Computational and Structural Biotechnology Journal 15 (2017) 161–167

COMPUTATIONAL AND STRUCTURAL BIOTECHNOLOGY JOURNAL

journal homepage: www.elsevier.com/locate/csbj

Mini Review

Mini-review: *In vitro* Metabolic Engineering for Biomanufacturing of High-value Products

Weihua Guo [1], Jiayuan Sheng [1], Xueyang Feng *

Department of Biological Systems Engineering, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, United States

ARTICLE INFO

Article history:
Received 4 November 2016
Received in revised form 12 January 2017
Accepted 15 January 2017
Available online 19 January 2017

Keywords:
Cell-free
Biosynthesis
Metabolic pathways
Design-build-test cycle

ABSTRACT

With the breakthroughs in biomolecular engineering and synthetic biology, many valuable biologically active compound and commodity chemicals have been successfully manufactured using cell-based approaches in the past decade. However, because of the high complexity of cell metabolism, the identification and optimization of rate-limiting metabolic pathways for improving the product yield is often difficult, which represents a significant and unavoidable barrier of traditional *in vivo* metabolic engineering. Recently, some *in vitro* engineering approaches were proposed as alternative strategies to solve this problem. In brief, by reconstituting a biosynthetic pathway in a cell-free environment with the supplement of cofactors and substrates, the performance of each biosynthetic pathway could be evaluated and optimized systematically. Several value-added products, including chemicals, nutraceuticals, and drug precursors, have been biosynthesized as proof-of-concept demonstrations of *in vitro* metabolic engineering. This mini-review summarizes the recent progresses on the emerging topic of *in vitro* metabolic engineering and comments on the potential application of cell-free technology to speed up the "design-build-test" cycles of biomanufacturing.

Contents

1. Introduction

For decades, scientists and engineers use metabolic engineering as a powerful approach to optimize industrial fermentation processes through the introduction of directed genetic changes using recombinant DNA technology. This has become an attractive, sustainable way to produce molecules [1–3], especially when chemical synthesis is difficult [4,5]. Metabolic engineering aims to endow cells with improved properties and performance [6] while synthetic biology could create new biological parts, modules, devices and systems, in addition to re-engineering cellular components and machinery that nature has provided [7]. Through the integration of metabolic engineering and synthetic biology, efficient microbial cell factories can be constructed to produce biofuels, biomaterials and drug precursors [8].

As high-valued products, biologically active compound is one kind of the most attractive engineering targets nowadays because many of them demonstrate important pharmacological activities or biotechnological significance [9]. However, due to the complexity of their structures which contains multiple chiral centers and labile connectivity [10], researchers seek microbial production instead of total chemical synthesis or semisynthesis from isolated precursors. However, these products often lack optimal production titer and high yield. Till now,

* Corresponding author.
E-mail address: xueyang@vt.edu (X. Feng).
[1] WG and JS have equal contribution.

http://dx.doi.org/10.1016/j.csbj.2017.01.006

except for a few examples such as introducing heterologous pathways into yeast for the large scale production of an anti-malaria drug artemisinin [11], few valuable biologically active compounds could be produced at high yield and reach into the stage of large-scale biomanufacturing. Commodity chemicals is another large group of chemicals that attracts researchers to use cell-based metabolic engineering for manufacturing, mainly due to concerns of depleting fossil fuels and climate changes [12]. Biomass produced from plants is the most abundant renewable resource and is considered to be the cost-competitive energy and carbon sources that could be converted to produce biofuels and biochemicals instead of fossil fuels [13]. Recent breakthroughs in synthetic biology and metabolic engineering led to the production of a series of bulk chemicals such as 1,4-butanediol [14] and isobutanol [15]. However, cell proliferation is the primary goal of microorganisms while bioconversions are the side effects. These inherent constraints of living microorganisms prevent them from implementing some important chemical reactions (*e.g.*, H_2 production from glucose and water) and prohibit them from achieving the theoretical yield of commodity chemicals.

The unsatisfactory results of large-scale biomanufacturing of high-value products and commodity chemicals are largely due to two challenges: complex cell-wide regulation of metabolic pathways, and difficulty in balancing biosynthesis of target products and innate cell physiology. First, a lot of organisms are difficult to be engineered because of unknown regulation patterns and the lack of engineering tools for non-model organisms [16]. Even for model microorganisms like *Escherichia coli* and *Saccharomyces cerevisiae*, which are well studied and equipped with a broad spectrum of biomolecular tools to allow metabolic engineering easily, the effects of heterologous expression of pathways are often unpredictable to guarantee a high productivity, as witnessed in metabolic engineering of *S. cerevisiae* to produce n-butanol [17] and engineering carbon dioxide fixation in *E. coli* [18]. In order to identify optimal biosynthetic systems and discover the best sets of enzymes, the "design-build-test (DBT)" cycles [19] are often used. However, the DBT cycles usually take months to finish, as culturing cells is time consuming. Second, a key challenge in metabolic engineering is balancing the tug-of-war that exists between the cell's physiological and evolutionary objectives on one side and the engineer's process objectives on the other [20]. Such conflict of resource allocation sometimes cannot be well addressed and toxic intermediates could be built up in the unbalanced pathway thus the manufacturing of high-value products often ends up with a low titer and yield and a high cost.

Many emerging technologies seek to address these challenges. Among them, cell-free biotechnology is one of the promising approaches that offer complementary advantages to *in vivo* metabolic engineering, especially in its potentials of speeding up the DBT cycles [21]. In general, the cell-free biotechnology bypasses the cell growth, and thus becomes time saving to permit more DBT cycles and avoids the conflict of resource allocation between cell growth and biosynthesis of target products. The cell-free biotechnology also uses an open reaction environment, which allows the easy and precise adjustment of components such as cofactors and intermediates during a biosynthetic reaction [22]. The cell-free biotechnology was first developed in 1961 for the purpose of elucidating the codon usage [23] and was repurposed for protein production since the end of the 1990s [24–27]. Recently in late 2000s, the cell-free biotechnology was further re-engineered to produce both biologically active compound and commodity chemicals [28,29,30]. In this mini-review, we summarized the experimental set-up and computational modeling of two *in vitro* metabolic engineering approaches: cell-free synthetic enzyme engineering and cell-free protein synthesis (CFPS)-based metabolic engineering (Fig. 1).

2. Cell-free Synthetic Enzyme Engineering

The principle of cell-free synthetic enzyme engineering is to purify the individual enzymes of a biosynthetic pathway, reconstitute the pathway and study its performance *in vitro*. For more than 100 years, biologists have sought to excise complete enzymatic pathways from their native cellular environments for biochemistry research [31]. *In vitro* analysis of metabolic pathways is becoming a powerful method to gain fundamental understanding of biochemical transformations, to reveal the mechanisms of enzymatic reactions and kinetics, and to identify key metabolites and feedback control of enzyme activities.

2.1. Functional Investigation of Natural Enzymes and Metabolisms

As a powerful method to investigate natural enzymes and metabolisms, some remarkable achievements have been reported. One remarkable example is the study of the bacterial fatty acid synthases. Although being investigated extensively at the genetic and enzymatic level, it is still not easy to manipulate enhanced production of specific fatty acids because of the complex cell-wide regulation of fatty acid synthesis. In 2010, Liu et al. revealed the strong dependence of fatty acid synthesis on malonyl-CoA availability and several important phenomena in fatty acid synthesis by a quantitative investigation of the fatty acid biosynthesis and regulation in a cell-free synthetic enzyme system [32]. Following these discoveries, Yu and colleagues reported an *in vitro* reconstitution of the fatty acid synthase derived from *E. coli* by overexpressing all nine fatty acid biosynthesis (Fab) enzymes and the acyl carrier protein (ACP) in the natural *E. coli* host, and purifying the enzymes to homogeneity. Upon supplementing the ten protein species with acetyl-CoA, malonyl-CoA and NADPH, C14-C18 fatty acids were observed in the system, evidenced by ^{14}C-isotope incorporation experiments and subsequently *via* UV-spectrophotometry [33]. The reconstituted multi-enzyme system has also highlighted that the fine-tuning of each individual components could substantially influence the partitioning between unsaturated and saturated fatty acid products. Similar to fatty acid biosynthesis, another pathway which synthesizes isoprenoids as key metabolites in both primary and secondary metabolisms, was reconstituted *in vitro*. Basically, in order to develop a route to synthesize the jet fuel farnesene, Zhu and colleagues reconstituted the mevalonate (MVA) pathway in a cell-free synthetic enzyme system *in vitro* by expressing and purifying eight enzymes of the MVA pathway as well as the α-farnesene synthase from an *E. coli* host [34]. The purified enzymes worked in tandem with the requisite NADPH and ATP cofactors to produce farnesene, as confirmed by gas chromatography–mass spectrometry. It was found that the isopentenyldiphosphate (IPP) isomerase was the most influential factor on the turnover rate of this pathway.

In addition to bacterial pathways, some eukaryotic pathways were also reconstituted *in vitro*. The biosynthetic pathways of dhurrin, which plays an important role in plant defense against pathogens [35], and camalexin, which is cytotoxic against aggressive prostate cancer cell lines [36], have been studied in cell-free synthetic enzyme system. Kahn and colleagues reconstituted the entire dhurrin biosynthetic pathway *in vitro* using enzymes from the natural host organism [37]. Through tedious enzyme purification processes, the researchers were able to obtain all three enzymes, CYP79, glycosyltransferase and P450ox, in the microsomal fraction of the *Sorghum bicolor* lysates. It was found that the microsomal environment could allow functional expression of catalytically active CYP79 and P450ox, and thus dhurrin synthesis was observed by radioactive TLC analysis when combining the three enzymes with ^{14}C-tyrosine, UDP-glucose, and NADPH. In another study, camalexin pathway was constructed *in vitro* by purifying three enzymes: CYP79B2, which catalyzes decarboxylation and N-hydroxylation of tryptophan to indole-3-acetaldoxamine (IAOx); a second P450 enzyme, which was previously unknown and is believed to catalyze an oxidative coupling of cysteine to IAOx; and CYP71A15, which decarboxylates and cyclizes the resulting cysteine-indole-3-acetonitrile (Cys-IAN) compound to form the thiazole ring structure within camalexin. By using a combination of gene expression data and protein sequence analysis, Klein and coworkers were able to identify a