IB Interview

A Conversation with Blake Simmons, Vice President, Deconstruction Division, and Jon Magnuson, Director, Fungal Biotechnology Group, Joint BioEnergy Institute, Emeryville, CA

Blake A. Simmons[1] and Jon Magnuson[2]

[1]Deconstruction Division, Joint BioEnergy Institute, Emeryville, CA; Biofuels and Biomaterials Science and Technology, Sandia National Laboratories, Livermore, CA
[2]Fungal Biotechnology Group, Joint BioEnergy Institute, Emeryville, CA; Chemical and Biological Processes Development Group, Pacific Northwest National Laboratory, Richland, WA

INDUSTRIAL BIOTECHNOLOGY: *The Fungal Biotechnology Group is a relatively new addition to the Deconstruction Division of the Department of Energy's (DOE's) Joint BioEnergy Institute (JBEI).*

BLAKE SIMMONS: At JBEI, we are focused on developing the science and technology around new ways to convert biomass into advanced drop-in fuels. We have teams working on all major elements of the biomass conversion process: Feedstocks, Deconstruction, Fuel Synthesis, and Technologies. In the Deconstruction Division, the group that I lead, we are tasked with developing new ways to liberate fermentable sugars from lignocellulose. One of the foundational technologies we have developed is based on ionic liquids, and the use of glycoside hydrolase enzymes remains a key feature in the production of sugars using that technology. In order to develop and optimize enzyme cocktails that perform well under industrially relevant conditions, you need a lot of enzyme to explore fully the parameter space.

In the first phase of JBEI we focused on the typical science activities around enzyme expression, essentially recombinant protein expression in *E. coli, Saccharomyces,* or an in vitro system. More often than not, however, we would have problems with an enzyme that would express well in one system but not in another, or that would express but at levels that were too low for our needs. To achieve adequate expression levels we needed to develop a better expression system. There is, in my opinion, no better system than fungal for protein expression. This is mainly due to evolution. Fungi are everywhere and they are very efficient at breaking down biomass, and to do that they have to express a lot of extracellular enzymes. They have evolved to express massive amounts of enzymes, sometimes to the tune of 100 g/liter. That is why fungi are the flagship systems for the big commercial enzyme companies such as Novozymes, DSM, Genencor, and Dyadic. So instead of trying to develop an *E. coli* that can compete with fungi, why not just use fungi?

It is also important to understand the science of recombinant protein expression, because not every enzyme can be expressed equally well in every fungal system. We saw this as one of the biggest gaps in our research portfolio at JBEI; if an enzyme does not express, it does not exist in any meaningful way to our research team. Moreover, you may be able to do something at the 100 microliter scale, but you really want to be able to implement it at the 100,000 liter scale in a biorefinery–it has to be expressed at scales relevant to industry. Although *E. coli* and *Saccharomyces* have industrial applications, they will probably not be applicable in cellulase and hemicellulase production in the near term.

INDUSTRIAL BIOTECHNOLOGY: *When was the Fungal Biotechnology Group established and why did the JBEI feel a dedicated fungal biotechnology group was an important addition at this time?*

BLAKE SIMMONS: The reason we have Jon Magnuson as the director of the Fungal Biotechnology Group and invited Pacific Northwest National Laboratory (PNNL) to join JBEI in phase 2 is because they have a worldwide recognized expertise in fungal biotechnology. Instead of trying to build another wheel, why not bring in a team of experts, which is what we did. The group was established on October 1, 2012, at the start of fiscal-year 2013, although Jon and the team were involved months prior to that and contributed to the renewal proposal for JBEI that was awarded by the Department of Energy (DOE). Fungal biotechnology was a new element within the Deconstruction Division in that renewal proposal.

