Into New Territory: Improved Microbial Synthesis through Engineering of the Essential Metabolic Network

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Abstract: Advances in synthetic biology and metabolic engineering offer the promise of next generation bioprocesses to produce numerous products including specialty and bulk chemicals and even biofuels sustainably from renewable feedstocks. A primary challenge is the optimization of product flux, within a much larger and complex metabolic network. While simple gene deletion methods can be used in the case of non-essential byproduct pathways, more sophisticated approaches are required when competitive fluxes are essential to host cellular functions. Engineering essential metabolic networks has been traditionally off-limits to metabolic engineers. Newer approaches to be reviewed include the rebalancing or rewiring of the metabolic network by tuning the levels of essential enzymes and the use of dynamic metabolic control strategies to conditionally reduce essential competitive fluxes.

Introduction

Petroleum is the primary feedstock, not only for the fuels used today, but the majority of the bulk and specialty chemicals consumed as well. The world is heavily reliant on this non-renewable resource. Replacement of petroleum with renewable feedstocks may ensure longer-term energy security and environmental sustainability. The production of numerous sustainable chemicals using engineered microbes can have a huge potential environmental impact with a significant reduction in green house gas emissions (GGEs) while offering the potential of advanced products with improved properties [1]. Despite the apparent and significant benefits of biological approaches, to date a relatively low percentage of industrial bioprocesses have been successfully commercialized using engineered microbes. Success stories have been limited primarily to amino acids, organic acids and select specialty and fine chemicals [2]. Outside of pharma, nutrition and specialty chemicals, the development of commercially viable

bioprocesses, which are competitive with traditional chemical catalysis and petroleum feedstocks remains a key barrier to the field. This is in large part due to the large technical challenges that are faced, many of which are unique to each specific product and market. These commercial hurdles are particularly large when considering the highest volume and lowest value products such as commodity chemicals and fuels, which require very low costs of production to be competitive, but also have the largest potential environmental impacts.

In face of these challenges, at its core, the general approach to metabolic engineering has remained largely unchanged since the field emerged as a defined discipline only a little over two decades ago [3]. Figure 1 illustrates the typical progression in engineering strain metabolism in order to improve product rates, titers and yields. First a heterologous pathway, capable of producing the desired chemical is identified and introduced into a chosen microbial host. This is followed by the deletion of any non-essential competitive metabolic pathways, which lead to undesirable byproducts and reductions in yield. After these initial standard and often simple approaches are taken, efforts to improve the heterologous production pathway are made in parallel with those to engineer a more optimal background metabolic network. Iterations of the process illustrated in Figure 1 have recently been rebranded as design, build, test (DBT) cycles, in reference to analogies between synthetic biology and electrical engineering and computer science [3, 4]. Numerous advances in technology have made achieving the “proof of concept” production of numerous non-native products increasingly facile [5-10], and technologies aimed at increasing the speed of iteration through DBT cycles have gained momentum in recent years [3, 11]. However technologies that have the potential to significantly push the boundaries on potential yields, productivities and titers are sorely lacking.

In order for bioprocesses utilizing engineered microbes to truly compete with current technologies for the large scale production of bulk and commodity chemicals as well as biofuels, step changes in performance are needed, which can lead to both reduced operating and capital costs. Technologies are not only needed to speed up and/or parallelize DBT cycle iterations, but also to systematically tackle the more complex challenges that currently limit production rates, titers and yields. This means creating new paradigms and challenging old assumptions. This review discusses several recent approaches that represent initial steps to push the boundaries of the field and approach problems that have been traditionally off-limits. Specifically, new strategies to engineer essential metabolism, ie metabolic pathways essential to cellular growth and viability, are discussed. Essential metabolic networks that compete for product formation have historically been difficult to engineer due to the negative impacts on growth, and represent boundaries limiting improvements in rates, titers and yields. Two general approaches have emerged over the past several years to push these boundaries and controllably modify essential competitive metabolic pathways. These are illustrated in Figure 2. These approaches include the static rewiring or rebalancing of the essential metabolic network as well as the dynamic regulation of essential fluxes. While not solely deployed toward the production of commodity chemicals and biofuels, these approaches, which push the boundaries of the field, have the potential to lead to significant improvements in performance metrics and as a result the competitiveness of numerous bioprocesses.

