“Metabolic Pathway Balancing and its role in the production of biofuels and chemicals”

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Keywords:

Pathway Balancing; Metabolic Engineering; Genetic Optimization; Post-translational Optimization

Abstract:

In the last decade, metabolic engineering benefited greatly from systems and synthetic biology due to substantial advancements in those fields. As a result, technologies and methods evolved to be more complex and controllable than ever. In this review, we highlight up-to-date case studies using these techniques, examine their potential, and stress their importance for production of compounds such as fatty acids, alcohols and high value chemicals. Beginning with basic rational control techniques and continuing with advanced level modern approaches, we review the vast number of possibilities for controlling metabolic fluxes. Our aim is to give a brief and informative insight about commonly used tools and universalized methodologies for metabolic pathway balancing and optimization.

Introduction:

Due to increasing energy demands and high ecological stresses placed on naturally derived natural products and petrochemicals, there is an ever-increasing need for the development of microbial-based production methods that can reduce or eliminate the need for chemicals derived from their native sources [1,2]. Many researchers have strived to answer this growing demand by using metabolic engineering techniques to develop microbes to produce many non-native biofuels and chemicals with varying degrees of success. Recently several researchers have demonstrated that major improvements in yield, titer, and productivity can be accomplished by balancing metabolic pathway gene expression [3–5]. We will discuss several approaches through which a pathway can be balanced, highlight some recent tools that have been developed to aid in modular pathway optimization, and review some of the most successful examples of metabolic pathway balancing published to date.

The principle objective of balancing a metabolic pathway is to produce more of a target product through reducing potential flux imbalances in the host organism. This is mainly accomplished by eliminating the production of excessive intermediate metabolites and precursors leading to efficient conversion of intermediates, substrates, and co-factors to desired products. Pathway balancing not only looks to reduce the build-up of pathway intermediates but also focuses on balancing components such as mRNA, which has been determined to be one of the primary components of metabolic burden in plasmid-based systems [6]. Several excellent reviews on systems metabolic engineering and synthetic biology have highlighted the motivation and need for pathway balancing [2,7,8].

Even though nature has optimized metabolite production for its needs, these specific optimal solutions are not compatible with industry-level overproduction demands. Thus, there is still a great need for further balancing efforts to meet the needs of industrial scale production. To this end, techniques for pathway optimization and balancing are often combinatorial and iterative in nature and require substantial screening for an optimal solution to be determined. Current high-throughput techniques are highly

amendable for fluorescent and colored products leaving a substantial demand for high-throughput screening assays that can detect modest improvements in titer for most non-fluorescent and non-colored products [9–11]. Even though there are novel approaches for automation, such as Multiplex Automated Genome Engineering (MAGE) and Yeast Oligo-mediated Genome Engineering (YOGE) [12–14], this hurdle still highlights one of the biggest challenges for the future of pathway balancing and optimization.

Techniques for Pathway Balancing:

Metabolic pathway balancing approaches can be summarized in six categories as highlighted in Figure 1:

1. DNA copy number modulation via plasmid overexpression or chromosomal integration,
2. Transcriptional modulation via promoter engineering,
3. Translational modulation via ribosome binding site (RBS) engineering,
4. Post-translational modulation using synthetic scaffolds to increase spatial orientation of substrate,
5. Dynamic Balancing using synthetic gene circuits to sense metabolite levels and adjust pathway flux in real-time,
6. Compartmentalization via subcellular organelles and/or protein shells.

Each level of modulation has its advantages and disadvantages and each application can benefit differently through implementation of different pathway balancing methods.

FIGURE 1 HERE

DNA-level Optimization

As a straightforward, quick and easy-to-apply technique, DNA copy number modulation is the most frequently applied method for pathway optimization. Many compatible vectors spanning a large range of copy-numbers and expression levels are available for most common industrially relevant microorganisms. As a brief summary, early metabolic engineering efforts mostly focused on rational approaches to increase the availability of precursors and intermediates by deleting competing pathways and over-expressing desired pathways on primarily high copy number vectors with strong promoters to achieve maximum production rates. In addition to ignorance of promoter, RBS, enzyme turnover efficiencies, and substrate availability, this more-is-better mentality led to only moderate strain improvements due to the propensity of the unbalanced pathways to build-up unnecessary and commonly toxic intermediate products [4].

