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Accumulation of lignin-breakdown products in several lignolytic and non-lignolytic bacteria and fungi

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Abstract: Valorization of lignin has the potential to significantly improve the economics of lignocellulosic biorefineries. However, efficient conversion of lignin to useful products has been elusive. One promising approach is depolymerization of lignin and subsequent biological conversion of the breakdown products. Microbial conversion of lignin in nature is efficient, but occurs very slowly. Engineering microbes to produce efficient and directed lignin conversion is a promising strategy (Linger et al., 2014). It is becoming increasingly clear that, in addition to metabolic engineering, engineering transport will be an important aspect of that engineering effort. Little is currently known about transport of lignin breakdown products into microbes. Indirect evidence such as growth and toxicity studies suggests lignolytic organisms may transport a wide range of mono-, di-, and possibly even higher molecular weight lignin breakdown products across the cellular membrane (Chaudhry et al., 2007; D'Argenio et al., 1999; Harwood et al., 1994; Jokela et al., 1987), but direct measurements of the substrate range and specificity are lacking (Nichols and Harwood, 1997). To that end, we developed mass spec and single cell imaging methods to quantify accumulation of lignin-like compounds in microbes. We applied these to characterize internalization of four mono-aryl compounds and a di-aryl compounds by the lignolytic organisms *P. chrysosporium and E. lignolyticus* (SCF1) and by the nonlignolytic organisms *S. cerevisiae* and *E-coli*. In some cases, consumption of compounds was followed through depletion of the substrates from the media using HPLC. Here we report native uptake results for several mono- and di-aryl compounds in lignolytic and non-lignolytic bacterial and fungal hosts.

Compounds tested to date:

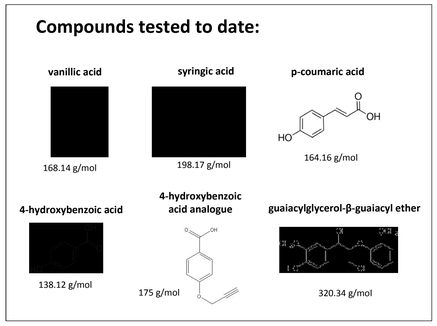

Fig. 1: A. Diagram of mono and di-lignol compounds tested in these native transporter studies. B. Organism and strain information for native hosts as well as the growth conditions for each organism.

Organism and Growth Conditions:

***P. chrysosporium* (IFO 31249, ATCC 34541)**: Grown at T=37 °C in Kirk's mineral salts and trace elements, 2 mg/ml microcrystalline cellulose, 20 mM ammonium tartrate. The uptake trials were initiated on day 5 after innoculation. Fungal media was replaced with media or PBS containing 4 mM of target compound. After a 4 or 72 hr incubation period, the contents of culture were collected, the pellet was washed 2 times with PBS, then vortexed for 2 hours with 70% EtOH and lysing matrix C, and filtered through 3KDa cutoff filter. Filtrate was analyzed by LC-MS.

***S. cerevisiae* (BY4741, ATCC 201388)**: The trial was initiated after cultures reached an OD of 1.0, grown at 30 °C. 4mM compound stock solution in the media (YPD) were added to each culture. Cells were grown for 4 hours then harvested, washed, lysed, filtered, and analyszed by MS protocols.

E. lignolyticus SCF1 and E. coli (MG1655): Cells were grown to mid log phase in minimal M9 media (*E-coli*) or rich media (*E. ligno*) and then compound stock solution in the media was added to 4 mM and allowed 4 hours for uptake. Harvesting, lysing, filtration, and MS protocols were the same.

Results:

Figure 1: Internalization of Compounds by LC-MS on Bacterial and Fungal Lysates

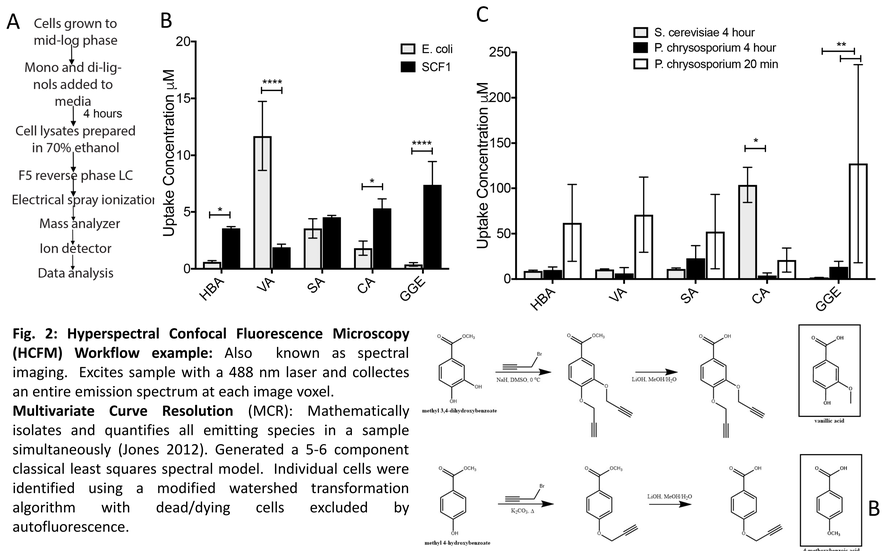

Fig. 2: Hyperspectral Confocal Fluorescence Microscopy (HCFM) Workflow example: Also known as spectral imaging. Excites sample with a 488 nm laser and collectes an entire emission spectrum at each image voxel.

Multivariate Curve Resolution (MCR): Mathematically isolates and quantifies all emitting species in a sample simultaneously (Jones 2012). Generated a 5-6 component classical least squares spectral model. Individual cells were identified using a modified watershed transformation algorithm with dead/dying cells excluded by autofluorescence.

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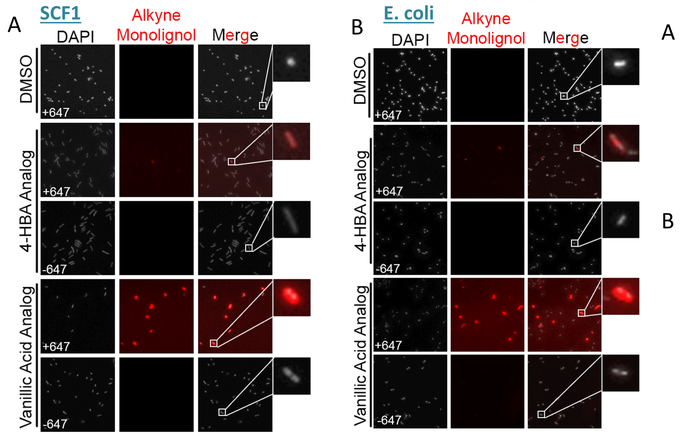
Figure 2: Internalization of Compounds and Single Cell Analysis in Bacterial Hosts by Fluorescence Imaging



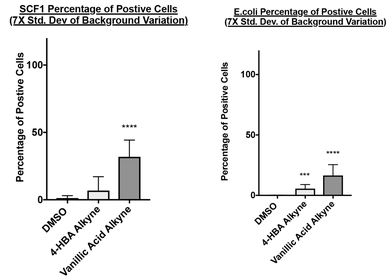
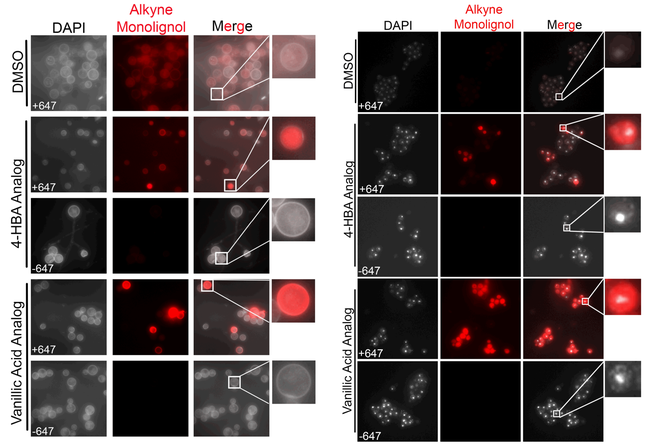


Fig. 3: A. Schematic representing experimental layout. R61 cells were exposed to high and low white light conditions for 1-5 weeks with samples taken and analyzed every 2 weeks. B. Image of cultures after 6 weeks growth (with regular passage) following continual high and low light exposure.