Rewiring the Metabolic Network

One approach to managing competitive essential metabolism is to rebalance or rewire the metabolic network. This often involves engineering reduced activity of key enzymes in competitive pathways while simultaneously optimizing the activity levels of production pathway enzymes. Metabolic engineers have utilized advanced approaches in gene regulation, introducing higher levels of transcript control in microbial cell factories, in order to reduce competitive pathway fluxes. Methodologies that have been leveraged include transcriptional “tuning” or repression via small inhibitory, antisense or regulatory RNAs [12-17], recently developed gene-silencing methods relying on CRISPR-cas systems [18-23], the use of feedback or dynamically regulated promoters [24-26] and engineered metabolic network branch points to enable controlled expression of essential competitive enzymes [27]. Together these approaches have resulted in anywhere from modest to significant fold improvements in production.

Tuning gene expression has recently been applied to multiple gene targets in parallel. For example, Na et al. recently reported using a library of synthetic small regulatory RNAs (sRNAs) to tune the expression levels of up to four candidate central metabolic and regulatory proteins improving the production of the amino acid tyrosine (up to 2g/L) and cadaverine, a potential commodity diamine, (up to 2.15 g/L) in *E. coli* [28]. In a similar approach, Nakashima et al. screened a library of synthetic small antisense RNAs to repress the expression of 71 genes involved in central carbon metabolism, aimed at improving the flux to the nutritional supplement pyruvic acid [29]. This effort led to a 5-fold improvement in pyruvate titers reaching over 25 g/L. Using CRISPR based interference in *E. coli*, Wu et al. engineered the parallel repression of up to six essential central metabolic enzymes resulting in significant increases in the flux of the central metabolite malonyl-CoA and as a result an over 7-fold increase in the production of the flavonoid naringenin [30] which has potential anti-cancer properties.

Tuning has also been engineered using natural metabolite responsive promoters. Yuan and Ching recently developed a system relying on a feedback regulated promoter to improve production of the terpenoid and antimalarial precursor, amorpha-4,11-diene in *S. cerevisiae* by over 3.5 fold [24]. In this yeast ergosterol is a natural essential metabolite but also a byproduct competing with amorphadiene for a common precursor: farnesyl pyrophosphate (FPP). Metabolic network rewiring was accomplished by replacing the native promoter for the gene encoding the first committed step in ergosterol biosynthesis from FPP with a promoter repressed by ergosterol, resulting in byproduct inhibition of byproduct formation and improved amorphadiene production.

Flux optimization has also been engineered by introducing non-native pathway branch points, which enable the tuning of competitive fluxes by controlling key enzyme expression levels. Solomon et al. recently engineered an *E. coli* strain with a 2-fold improvement in the production of the bulk chemical gluconate from glucose [27]. In order to be able to reduce the utilization of glucose for cellular processes, the approach involved engineering an alternative non-phosphotransferase, permease dependent, uptake system, by which glucose enters the cell. After entry, glucose can either be utilized for gluconate production via a single enzyme or alternatively phosphorylated to glucose-6-phosphate by the *E. coli* glucokinase encoded by the *glk* gene. Glucose-6-phosphate is

then consumed in primary metabolism. By tuning expression levels of the *glk* gene in a strain with an altered glucose uptake system improvements in gluconate flux were observed.

Dynamic Flux Control

While enabling manipulation of essential metabolic pathways, a major remaining challenge to approaches involving network rewiring is balancing the complex needs of the microbial host while redirecting flux to a desired product. Dynamic metabolic control offers an alternative approach, wherein fluxes are engineered to switch from a state more optimal for cellular growth to one more optimal for product formation. This strategy can allow for the decoupling of growth from product formation, theoretically enabling higher maximal yields and simplifying engineering strategies for the optimized production state [31, 32]. Key to dynamic metabolic control is the development of genetic systems, which enable dynamic flux redirection. Similarly to the case of static network rebalancing, metabolic engineers have developed methods for the dynamic control of enzyme levels by engineering transcriptional control. In addition, methods have been increasingly developed to engineer enzyme activities by directly modifying protein levels or activities post-translation.

