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Abengoa Research/JBEI Methyl Ketone Flux Analysis:

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Author

Garcia Martin, Hector

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SUMMARY REPORT

JBEI's mission is to produce fundamental scientific discoveries and major technological advances to enable the development of cost-effective, energy-efficient, and commercially viable processes for large-scale conversion of lignocellulosic biomass into fuels. Collaboration with Abengoa, a world leader in lignocellulosic biofuel, allows the translation of technology developed at JBEI into industry and help make a lignocellulosic biofuel based economy into reality. Abengoa Research will capitalize on the results of JBEI's leading biofuels research and IP through developed technologies.

JBEI Project Team: Hector Garcia Martin, Research Scientist (PI); Ee-Been Goh, Research Scientist, Harry Beller, Senior Scientist.

Abengoa Project Team: Rosario Gomez, Senior Investigator

Objective

Abengoa is committed to developing advanced biofuels technology for deployment in the US market. Abengoa intends to continue to innovate in cellulosic biofuels production and to commercialize innovative solutions, including those that may be developed in collaboration with JBEI under the CRADA. The collaboration between JBEI and Abengoa will most likely lead to new metabolically engineered microorganisms and perhaps even novel fuel molecules. If the features of these new molecules suggest potential improvements, the next step would be to scale-up the production in a pilot plant and later in a demonstration facility affiliated with Abengoa Bioenergy NT in different US locations to demonstrate the feasibility of the commercial process. It then intends to implement these solutions on a commercial scale where economically promising in the US. Both steps would lead to the creation of new farming, construction, supply chain, and plant operation jobs in US.

Product yields and low product margins challenge current biofuel production. To address these challenges, the proposed CRADA research will investigate the construction in microorganisms of biofuel synthesis pathways that are more economically viable than those sometimes being pursued by industry today. Several laboratories in the world have demonstrated the feasibility of manipulating microbes to produce molecules similar to petroleum-based products, albeit at relatively inadequate productivity.

Some of the obstacles to achieving high yields are the result of the interdependence of metabolic networks, which are strongly influenced by the global levels of handful metabolites. These central metabolites play an important role in regulating multiple pathways in the cell, because the cell uses the relative ratios of these metabolites to regulate a pathway's activity and ultimately the physiology of the cell. The incorporation of new pathways for biofuel synthesis can destabilize the balance of these important metabolites leading to the production of undesirable byproducts and a decrease in yield. One method for predicting the impact of a new metabolic pathway on growth and product formation is through the use of metabolic models. Genome-scale models like the one being developed at JBEI are playing a larger role in directing metabolic engineering efforts with more rational and systematic approaches. The CRADA research aims to take advantage of JBEI's modeling to make methyl ketone producing strains more economically viable.

The ability to generate microorganisms that can produce biofuels similar to petroleum-based transportation fuels would allow the use of existing engines and infrastructure and save an enormous amount of capital required for replacing the current infrastructure to accommodate biofuels that have properties significantly different from the petroleum-based fuels. Since some methyl ketones can be used as drop-in replacements for diesel, the CRADA research will advance the US goal of becoming less dependent on petroleum while keeping the existent fuel pipelines and engine infrastructure intact.

Strain optimization

During JBEI's first five years of existence, the Fuel Synthesis division has developed a variety of strains producing gasoline, diesel or jet-fuel molecules. In terms of the collaboration with Abengoa there is a trade-off regarding strain development: the more developed strains have high titers and may be the subject of industrial use in the near future but their patent licenses are more hotly contested and their biofuel production may be harder to further optimize. The less developed strains have low titers and will require more work before they become industrially relevant, but there is less competition for patent licenses and, typically, more options in terms of improving titer.

After careful deliberation JBEI and Abengoa have decided that this project will focus on the methyl ketone producing *E. coli* strains EGS1370 and EGS1710 developed in the JBEI Biofuel Pathways directorate. The titers are high enough that these strains could be susceptible of industrial use if licensed by Abengoa and, at the same time, there is plenty of unexplored territory in our understanding of their metabolism to produce fundamental knowledge and publications. Strain EGS1370 has been engineered to produce methyl ketones by modifying the β -oxidation pathway (specifically, the overexpression of a heterologous acyl-CoA oxidase (ACO) and native FadB, and chromosomal deletion of *fadA*) to overproduce β -ketoacyl-CoA, overexpression of native enzymes, FadM (a thioesterase), FadD (fatty acyl-CoA synthetase), and FadR (fatty acid regulator protein), as well as the chromosomal deletion of *poxB* (pyruvate oxidase). Strain EGS1710 is similar to EGS1370, except that the overexpressed pathway genes that were originally borne on

two plasmids have been consolidated into a single plasmid and the heterologous acyl-CoA oxidase gene has been codon-optimized for expression in *E. coli*.

