A Bioengineering Platform to Industrialize Biotechnology

Stephanie Culler
Genomatica, Inc.

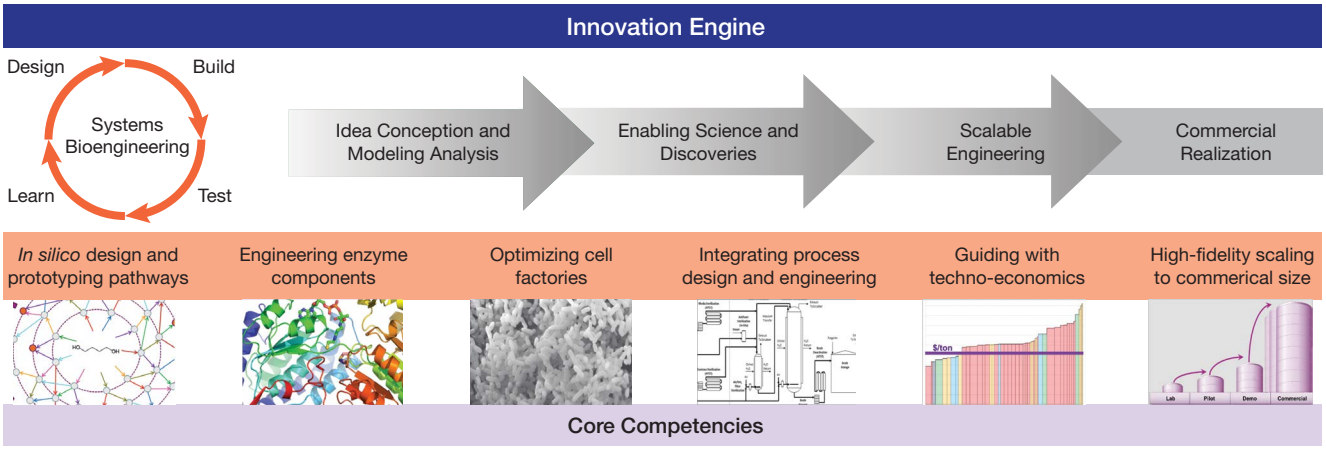
An integrated bioengineering platform that harnesses the synthetic biology toolbox is the key to an economically viable commercial bioprocess.

Industrial biotechnology enables the replacement of traditional petrochemical processes with benign bioprocesses that harness renewable feedstocks. Such sustainable bioprocesses have the potential to reduce safety risks and overall costs.

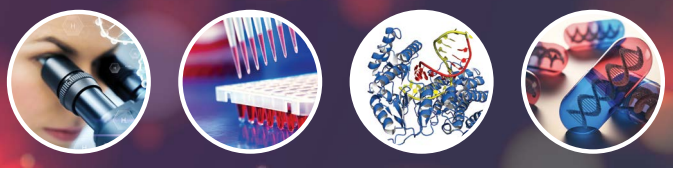
Growing concerns over greenhouse gas emissions, volatile fossil energy costs, high capital requirements, and waste from processes using fossil feedstocks (*i.e.,* oil, coal, or natural gas) have inspired scientists and engineers to develop alternative processes that use engineered microorganisms *(1)*. Microorganisms have the ability to generate products from a variety of renewable feedstocks, such as sugars, biomass, and fats and oils. The feedstock flexibility of microorganisms is a hedge against the volatility of petroleum supplies and prices.

Most of the reaction steps required to convert a feedstock into a desired product (chemical or fuel) can occur inside a microbial host cell, which enables direct production via a single fermentation step. As a result, bioprocesses typically have lower capital costs and risks, smaller environmental footprints, and lower energy consumption than petrochemical processes.

Genomatica has established an integrated computational and experimental bioengineering platform to design, create, and optimize novel high-producing organisms and bioprocesses (Figure 1) that can compete with traditional



▲ **Figure 1.** This complete platform to industrialize biotechnology includes tightly integrated core competencies across a broad range of disciplines, including computation, experimentation, and bioprocessing.



petroleum-based processes. The platform covers the entire workflow from idea generation to commercialization. A key feature of the platform is a systems biology approach that enables more-informed and faster decisions, increasing overall efficiency and the speed of strain engineering design-build-test-learn (DBTL) cycles that can be completed.

A broad range of core competencies provides the backbone of the platform, including *in silico* design and biopathway prediction, optimization of microbial factories using synthetic biology tools, process design, guided techno-economics, and commercial scaling of pathway fermentative processes.

The GENO-BDO™ technology, a sustainable bioprocess for the production of the industrial chemical 1,4-butanediol (BDO) from renewable feedstocks, embodies this platform and whole-process approach best. BDO is a chemical intermediate (with a global market of about two million metric tons) that goes into a variety of products, including plastics manufactured for automobiles, electronics, and apparel. It is currently produced commercially through energy-intensive petrochemical processes using hydrocarbon feedstocks.

A whole-process approach for delivering a commercial bioprocess

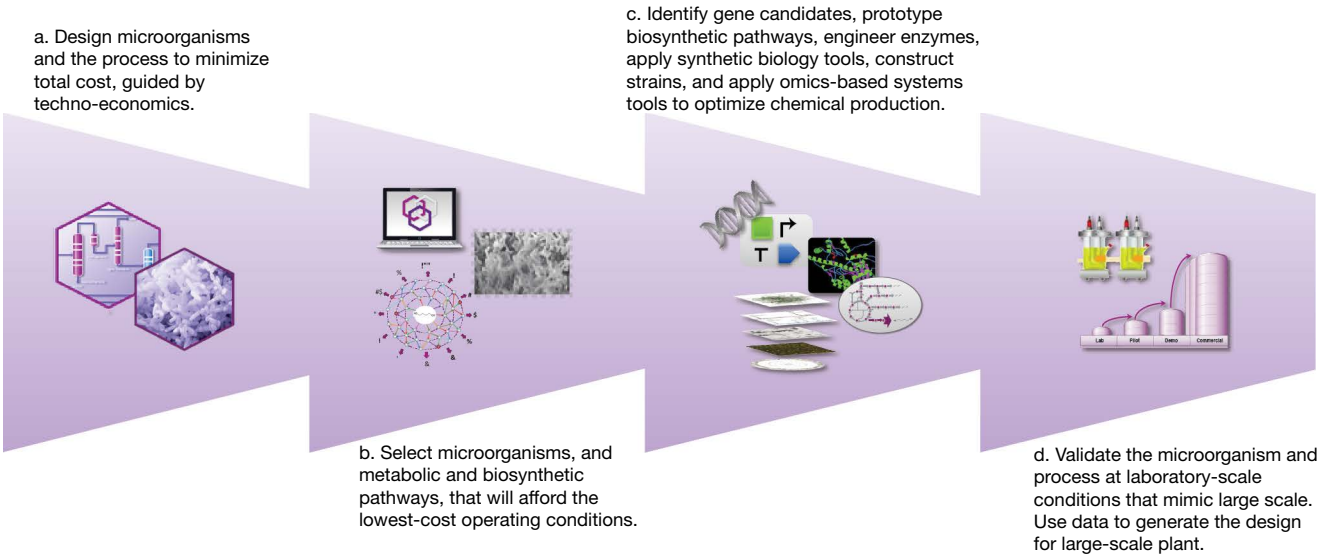
Genomatica engineers adopted a process-driven strategy for developing a commercial BDO bioprocess, and envisioned the entire process at full-scale (*e.g.,* feedstock, conversion, separation, etc.) prior to conducting experimental work *(2, 3)*. This approach enabled parallel development and optimization of the engineered microorganism with the overall process.