Transcriptional Toggle Switches

One set of tools for dynamic metabolic engineering are genetic “toggle” switches, which enable the inducible repression of genes including essential enzymes responsible for competitive metabolic fluxes [33-40]. A common essential and competitive metabolic pathway is the citric acid or tricarboxylic acid (TCA) cycle. This pathway consumes key metabolites resulting in reducing power (NADH and NADPH), energy (in the form of ATP) and carbon dioxide (CO₂) as a key byproduct. Flux through the TCA cycle can often compete with the flux to product, in many aerobic production pathways. Citrate synthase is the first committed step of this cycle. Soma et al developed a “toggle switch” to dynamically repress expression and therefore levels of the citrate synthase of *E. coli* encoded by the *gltA* gene. This was implemented to improve the production of the commodity chemical isopropanol [41]. The toggle switch resulted in an over 3 fold improvement in both yield and titer which reached ~3g/L.

Glyceraldehyde-3-phosphate dehydrogenase is another essential enzyme, involved in glycolysis, which has been successfully dynamically repressed. Tsurono et al. recently implemented a toggle switch to repress the *E. coli* glyceraldehyde-3-phosphate dehydrogenase (encoded by the *E. coli gapA* gene) in order to redirect flux to the platform chemical 3-hydroxypropionate (3-HP), a precursor to several commodity chemicals including acrylic acid, from the feedstock glycerol. This strategy resulted in over a 40% improvements in 3-HP titer [42].

Alterations in enzyme activities have also been used to create sinks for key metabolites, enabling flux redirection and optimized product formation [43, 44]. Wu et al. were able to optimize the production of the bulk chemical, lactic acid, which is normally an anaerobic fermentation product, under aerobic conditions [44]. This was accomplished by the heterologous expression of a pathway to consume and as result reduced the

quinone pool, which is essential for the transfer of reducing equivalents from NADH to molecular oxygen. This strategy, which the authors termed a “metabolic transistor”, resulted in elevated NADH levels and improved lactic acid yields.

Metabolic Valves

Recent successes have also been achieved using dynamic metabolic control strategies that rely on the post-translational control over enzyme activity. These systems, often referred to as metabolic valves, rely on controlled inactivation of key enzymes or in many cases controlled or tunable proteolytic degradation [45-47].

Lynch et al. engineered a modified strain of *E. coli* to produce high-levels of 3-HP, through the metabolic precursor malonyl-CoA, using a two stage fermentation process and a metabolic valve relying on a temperature sensitive essential enzyme [48]. In this approach a temperature sensitive enzyme involved in fatty acid biosynthesis, the enoyl-ACP reductase, encoded by the *fabI* gene, was utilized. At the permissive temperature flux through fatty acid biosynthesis proceeds normally, at elevated temperatures, the mutant *fabI* allele is nonfunctional and flux is eliminated. This approach not only enabled the temperature-controlled elimination of a competitive essential metabolic pathway, but the simultaneous activation of precursor synthesis. As a consequence of eliminating fatty acid biosynthesis, levels of acyl-ACP intermediates, potent inhibitors of acetyl-CoA carboxylase (*accAse*), the enzyme producing malonyl-CoA, are greatly reduced. This resulted in higher *accAse* activity and malonyl-CoA fluxes. This approach led to an over 25-fold improvement in 3-HP biosynthesis in fed batch fermentations, with titers up to 40 g/L. Strains based on this platform were further engineered to produce over 70g/L of 3-HP in two-stage fermentations [49]. A similar approach was implemented by Cho et al., which involved engineering glyceraldehyd-3-phosphate dehydrogenase (*gapA*) mutants with altered activities at different temperatures [50]. In this case a downshift in temperature resulted in inhibition of *gapA* activity and reduction in glycolytic flux.