INDUSTRIAL BIOTECHNOLOGY: *Was the Fungal Biotechnology Group formed mainly by consolidating projects, resources, and staff already present and in progress at the Institute, or was it initiated with a broader mission?*

BLAKE SIMMONS: This was both an assimilation and an integration activity. We did not have strong fungal biology expertise within JBEI, because our mission in the first 5 years was focused on fundamental science and the more traditional

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approaches to protein expression in a laboratory setting. We have analytical support, bioinformatics support and high-throughput screening resources, and some wet biology expertise that was working with fungi, and we brought all of those together, but under new management. So the group is, for all intents and purposes, a new team element for JBEI, but it leverages what had been built during the first 5 years.

To Jon's credit, it involved hiring people through PNNL, but stationing them at JBEI, which is in Emeryville, CA. and Jon, as the director of the team, has to make frequent trips to Emeryville to lead that team. The group is centralized in one physical location, but it extends back to the partner institutions, so we have an open and transparent flow of information and data from PNNL and JBEI, and vice versa. The personnel retain ''dual citizenship,'' so to speak. PNNL signed off on that institutionally, Jon agreed to that arrangement personally, and JBEI had to commit the resources to enable it.

JBEI is not a legal entity. All of the people who work at JBEI have dual roles, one at JBEI and one at the institution that hires and pays them. We wanted to co-locate at one facility, because having the face-time and the connectivity, and being able to interact in the hallways, the labs, and the conference rooms between the different divisions and disciplines enables breakthroughs in science. We have already seen that scenario play out in significant ways during the first 5 years of JBEI.

INDUSTRIAL BIOTECHNOLOGY: *What is the scope and focus of the Fungal Biotechnology Group?*

BLAKE SIMMONS: The basic tenets of the group are to understand and manipulate fungi and fungal expression systems to obtain high protein expression yields of enzymes we care about, and to generate enough of them so we can achieve cocktail optimization in a biorefinery-relevant setting. By that I mean high solids loading, the appropriate environmental conditions, and the first glimmers of scale-up from bench science to something that would be meaningful to the industrial sector. And because we still do not know *a priori* whether an enzyme will express well in one system versus another, a lot of fundamental research still needs to be done in terms of understanding the sequences of genes, the translation mechanisms, where the proteins go during the folding events, and where they end up.

That is why we have chosen *Aspergillus niger* as our benchmark system of interest at JBEI. There is a lot known about that system, including its genetic make-up. Also, Jon's area of expertise is around *Aspergillus niger*, and it is an industrially relevant species.

INDUSTRIAL BIOTECHNOLOGY: *What types of applications are the Group targeting?*

JON MAGNUSON: We are looking at protein production, specifically of heterologous proteins that are being discovered within the other groups in the Deconstruction Division. That is a challenge for any system, including fungi–expressing proteins that are not native to the system you are studying. We want to increase the expression of those proteins and understand the fundamentals of why they do or do not express well from gene-to-gene or protein-to-protein. If we can understand that we may be able to shorten the time it takes to increase the expression of any gene someone would hand us.

The attractiveness of these new enzymes is that they function well within the integrated JBEI process. They work well with high solids loading, with the particular pretreatment process we use, and with the polysaccharides produced that we want to hydrolyze into sugars for fuel production downstream.

BLAKE SIMMONS: They also work well at higher temperatures and targeted pH ranges. These are not ''your daddy's enzymes.'' These are new classes of enzymes that we have targeted from different environmental screens and bioinformatic scans to be fully operational and efficient in our targeted environment. We have not only focused on and optimized for gene-to-gene and gene-to-gene-product differences, but they also perform well in our particular environment and biomass conversion processes.

We are not focused on creating a ''superzyme.'' We do not adhere to the approach that we will be able to create one enzyme that will be able to do all the steps of cellulose hydrolysis in all the environments we are investigating. Just like it takes a village to raise a child, it takes a village of enzymes to do this job. We are focused on cocktail optimization for the enzymes and understanding the potential synergies between the different types of enzymes present. To be able to understand that level of nuance and detail you need large amounts of the enzymes to carry out a robust design of experiments and fully optimize the system. That is one of the core motivations for establishing the fungal biotechnology group.