A key foundation for this approach was a techno-economic analysis (TEA), which generated a complete picture of the total capital investment and production costs. TEA models are generated at the beginning of a project and are used throughout as a guide, enabled by iterative assessment of the project's technical parameters weighed with economic prioritization.

The first step in this engineering-focused approach is to design the overall chemical production process and microorganism to maximize operation at scale and minimize total costs, guided by the TEA model (Figure 2a). During this stage, multiple organisms are evaluated. Bioengineers use thermodynamics to guide the identification of the best metabolic stoichiometries, fermentation rates, and fermentation conditions, and to select the microorganism, the metabolic pathways, and biosynthetic pathways that will afford the lowest-cost operating conditions (Figure 2b).

The chosen biosynthetic pathways, which encode multiple enzymes that catalyze conversion of a renewable feedstock into the final chemical product, are then engineered into the host microorganism. Bioinformatic analysis is used to identify gene candidates based on information about known enzymes that catalyze similar reactions *(4)*. Introduction of the biosynthetic pathway is facilitated by genetic engineering tools.

Bioengineers typically evaluate the fermentative performance of the engineered cell factory through three essential process metrics: titer (concentration), rate (productivity), and yield (feedstock required to produce a given amount of product), which together are referred to by the acronym TRY. Yield is the most important of the three metrics, as large-



▲ **Figure 2.** A process-driven approach for developing a commercial bioprocess begins with the design of microorganisms, biopathways, and the overall process to minimize operational costs guided by TEA models (a, b). Next, engineers create the production strains and validate their performance under commercially relevant conditions, and generate data for the design of a large-scale plant (c, d).

scale bioprocesses must operate at 80% or more of theoretical yield to be economically competitive *(2)*. It is not possible for bioprocesses to achieve theoretical yields, because a portion of the carbohydrate feedstock is required for cell growth and maintenance energy. TRY metrics are interdependent and their maximization — through the reduction of byproducts, for example — is key to reducing overall costs.

Next, pathway enzyme engineering and synthetic biology tools are applied iteratively to maximize pathway flux and reduce byproducts (Figure 2c). The systems biology platform uses extensive omics tools — transcriptomics, proteomics, metabolomics, and fluxomics — to probe cellular function.

Transcriptomics and proteomics examine the global expression of genes, while targeted metabolomic approaches analyze pathway intermediates from sugar to product, as well as redox and energy ratios. Fluxomics tools that use ^{13}C-based metabolic flux analysis *(5)* combine stoichiometric models of metabolism with mass spectrometry methods to trace the conversion of isotopically labeled metabolites. This analysis provides quantitative information on metabolic reactions *in vivo* and provides the ability to evaluate fluxes through native, engineered, and heterologous pathways.

These tools are applied at laboratory scale to develop and optimize the microorganism and fermentation process under the conditions anticipated at large-scale. The process is then scaled up to generate data for plant design (Figure 2d). The result is a microorganism and fermentation process that translate well to the design of the large-scale production plant with minimal additional capital costs.

Bioengineering a BDO pathway in *E. coli*

The initial development of the *Escherichia coli* (*E. coli*) BDO production strain achieved titers of several g/L BDO from fermentations that lasted nearly five days *(6)*. Because BDO is not a natural chemical, much of the early effort involved the identification of new biochemical pathways to BDO, as well as computational modeling to generate a blueprint of a host strain that would maximize carbon flux to the pathway while balancing overall energy and redox.

Early adoption of the whole-process approach narrowed down the candidate biosynthetic pathways to one that required five heterologous steps to convert the tricarboxylic acid (TCA) cycle intermediate succinyl-CoA into the desired product (Figure 3) *(6)*.

The pathway uses a CoA-dependent succinate semialdehyde dehydrogenase (sucD) enzyme to generate succinyl semialdehyde, which is further reduced to 4-hydroxybutyrate (4-HB) by the 4-hydrobutyrate dehydrogenase (4hbD) enzyme. The 4-hydroxybutyrl-CoA transferase (cat2) enzyme adds CoA to activate 4-HB. The final two reduction steps are catalyzed by the aldehyde dehydrogenase (ALD) and alcohol dehydrogenase (ADH) enzymes to reduce 4-HB-CoA and produce BDO.

The BDO pathway is redox-intensive. In order to balance cellular energy and overall redox demands, a microaerobic fermentation process was implemented. This process provided sufficient reduced cofactors to drive the BDO pathway, while respiring excess redox to generate the adenosine triphosphate (ATP) needed for cell growth and maintenance.