A modern-era metabolic engineering example, Multivariate Modular Metabolic Engineering (MMME) must be highlighted as a method for DNA-level optimization that

was recently used to maximize the production of free fatty acids, a biofuel precursor, in *E. coli* [5]. In this study, overexpression targets for the production of the primary precursor, malonyl-CoA, as well as the fatty acid synthesis pathway genes were broken into three modules. These three modules were balanced using three different copy number ePathBrick vectors [15] to determine the best expression levels yielding the highest fatty acid production. Screening of eight strains, each with different expression levels for each module, demonstrated an approximate 4-fold improvement in titer solely via DNA-level optimization (Figure 2).

FIGURE 2 HERE

Chromosomal integration is an alternative and powerful technique to reduce metabolic burden (no plasmid replication and maintenance), improve strain growth and decrease fermentation costs (no antibiotic selection). For these reasons, techniques for *E. coli* chromosomal integration have gained much interest in the literature. Chromosomal integration of pathway genes have shown greatly improved titers for the production of lycopene [16] and taxadiene [3] over their corresponding plasmid based systems.

Chromosomally integrated constructs are also subject to the same modulation and screening as plasmid based systems to determine the appropriate expression rates for the highest product titer. The difficulty of creating these libraries of chromosomal integrations (as compared to plasmid-based libraries) is the primary factor holding back substantial chromosomal integration-based pathway optimization studies. Many methods utilizing a wide range of techniques have been developed for site-specific chromosomal integration in *E. coli* [17–21], but have yet to produce a system for high-throughput, easy-to-apply, and stable incorporation of entire pathways into the chromosome. Initial studies on integration based-technologies to produce double stranded DNA breaks [21,22] as well as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-based technologies [23] show promising results towards the production of chromosomal integration libraries in both *E. coli* and yeast. The ability to create libraries of chromosomally integrated constructs for screening will be of utmost importance in the future of pathway optimization.

RNA-level Optimization

Transcriptional level pathway optimization is accomplished by using a library of different strength promoters to vary the mRNA levels of individual genes, transcripts, or modules *in vivo*. RNA-level optimization has several advantages over optimization at other levels due to substantial work done to create promoter libraries, both constitutive [24–28] and inducible [29,30], with a wide range of activities. A recent excellent review on all aspects of promoter engineering has been published by Blazeck and Alper [31]. With organizations such as International Genetically Engineered Machines (iGEM) foundation and The BioBricks Foundation, recent growth of public-domain standard biological parts has been steadily increasing over the past several years (although not without

controversy) [32]. The up-front balancing accomplished using transcriptional-level optimization has an advantage over translational and post-translational balancing because optimization at the RNA-level minimizes the unnecessary overproduction of mRNA and subsequently protein inherent to post-transcriptional balancing which in turn will help to reduce metabolic burden and exhaustion of ribosomes. One characteristic example that highlights the potential of promoter engineering combined a library of constitutive and inducible promoters in *Saccharomyces cerevisiae* creating synthetic hybrid promoters to expand and enhance promoter activity and control over metabolic flux [33]. Furthermore, combinatorial promoter libraries have been applied to individual genes, rather than modules and operons, to give fine-tuned control over pathway flux for violacein production [34] and xylose utilization [35,36] in *S. cerevisiae*.

Protein-level Optimization

Translational balancing by ribosome binding site (RBS) modification is similar to that of transcription optimization in that it is another well-studied method. The Salis Lab at Penn State University and others have championed the groundwork for understanding the RBS sequence to RBS strength relationship [37–39]. Their development and continuous improvement of the RBS Calculator is a useful tool for all metabolic engineers and systems biologists attempting to optimize a pathway via translational balancing [40,41]. The RBS calculator predictions have been vetted against experiential findings and one such study found the data to have an $R^2 > 0.98$ indicating a superb correlation across a wide range of predicted RBS strengths [42]. Despite the aforementioned advantages of DNA and RNA-level optimization over protein-level optimization, several investigators have shown success using RBS libraries to increase product titers [5,29,43,44].