INDUSTRIAL BIOTECHNOLOGY: *How do fungi and the study of fungal biology complement, add value to, and integrate with the other groups within the Division–Biomass Pretreatment, Enzyme Optimization, and Microbial Communities?*

BLAKE SIMMONS: We spent a lot of time figuring out how these teams would work together. The Biomass Pretreatment team defines the upstream process that generates the product that defines how the enzymes need to function. We then have a trio of teams that work in concert with one another to make the enzymes work in that environment. Microbial Communities works on the discovery side of identifying new proteins and genes that function well in these targeted environments, through a bioprospecting approach. Enzyme Optimization works on optimizing the enzymes and cocktails through engineering approaches to improve performance. And the Fungal Biotechnology team expresses the enzymes so we can generate them at the desired levels while maintaining activity in the appropriate process conditions.

The biology folks outnumber the pretreatment folks by quite a bit, and this is because enzyme costs are a huge challenge to the development of commercial biorefineries. By some estimates in the literature, enzymes are projected to account for $0.75-1.50 per gallon of fuel produced in terms of the operating expense. So if you can develop better enzymes that are more efficient and are recyclable, do not need to function as long, or can be used at much lower doses, that can have a big impact on the cost of fuel

produced. That is why we have three teams devoted to trying to address this problem.

JON MAGNUSON: Another aspect of this integration at JBEI relates to the personnel. Both Steven Singer, who is the director of Microbial Communities, and John Gladden, who is my deputy director of the Fungal Biotechnology Group, were and are working in fungal communities. They were working on the fungal technology being developed at JBEI during years one through five of phase 1.

BLAKE SIMMONS: We want to achieve success quickly, so we needed to hit the ground running. By having the resource integration, the personnel integration, and the expertise we feel that we have set the stage to do just that.

INDUSTRIAL BIOTECHNOLOGY: *What would you identify as some of the biggest research challenges in the area of fungal biology?*

JON MAGNUSON: In the area of protein production, there is still a lot of fundamental understanding needed–in the protein secretion process, for example. These are complex organisms. They are in the same domain as humans, so they have the same degree of compartmentalization and organelles. It is a complex process from gene expression, to protein production, to protein secretion, and we need to understand the whole process within our application-driven focus of producing multi-gram per liter quantities of new and better enzymes. Fortunately we have a lot of new tools to help accelerate research in that area.

INDUSTRIAL BIOTECHNOLOGY: *Broadly speaking, what types of strategies and innovative approaches are being explored to overcome these challenges?*

JON MAGNUSON: We are using traditional types of approaches such as mutagenesis and screening, which the large enzyme companies and others have used to identify better protein secreters for their particular enzymes of interest. Another example would be targeted gene deletion. But we are combining these approaches with the modern tools of systems biology, such as genomics, RNA-Seq for looking at the expression of mRNAs, proteomics to look at the glycosylation of proteins and identify important genes involved in high-level protein secretion, especially for expression of heterologous genes and foreign proteins. So we are taking more traditional approaches to identify a random mutant–which may work but you don't know why–and then applying modern analytical tools such as targeted genome resequencing to find the genes associated with the productive mutation. Then we can knock out or over-express genes associated with the beneficial mutations in the wild-type strain. In this way we can stack up important genes without getting all of the collateral damage that occurs when you just take a random mutagenesis and selection approach.

BLAKE SIMMONS: Jon also touched on the important point of taking a systems biology or synthetic biology approach to defining a ''parts list'' of enzymes. The fungal biology community has developed cassette systems in which you can parse out genes and insert them into fungal expression systems, but there has not been a real community-based repository of that kind of information specific to fungi. At JBEI we have developed new tools for the purpose of engineering metabolism for biofuels production, and if we can take something from that school of thought and apply it to fungal biology and fungal expression of proteins, I think it would be a new way to approach the problem using the same tools and foundational knowledge, and the explosion in genomic knowledge to enable it.