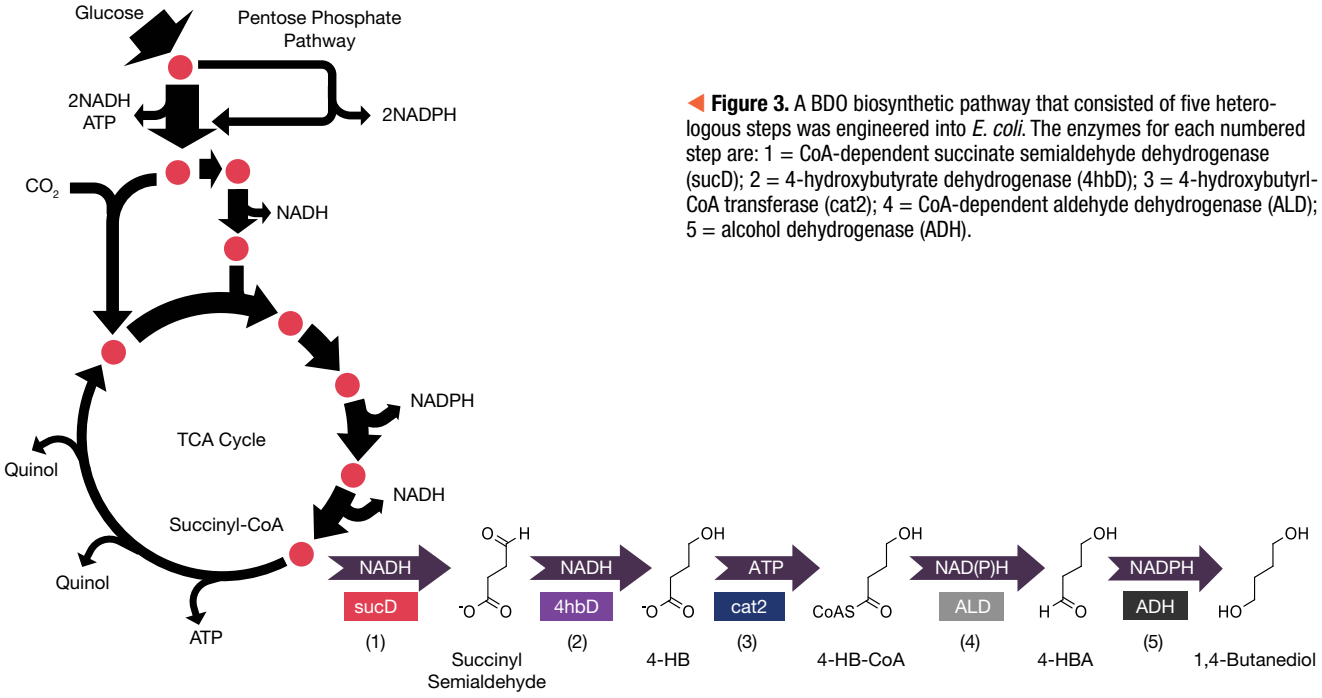


Figure 3. A BDO biosynthetic pathway that consisted of five heterologous steps was engineered into *E. coli*. The enzymes for each numbered step are: 1 = CoA-dependent succinate semialdehyde dehydrogenase (sucD); 2 = 4-hydroxybutyrate dehydrogenase (4hbD); 3 = 4-hydroxybutyrl-CoA transferase (cat2); 4 = CoA-dependent aldehyde dehydrogenase (ALD); 5 = alcohol dehydrogenase (ADH).

To make significant improvements on early BDO titers, we rewired the central metabolism to eliminate drains of redox and ATP, thereby forcing flux to the product. The resulting strain produced up to 18 g/L BDO in fed-batch fermentation *(6)*.

In order to create an industrially relevant microorganism, in further BDO production strains we also improved the BDO pathway enzymes, host metabolism, fermentation, and downstream processes *(7)*. The combined effects of these improvements ultimately allowed commercial production to begin in late 2012, five years from project startup (Figure 4a).

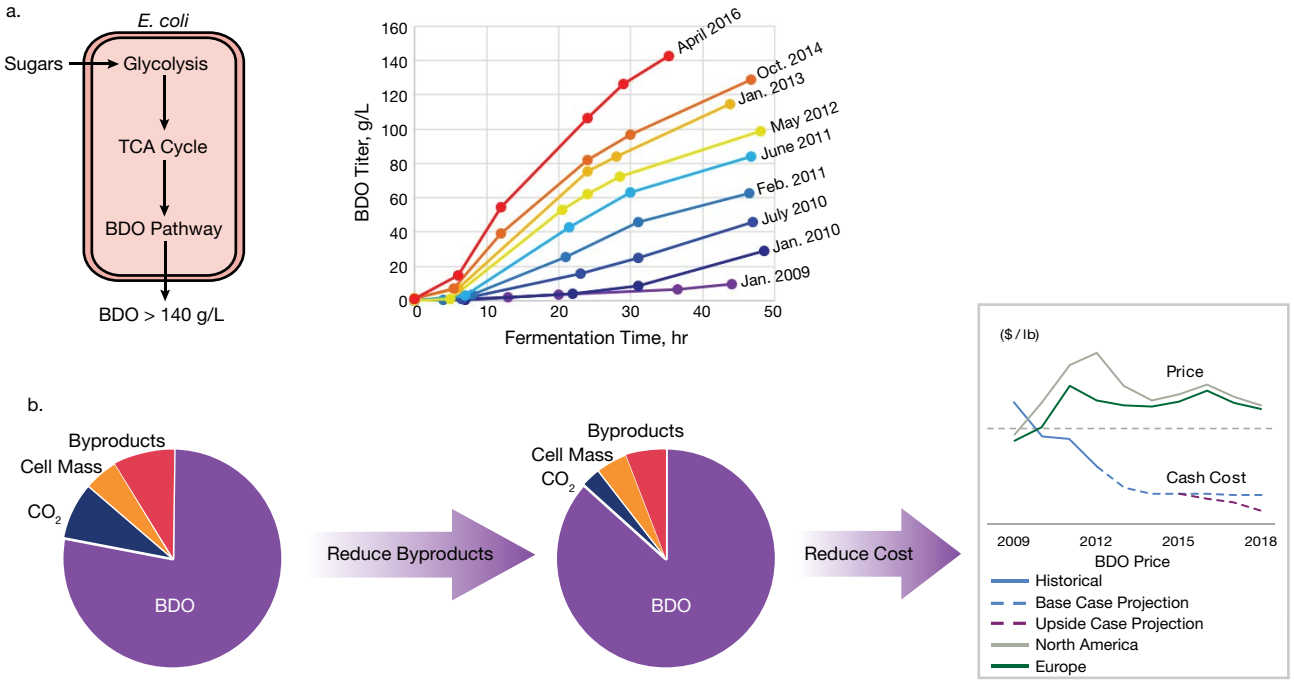
Genomatica engineers have focused on reducing metabolic and biosynthetic byproducts, as well as CO_2 from respiration, to reduce the overall production costs. This effort has also demonstrated the economic viability of the process through favorable TRY metrics, including titers greater than 140 g/L, rates greater than 3 g/L-h, and yields that exceed the commercial target (Figures 4a, 4b). TEA models were used to calculate metrics showing the cost advantage of the GENO-BDO process over conventional processes for making BDO from fossil feedstocks (Figure 4b).