Post-translational Optimization

The co-localization of pathway enzymes to facilitate substrate tunneling is the primary method employed for post-translational pathway optimization. This is accomplished through the use of protein fusions for enzyme cascades and synthetic scaffolding proteins to dock enzymes in close proximity to one another [45]. The development and implementation of synthetic protein scaffolds has achieved a 77-fold improvement in mevalonate production [46], a 5-fold improvement in glucaric acid production [47], and a 3-fold improvement in butyrate production [48] over solely plasmid-based expression of pathway enzymes. Like other methods of pathway optimization, post-translational balancing does not benefit from reducing the production of surplus quantities of RNA or protein, but rather, by improving the efficiency of substrate transfer from enzyme to enzyme, minimizing diffusion, before the substrate reacts with the enzyme. Scaffold based optimization techniques benefit from the formation of microdomains with extremely increased metabolite concentrations in the cytosol [49]. If substrate trafficking and diffusion is not limiting, the application of scaffolding proteins for pathway optimization will show little to no improvement, or potentially worsen

production due to the metabolic burden associated with scaffolding protein production or blocking the access of substrate to active site due to complicated 3-D structures of scaffold-enzyme complexes in overexpressing strains.

Similar to synthetic protein scaffolds, the importance of DNA- and RNA-based scaffolding for spatial and temporal control over metabolic flux has been shown recently [50,51]. In 2010, an iGEM team from Slovenia won the grand prize in the jamboree by developing zinc-finger protein-DNA scaffolds, demonstrating the elimination of side products in the violacein pathway, and improving catalytic efficiencies in mevalonate, 1,2-propanediol, and *trans*-resveratrol pathways [52]. Likewise, RNA-based scaffolds are used to improve microbial alkane production through several different 1-D and 2-D orientations co-localizing pathway enzymes [53]. A recent excellent review covering the brief history of nucleic acid based scaffolding and its potential in metabolic engineering and synthetic biology has been published [54].

Dynamic Balancing and Dynamic Sensor Regulatory Systems (DSRS):

It is commonly accepted that the application of a closed loop control system to dynamically modulate a given system based on feedback from external conditions can greatly improve the process efficiency and reduce sensitivity to disturbances. Traditional static pathway balancing techniques, presented above, enhance the open loop dynamics whereas other synthetic biology techniques, presented below, are used to “close the loop”. Early synthetic biology applications of genetic circuits and promoter regulatory systems [55,56], caused metabolic engineers to give more importance to real time response to intracellular metabolite levels. Dynamic balancing or the process of altering *in vivo* gene expression through applications of synthetic biology and genetic circuits is the most recent development in the field of pathway optimization and balancing. The ability to use genetic feedback systems to, in real-time, up-regulate or down-regulate pathways by internally sensing metabolite levels potentially demonstrates the most powerful example of pathway balancing technology.

Substantial work has been done to create regulatory components for quorum sensing and dynamic control of cellular function [57–60]. Using this available knowledge, investigators have shown marked improvements in product titers of lycopene [61] and fatty acids [62–64] by implementation of dynamic balancing systems. The development of an anti-sense RNA and dynamic inverter regulated metabolic valve for glucose uptake in *E. coli* demonstrated the ability to redirect glucose flux away from central carbon metabolism for direct use as a substrate [65]. This technology enabled a 50% reduction in specific growth rate without altering biomass accumulation. Most recently, the malonyl-CoA sensing, regulator protein FapR from *Bacillus subtilis* and native *E. coli* promoter, pGAP, were incorporated into a dynamic control circuit to control fatty acid production in *E. coli* [62]. This was accomplished through regulation of the intracellular malonyl-CoA pool using overexpression of acetyl-CoA carboxylase (ACC) to increase intracellular malonyl-CoA (source pathway) and overexpression of the fatty acid

synthesis (FAS) pathway to utilize malonyl-CoA (sink pathway). This documented a 3-fold improvement in fatty acid production over static over-expression of ACC and FAS modules.

