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Systems biology in biofuel

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1 Introduction

Biotechnology has played an important role in society, life, and economics since ancient times. Application of microorganisms in biotechnology demonstrates the use of cell factories for the production of a wide variety of chemicals used for fuels, commodities, specialty chemicals, polymers, and drugs [1, 2]. Current global problems, including environmental pollution, global warming, and energy security, have led to an increasing interest in renewable production of fuels and other chemicals currently derived from petroleum, especially the production of biofuels from lignocellulosic biomass [3]. The International Energy Agency (IEA) has predicateda more than 10-fold increase in biofuel demandfrom2010 to 2050, resulting in a total demand of more than 30 exajoules [4]. The currently availability of biofuels, such as bioethanol and plant-oil derived biodiesel, is not sufficient to meet current demand. Additionally, due to its physical properties, such as low energy density and high hygroscopy, bioethanol is not an ideal substitute for gasoline. Advanced biofuels with superior fuel and operational properties, including a diversity of C_4-C_5 chain alcohols, biodiesel (fatty acid ethyl esters (FAEEs) and farnesane) and jet fuels (alkanes, olefins, and terpenes), are not naturally produced in preferred cell factories, e.g. *Escherichia coli* and *Saccharomyces cerevisiae*. Therefore, novel pathways have to be introduced to enable microorganisms to produce new and advanced biofuels.

Since the advent of recombinant DNA technology, the production of desired natural products has been greatly enhanced. Especially in the past three to four decades, metabolic engineering has been developed as a powerful approach to increase production of these useful chemicals through directed genetic engineering [2, 5]. In the early stages of metabolic engineering, the development of this technology was mainly focused on improving the product yield and range of natural and unnatural chemicals, as well as the substrate utilization rates by targeted modification of metabolic pathways in host microorganisms. With recent advances, metabolic engineering has been applied to promote the design and development of novel cell factories [6–[10], which, through its requirement of a cell-wide understanding and modification of microorganism metabolism, contributed to the growth of synthetic biology. To promote the production of biofuels using microorganisms, a significant modification of intracellularmetabolismis required [1, 11, 12].

The ability for cell-wide metabolic engineering and synthetic biology, however, requires a system-level understanding of cellular metabolism. With the development of omics techniques, metabolic toolkits are needed to modify or build large numbers of metabolic pathways to produce chemicals in a feasible manner [13, 14]. The application of such techniques has severe consequences on metabolisms of the host strains. Therefore, a deep understanding of cellular metabolism is necessary for the design of strategies. With the advancement of omics techniques, thousands of parameters can now be monitored simultaneously [15, 16], making diagnosing and fixing metabolic engineering problems feasible. To analyze the vast amounts of data, system-wide models of various aspects on genetic regulation and metabolism as well as corresponding computational tools are needed, which introduce systems biology [17, 18].

Governments and companies show high interest in the production of biofuels. This indicates a successful and fast development of biofuel production in the near future. This review will cover the recent developments in biofuels from a technological point of view supported by systems biology. The development of biodiesel, jet fuels and butanol will be discussed in more detail, especially with regard to how tools from systems biology may advance the implementation of metabolic engineering processes and promote the development of novel biorefinery processes.

2 The importance of systems biology

In traditional molecular biology approaches, only a few metabolic engineering modifications would be evaluated simultaneously, making a system-level perspective difficult to obtain. Understanding the metabolisms in

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a system-wide view, however, is strictly necessary for the success of metabolic modification strategies. With the application of systems biology, cellular phenotypes can be analyzed in more detailed and in a whole view, further leading to the improvement of cell-factory design through detailed metabolic modeling. Today, by introducing different omics techniques for cell factories, a large number of cellular components can be analyzed [18, 19]. The omics technologies show their deep influence on the development of metabolic engineering strategies. For example, the complex regulatory pathways involved in controlling metabolism, such as the protein kinase in yeast [20, 21], identification of transcriptional influence to metabolic fluxes in yeast and *E. coli* [22, 23] and the interaction among protein kinases in yeast [9]. With the integration of the omics techniques and system-level modeling approaches, metabolic engineering strategies can be drawn out rationally (Figure 1). In the following parts, the omics techniques and system-level modeling are brief discussed.

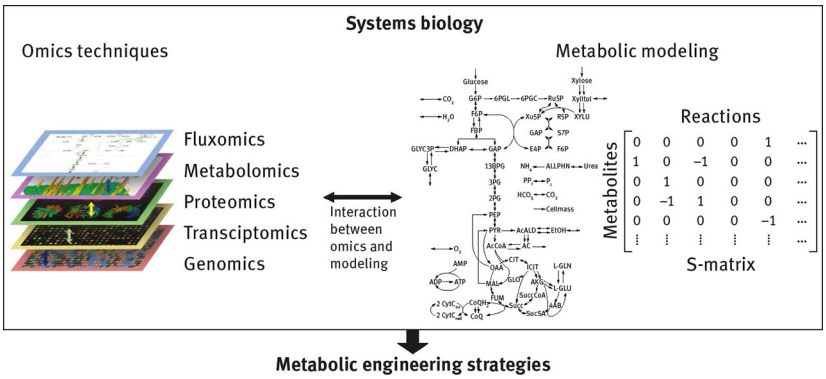

Figure 1: The derivation of metabolic engineering strategies from systems biology. The interaction between omics data and metabolic modeling would promote the understanding of the target organisms and lead to the implementation of rational metabolic engineering strategies.

1 Genomics

The study of genomics provides information for acquiring and interpreting genomic sequence data. A staggering amount of genomics now exists for hundreds of different microbial and nonmicrobial systems, providing information on mutation, adaption, and growth conditions based on cell-wide data [24]. With the recent development in techniques and reduction in sequencing costs it is possible to use genome sequencing and RNA sequencing to obtain information at the genome level as well as improved annotation and expression profiling [25]. The technology has also enabled rapid profiling of microbial diversity [26] and gene function [27] from complex, environmental samples. The genome-sequencing technique has been used to identify driving mutations among adaptively evolved strains in studies of *E. coli* [28] and of yeast [29], which proved its power. The demand to obtain and process the large amount of data obtained from transcriptional array studies have raised the need for entirely new disciplines combining bioinformatics and computational biology [17], and therefore required the development of protocols for rigorous reporting [30]. However, with the possibility of identifying genomic mutations, genome data alone has difficulty to identify governing mutations without help from additional omics technologies for detailed phenotypic analysis [29]. This kind of difficulty is usually caused by the appearance of silent mutations resulted from adaptive evolution. Therefore, it is desired to integrate the genomic data with other omics data [31].

2.2 Transcriptomics

Genome-wide transcription analysis is the most widely used omics technology in metabolic engineering, which has been applied to various industrially relevant microorganisms, such as yeast [32], *E. coli* [33, 34] and *Aspergilli* [35]. The value of transcription analysis lies in its scale, which is genome-wide, and therefore can be integrated directly with genome-scale metabolic models via different approaches [36–[38]. Transcription and protein expression data correlated relatively well for detailed pathways [17], however, the correlation is usually poor for cell-wide analyses [39, 40], which indicates regulatory processes have a higher importance than transcriptional control. A study on mRNA synthesis and degradation in *S. cerevisiae* showed that both of them can influence protein levels significantly [41]. Meanwhile, stressors could also affect mRNA stability [42, 43]. With the integration of proteomic data, in addition to transcription, post-translational modification [44], protein localization [45, 46], and protein-protein interactions showed importance to cellular responses to stress [47]. Functional anal-

ysis of *S. cerevisiae* under particular stress conditions showed that candidate genes essential for growth did not show significant change [25], which emphasizes the importance of responses beyond differential expression.

2.3 Proteomics

Just as mentioned above in transcriptomics, proteomics plays an importance role in metabolism and has advanced significantly in the past decades [48]. As the primary approach to capture regulation of cellular response beyond mRNA level, proteomics provides important information for functional genomics studies. Traditionally, proteomics are measured with two-dimensional electrophoresis [49] while nowadays high-throughput techniques via mass spectrometry have become the foundation for research. With the development of strategies for protein identification [49, 50] and workflow for protein abundance quantification [51], proteomics provides wide-ranging information such as composition, function and location. Among the various techniques, the iTRAQ technique, which can simultaneously label samples with up to eight conditions via isobaric tags [52], has been applied to detect protein changes [53] and to determine members of protein complexes [54]. Besides the progress of measurement approaches, computational analysis has to be improved to reduce artifacts in information and false positives [55–[57]. With decades' development, the standardized approaches that can report and handle proteomics data statistically soundly are still evolving [58, 59].

2.4 Metabolomics

In order to wholly understand the enzyme activity and substrate turnover, monitoring transcriptome and proteome alone do not provide enough information. Metabolomics are providing true measurements of the cellular response to stress and manipulation [9, 60], and there has been a huge increase both in fields of application and the number of metabolites that can be measured [61, 62]. Methods have been developed for metabolome analysis of microorganisms [63–[66], but key challenges still exists in sampling and extraction [67, 68]. Meanwhile, the absence of reliable and standardized databases, which should contain all of the existing spectral information and allow correct identification of unknown detected metabolites, is still one of the problemsaffecting metabolomics research. Additionally, the data on metabolite levels from different laboratories based on various experimental methods are not quantitatively consistent while the relative levels are comparable [69].

2.5 Fluxomics

Additional to the above omics, fluxomics has shown its usages in studies of a range of various industrial microoganisms [70, 71]. The fluxome integrates information on cellular processes, and shows the unique phenotypic characteristics of the metabolisms of the cells [72]. The metabolome can be captured by flux analysis through its functional interactions with the environment and the genome [73]. Therefore, the fluxome is always accompanied by knowledge of the metabolome. With current techniques, it is still not that easy to measure intracellular fluxes. Thus, computational methods are combined together with experimental methods, in which the most reliable approaches are based on isotope-labeled precursors of metabolic pathways, mainly ^{13}C-labeled substrates [32, 74]. Via metabolomics analytical platforms, the concentration and isotopomeric distribution (or labeling pattern) of the labeled metabolites can be determined [72, 75]. However, the estimation of fluxes based on tracer metabolomics data requires *a priori* knowledge of possible distributions of the tracer used within the network, i.e. the structure and components of the network. Therefore, the lack of information on reactions and metabolites might lead to erroneous results, whichmeans that a further understanding of other omics information is very important to the accuracy of fluxomes [76].

2.6 Computational Methods

Metabolic modeling has been an integral part of metabolic engineering since its formation. With recent developments, by integration of these system-wide data from omics technology, mathematical modeling has been applied to design cell factories, which is another key contribution of systems biology to metabolic engineering [77]. Integrated models have been applied in many aspects for metabolic engineering, such as identifying essential genes [78], transcriptional elements [16], regulatory circuits [79], and stress response [15]. The models, therefore, provide valuable information about intracellular activities such as reaction fluxes and may lead to the discovery of the as yet unrevealed reasons preventing optimal production [80]. To integrate the omics data

with metabolic models effectively, various computation methods have been developed to try to address issues with data integration [81]. Valuable tools have been published with open sources, which has made it much easier for researchers to analyze, compare, and mine genomics data [82–[84]. The development of the tools has recently been reviewed extensively [14, 36], 85–[87], while model repositories and on-line modeling tools [88, 89] will significantly promote the application of these models in the field of industrial biotechnology.

3 Applicability of systems biology in biofuels

In order to combine native and heterologous genes in metabolic engineering [90], understanding how the incorporation of an engineered exogenous pathway perturbs the host system is important for overcoming pathway bottlenecks. Reaching desired production levels of metabolites requires a significant amount of pathway optimization. The application of systems biology in strain development for biofuel production is critical to identify potential bottlenecks and reveal detrimental side effects [21]. With the development of computational optimization approaches, *in silico* simulation may prove a product of interest possible to be produced *in vivo* with rational metabolic engineering strategies. Therefore, the undesired side products can be depressed while the carbon flux towards the target product can be maximized. This approach has been appliedin the metabolic engineering improvement of bio-ethanol production from *E. coli* [5]. In the following parts, applications of metabolic engineering strategies based on integration of omics techniques and systems-level modeling are briefly reviewed in the production of advanced biofuels, specifically, biodiesels, jet fuels and biobutanol.

3.1 Biodiesels

The development of biodiesel techniques, just as other biofuels, focuses on a consistent, scalable and renewable commodity supply and a cost-efficient production based on the requirements of a sustainable society. To reach the target, metabolic engineering strategies, derived from understanding metabolism with the help of systems biology, have to be applied to construct a single cell factory. For biodiesel synthesis, usually, two metabolic pathways, the lipid and isoprenoid pathways, are employed [91].

Several research groups have investigated the metabolic engineering approaches to improving FAEE production in *E. coli* as well as the fatty acid metabolism [92–[96]. By introducing cytosolic expression of thioesterase (TesA), modulation of β-oxidation (by overexpression of *faaD* and deletion of *fadE*) and introduction of wax ester synthase/diacylglycerol acyltransferase (WS/DGAT) from *Acinetobacter sp*. ADP1, an engineered *E. coli* strain has been constructed which produced 400 mg/l FAEEs on glucose and 2% ethanol in 2 days [96]. To further promote the production of FAEEs, the alcohol synthesis pathway (*pdc* and *adhB*) from *Zymomonasmobilis*and a second copy of *atfA* and acetyl-CoA ligase from *S. cerevisiae* were further introduced, which increased the production of biodiesel up to 674 mg/l based on glucose [96]. By employing different thioesterases, the distribution of product chain lengths was under control. Additional overexpression of acetyl-CoA carboxylase (accBACD) led to a final concentration of 922mg/ l FAEEs in a scaled up fed-batch fermentation [97]. The main products from the fermentation consisted primarily of ethyl palmitate, ethyl oleate, ethyl myristate and ethyl palmitoleate. In order to further improve the production, the pyruvate dehydrogenase complex (*aceEF*) could be overexpressed. Meanwhile, the interruption of pathways to lactate, formate and acetate by deleting corresponding genes *ldhA*, *pflB*, *poxB* and *ackA* respectively, as well as phospholipid pathway through deleting *gpsA* and *plsB*, have been applied to reduce byproduct formation.

Besides the work on *E. coli*, *S. cerevisiae* has also been modified to study its capacities on the production of biodiesels [98]. The removal of genes *DGA1*, *LRO1*, *ARE1*, and *ARE2* led to the loss of capacity of storage lipids (TAGs and steryl esters) synthesis. Low FAEEs could be produced by the strain with the introduction of WS/DGAT [99]. An additional approach has also been applied by recycling glycerol into biodiesels by yeast. During the process, the introduced glycerol was converted into ethanol and was further converted into FAEEs by external addition of fatty acids and introduction of endogenous transesterification reaction catalyzed by WS/DGAT from *Acinebacter sp*. ADP1. The modification of the metabolic pathways led to a minimized glycerol secretion with 0.52 g/ l FAEEs production rate and 17 g/ l glycerol consumption rate [100].

Farnesane production has also been studied systematically, which requires introducing farnesene synthase to convert farnesyl pyrophosphate (FPP) to farnesene and further from farnesene to farnesane. By overexpressing the mevalonate pathway and fusing heterologous FPP synthase (*ispA*) and the apple α-farnesene synthase gene (*wFS*), *E. coli* produced a final concentration of 380 mg/l α-farnesene [101]. Besides, by engineering the mevalonate pathway and introducing bisabolene synthase from *Abies grandis* Ag1, both *E. coli* and *S. cerevisiae* reached a production of bisabolene higher than 900 mg/l, which is a precursor of bisabolane. The latter has been identified as an alternative fuel compound [102].

3.2 Jet fuels

For a jet fuel, the must-have characteristics include low freezing point (–40 °C), high energy density (> 53.4 MJ/l) and comparable net heat combustion [103]. The commonly recognized candidates include n-alkanes, n-alkenes (n-olefins) and terpene compounds (pinene, sabinene and terpinene) [104]. Among the above chemicals, alkanes/alkenes have received further attention. By applying systems biology approaches, several pathways in bacteria for producing long-chain ($C_{10} < x < C_{20}$) and very-long-chain ($> C_{20}$) alkanes have been discovered. Comparison of cyanobacteria with and without alkanes product capacity led to the discovery of two genes that encoded enzymes for producing fatty aldehydes and converting further to alkanes by removing the carbonyl carbon [105]. The two encoded enzymes have been proven to be acyl-ACP reductase and aldehyde deformylase respectively [106, 107]. The functions of the two enzymes have been proven by introducing into *E. coli*, which led to a production of up to 300 mg/l of pentadecane, pentadecene, and heptadecene. Further adaptation of metabolisms by the introduction of the enzymes include increasing NADPH consumption and balanced protein reductive system for aldehyde deformylase. Meanwhile, acyl-CoA reductase can also produce fatty aldehydes (and theoretically alkanes) [108]. FFA can be converted to acyl-CoA synthetases and further into fatty alcohols, which have better price and performance than diesel [109]. A second route for alkane production, which was identified in a *Jeotgalicoccus sp*. by a reverse genetic approach, is catalyzed by P450 enzyme OleTJE [110]. When heterologously expressed in *E. coli*, the protein led to production of 1-pentadecene and 1,10-heptadecadiene as well as 1-heptadecene with addition of stearic acid (18 : 0). The results suggest that OleTJE can utilize both acyl-ACPs and FFA [109]. In addition to the two pathways mentioned above, *Shewanella oneidensis* can head-to-head condensate fatty acids to long-chain alkenes (C_{23}–C_{33}) [111, 112].

Besides alkanes, olefins have been produced in several bacteria, which can be blended with diesel as well as be used as chemical building blocks. *Synechococcus sp*. PCC7002 produces 1-nonadecene and 1,14-nonadecadience [113]. Ols was found to be responsible for the synthesis of the olefins. Sulfation is the activation center for the protein. Its current titers are still very low while engineering the gene might lead to a set of pathways for producing a wide range of olefins [109].

3.3 Biobutanol

As the natural butanol producer, *Clostridium acetobutylicum*has been recognized as an industrial cell factory for 1-butanol production and has been extensively studied [114]. Recently, progress on genetic engineering of *Clostridium* has been made [115]. Metabolic engineering strategies have been applied to eliminate byproducts (such as ethanol, acetone) [116, 117], to improve the oxygen tolerance and to reduce growth inhibition [118, 119].

While the study of *Clostridium* revealed its metabolic mechanisms, the heterologous strains were also built based on the results. Two different approaches have been applied to *E. coli*. The first approach is to modify *E. coli*'s highly active amino acid biosynthetic pathway, which led to a 1-butanol production of 0.5 g/l [90]. Another method is to introduce the 1-butanol pathway from *C. acetobutylicum* [120]. The host strain produced a similar amount of 1-butanol as the first method. In order to further promote 1-butanol production, several pathways have been introduced into *E. coli*. With the introduction of enzyme Crt and AdhE2 from the 1-butanol pathway of *C. acetobutylicum*, PhaA and Hbd from *Ralstonia eutropha* and Ter from *Treponema denticola* as well as the overexpression of *aceEF* to increase the acetyl-CoA pool, the constructed *E. coli* showed an evaluated metabolic system improvement and produced 1500-fold increase of 1-butanol titer from 3 mg/l to 5 g/l [121, 122]. Additional modification of metabolic pathways by blocking NADH reducing pathways (i.e. deleting *frdBC*, *ldhA*, *adhE*, and *pta* to block succinate, lactate, ethanol and acetate pathways respectively), implementing an NADH drain in 1-butanol pathway through the enzymes from *C. acetobutylicum* (inserting Hbd, Crt and AdhE) and introducing highly active endogenous acetyle-CoA acyltransferase (AtoB) and irreversible trans-enoyl-CoA reductase (Ter) from *T. denticola*, led to a 15 g/ l 1-butanol production. *In situ* removal of product from fermentation broth further doubled the yield to 30 g/ l [123], which indicated that the product toxicity-level has to be kept low for proper cell functioning [124, 125].

Meanwhile, *S. cerevisiae* is also considered as a better host strain for 1-butanol than *E. coli* due to its higher butanol tolerance and long application in industrial ethanol production [126, 127]. However, with systems biology approaches, *in silico* simulations suggested lower butanol and propanol yields in yeast compared to *E. coli*, which was partially due to the limited flexibility of the central metabolism[128]. By introducing various enzymes to construct corresponding butanol production pathways from *E. coli*, *Clostridium beijerinckii* and *Ralstonia eutropha*, the constructed *S. cerevisiae* produced a 1-butnaol concentration of 2.5 mg/l [129]. Further modifications, such as increasing the acetyl-CoA pool by overexpression of pyruvate dehydrogenase multienzyme complex (*lpdA*, *ace*, *aceF*) and downregulating pyruvate decarboxylase (PDC), did not show much better

results [111]. Therefore, more modification based on systems biology is still needed to construct engineering strains with high 1-butanol production.

4 Conclusions and outlook

Cell factories for the production of biofuels, including biodiesel [93] and isobutanol production [90], have made substantial progress in recent years. Further improvements on the metabolism of the microorganisms, however, are still needed to meet the industrial requirements for advanced biofuels in terms of yield, titer and productivity. A systems-level understanding and analysis of metabolism will reveal strategies that can help to improve the productivities as well as other important factors such as tolerance towards the product of interest and the ability to use complex feedstocks derived from biomass. Many strategies for metabolic engineering have been proposed based on recent cell-wide studies to improve various aspects of biofuel production with the developments on omics techniques [130–[132]. Systems biology is necessary to fully understand the regulation mechanisms of the underlying metabolic network and the response to the overproduction of molecules for the host in order to deduce pathway bottlenecks and optimize the design of cell factories. With the advancement of systems biology methods and tools, the application of systems biology approaches in metabolic engineering will become a key technology for the future creation of robust and efficient cell factories for industrial applications.

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