A tightly integrated technology platform that encompasses metabolic modeling, synthetic and systems biology, fermentation, and process engineering enabled us to tackle the process and strain performance challenges encountered along the path to commercialization of the bio-BDO process. Development and application of new synthetic biology tools played a significant role in this journey. Several high-impact developments made by our bioengineers helped reduce the overall costs for the commercial development of the bio-BDO process. These included:

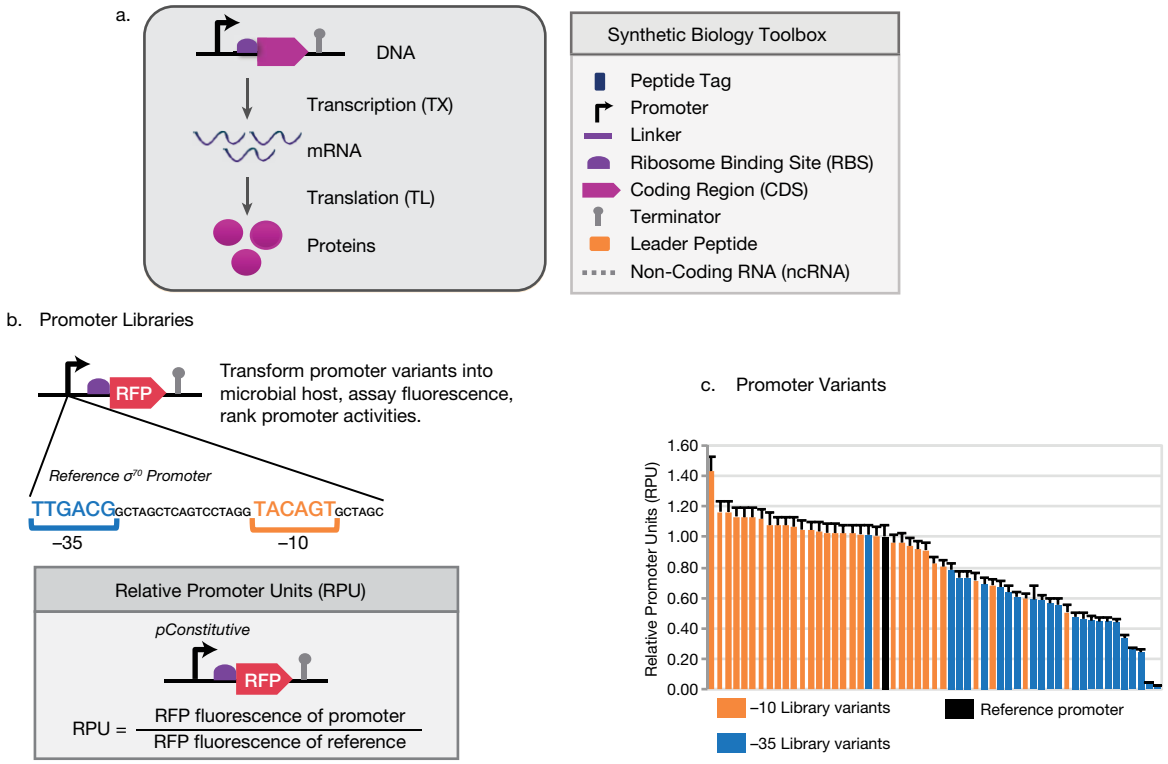
- creation of a synthetic biology toolbox, parts registry software, and automated cloning platform
- elimination of costly process dependence on antibiotics and genetic inducers
- optimization of pathway flux, and improvements in redox availability, energy efficiency, and pathway debottlenecking, as well as reduction of byproducts through expression tuning of enzymes
- development of a cell-free expression platform for the high-throughput (HT) prototyping and debottlenecking of biosynthetic pathways and enzyme screening.

Developing a framework for generating and characterizing biological parts

Synthetic biology facilitates the engineering of biological systems. DNA sequencing (reading) and DNA synthesis (writing) are at the heart of synthetic biology. DNA sequencing allows scientists and engineers to understand the genetic architecture and components of natural biological systems. The writing, or synthesis, of DNA gives researchers the abil-



▲ **Figure 4.** (a) Significant improvements in BDO strain performance have been achieved since 2009. (b) Carbon is distributed among BDO, biomass, and byproducts. Reduction of byproducts reduced the overall cost of goods sold (COGS).



▲ **Figure 5.** A synthetic biology toolbox enables engineering of chemical-producing microorganisms. (a) In bacteria, DNA is transcribed into messenger RNA (mRNA) by the RNA polymerase enzyme. The mRNA is translated into proteins by the ribosome. The synthetic biology toolbox contains a variety of genetic parts that can be used to probe gene expression at all levels. (b) Promoter libraries were created by mutagenesis of a reference sigma 70 (σ70) promoter, and variants were ranked by fluorescence output. σ70 refers to the sigma factor with a molecular weight of 70 kDa. (c) The library was cloned in front of a reporter gene encoding for the red fluorescent protein (RFP). Promoter variants are transformed into a microbial host of interest, assayed for fluorescence, and ranked by activity in terms of relative promoter units (RPU) — the fluorescence of the promoter variant relative to the parent reference promoter. A broad range of promoter sequences and activities was observed for the variants. However, –10 variants were on average stronger and –35 variants weaker than the reference promoter.

ity to rapidly design, build, and test new synthetic biological parts and systems. We envisioned synthetic biology as a biology-based toolkit that uses standardization and automated construction of biological parts to assemble biological systems for the production of sustainable chemicals.

The development of our synthetic biology toolkit was motivated by the need to eliminate the use of antibiotics and pathway inducers, balance metabolic and pathway enzymes, and facilitate the reduction of byproducts in our production strains. To accomplish those goals, we needed to develop tools that would enable the tuning of gene expression at all levels, from DNA to protein, both within the molecule (*cis*) and outside of the molecule (*trans*).

In bacteria, genes (regions of DNA that encode function) are transcribed (TX) into messenger RNA (mRNA) by the RNA polymerase enzyme and an associated sigma factor, which enables specific binding of the RNA polymerase. Transcription factors, proteins that bind to specific DNA sequences, may also be involved in this process, by controlling the rate of transcription. The resulting mRNA is then translated (TL) by the ribosome into protein (Figure 5a).