Compartmentalization:

As further improvements to scaffold-based systems are obtained, compartmentalization of those components rises as a viable tool for metabolic engineering and systems biology applications [66]. Several successful applications of this technique for farnesyl diphosphate (FDP) control in terpenoid production and redirection of the Ehrlich pathway to produce branch-chained alcohols by employing compartmentalization in the mitochondria of yeast have highlighted the potential of this technique in eukaryotic systems [67,68]. The target location for compartmentalization will vary with the needs of each application. Eukaryotic organisms contain a variety of organelles with various conditions and intracellular metabolite availabilities while, prokaryotic systems consist of a variety of encapsulins, lumazine synthase complexes, and bacterial micro-compartments (BMCs) with diameters from 20 to 100nm [49,69,70]. Despite successful regulation over formation of empty protein shells, this field is still in its infancy for prokaryotic systems [71]. However, recent advancements in control of encapsulated enzyme activity represents a promising step towards *in vivo* application [72]. Control of substrate and product solubility, selectivity, and intermediate product diffusion through pores in the protein shell highlight the major hurdles for the successful utilization of compartmentalization for pathway optimization and balancing. Nevertheless, compartmentalization shows promise to give superior control over pathway bottlenecks and to allow for reduction in the effects of toxic compounds. This would permit higher titer production of toxic products with reduced affect on cell viability and growth. As metabolic engineering efforts push the limits of cells toxicity with ever-higher product titers, methods such as compartmentalization will gain popularity as a technique to further increase production.

Bottlenecks and Limitations in Pathway Optimization:

The primary global disadvantage is the number of configurations that must be considered to determine a global optimal solution. When traditional cloning is utilized, the ability to construct hundreds of configurations of pathway genes with varying copy number, promoter, and ribosome binding site (RBS) strengths is a daunting and time consuming task even for small pathways. Luckily, with advances in metabolic modeling, liquid handling capabilities, and high-throughput screening many configurations can be eliminated from the solution space and the time required for testing and screening has been reduced substantially in the past several years. Also, depending on the application, not all methods of pathway optimization need to be applied concurrently.

Although there have been substantial improvements in high-throughput screening technologies, many desirable products still lack amenability to high-throughput

screening [34]. To counter this problem, algorithm-aided mapping, various mechanistic or non-mechanistic modeling approaches, and computational optimization strategies have been employed to model the production landscape across all possible configurations of genetic elements [73]. In one unique example, the predictability of multi-enzyme pathways was investigated by using sequence activity maps (SEAMAPs) and the RBS Library Calculator (to generate necessary expression space) to demonstrate an approximate 100,000-fold range in protein expression [73]. Furthermore, this approach could easily be used in an iterative optimization study by balancing the flux control coefficients (FCC) of the pathway enzymes to reach an “optimally balanced” pathway [73].

A secondary disadvantage to pathway optimization is the inability for the optimized solution to be conserved between small-scale screening and fermentation conditions. Many pathways, such as the fatty acid synthesis pathway in *E. coli*, are full of regulatory mechanisms that govern flux through the pathway as a function of internal (i.e. repression by build up of product) and external conditions (i.e. temperature, oxygen saturation, cell density, etc.). These regulatory conditions can vary drastically from small scale screening to large-scale high cell density cultures obtained from fed batch fermentation studies. It has been shown many times in the literature that the scale-up optimizations are as much important as stable, overproducing, genetically optimized strain. To this end, much work still needs to be done to develop general guidelines for the scalability of carefully balanced pathways.

Conclusion:

Regardless of pathway, not every optimization strategy will result in higher titers, yield, or productivity. The choice of correct optimization level is central to the success of any pathway-balancing project. Recent work has demonstrated that substantial improvements can be obtained through many different avenues of optimization. Further advances in high-throughput screening of large static libraries and creative development of dynamic balancing systems represent the largest needs for the field of pathway optimization as well as computer-aided modeling approaches. It is clear that balancing and optimization merges the fields of metabolic engineering and systems biology to develop tailor-made microbial factories for the efficient production of chemicals and biofuels at titers that are now approaching feasible levels to replace products derived from natural sources.

Acknowledgements:

The authors would like to acknowledge Daniel P. Howsmon for discussions related to control theory and dynamic balancing. This work was supported by the ARPA-E REMOTE program grant number DE-AR0000432 awarded to Mattheos Koffas and the Fulbright Scholars Program grant awarded to Ö. Duhan Toparlak.

