"Development of an Integrated EMSL MS and NMR Metabolic Flux Analysis Capability In Support of Systems Biology: Test Application for Biofuels Production"

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Science Goal and Impact of MFA on Biosystem Dynamics and Design.
An important element in the production of biofuels is the metabolic engineering process required to maximize the production of renewable biofuel molecules from fermentable substrates. Product yield is a major determinant of final price; any improvement in this area translates into immediate economic benefits. Biofuel production rates are, in simplest terms, the flux through the particular reaction creating the desired metabolite product. However, understanding the level of production of a given metabolite requires understanding the integrated network response encompassing the entire genome of the host. Metabolic fluxes cannot be measured directly, and so must be inferred from other measurements; much work has been done in the area of quantifying metabolic fluxes using stable isotope labeling and either nuclear magnetic resonance (NMR) or mass spectrometry (MS) detection methods. Metabolic flux analysis (MFA) then employs various formal mathematical approaches including stationary or quasi-steady state approaches (1-13) including cumulative isotopomer (cumomer) (8-11) and elementary metabolite unit (EMU) analyses (5, 8, 12), as well as non-stationary or dynamic approaches (13-23).

EMSL/PNNL presently lacks MFA capabilities commensurate with its stature as a world-class research facility. We do possess state-of-the art instrumentation in both Mass Spectrometry (MS) and Nuclear Magnetic Resonance spectroscopy (NMR), and over the past several years have been active in MS and NMR metabolomics data collection and analysis. However, our approach to MFA thus far has consisted of data collection only; we then send the collected data to other entities for analysis. Developing a resident MFA capability at EMSL integrated with our NMR and MS capabilities will be vital to pursue future collaborative research in systems biology and metabolic engineering. It will allow us to provide a complete package of rational experiment design, NMR and MS data collection and analysis. Further, development of MFA at EMSL will enhance our established competencies in metabolomics, transcriptomics, proteomics and genomics. Establishing an EMSL base of MFA software and expertise that integrates into an overall complete systems biology platform will be crucial to fulfilling the Biosystem Dynamics and Design research goals of EMSL, to “understand intra- and intercellular complexes and dynamic processes in microbes, fungi and plant roots to advance systems biology for bioenergy and bio-renewables,” and will enable us to partner more effectively with new and existing EMSL users to advance research in systems biology relevant to the goals of our primary client, DOE-BER.

We propose establishment of a new EMSL MFA capability in the context of a microbial biofuel production system. Implementation will be accomplished by working in partnership with Wolfgang Weichert and Katharina Noeh, the developers of the 13CFLUX2 metabolic flux analysis software suite (8). We plan for this project to utilize 13CFLUX2 to implement stationary (quasi-steady-state) approaches that are best suited to bacterial cultures in defined media. This work will set the stage for future development and implementation of EMSL fluxomics non-stationary or dynamic approaches that would allow us to explore flux in eukaryotic organisms such as fungi, using: 1) simplification protocols such as pooling of metabolites in compartments, assuming rapid equilibrium based on continuous action of transporters, or 2) dynamic approaches, which, as there is no publicly available software at this point, requires tracer experiments to test whether a given pathway is active. (13, 20)

Research Approach for Main Objectives and Scope of Work.
Our plan is to obtain the current release of 13CFLUX2 and develop resident expertise in experiment design, data collection, data processing, and complete flux balance analysis using 13C isotopomer data. 13CFLUX2 is the only major software package that is currently capable of handling both NMR and MS data. Drs. Wiechert and Noeh have kindly offered to provide a five year academic license (no cost to PNNL), if we will travel to Germany to take a training class; they have further offered to waive the tuition for this training course for two persons, one representing bioinformatics, and one representing

either NMR or MS. Once we have installed and tested 13CFLUX2, we will work with Dr. Hector Garcia-Martin of the Joint Bioenergy Institute (JBEI, current Science Theme Proposal, EUP47558,) to apply our new capability to model-guided biofuel production enhancement of metabolically engineered *E. coli*. This offers us two advantages: 1) Dr. Garcia-Martin will acquire 13C isotopomer data at EMSL as part of his ongoing project, meaning we can utilize already-planned datastreams; and 2) Dr. Garcia Martin will independently employ Two-Scale 13C Metabolic Flux analysis (TS 13C MFA) that was developed at JBEI Quantitative Modeling Directorate, giving us a valuable check on our newly established EMSL fluxomics methodologies.

While TS 13C MFA is a promising and novel approach, its algorithm is currently unpublished, and it is not available to support the EMSL general user community. 13CFLUX2 has been in production use for several years, with development of the original version dating back to 2001, and is a world leader in this area. Its algorithm has been published (8), and we have the strong support of its developers, who recently visited EMSL (June 2014). Thus, we believe that installation of the 13CFLUX2 package is the best way at present for EMSL to move into this field of analysis. We will compare our flux reports with TS 13C MFA results for Dr. Martin's mass spec data, which should support better and easier calibration of 13CFLUX2 (and vice versa) on our EMSL instruments. Also, we will extend the data analysis to new NMR samples, funded in this proposal, so that 13CFLUX2 will be tested on both mass spec and NMR samples. This will support integration of NMR and MS resources at EMSL, and will also benefit Dr. Garcia-Martin, who will be provided with additional flux profiles from NMR experiments to further the research in his current and future EMSL User Proposals.

Science Driver: In phase 1 of his current EUP, Dr. Garcia-Martin will be testing a variety of flux balance analysis methods, each of which uses different ‘omics data sets to constrain the metabolic models. He will do this to find the most accurate predictive method to use in making the genetic manipulations in phase 2, wherein he alters gene expression of genes encoding global regulators (such as arcA) or alternate routes for carbon excretion (poxB) to see the effect on flux profiles and isopentanol (gasoline molecule) production. The output of phase 2 will be knowledge that can be translated into quantitative models that will indicate new approaches to increase yield. In phase 1, the various modeling methods will be compared against the flux measurements from TS 13C MFA, such measurements being a "golden standard" .We will be able to provide a second means of comparison, using 13CFLUX2. As Dr. Garcia-Martin stated in his proposal, the work in Phase 1 is itself very important - it will provide guidance as to the fastest and easiest method to use to build accurate metabolic models. Our work here will aid in this effort, as well as assisting Dr. Garcia-Martin in phase 2 by providing another validation of flux predictions at selected data points for the prediction method selected for use in phase 2.

Most relevant to the current application (Selected from over 35 peer-reviewed publications)

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