Biological parts that drive both processes include:

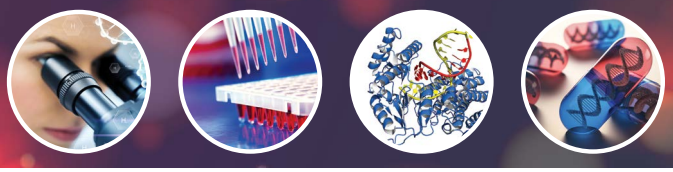
• a promoter — a DNA sequence that recruits the transcriptional machinery, which, in turn, initiates transcription

• a ribosome binding site (RBS) — an RNA sequence found in mRNA that initiates translation.

Synthetic RBSs to control protein expression can be quickly generated with RBS calculator software *(8)*.

Promoters can be either inducible or constitutive. If an inducible promoter is applied, a transcription factor controls the rate of transcription. If a constitutive promoter is used, transcription is regulated by the availability of RNA polymerase and corresponding sigma factors. The specific DNA sequence of the promoter also determines the promoter's overall strength; a strong promoter will have a high rate of transcription.

Genomatica's synthetic biology toolbox contains parts generated in-house as well as parts from the Registry of Standard Biological Parts (http://parts.igem.org). Synthetic biology standards were applied for defining biological



parts *(9)*, and corresponding sequence information was entered into our laboratory information management system (LIMS). A framework was developed that would allow bioengineers to construct biological systems composed of parts from the toolbox using an integrated platform that couples design software with the parts registry. Corresponding designs are then sent to an automated HT-cloning platform, which rapidly stitches together DNA parts that encode for a desired function or biosynthetic pathway.

With this framework in hand, the initial focus was on challenges faced by the first-generation BDO production strains. These early strains relied on costly antibiotic selection to maintain plasmids (circular DNA) that express pathway genes with an inducible promoter *(6)*.

Relying on the small molecule inducer isopropyl β-D-1-thiogalactopyranoside (IPTG) for pathway expression is cost-prohibitive at scale. To solve this challenge, and enable transcriptional-based tuning of genes, inducible promoters were replaced with constitutive promoters.

We constructed promoter libraries by random mutagenesis of the two most conserved sequences, the –10 and –35 sequences of a parent constitutive promoter (Figure 5b). Mutation of these sequences has an impact on overall promoter strength. A library of these sequences could produce promoters that vary in activity *(10)*. A broad range of promoter sequences and activities were observed for the variants: –10 variants were on average stronger and –35 variants weaker than the reference promoter (Figure 5c). We pursued chromosomal integration of pathway genes under constitutive promoters of varying strength to improve overall BDO strain robustness and eliminate the need for costly antibiotics and transcriptional inducers *(6)*.

Optimizing BDO production with biological parts

BDO fermentation performance neared commercial targets as we conducted diagnostic and systems-based experiments to prioritize strain optimization and enzyme engineering efforts *(11)*. Fermentations of these strains performed well until the BDO titer approached 80–90 g/L, at which point BDO productivity slowed simultaneously with a spike in the production of the upstream pathway byproduct 4-HB. These results suggested that flux into the downstream BDO pathway was limiting.

Fine-tuning of exogenous pathway expression at the transcriptional and translational levels is often used to optimize flux to a product of interest and minimize metabolic burden on the host *(12)*. We explored constitutive promoters of various strengths to tune the expression of the ALD enzyme in the downstream BDO pathway (Figure 6a). Fermentation production of BDO is significantly influenced by ALD protein levels, suggesting the ALD may be an enzyme-level bottleneck. However, tuning the expression of single genes has often produced undesired consequences, such as reduced expression of other pathway enzymes *(11)*.

We employed a systematic and combinatorial approach for altering the expression of the downstream BDO pathway genes (Figure 6b). To optimize expression of the ALD, cat2, and ADH enzymes, we tuned both transcription and

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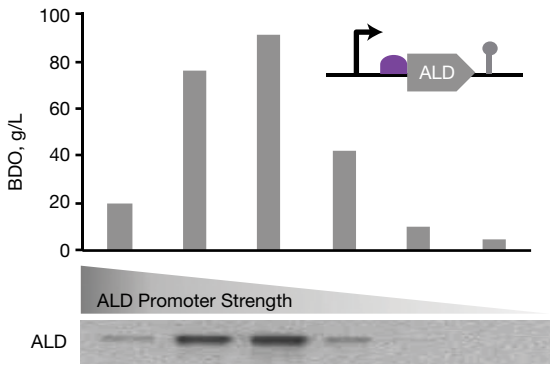
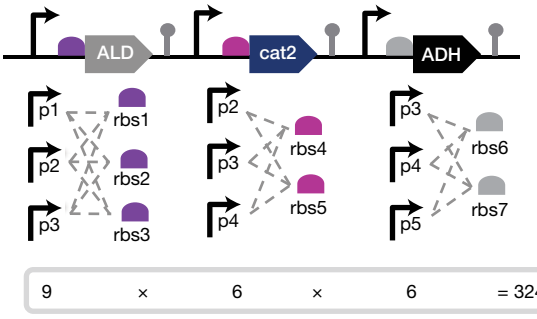


b.



(ALD)	(ALD)	(cat2)	(cat2)	(ADH)	(ADH)	BDO (g/L)
p3	RBS2	p2	RBS5	p5	RBS6	10.4
p3	RBS1	p2	RBS4	p5	RBS6	4.4
p3	RBS1	p2	RBS4	p5	RBS7	4.5
p3	RBS2	p2	RBS4	p5	RBS6	3.4
p3	RBS2	p2	RBS4	p5	RBS7	2.4
p3	RBS2	p2	RBS5	p5	RBS7	0.0
p3	RBS1	p3	RBS4	p3	RBS6	14.8
p3	RBS1	p3	RBS4	p3	RBS7	12.8
p3	RBS1	p3	RBS5	p3	RBS6	11.7
p3	RBS1	p3	RBS5	p3	RBS7	13.2
p3	RBS1	p3	RBS4	p4	RBS6	14.3
p3	RBS1	p3	RBS5	p4	RBS6	11.8
p3	RBS1	p3	RBS5	p4	RBS7	12.8

▲ **Figure 6.** (a) To enhance BDO pathway gene expression, constitutive promoters of various strengths were used to optimize ALD protein levels. Lab-scale (2-L) fermentation of the resulting strains demonstrated that BDO production levels are strongly influenced by ALD protein expression — higher soluble ALD expression increased BDO production. However, ALD solubility and expression did not correlate with promoter strength. (b) To tune the downstream BDO pathway, 324 pathway variants were constructed using HT-cloning techniques and assayed in small-scale (mL) fermentations for BDO production. A subset of the results is shown.

translation by altering the strength of promoters and synthetic RBSs in front of each gene within the multi-gene construct (Figure 6b). Five promoters, from strong to weak (p1 > p2 > p3 > p4 > p5) were chosen for transcriptional tuning, and synthetic RBS sequences were designed for each gene in the downstream pathway, likewise of different strengths (rbs1 > rbs2 > rbs3 [ALD], rbs4 > rbs5 [cat2], rbs6 > rbs7 [ADH]). In total, 324 pathway combinations were constructed and transformed into a BDO production host with upstream enzymes sucD and 4hbD.