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Figure Legends:

Figure 1. Six major approaches to optimize metabolic pathways in common laboratory organisms such as *E. coli* and *S. cerevisiae*. The left and right hand side of the figure represent modern and classical approaches, respectively. Modern techniques can be summarized as dynamic metabolite monitoring and balancing through critical intermediate chemicals, spatial organization of enzymes by using synthetic scaffolds or fusion proteins, and organelle-level compartmentalization of both metabolites and pathway enzymes to take advantage of elevated concentrations of substrates and enzymes. On the other hand, classical techniques include utilizing plasmid copy number or chromosomal integration modularity by combinatorial approach, gene expression level control through promoter engineering, including synthetic hybrid promoters (e.g. regulation through toxic chemicals or specific precursors) and lastly, ribosome binding site engineering for each different pathway gene to optimize and normalize their translational efficiencies.

Figure 2. Modular pathway optimization using plasmid copy number. The overexpression targets for fatty acid production were broken into three modules: Glycolysis (GLY), Acetyl-CoA Activation (ACA), and Fatty Acid Synthesis (FAS). Each module was expressed at one of three expression levels: High (pETM6 or pRSM3), Medium (pCDM4), or Low (pACM4 or pCOM4) using ePathBrick expression vectors. Data presented as free fatty acid titer normalized to lowest producing configuration. This result highlights the power of pathway balancing and the ability for maximized production from lower copy number overexpression vectors.

Annotated References:

(●) Special Interest

- [15] The authors developed a modular platform for DNA assembly using isocaudomer restriction enzyme pairs. This set of duet-based vectors allows for cloning and expression of entire pathways in three operon configurations.
- [28] The authors characterize standard biological parts in an attempt to predict quantitative behavior for novel combinations of genetic elements. Using their method they estimate, with 93% reliability, to within 2-fold of target expression. This work focuses on increasing the reliability of forward engineering projects using public-domain standard biological parts.
- [33] In this paper the authors created a synthetic hybrid promoter library by combining constitutive and inducible promoters in yeast. Even though they did not test every single promoter activity within a metabolic pathway, the library they created spans a great range of expression levels to control metabolic pathway genes. Promoters can be regulated by different chemicals, such as galactose, so that the metabolic pathway can be optimized and balanced.
- [48] Application of pathway optimization using protein scaffolds resulted the highest reported titers of butyrate of 7.2g/L after fed batch fermentation.
- [58] Utilizing the natural fatty acid regulator protein, fadR, the authors developed a dynamic sensor-regulator system for the production of fatty acid ethyl esters in *E. coli*. This dynamic sensor backbone can be applied to work with a wide variety of biosynthetic pathways for which natural sensors exist.

(●●) Outstanding Interest

- [5] This paper shows an elegant example of DNA and RBS optimization in *E. coli* producing 8.6g/L free fatty acids, the highest reported titers to date.
- [29] The authors have created an inducible, high-level, T7-based expression system that is orthogonal to traditional T7 expression. They demonstrated this orthogonal system through the production of two colored products, lycopene and deoxychromoviridans. This technology opens exciting avenues into future applications in metabolic engineering and systems biology.
- [53] The authors have designed RNA-based scaffolds to co-localize enzymes in 1-D or 2-D geometries to increase production of alkanes and succinate. They have shown that due to a labile intermediate in the alkane pathway, pentadecane synthesis is dependent on geometry of co-localized enzymes via their RNA-binding-domain and aptamer orientations.
- [62] Using the malonyl-CoA sensing ability of the FapR protein, the authors developed a genetic circuit capable of regulating the intracellular malonyl-CoA pool through the controlled expression of both source and sink modules. This *in vivo* balancing led to 15.7 fold improvement in fatty acid titer.

- [68] These authors' work represents one of the first remarkable and inspiring examples of compartmentalization. By using localization signals, they redirected the overexpressed pathway genes into mitochondria of yeast to take the advantage of high levels of Ehrlich pathway precursors to convert them into branched chain alcohols. By doing that, they have shown 260% increased levels of alcohol compared to cytoplasmic overexpression strain.
- [73] The paper presents a revolutionary method for metabolic pathway modeling. Using SEAMAPs, the production landscape was measured to cover a 100,000-fold range of protein expression. Biological characterization results show agreement with theoretical predictions and significant improvement over previous efforts. The work presented here has profound implications for future studies of metabolic network modeling and one day may lead to the ability for optimized *a priori* design.

Figure 1

Figure 2:

Graphical Abstract: