 mL aliquots were transferred to fresh

MM. In total, five successive transfers were performed after which 100 µL of the cultures was streaked onto Luria Broth (LB) agar to obtain pure cultures.

From the pure cultures, total DNA was isolated using the QIAamp DNA Stool Mini Kit (Qiagen). Partial 16S rRNA gene sequences were amplified by polymerase chain reaction (PCR) using primers Fwd16S: AGAGTTTGATCMTGGCTCAG and RP1/2: ACGGYTACCTTGTTACGACTT,[4] using Phusion High-Fidelity DNA Polymerase (Finnzymes). The resulting PCR products were sequenced by GATC Biotech and a Basic Local Alignment Search Tool (BLAST) analysis was performed using these sequences as query to determine the identity of the bacterial isolates.

The bacteria isolated from the enrichment cultures (*Ralstonia* sp. LD1, *Pseudomonas* sp. LD2, *Enterobacter* sp. LD3, *Shigella* sp. LD4, *Streptonomonas* sp. LD5, *Propionibacterium* sp LD6, *Exiguobacterium* sp. LD7 and *Delftia* sp. LD8) were routinely cultured in Luria broth (LB) containing 5 g/L NaCl, 10 g L^{-1} tryptone and 5 g L^{-1} yeast extract at pH 7. To monitor growth of the isolates on lignin substrates, minimal medium was supplemented with either the natural, synthetic, 50 % dearomatized or 100 % dearomatized lignin to a final concentration of 5 g L^{-1}. The cultures were incubated at 30 °C with shaking and samples were drawn at regular intervals to monitor growth. In case of natural and synthetic lignin, the employed substrate concentrations prevented accurate optical density measurements. Therefore, growth was monitored by colony-forming unit (CFU) counts. For CFU-counts, the cultures were serially diluted in 0.9 % (w/v) NaCl and plated on LB agar plates. CFU determinations were performed daily until no further CFU increase occurred. In the case of pure cultures supplemented with 50 % or 100 % dearomatized lignin, the $OD_{600\ nm}$ was recorded daily.

Laccase, peroxidase and β-etherase activity measurements.- Laccase-catalyzed oxidation of 0.5 mM 2,2´-azinobis(3-ethyl-benzothiazoline-6-sulfoniate) (ABTS) to the corresponding radical cation ($\varepsilon_{420} = 3.6 \times 10^4\ M^{-1}cm^{-1}$) in 100 mM sodium acetate buffer at

pH 4.5 was monitored by the increase in absorbance at 420 nm.[5] Lignin peroxidase-catalyzed oxidation of 32 μM Azure B (ε_{651} = 4.88 × 10^4 $M^{-1}cm^{-1}$) was monitored by the reduction in absorbance at 651 nm in 50 mM sodium tartrate buffer at pH 4.5 containing 0.1 mM H_2O_2.[6] MnP activity was generally determined by oxidation of 2,6-dimethoxyphenol (DMP) to coerulignone (3,3′,5,5′-tetramethoxydiphenoquinone) at a wavelength of 469 nm (ε_{469} = 4.96 × 10^4 $M^{-1}cm^{-1}$).[7] β-Etherase activity was measured fluorometrically with the substrate MUAV (α-*O*-(β-methylumbelliferyl)-acetovanillone) in a plate reader using an excitation wavelength of λ = 360 nm and an emission wavelength of λ = 450 nm as described previously.[8]

Dye decolorization assays.- Decolorization of lignin-mimicking dyes Azure B and methylene blue was assessed in solid-phase assays. Bacteria were streaked onto dye-containing agar plates with various media. Either LB or MM was used, the latter one supplemented with citrate (20 mM) as a carbon source and yeast extract (0.5 g L^{-1}). Dyes were added at a final concentration of 25 mg L^{-1}. The plates were monitored daily over a period of 120 h for growth and the development of decolorization zones.

References

1.- Wiermans, L.; Schumacher, H.; Klaassen, C.M.; Domínguez de María, P. Unprecedented catalyst-free lignin dearomatization with hydrogen peroxide and dimethyl carbonate. *RSC Adv.*, **2015**, *5*, 4009-4018.

2.- Nakagame, S.; Chandra, R.P.; Nadla, J.F.; Saddler, J.N. Enhancing the enzymatic hydrolysis of lignocellulosic biomass by increasing the carboxylic acid content of the associated lignin. *Biotechnol. Bioeng.* **2010**, *108*, 538-548.

3.- Wiermans, L.; Pérez-Sánchez, M.; Domínguez de María, P. Lipase-mediated oxidative delignification in non-aqueous media: Formation of de-aromatized lignin-oil and cellulase-accessible polysaccharides. *ChemSusChem*, **2013**, *6*, 251-255.

4.- Weisberg, W.G.; Barns, S.M.; Pelletier, D.A.; Lane, D.J. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.*, **1991**, *173*, 697-703.

5.- Soden, D.M.; O'Callaghan, J.; Dobson, A.D.W. Molecular cloning of a laccase isozyme gene from Pleurotus sajor-cadu and expression in the heterologous *Pichia pastoris* host. *Microbiology*, **2002**, *148*, 4003–4014.

6.- Archibald, F.S. A new assay for lignin-type peroxidases employing the dye azure B. *Appl. Environ. Microbiol.*, **1992**, *58*, 3110–3116.

7.- de la Rubia, T.; Linares, A.; Pérez, J.; Muñoz-Dorado, J.; Romera, J.; Martínez, J. Characterization of manganese-dependent peroxidase isoenzymes from the ligninolytic fungus Phanerochaete flavido-alba. *Res. Microbiol.*, **2002**, *153*, 547–554.

8.- Picart, P.; Müller, C.R.; Mottweiler, J.; Wiermans, L.; Bolm, C.; Domínguez de María, P.; Schallmey, A. From gene towards selective biomass valorization: Novel bacterial beta-etherases with activity on lignin-like polymers. *ChemSusChem*, **2014**, *7*, 3164-3171.