BDO production results varied widely among combinations. In general, medium-strength promoters produced higher levels of BDO and lower levels of 4-HB (p3/p4). Combinations that produced little BDO were noted, as they also provided insight into flux-limiting enzymes. We incorporated the results of this study into a strategy that combined enzyme engineering and expression optimization to effectively reduce the levels of 4-HB *(7)*. Furthermore, data generated from similar combinatorial studies can be fed into machine-learning algorithms *(13)* to generate models to improve the overall robustness of strain design.

Reducing byproducts with protein degradation tags

Minimizing overall process costs through the reduction of byproducts is critical for the development of a commercially viable bioprocess with high product yields and low downstream separation costs. Following the critical reduction of 4-HB, we focused our strain engineering efforts on increasing flux into the TCA cycle and upstream BDO pathway *(7)*.

An evaluation of these enhanced TCA cycle strains suggested that excess CO_2 had become a significant byproduct, and accounted for more than 10% of the carbon substrate. Excess CO_2 reflects how much substrate is directly respired and is a sign that the cell has an energy limitation (*e.g.,* not enough cellular ATP).

Our attempts at reducing the oxygen in fermentations — with the goal of limiting excess CO_2 — were unsuccessful, due to reduced biomass propagation and therefore lower volumetric productivity. ^{13}C-flux analysis of these strains revealed that the pentose phosphate pathway and the TCA cycle were the major sources of excess CO_2. Initial efforts to eliminate CO_2 production from the TCA cycle by deleting succinyl-CoA synthetase (sucCD) — an ATP-producing enzyme complex — created severe growth defects and reduced BDO production (Figure 7a).

The observed growth defect suggested that during the growth phase, the complete TCA cycle is necessary to satisfy the overall energy requirements for biomass propagation. However, an active TCA cycle during BDO production creates excess CO_2. Temporal regulation of the TCA cycle might solve this problem, because TCA flux would be provided when needed during fermentative growth and would be turned off during the BDO production phase.

Degradation tags, which are short peptide sequences that target a protein for degradation by intracellular proteases (enzymes that break down proteins), are one way to temporally regulate the TCA cycle *(14)*. The degradation tag effectively decreases the half-life and the overall concentration of the targeted protein. Degradation tags that target sucCD would enable sufficient production of sucCD in the growth phase, while coupling the reduced protein half-life with the normal decrease in protein translation that occurs as the cells complete growth and transition to BDO production (Figure 7b).

We exploited *E.coli's* natural protein degradation system, which is based on ssRA (small stable RNA A)-mediated tagging of prematurely terminated proteins. Peptide sequence variants of the ssRA tags can be used to generate targeted protein variants with a broad range of half-lives *(15)*.

Two ssRA tags of different in strengths — very fast ssRA (vfssRA) and fast ssRA (fssRA) tags — from the Registry of Standard Biological Parts were added to the C-terminus of the sucD subunit of sucCD to provide temporal degradation. The relative sucD protein levels were assessed for the strains containing sucCD-vfssRA, sucCD-fssRA, and sucCD throughout the course of a standard fermentation (Figure 7c). The fssRA tag achieved the desired temporal regulation — sufficient levels of sucCD were produced during growth (measured at 10 hr), and then sucCD production subsequently decreased during the BDO production phase (24–48 hr). The vfssRA tag was too fast, producing barely detectable levels of sucCD.

The fssRA tag was further explored in a side-by-side fermentation with the sucCD plus and minus strains. The growth of the degradation tag strain was less than that of the sucCD plus strain, as expected, but better than that of the sucCD deleted strain. Therefore, this strategy achieved the desired temporal regulation without compromising BDO production (Figure 7d). These data demonstrated that protein degradation tags are a viable approach toward achieving temporal regulation of gene expression.

Cell-free technologies for industrial metabolic engineering

Despite the number of systems and synthetic biology technologies for the engineering of biological systems, most industrial-scale bioprocesses take significantly longer than five years to develop and require tens of millions of dollars in funding *(16)*. Much of this is the result of a tug-of-war that exists between the bioengineer's objectives and the host organism's objectives. This challenge encouraged us to find new ways to reduce timelines, increase the speed of DBTL

cycles, and improve methods of prototyping biosynthetic pathways.

We were consequently driven to explore cell-free expression systems for a variety of reasons. Recent advances in cell-free technologies have allowed for rapid expression and activity screening with minimal molecular biology effort *(17)*. Cell-free technologies have no cellular barriers, which makes them suitable for screening toxic metabolites and proteins, and they provide flexibility in constructing and characterizing complex biochemical systems. Most importantly for metabolic engineering, a cell-free environment is similar to the cellular cytoplasm, and oxidative phosphorylation and protein synthesis can be co-activated *(18)*.

As an industrial partner on a Defense Advanced Research Projects Agency's (DARPA) Living Foundries grant, we demonstrated through a proof-of-concept project the industrial applicability of cell-free transcription-translation (TX-TL) technologies.

