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A Review of Computational Approaches for *In Silico* Metabolic Engineering for Microbial Fuel Production

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Abstract: High energy consumption nowadays alongside with concerns on the environment had caused rising demand for synthetic alternative fuels. These include biofuels that can be produced from a variety of engineered microbes such as *Escherichia coli*. In the metabolic engineering field, this is done by genetically modifying the target microbes to obtain optimal production of a particular biochemical. Conventional metabolic engineering approaches often intuitive, but with advancements in modern biology, vast amount of informative data generated from time to time to describe the metabolism system of the microbes more thoroughly. Discoveries from interpreting these available data using computational approaches are highly beneficial to metabolic engineers, especially professionals working in the *in silico* metabolic engineering field. Within the past decade, many computational approaches and routines have been proposed and developed in providing a platform to discover rational strategies to aid biologists in engineering the metabolic network. Here, efforts to find the optimal butanol production route in *E. coli* as well as several optimization algorithms currently available for finding optimal solution to enhance biochemical production in designated target microbe are discussed. This review aims to show different optimization algorithms developed for *in silico* metabolic engineering and their applications in microbial fuel production.

Keywords: Biofuels, computational biology, *in silico* metabolic engineering, metabolic networks, microbial strain improvement, optimization, system biology.

1. INTRODUCTION

Rapid advancements in transportation and technology have increased the demand for more liquid fuels. At the present time, the major source is from fossil fuels, but depleting resources coupled with increasing demand have urged for the development of alternative sustainable fuels to suffice the need. Being one of the alternative fuels, biofuels have garnered considerable attentions where one of the common solutions is through conversion of biomass to ethanol. As the very first biofuel being adapted, ethanol is unfortunately impractical due to its low energy density, high hygroscopicity and inability to blend well with gasoline [1]. Thus, higher chain alcohols such as butanol, which possess higher energy density and lower hygroscopicity, have become the main focus of scientists. These potential alternative fuels are natively produced by certain microorganisms such as *Escherichia coli*, *Saccharomyces cerevisiae*, *Clostridium acetobutylicum*, and so on. However, native production of the aforementioned biochemical compounds by microorganisms is insufficient to meet industrial production level. This has become a challenge for metabolic engineers to create a cellular system that can give optimal yield efficiently and economically [2].

Metabolic engineering was introduced almost 20 years ago. It is a distinct field from genetic engineering that focuses on biosynthetic and metabolic pathways [3] and acts as the main platform to improve the design of microbes strain including those in biofuels production. To date, efforts have been poured into engineering both native and non-native production host in biofuel production. In order to achieve large scale production with ease of modification, industrial microorganisms such as *E. coli* and S. cerevisiae are often used since there are well-established genetic tools and long track record of successful industrial applications for these microorganisms [4]. Most early endeavors are often intuitive and with the large amount of omics data being generated these days, intuitive analyses and interpretations are no longer relevant to aid the illustration and understanding of the metabolic behavior of target microbes. Moreover, it is also crucial that the analyses and interpretations performed can further aid in finding optimal and rational strategies in engineering these microbes; this field of research is known as computational or *in silico* metabolic engineering. *In silico* metabolic engineering involves the modeling, optimization and simulation of related microorganisms to computationally obtain valuable prior knowledge on the metabolic system [5] so that rational interventions strategies can be proposed (Fig. **1**). To date, vigorous attempts have been engaged to develop numerous approaches, but studies on biofuel (ethanol or butanol) productions are considerably lesser.

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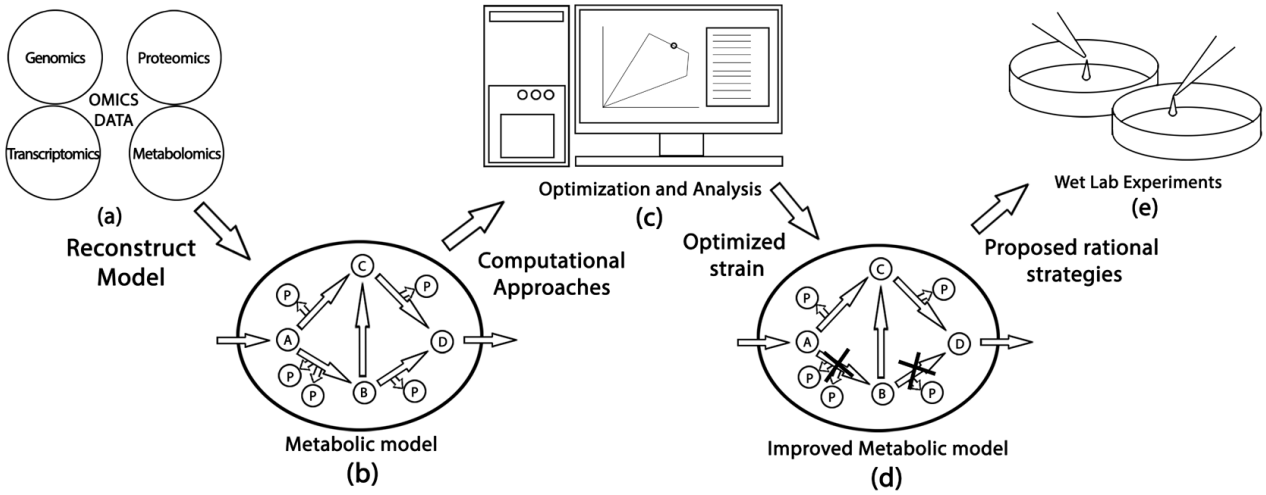

Fig. (1). Illustration of *in silico* metabolic engineering where (**a**) omics data are utilized to reconstruct the genome-scale metabolic model; (**b**) the metabolic model is then used as an engineering platform using computational approaches which involve (**c**) optimization methods and analyses to predict the interventions to get optimal phenotype. (**d**) The improved metabolic model will be validated and the results will decide the feasibility of the strategies predicted. The system will also propose the strategies for (**e**) wet lab experiment for real industrial production.

Here, a brief review will be given on these approaches and other available approaches to facilitate better understanding on current advances in *in silico* metabolic engineering.

2. *IN SILICO* GENOME-SCALE METABOLIC MODEL

Recent advancements in genomics have increased the availability of complete genome sequences of many organisms. These data have spurred the interest of researchers in constructing *in silico* genome-scale metabolic models to decipher genome-scale metabolic characteristic and behavior. Such simulation can save time, labor and research expenditure as it minimizes the amount of real laboratory experiments needed to be performed.

Reconstructions of these metabolic models are initiated by compilation of related stoichiometric reactions in gene annotation data [6]. To perform the compilation, linear mass balance equations for the metabolites have to be set, and the gaps within the network have to be filled according to information provided by literature and experiments as well as from the well-known databases such as KEGG [7]. The models then make suggestions for strain improvements after being validated through comparing the simulated data with actual experiments. *In silico* genome-scale metabolic model plays an important role to provide "virtual" microbes for strain improvement and optimization.

Currently, several genome-scale metabolic models have been used in microbial fuel production related research and Table **1** shows some examples of them.

3. *IN SILICO* METABOLIC ENGINEERING ON RELATED MICROBIAL FUEL PRODUCTION RESEARCH

Based on a comprehensive review [13] done previously, there are basically three metabolic engineering strategies in production host selection. The first strategy is related to using organisms that are capable of forming native products of biofuels compounds (ethanol or butanol). The second strategy is about utilizing organisms with native substrate; these organisms use a wide range of substrates such as lignocellulose or syngas that can be further manipulated to form biofuels. The third strategy deals with the utilization of industrial organisms such as *E. coli* as a platform to integrate biosynthetic pathways to achieve desirable chemicals production capabilities. To date, in related researches in *in silico* metabolic engineering for microbial fuels production, there are basically two different directions which is optimizing the models of native production host to achieve such desirable production rate [14, 15] and the integration of identified biosynthetic pathways into industrial microbial models and manipulated for optimal production of biofuels [16, 17].

Table 1. Available Genome-Scale Metabolic Models Used in Microbial Fuel Production Related Researches

Species	Model Reaction Count	Refs.
Escherichia coli	2077	[8]
Saccharomyces cerevisiae	1149	[9]
Clostridium thermocellum	577	[10]
Clostridium acetobutylicum	552; 502	[11,12]

Generally, *in silico* metabolic engineering approaches are divided into two categories, namely determinative and predictive approaches. Within the predictive approaches, there are another two categories - pathway-based approaches and optimization-based approaches. However, this review paper only focuses on the optimization-based predictive approaches and related applications in microbial fuel production research. Details regarding the other aforementioned approaches can be found in [18]. Table **2**

gives an overview of the methods being discussed in the following sections (3.1 and 3.2).