Figure 3: Internalization of Compounds and Single Cell Analysis in Fungal Hosts by Fluorescence Imaging



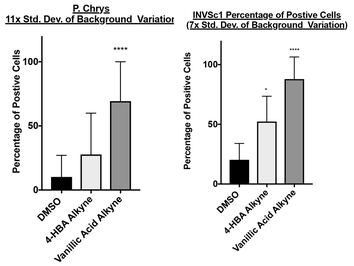
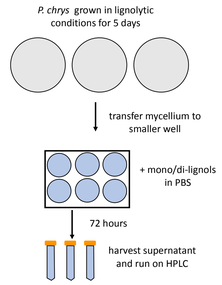


Fig. 5: A. Representative HCFM images of *Acaryochloris* sp. R61 under high and low white light exposure over a time course of 5 weeks. Individual spectral components can be separated out and analyzed for localization and intensity/concentration. B. Single cell concentrations of each of the main spectral components at 5 week timepoint.

Figure 4: Consumption of Compounds by 72 hours in *P. chrysosporium*



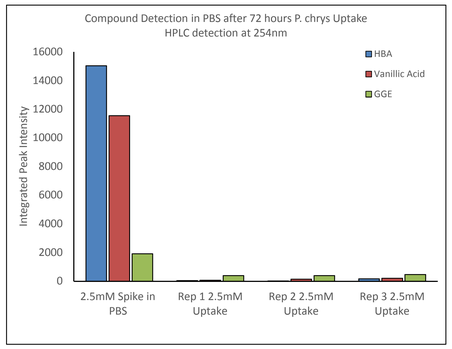
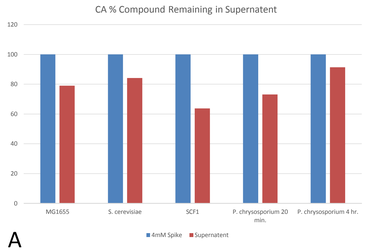


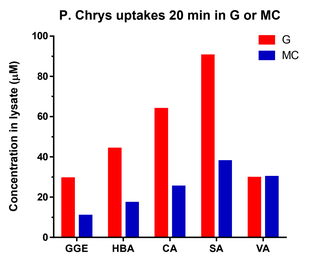
Fig. 6: A. Graphs of spectral component concentration ratios at 5 week timepoint. B. Representative images of R61 cells exposed to high and low white light conditions for 1, 3, and 5 weeks. Cells were imaged using hyperspectral microscope and analyzed using MCR workflow represented in Fig. 2. The overlaid images highlight changes in localization pattern that aren't necessarily reflected as whole cell concentration changes. Single cell concentration and localization analysis allows for total picture of spectral behavior in cells.



A

Same graph as to the left but for GGE results

Fig. 7: A. Representative HCFM images reveal localization changes between Chlorophyll A and Chlorophyll B in low light conditioned cells at 5 week timepoint. Several examples of polar localization patterning can be observed between the two types of chlorophyll that aren't necessarily reflected in the whole cell concentration of each pigment (B.).



B

Conclusions

- Pigments from *Acaryochloris sp.* R61 cultures grown under high and low white light conditions can be separated spectrally using MCR.
- Investigated spatial-temporal response of *individual* pigments to light condition changes (PC&APC, ChlA, and ChlB). ChlB expression was previously unknown.
  - Identified decrease in phycobilisome and ChlA proteins over timed exposure to high white light.
  - Observed localization changes in ChlA and ChlB in response to high light conditions over time.
  - Observed localization changes between ChlA and ChlB in low light at 5 weeks.

References

Jones, et al. (2012) Chem. & Intel. Lab. Systems. 117:149-158. Ruckebusch, et al. (2013) Analytica Chimica Acta, 765(2013)28-36