INDUSTRIAL BIOTECHNOLOGY: *How has the ''genomics era'' and the emergence of next generation sequencing technology changed how fungal biology research is being done?*

BLAKE SIMMONS: With these next-generation sequencing technologies and bioinformatics we can dig deeper and get to the fundamentals of linking genotype to phenotype, which may allow us to develop a more robust engineering approach to making processes work even better and be more versatile in terms of which genes you put into the system. We are pretty excited about what the next 5 years holds because of this confluence of events that we happen to be lucky enough to leverage.

Data management and bioinformatics are probably among our biggest challenges at present. We can generate mountains of data, and if you are looking at that through a pinhole camera, it is going to take a very long time to understand what is going on. We need new ways of integrating these massive data sets, visualizing them, and interpreting them into actionable data. Bioinformatics is a critical element, coupled with next-generation sequencing technology, and the omics analytical platforms. It can be daunting. But when we set up the team, we understood the importance of bioinformatics, and we have an element within the resource distribution for the fungal biotechnology team that is dedicated to bioinformatics, explicitly to support the needs of the activity.

JON MAGNUSON: To me the genomics era and the next-generation sequencing have been revolutionary for biology in general, but the next-generation sequencing especially for fungal biology, because fungi have large genomes and transcriptomes compared to bacteria. The ability to do targeted resequencing, as I mentioned before, and to discover mutations has been very important. And the access to genome sequences has made our development and use of molecular biology and genetic tools in fungal systems much stronger and faster. To be able to target genes to particular sites in multiple places in chromosomes when designing fungi you need to have the sequences around the targeted areas of the genes you want to knock out or where you want to insert a gene. The new technologies are allowing us to build the constructs we need with long, 1,000-base pair flanking regions to enable targeted gene insertion. The technology has revolutionized the amount of information and the types of information we can now collect to increase our understanding of fungal biology and protein production, and it has also improved our ability to go in and modify a fungus to enhance the properties we are after.

INDUSTRIAL BIOTECHNOLOGY: *How are advances in synthetic biology and tools and techniques for improving and controlling gene expression and protein production contributing to progress in fungal biology research?*

BLAKE SIMMONS: Although we have mentioned the role of synthetic biology previously, there is another opportunity we did not discuss. There is a way to use synthetic biology and systems biology to develop these consolidated bioprocessing hosts–a bug that can deliver the enzymes, liberate the sugars or the dimers, and then convert those sugars into fuels. Through synthetic biology and the development of parts registries, if we can understand the fundamentals of protein expression for a certain class of enzymes and a fungal system and then adapt it for *Saccharomyces*, we could then integrate those genes more efficiently within that system. But you do not want to try to optimize the metabolic pathway and protein expression at the same time. You would rather solve those two challenges independently and then bring them together under one host engineering effort. That is another unique opportunity that synthetic biology offers us that we did not have 5 or 10 years ago.

INDUSTRIAL BIOTECHNOLOGY: *Fungi are excellent protein production hosts; can they also be used for the production of "next generation" infrastructure-compatible biofuels?*

BLAKE SIMMONS: The great thing about synthetic biology is not what you *can* do but what you *should* do. You have to act on the best science and information that you have. In addition to fungal biotechnology being relevant for deconstruction at JBEI, I think it also has far-reaching implications for fuel synthesis and technologies, and that is all driven and enabled by this synthetic biology approach.

JON MAGNUSON: This is an emerging area in which PNNL wants to contribute to JBEI. And looking at this from the other direction, at PNNL we are working with the applied side of DOE, the BioEnergy Technologies Office, which is focused on infrastructure compatible biofuels and production of biofuel precursors in fungi, including yeast. We are looking at producing lipids as hydrocarbon precursors, at polyketides, and at a variety of hydrocarbon-like molecules that can be produced by fungi.

Much of our previous research on fungi, until the past year or so, was on over-production of small molecules–organic acids that are not biofuels, but rather bioproducts. Still, central metabolism is involved in achieving high flux from the sugars the fungi are taking in to the production of small molecules, whether it is a bioproduct or a biofuel. The use of synthetic biology is similar for both in terms of building better, more efficient and productive pathways to increase that flux from the sugar to the bioproduct or biofuel.