3.1. Optimization of Native Production Host

As mentioned in the previous section, a native production host is one that can natively provide desired product formation, and an optimization-based approach is one mainly used to identify mutations in the production host to improve the desired phenotype. In this case, the production of desired biochemical compound and final solution are identified by maximizing or minimizing a specified objective. In cellular metabolism, regulatory network remains important during the engineering of microbes because genetic modification without considering regulatory network will cause fatal functionality and is lethal to the microbes [14].

A bi-level computational framework, OptReg, has been developed to determine optimal activation or inhibition and elimination to achieve targeted biochemical overproduction [15]. By using OptKnock as the starting point, OptReg extends the framework with several algorithmic and modeling changes. One of the main changes is the introduction of a parameter that can predict the up- and/or down regulation in addition to gene-knockouts. Conceptually, the introduced regulation strength parameter, C, has a value ranging from zero to one and quantifies the threshold to determine whether a reaction is up- or down regulated. However, previous researches [19, 20] have shown that majority of the reactions could not obtain unique solutions at steady state when maximization of biomass or any other cellular objectives are used. Furthermore, due to redundancy in network, a range of flux values are often identified for corresponding alternate optima other than the biomass. This resulted in the development of another extension, which is the implementation of flux measurements (range of flux values based on experimental data) to describe the base state of the network. This means that a range of values, rather than a single value, is used to represent the network's base state before any genetic modification applied. Such method has been used in optimizing ethanol production in *E. coli* using the wild-type network of *E. coli* [21] with some fixes done to the central metabolism based on the flux measurements extracted from previous work [22]. The results were promising where down regulation of phosphoglucomutase alongside with deletion of oxygen uptake and pyruvate formate lyase of up to 99.8% of the maximum theoretical yield of ethanol in *E. coli*.

Other similar approaches which are based on gene deletion with considerable attention placed on regulatory networks have also been developed [14]. These include the OptORF that had been used to predict engineering strategies for ethanol production in *E. coli*. OptORF is different from other approaches because it can identify metabolic engineering strategies based on gene deletion and overexpression, not on reaction deletion. The solution, which also considers transcriptional regulatory networks, is thus more feasible. OptORF has also been used as an integrated model for the metabolism and regulation of *E. coli*, iMC1010v2 [23] which has 906 metabolic genes and 104 transcription factors (TF). In the study, OptORF introduced a new bi-level optimization formulation where GPR association (gene-protein-reaction) and transcriptional regulations were used as constraints. GPR represents the relationships among particular reactions with corresponding proteins and specific genes. The transcriptional regulations consist of regulatory rules formulated in Boolean. The final predicted yield was approximately 86.2% of maximum theoretical yield, and it should be noted that transcriptional regulatory effects were taken into account.

OptReg is powerful in the sense that it considers the regulations of genes as well as the flux measurements. However, the downside of this model is that it does not consider regulatory network like that considered in OptORF. Furthermore, the results from OptReg need prudent interpretation because the reversible reactions are separated into two, which are the forward and backward counter-parts. Unlike OptReg, as demonstrated in the integrated model of *E. coli*, OptORF emphasizes on the relationships among reaction, protein and genes. Moreover, it can provide highly feasible results suitable for real world experiments. Nevertheless, improvement can still be done, for example in the formulation of regulatory rules, to create more dynamic environment.

3.2. Search and Optimization of Biosynthetic Pathways for Non-Native Production Host

Identifying the native production host can simplify the process without integrating complex biosynthetic pathways into the microbes. However, currently there are no microbes that can produce desired products in a massive scale and have good natural tolerance. Therefore, industrial microbes are introduced with foreign genes and biosynthetic production pathways to achieve the desired phenotype. Despite of that, our knowledge on biosynthetic pathways is still limited and even for well-known microorganisms such as *E. coli*, more alternative pathways continue to be discovered and used in biofuel synthesis [24]. Furthermore, with supportive information provided by rapidly expanding compilation of biotransformations such as KEGG [7] and BRENDA [25], computational discoveries of novel pathways have become even more intense. These novel pathways can be further implemented into genome metabolic models for *in silico* strain design.

Few approaches have been used for pathways identification such as the use of shortest pathways algorithm by Ma and Zeng [26] to observe the reaction of genome-scale metabolic model during reconstruction. A scoring algorithm [27] has also been developed to evaluate and compare novel pathways based on enzyme-reaction rules. In the context of biofuels production, fewer approaches have been developed and used in 1-butanol production.

Recently, graph theory-based method alongside with an optimization-based approach has been used to identify non-native production route and metabolic interventions for 1-butanol production in *E. coli* [16]. With the reaction data obtained from KEGG, BRENDA, and the genome-scale metabolic model of *E. coli*, iAF1260, a min-path graph procedure was used to locate all possible pathways that would link the desired source to a desired target molecule. The process remained traceable even while handling large amount of data. The novel pathways identified were then evaluated using OptFORCE [28] to identify possible genetic

interventions that led to overproduction of 1-butanol. OptFORCE is an optimization approach which utilizes metabolic flux measurement to identify the necessary changes in fluxes to achieve a desired optimal solution. OptFORCE divides the fluxes into two categories - MUST set, which consists of fluxes that must be changed to meet overproduction target, and FORCE set (extracted out from MUST set), which is a minimal set of direct interventions (i.e., knock-outs) that guarantees a pre-specified yield for 1-butanol. The utilization of flux measurements provides a better representation of the metabolic networks. Results from the research included the identification of some novel pathways for 1-butanol production other than conventional fermentative pathways like the ketoacid pathway and thiobutanoate pathway.

Combination of a graph-based method called Biochemical Network Integrated Computational Explorer (BNICE) framework [29-31] with structural-based screening method was also used to identify novel pathways for 1-butanol production in *E. coli* [17]. BNICE framework is formulated based on the graph theory to create complex networks of compounds and reactions using the generalized reaction rules or operators. By setting pyruvate as the starting carbon source, BNICE framework is used to search all possible linear routes that produce 1-butanol from pyruvate up to hundreds and thousands of novel pathways.

Different from the research done by Ranganathan *et al.* [16], Wu *et al.* suggested a structural-based screening method to discover novel pathways using molecular docking. The authors also introduced an enzyme fitness function to characterize the quality of the docked poses. Moreover, the enzyme fitness function is able to distinguish the native substrate and other native ligands from inactive decoys for a diverse set of enzymatic reactions. This introduced structure-based screening method is able to identify specific proteins within a given enzyme class that are most likely to catalyze a given novel reaction as well. This provides an ideal balance of accuracy and throughput to determine the feasibility of the strategies predicted.

Both Ranganathan *et al.* and Wu *et al.* demonstrated the application of combined methods in microbial fuel production. However, k-shortest path algorithm was employed by Ranganathan *et al.* to search novel pathway and several other pathways like non-fermentative pathways were identified while Wu *et al.* used pathway generation algorithm in BNICE to search for novel pathways without considering nonlinear branched pathways that converge pathways with different intermediates. The results generated only had fermentative pathways. In order to assess the discovered pathways, both researches used OptFORCE and molecular docking respectively.

The advantage of using OptFORCE is that it is able to identify and classify essential flux for desired overproduction. As for the molecular docking method, it can be used to assess feasibility on the molecular level, and thus provides reliable support for the assessment. Despite of that, both methods do not consider regulatory networks, and although the solutions put forth by OptFORCE are reliable, these solutions are provided on the metabolic flux level only. Therefore, multiple rounds of experimental strain improvements may be needed to map the FORCE set reaction to corresponding gene expression levels.

4. OTHER ADVANCES IN *IN SILICO* METABOLIC ENGINEERING

Besides the computational approaches discussed above, there are several other computational approaches available in *in silico* microbial strain design. However, these approaches are yet to be used in the case of microbial fuel production.