In addition to strains, JBEI has been developing metabolic engineering tools in an effort to enhance and speed up metabolic engineering. These tools include flux analysis methods that integrate ¹³C labeling experimental data to measure and predict metabolic fluxes for genome-scale models (Two Scale ¹³C Metabolic Flux Analysis, 2S ¹³C-MFA), proteomic-based pathway optimization, DNA synthesis tools (j5, DIVA, the JGI DNA foundry) and data storage (EDD) and omics visualization software (Arrowland). This collaboration will focus on using genome-scale models to study the physiology of methyl-ketone producing strains and try to improve them.

Results summary

- 1. Flux Balance Analysis (FBA) for production strains.** We initially characterized Strains EGS1370 and EGS1710. Profiles for CO₂ output, O₂ consumption, OD, and concentrations of acetate, glucose, lactate, ethanol, formate, fatty acids, succinate, and methyl ketones were collected. We used this data to perform an exploratory FBA study for both strains (1% glucose) at time points corresponding to 15 (exponential phase) and 20 hours (late exponential phase). An obvious carbon drain for both time points was shown to be acetate, and this flux is channeled through the PTAr (phosphotransacetylase catalyzed by genes *pta* and *eutI*) and ACKr (acetate kinase catalyzed by genes *purT*, *tdcD* and *ackA*) fluxes) reactions.
- 2. KO predictions using MoMA.** We simulated knocking out the genes corresponding to reactions PTAr and ACKr through the Minimization of Metabolic Adjustment (MoMA) method in order to investigate the possible impact on methyl ketone yields. The results show no improvement on yield by knocking out the PTAr or ACKr reactions, but a 66% improvement if PTAr, ACKr and the acetate transporter reaction ACt2rpp are knocked out. Furthermore, we used MoMA again to independently knock out all the fluxes in the model in order to suggest future (less obvious) targets. As part of the project, we are proceeding with the construction of the following KOs as suggested by the MoMA analysis: GND (phosphogluconate dehydrogenase): b2029, FUM (fumarase): b1612 and b4122 and b1611, SUCDi (succinate dehydrogenase): b0721 or b0722 or b0723 or b0724, RPE (ribulose 5-phosphate 3-epimerase): b4301 and b3386, PGI (glucose-6-phosphate isomerase): b4025, GHMT2r (glycine hydroxymethyltransferase): b2551, MTHFC (methenyltetrahydrofolate cyclohydrolase): b0529, MTHFD (methylene tetrahydrofolate dehydrogenase (NADP)): b0529.
- 3. Experimental test of KO predictions:** Experimental results obtained outside of the scope of this project showed a ~100% production increase for the PTAr and ACKr knockouts (in M9 medium), validating the predictive technique. Within this project, the knockouts suggested by the MoMA analysis listed above were constructed. Deletions of the two ribulose-5-phosphate 3-epimerase homologs, *rpe* and *sgcE* (RPE reaction), were the most promising, initially resulting in > 50% improvement in MK production.
- 4. 2S ¹³C MFA for KO predictions.** We decided to study the *sgcE* KO in more detail using 2S ¹³C MFA, which leverages data from ¹³C experiments to provide better information on internal metabolic fluxes. To our surprise, this analysis found that knocking out *sgcE* seems to

significantly increase flux through the pentose phosphate pathway. It is possible that the concurrent NADPH production increase causes the increase in methyl ketone production.

Conclusion

We have demonstrated the use of flux analysis and genome-scale models to obtain a glimpse of the internal metabolic state can lead to actionable items to increase production. We have used several techniques to suggest gene knockouts in order to increase methyl ketone production on JBEI research strains. Furthermore, the use of these techniques also produces mechanistic insights into why these knockouts increase production. We are working on preparing the results for publication, and repeating some key experiments since it seems that temperature fluctuations produced a lower than expected production for the control strain.