INDUSTRIAL BIOTECHNOLOGY: *How does the work of the Fungal Biotechnology Group benefit from collaborations between the JBEI and other research organizations such as the DOE's Environmental Molecular Science Laboratory (EMSL) and the Joint Genome Institute (JGI)?*

BLAKE SIMMONS: The three DOE BioEnergy Research Centers in the US have a link to the JGI, which is an incredible resource for the high bandwidth genome sequencing operations we need to conduct our research. Without the JGI we would be in a difficult place both in terms of cost and sequencing throughput. JGI is a unique resource for us to leverage within the science landscape to do these massive gene sequencing projects in terms of breadth and depth. Let's say we want to sequence hundreds of different fungi and compare the unique sequences and understand what makes some work and some not work. The genes define the potential of the system.

Then we need to get into the functional aspects of the system and to access transcriptomics and proteomics datasets. We have links, in the form of approved user proposals to EMSL at PNNL. EMSL has very powerful proteomics and imaging capabilities that help us understand how these organisms do what they do in the environments in which we are interrogating them. By cross-referencing the datasets of the proteins and transcripts to the genes–although it is not quite as straightforward as that–hopefully we can create a library of knowledge around how these fungal systems work and why. To be able to visualize that we would like to be able to use projects such as the DOE Systems Biology Knowledgebase (KBase), the new software and data environment the DOE is rolling out, which is now about 2 years old. It will provide bioinformatics capabilities that we cannot build in-house at JBEI and could provide alternative and insightful ways of interpreting and understanding the data generated by JGI and EMSL.

The access JBEI has to these three entities enables high impact science to be done. We are putting these pieces into place; we are starting to correlate these datasets at JBEI, and in fungal biotechnology right now, it is all about resequencing and looking at the impact of mutations, and understanding the impact of the natural diversity out in the environment in terms of the function of the fungi. That has not been done at the level we are proposing in a public way to date. I am sure that companies have done some of that, but our function at JBEI is to do the science and then communicate it and share it with the broader scientific community to enable everyone in the fungal biotechnology realm.

JON MAGNUSON: All of these organizations are funded by the DOE's Office of Science, and specifically the Office of Biological and Environmental Research. There are various top-down and bottom-up directives to get these user facilities and the bioenergy research facilities to work together and leverage the resources that are there, and we are certainly doing that. Before JBEI, PNNL had a longstanding relationship with JGI to sequence the fungal genomes that we were interested in. They provide the genomic data and some of the transcriptomic data that is really foundational to applying some of these other high-throughput tools for understanding and genetically manipulating the fungi.

EMSL, which is located at PNNL, has tools for imaging and proteomics that will help in understanding heterologous protein

production and is developing new techniques to find glycosylation sites and signaling sequences that impact protein secretion in fungi. The data we get from JGI are necessary and complementary to the tool development at EMSL.

Blake A. Simmons, PhD, is Vice President, Deconstruction Division, Joint BioEnergy Institute, Emeryville, CA, and Senior Manager, Biofuels and Biomaterials Science and Technology, Biofuels Program Lead, Sandia National Laboratories, Livermore, CA. Mail: Sandia National Laboratories, 7011 East Avenue, MS 9291, Livermore, CA 94551; or Joint BioEnergy Institute, 5885 Hollis Street, Room 4201, Emeryville, CA 94608; Phone: 415-235-3170; Email: basimmo@sandia.gov

Jon Magnuson, PhD, is Director, Fungal Biotechnology Group, Joint BioEnergy Institute, Emeryville, CA, and Staff Scientist, Pacific Northwest National Laboratory, Richland, WA. Mail: 902 Battelle Blvd., MS: P8-60, Richland, WA 99352; Phone: 509-372-4119; Email: jon.magnuson@pnnl.gov