In metabolic engineering, knowledge regarding the distribution and behavior of the metabolic fluxes is critical. In order to predict how the metabolic fluxes are distributed, a number of approaches have been developed such as the flux

Table 2. Overview of the Advantages and Disadvantages of Computational Approaches to *In Silico* Metabolic Engineering Used to Enhance Microbial Fuel Production

Method	Ref.	Advantages	Disadvantages
Production in Native Production Host (Ethanol)			
OptORF	[14]	✓ Considers the regulatory effects, as demonstrated by the integrated model of *E. coli* ✓ Functions based on gene deletion.	× Regulatory effects are modeled in Boolean and do not fully represent dynamic nature of metabolism.
OptReg	[15]	✓ Regulation strength parameter for regulation prediction (on/off) ✓ Used flux measurements to describe the state of the metabolic network	× Reversible reaction are separated into two counterparts (forward and backward) and thus requires careful interpretation × No actual integration of regulatory network.
Production in Non-Native Production Host (1-Butanol)			
k-shortest path + OptFORCE	[16]	✓ Identifies possible novel pathways (fermentative and non-fermentative). ✓ Uses flux measurements to identify essential fluxes for desire overproduction.	× Provides solution on metabolic flux level only. × Lack of mapping between gene expression and flux levels.
BNICE + molecular docking	[17]	✓ Structure-based screening for identified pathways. ✓ Enzyme fitness function gives reliable and accurate results to assess the feasibility of the solution.	× Screening method is not automated. × Lack of consideration on dynamicity of the metabolic networks.

Table 3. Computational Approaches Available for *In Silico* Metabolic Engineering

Method	Ref.	Objectives
FBA	[32]	Predicts intracellular flux distribution at steady state.
MOMA	[33]	Predicts metabolic flux distribution in un-evolved mutants.
ROOM	[34]	Predicts the steady state behavior of metabolic network in response to gene knockouts.
Dynamic FBA	[35]	Extension of FBA, allows model to update and change over time, more dynamic.
Dynamic ROOM	[36]	Extension of ROOM, allows model to update and change over time, more dynamic.
OptGene	[37]	Predicts gene-knockout using genetic algorithm and flux balance analysis.
OptKnock	[38]	Predicts gene-knockout through bi-level optimization framework.
BiMOMA	[39]	Application of MOMA through bi-level optimization framework.
SimOptStrain	[39]	Extension of OptStrain, simultaneously considers gene deletion and non-native reaction addition.
OptStrain	[40]	Predicts solution by consider gene deletion and non-native reaction addition from a universal database.

balance analysis (FBA) that has been used to optimize alternative objective functions, such as maximizing the targeted biochemical production [32].

Other approaches include minimization of metabolic adjustment (MOMA) [33] and regulatory on/off mechanism (ROOM) [34]. These approaches are used to predict the immediate behavior of knockout strains. Extensions of these approaches that focus on the dynamicity (internally perturbed) of the model have also been developed so that the model can be updated and changed over time [35, 36]. Efforts have been put into using genetic algorithm in predicting gene knockout to obtain desired production of targeted biochemical as well [37].

4.1. Bi-Level Optimization

In order to identify the metabolic engineering strategies with highest production level, huge amount of possibilities is needed, particularly when the size of the genome-scale metabolic model is big and due to the combinatorial nature of the problem. This is where the bi-level approach comes handy because it can efficiently identify the mutations needed to achieve the highest production rate. In fact, many of the optimization-based approaches are using this bi-level optimization framework, such as OptKnock [38] that identifies reaction deletion coupled with cellular growth and biochemical production. In order to increase the biochemical production, identification and enhancement of mutant growth is required.

Another approach, OptStrain, consists of multiple steps that identify non-native reactions to improve production capabilities. The reactions found can be coupled with production and growth in the mutant metabolic network. A more recent bi-level approach (SimOptStrain) [39], which is an improved version of OptStrain [40], is used to identify metabolic engineering strategies by simultaneously considering gene deletion and novel reaction additions. An extension of MOMA [33], named BiMOMA [39], is used to predict knockout behavior in MIP-based bi-level problem. The aforementioned computational approaches in this section are briefly shown in Table **3**.

5. CONCLUDING REMARKS

The goal of *in silico* metabolic engineering is to produce a useful platform that can provide rational engineering strategies from the simulated results of the computationally optimized strains. In related research endeavors of microbial fuel production, *in silico* metabolic engineering has contributed in finding both connected mutation for native production host and biosynthetic pathways that can be integrated into non-native production host. As mentioned before, many available approaches have been developed for *in silico* metabolic engineering (as shown in Table **3**) and alongside with on-going advancement on omics researches, more rigorous discoveries of new engineering strategies and biosynthetic pathways are possible. With these available sources of knowledge, current advances in *in silico* metabolic engineering can be further expanded and to benefit biofuels production and to ease fuel crisis.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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