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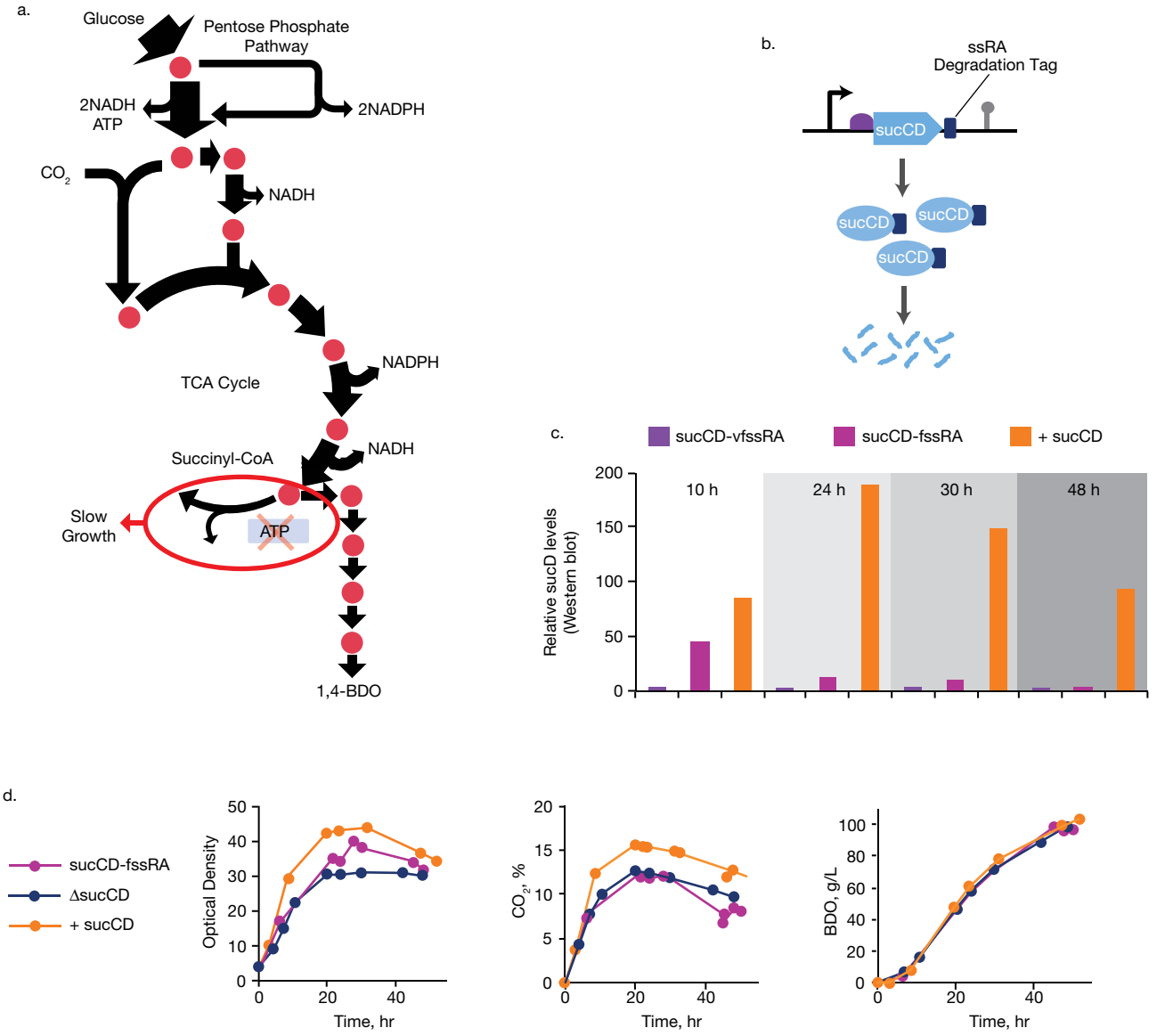


▲ **Figure 7.** (a) To reduce the amount of excess CO_2 generated in the TCA cycle, strain engineering efforts focused on eliminating the production of CO_2 from the TCA cycle through the deletion of succinyl-CoA synthetase (sucCD). This created severe growth defects and reduced BDO production. (b) Temporal regulation of the sucCD enzyme complex was achieved with ssRA-mediated degradation tags. Very fast (vf) and fast (f) ssRA degradation tags were appended to the C-terminus of the sucD subunit. (c) Relative sucD protein levels were assessed by Western blot analysis for the strains containing sucCD-vfssRA, sucCD-fssRA, and sucCD throughout the course of a BDO lab-scale fermentation. (d) Lab-scale fermentations of the sucCD-fssRA achieved the desired temporal regulation without compromising BDO production.

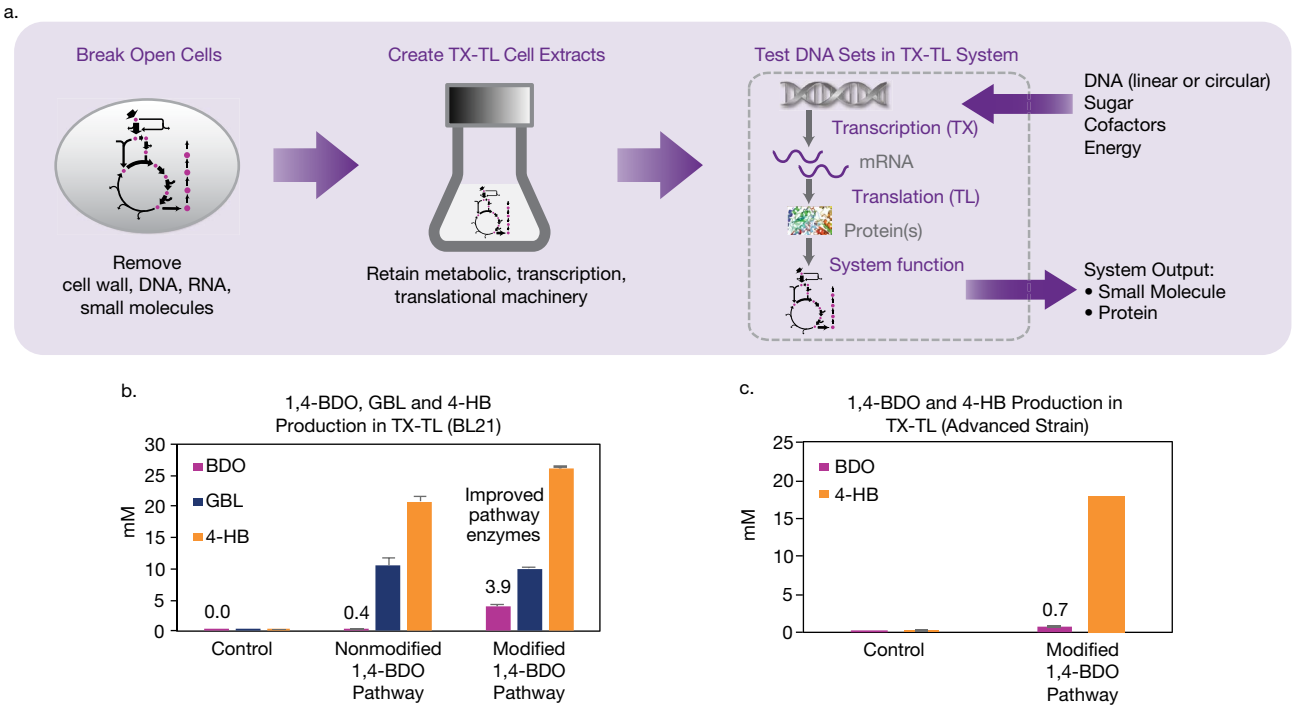
Preparation of cell-free TX-TL extracts begins with the selection of a microorganism of interest (Figure 8a). The bioengineer grows the organism under conditions that support robust transcriptional and translational activities. The cells are broken open and the cell wall, endogenous DNA, RNA, and small molecules are removed. The retained proteins are metabolically, transcriptionally, and translationally active.

