ISEN Final Report

Fungal Macromolecular Products as Potential Novel Biofuel Source

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Introduction

Fungi are key agents in the carbon cycle because of their collective abilities to efficiently decompose the three most globally abundant biopolymers, lignin, cellulose and chitin (Baldrian et al. 2011). While it is clear that a significant global flux of C flows through fungal-mediated pathways, surprisingly the organic geochemical record of the flow is not well recognized. Most analyses of fungi have focused on extractable components such as lipids, pigments and some proteins, as well as the standard hydrolysable protein and carbohydrate pools. Bound fractions of lipids that are released only by base- or acid-hydrolysis are rarely studied in fungi and systematic searches for non-extractable hydrolysis-resistant components have yet to be undertaken. Analyses of extractable and bound lipids in sediments have indicated different sources for each, thus illustrating the need to consider both fractions when interpreting organic geochemical records (Wakeham 1999). Hydrolysis-resistant macromolecules, once they are sequestered from oxic conditions, can be important contributors to sedimentary organic geochemical records (Dereene and Largeau 2001).

Accordingly, one objective of the ISEN project was to determine the presence of a bound lipid fraction in soil fungi isolates and begin its characterization. This preliminary information would then be used as a basis for proposals to external funding agencies.

The efficient conversion of lignocellulosic crop waste and/or chitin-rich debris from the shellfish industry to a liquid hydrocarbon fuel could partially supplant the use of fossil fuels, especially in local situations near the production of the waste stream. In the case of shellfish processing plants, crab and shrimp shells must be disposed of in landfills thus the conversion to fuel would serve a double benefit. The ability of fungi to depolymerize lignin, cellulose and chitin and produce fatty acids makes them a potential target for biodiesel production. Some fungi have been found to produce biomass that can be as high a 50-60% lipid for some species and are frequently easy to culture and archive (they produce resilient spores; Feofilova et al. 2010). The second objective of this project thus was to evaluate the extractable fatty acid component of soil fungal isolates in an effort to identify potential target species for biodiesel synthesis.
Results

Bound lipids

We subjected a soil fungal isolate, *Rhizopus stolonifer*, to an extraction-hydrolysis sequence to determine the presence of bound fatty acids. The fungi biomass was treated with multiple organic solvent extractions that varied in polarity (hexane, chloroform, 2:1 dichloromethane:methanol) and hydrolysis steps using increasing concentrations (2 M, 4 M, 6 M) of trifluoroacetic acid (TFA). Our analyses indicate that a substantial fatty acid fraction is not extractable until after the TFA hydrolysis steps that remove most carbohydrate and many protein components (Table 1). There is also a fatty acid component that is not extractable from the TFA residue but is released by transmethylation with tetramethyl ammonium hydroxide at 300°C. The residue is primarily chitin-chitosan as determined by FTIR and pyr-GCMS.

Chitin-protected fatty acids are likely to be a ubiquitous contributor to the soil bound lipid pool because all known fungi produce chitin (Feofilova 2010) and fungi are the dominant source of that biopolymer in soils (Joergensen and Wichern 2008). The chitin-fatty acid pairing is especially intriguing because studies of arthropod fossilization suggest that the chitin exoskeleton and/or its diagenetic product may play a role in the selective preservation of long-chain alkyl moieties by protecting fatty acids until they can be cross-linked and integrated into the developing geopolymer (Cody et al. 2011). Lipids bound within biopolymers such as chitin are a small component of the total lipid pool within an organism. However, if these bound fractions turn over more slowly within sediments because the macromolecular matrix impedes degradation, they become important over time as a part of the diagenetic process.

This discovery may have significant implications for the formation of petroleum. The source of the long-chained hydrocarbon functionality of the petroleum precursor material in sedimentary rocks is a long-standing mystery in the field of organic geochemistry. Preferential preservation of lipids relative to other biochemicals is the current explanation in the literature but the proposed mechanisms are unpersuasive in some cases and not well supported with data. The ability of ubiquitous soil fungi to convert a portion of abundant biomaterials such as lignin and cellulose to an insoluble hydrolysis-resistant chitin-fatty acid complex may be the missing mechanism for the sequestration of hydrocarbon functionality. The recognition of the novel and potential importance of this process led to funding from the Petroleum Research Foundation administered by the American Chemical Society. The ACS-PRF project supports a broad survey of fungal bound lipids as well as an assessment of the environmental stability of those compounds.

Table 1: *Rhizopus stolonifer* fatty acids (Wilson et al., in prep).

|   | 12:0 | 13:0 | 14:0 | 15:0 | 16:0 | 16:1 | 16:2 | 17:0 | 18:0 | 18:1 | 18:2 | 18:3 | 20:0 | 20:1 | 22:0 | 23:0 | 24:0 | 24:1 | 25:0 | 26:0 |
|---|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|
| extractable | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x |
| bulk bound | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x |
| chitin bound | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x |


Extractable lipids

Over sixty distinct soil fungal isolates were assayed for fatty acid ester content. DNA analyses are currently underway to identify the fungi with special attention to the lipid-rich species. The genera Rhodotorula, Fusarium, Aspergillus and Rhizobium have been identified thus far. Fatty acid ester contents ranged from 0.3 to 14% (Fig. 1). Lipid contents of the Rhodotorula and Fusarium isolates increased to >25% upon manipulation of the C/N ratio of the culture media. We anticipate future work will focus on optimizing fatty acid production from lignocellulose waste using the appropriate fungi. We will be targeting NSF and DOE funding opportunities.

Project participants

This project is a collaborative study with Dr. Louise Egerton-Warburton of the Chicago Botanical Garden. It has provided partial support for a post-doctoral associate, Dr. Thea Wilson., who isolated the chitin-fatty acid complex. Kenny Fournillier, a Ph.D. student in Environmental Engineering, was also supported and provided measurements of the bound fatty acids that will be a part of his dissertation. Ilana Golbin with the assistance of Dr. Wilson developed a rapid, small sample FTIR assay for lipids in fungal biomass and performed the lipid survey of over 60 isolates. This work was the focus of Ms. Golbin’s senior thesis in Environmental Science and contributed to her receiving honors at graduation. William Levinson, an undergraduate at Lake Forest College and intern at the Chicago Botanical Garden, assisted with the isolation and culturing of the fungal species, and performed the C/N manipulation experiments under the guidance of Dr. Egerton-Warburton. This research will contribute to his senior thesis. Elizabeth Piotrowski Conger, a sophomore in Environmental Engineering, joined the group this summer and is developing a method to characterize the individual fatty acids.
Literature cited


Submitted proposals resulting from this ISEN project

Collaborative Research: Survey and Characterization of Fungal Bound Lipids from an Organic Geochemical Perspective, Blair and Egerton-Warburton, NSF, requested amount $393,979, not funded.

A Potential Fungal Contribution to the Selective Preservation of Long-chain Hydrocarbon Functionality in Soils and Sediments, Blair (w/Egerton-Warburton as collaborator), ACS-PRF, $100,000, 9/1/12-8/31/14, funded.

Conversion of Lignocellulose and Chitin to Biodiesel via a Novel Agent, the Labyrinthulomycetes, Blair and J. Collins (SUNY- Stony Brook), Concept paper to ARPA-DOE, not selected for final submission.