Metabolic valves, relying on controlled proteolysis, have also been recently implemented to improve product fluxes. Brockman et al. improved microbial metabolism toward the production of glucaric acid utilizing controlled degradation of the essential glycolytic enzyme phosphofructokinase, encoded by the *pfkA* gene of *E. coli* [51]. This approach involved tagging the enzyme with a C-terminal tag, which targeted the enzyme for proteolysis by the *clpXP* protease only in the presence of a chaperone that recognizes the tags. Induction of chaperone expression resulted in proteolytic degradation of phosphofructokinase. By reducing glycolytic flux, glucaric acid produced from upstream glycolytic intermediates was improved by approximately 3-fold reaching about 1.5g/L. This approach is generalizable to the dynamic control of many essential metabolic pathways.

Conclusions

The standard of skill and expertise in synthetic biology and metabolic engineering has made significant strides over the past 25 years. These fields have matured to the point, where currently, scientists skilled in these disciplines can readily engineer

microbes allowing for the measurable (“proof of concept”) production of numerous chemical products with a range of market applications. Commercial feasibility can be envisioned readily for products with very high value. However, the development of competitive bioprocesses for bulk and commodity chemicals as well as biofuels has remained largely out of reach. It is in these areas where metabolic engineering approaches have the greatest potential for environmental and societal impact. As the field moves into its third decade, a key challenge is to push the boundaries that limit current process performance, which include the boundaries imposed by the microbial cell factories themselves.

This review has highlighted several recent and more advanced approaches to engineering essential aspects of the cellular metabolic network, with the goal to overcome production limits imposed by essential cellular processes and more efficiently redirect carbon toward product formation. These approaches, including static rewiring and dynamic metabolic control, have been largely implemented in the well-characterized platform hosts *E. coli* and *S. cerevisiae*, and applied to a variety of products including fine, specialty, bulk and commodity chemicals. Together these approaches have resulted in a range of process improvements, from modest (10-20% improvements) to impressive (> 40 fold). Conceptually, these approaches alone and potentially in combination can be adapted to multiple additional products and microbial hosts. Particularly, the generalizable nature of newer gene silencing technologies, including CRISPR interference, make transcriptional tuning an attractive platform for any desired microbe. Better metabolic models will likely be needed in order to choose the optimal combinations of both tuning targets and repression levels, for optimal production. This will also be true when deploying dynamic control strategies in other hosts and for other products. To date the successful demonstration of metabolic valves, have relied on the choice of readily identified enzyme targets, which are directly competitive for a particular production pathway. For example, targeting fatty acid biosynthesis for the production of 3-HP from the shared precursor malonyl-CoA.

While these technologies represent promising initial steps in pushing important boundaries imposed by the cell themselves, significant advancements are still needed to achieve the lofty performance metrics required for many potential bioprocesses. It is good to see that experts in the field are moving past easy problems and simple solutions and tackling more difficult challenges related to the engineering of complex, essential metabolic pathways. Future areas for promising research include the engineering of other essential metabolic and physiologic systems that currently limit bioprocess performance. Natural barriers still include limitations in sugar or other feedstock uptake rates and product tolerance levels, which may need to be well above 2 molar in many cases. Although many challenges still remain, these recent efforts further support the potential of this discipline in making a significant impact in the production of high volume industrial products, with the potential to displace petroleum with more sustainable alternatives.

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Figure 1: Typical Industrial Strain Engineering Process. (LEFT) First a heterologous pathway is introduced to produce the product of interest. (A) Next pathway expression is optimized and key non-essential byproduct producing pathways are deleted. (B) Host metabolic network optimization. The host's metabolic network is engineered to supply the precursors, cofactors and energy needed for optimal product formation while minimizing unwanted competitive metabolic fluxes.

Figure 2: Engineering Essential Competitive Metabolic Pathways. **Static Rebalancing** involves optimizing heterologous pathway expression while tuning or repressing the levels of key competitive essential enzymes. Controlled repression can be accomplished with RNAi and CRISPRi. Transistors involve the induction of enzymes to consume key essential metabolites resulting in the redirection of fluxes. **Dynamic Control** can be engineered through either toggle switches or metabolic valves. Toggle switches enable controlled and dynamic repression of competitive essential enzymes or pathways. Metabolic valves entail controlled proteolytic degradation or inducible inactivation of essential enzymes.

Graphical Abstract