TX-TL reactions can be set up at the µL scale, and either linear or circular DNA can be added as the template for protein production. The output of the reaction can be a protein or multiple proteins that catalyze the conversion of a substrate into a small molecule. We examined the ability to rapidly prototype, screen the expression, and debottleneck biosynthetic pathways with TX-TL using the 1,4-BDO pathway.

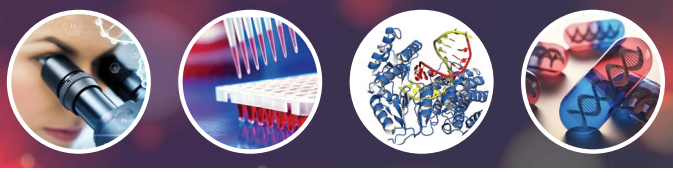
Automated HT-cloning was used to construct linear fragments for each gene in the BDO pathway. The original BDO pathway and the modified pathway, including engineered cat2, ALD, and ADH enzymes, were examined in TX-TL using an extract made from the BL21 protein production host (Figure 8b). TX-TL reactions ran for approximately 8 hr and were analyzed by liquid chromatography-mass spectrometry (LC-MS). Expression of each gene was confirmed (data are not shown) and BDO production was observed for both pathways, with a tenfold difference between the nonmodified and the engineered pathways.

The difference in BDO production with the engineered downstream pathway enzymes suggests applicability of TX-TL as an enzyme engineering platform. Moreover, this demonstration of BDO production indicates that the TX-TL reaction can significantly reduce timelines for prototyping biosynthetic pathways, as it took the BDO team six months to generate a similar amount of BDO *in vivo*. Appearance of the byproduct γ-butyrolactone (GBL), a spontaneous lactonized form of 4-HB produced from 4-HB-CoA and 4-HB, suggested the downstream BDO pathway was limiting. This same observation was made after months of systems-based studies of *in vivo* strains, which validated the potential for TX-TL as a means to debottleneck pathways.

Optimally, pathway prototyping in TX-TL should be conducted in extracts from the *in vivo* host strain. We created extracts from an advanced chemical production strain with over 50 metabolic modifications and likewise demonstrated



▲ **Figure 8.** Cell-free transcription-translation (TX-TL) technologies may reduce industrial timelines. (a) To create TX-TL cell extracts, selected microorganisms are grown under conditions that support robust production of the TX-TL machinery. Cells are broken apart (lysed), and the cell wall, DNA, RNA, and small molecules are removed. Metabolic, transcriptional, and translational machinery are retained in the prepared extracts. Bioengineers initiate reactions by adding a DNA template, substrate (sugars), cofactors, and an energy source. The system output can be an expressed protein or a small molecule. (b) The BDO pathway was prototyped in the TX-TL system. Linear DNA encoding the nonmodified and modified BDO pathways was added to a BL21-based extract. The BDO, γ-butyrolactone (GBL), and 4-HB concentrations were measured 8 hr later via LC-MS analysis. (c) The modified BDO pathway was assessed in extracts made from an advanced strain. This strain contained a novel esterase that cleaves GBL into 4-HB, so GBL production was not detected. BDO and 4-HB was similarly assessed by LC-MS.



BDO production, albeit at a lower concentration than was observed in the BL21 background (Figure 8c). These results suggested that pathways could be prototyped in TX-TL extracts from corresponding *in vivo* host strains.

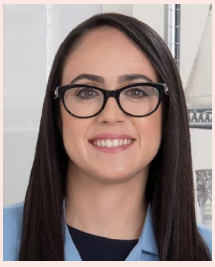
Closing thoughts

A whole-process approach combined with a tightly integrated technology platform has given Genomatica the tools to deliver an economically viable commercial bioprocess. However, the path toward commercialization of this and other processes is often challenged by our ability to maximize DBTL cycles. Complementing a systems biology mindset with a synthetic biology toolbox enables the bioengineer to rationally probe and rapidly manipulate biological organisms, thereby increasing the speed of such cycles. New opportunities exist for technologies such as TX-TL and the synthetic biology toolbox, which can be adapted to explore nontraditional but phenotypically advantaged bioproduction hosts.

Future efforts will explore the development of TX-TL as an enzyme discovery and screening platform, which is critical for early-stage programs in determining the best combination of enzymes for a desired product. Moreover, a rigorous systems biology study to develop a map of the metabolism in TX-TL extracts will be necessary to improve *in vivo* performance of TX-TL pathways. Over time, the seamless integration of data management and machine-learning algorithms will improve the robustness of strain development for commercial bioprocesses, and generated predictive models, rather than trial-and-error, will facilitate more informed R&D decisions. CEP

In Memory

This article is dedicated to the bright memory of Eric Van Name, a brilliant colleague and true friend. His relentless and innovative spirit lives on through the technologies described here.



STEPHANIE CULLER, PhD, is a senior scientist at Genomatica, Inc. (Email: sculler@genomatica.com), where she has developed and applied synthetic biology tools toward the engineering of microorganisms as biocatalysts for the industrial production of sustainable chemicals. She has led the development of cell-free transcription-translation (TX-TL) technologies for industrial metabolic engineering and successfully demonstrated the production in TX-TL of Genomatica's platform chemicals. Since joining the company in August 2010, she has led two major programs focused on delivering bioprocesses utilizing syngas as a feedstock for the production of chemicals and the direct production of bio-butadiene from renewable sugars. Culler received her BS from the Univ. of California at San Diego and her PhD from the California Institute of Technology, both in chemical engineering.

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