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Review Article

Cell-free protein synthesis enabled rapid prototyping for metabolic engineering and synthetic biology

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ABSTRACT

Advances in metabolic engineering and synthetic biology have facilitated the manufacturing of many valuable-added compounds and commodity chemicals using microbial cell factories in the past decade. However, due to complexity of cellular metabolism, the optimization of metabolic pathways for maximal production represents a grand challenge and an unavoidable barrier for metabolic engineering. Recently, cell-free protein synthesis system (CFPS) has been emerging as an enabling alternative to address challenges in biomanufacturing. This review summarizes the recent progresses of CFPS in rapid prototyping of biosynthetic pathways and genetic circuits (biosensors) to speed up design-build-test (DBT) cycles of metabolic engineering and synthetic biology.

Contents

1. Introduction

Metabolic engineering and synthetic biology are one of the most promising solutions to address sustainability and global climate change challenges through the development of efficient cell factories for producing fuels, chemicals, and pharmaceutics. Introduction of a biosynthetic pathway containing multiple heterologous genes is generally the first step to enable microbial synthesis. For practical applications, pathway gene expression levels must be carefully fine-tuned and balanced to maximize product titer, rate, and yield (TRY) [1]. However, iterative design-build-test (DBT) cycles are generally required to optimize metabolic pathways, making the development of efficient cell factories rather time-consuming. Although numerous synthetic biology tools have been developed, such as gene copy number tuning [2,3] and combinatorial transcriptional regulation [4], the build and test of the combinatorial biosynthetic pathway library remains a grand challenge for metabolic engineering. For example, it is reported that more than 100 person-years are needed to commercialize biobased 1,3-propanediol [1]. Therefore, the development of novel enabling synthetic biology tools to accelerate

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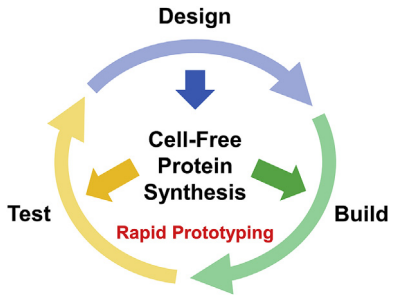

Fig. 1. CFPS as an enabling platform to accelerate design-build-test cycles of metabolic engineering and synthetic biology.

DBT cycles will be critical for the construction and optimization of microbial cell factories.

Cell-free protein synthesis system (CFPS) has been widely used for recombinant protein expression, particularly toxic proteins and membrane proteins that are difficult to express *in vivo* [5–9]. Recently, CFPS is further developed as an enabling platform for rapid prototyping of biosynthetic pathways and genetic circuits to address the challenges in metabolic engineering and synthetic biology. Compared with the conventional *in vivo* systems, CFPS activates biological machineries without the boundary of cell membranes and cell walls, and the open environment allows for direct monitoring and manipulation of transcription, translation and metabolism [10]. In addition, CFPS uses linear DNAs (i.e. PCR products) as templates for transcription, which bypasses the time-consuming and laborious gene-cloning and microorganism transformation steps and allows rapid prototyping in a high-throughput manner [11]. Different from the *in vivo* strategies by manipulating the complex transcription and translation machineries, metabolic pathway gene expression levels can be simply controlled by adjusting linear DNA concentrations supplemented to CFPS with coupled transcription-translation reactions under no resource limitation conditions [11]. Currently, CFPS has been successfully applied to the construction and optimization of metabolic pathways [12–17] and genetic circuits [18–22]. It has been reported that the CFPS can reduce the time to build metabolic pathways and genetic circuits from days to hours. Notably, biosensors based on the optimized genetic circuits hold great potentials for rapidly testing the constructed metabolic pathway libraries in both *in vivo* and *in vitro* metabolic engineering systems in a high throughout manner [23,24]. The combined rapid prototyping of metabolic pathways (build) and genetic circuits (test) open unique opportunities to accelerate DBT cycles for metabolic engineering and synthetic biology (Fig. 1).

As protein synthesis in CFPS has been reviewed in detail [25–27], this review covers the most recent update on the applications of CFPS in rapid prototyping for metabolic engineering and synthetic biology. More specifically, we focus on the construction and optimization of metabolic pathways and genetic circuits (biosensors) using CFPS. The methods to prepare the CFPS extracts and the corresponding energy regeneration systems will be briefly reviewed as well. Finally, the challenges and future perspectives on CFPS for metabolic engineering and synthetic biology applications will be discussed.

2. Construction of CFPS systems

CFPS systems have been developed for decades due to its versatility and broad applications [26]. Transcription-translation process can be accomplished by cell extracts from *Escherichia coli* and some other organisms [27]. Due to the high efficiency, flexibility, and low cost, *E. coli* CFPS system is the most commonly used. The traditional *E. coli* S30 extract preparation method have been simplified and streamlined to decrease the cost while maintain the productivity [28,29]. Notably, most of the CFPS systems rely on T7 bacteriophage mechanism (T7 promoter and T7 RNA polymerase pair), enabling high yield production of recombinant proteins [28,29]. Recently, a more versatile and flexible *E. coli* crude cell extract was developed to allow expression and regulation using both endogenous (i.e. sigma70-based promoters) and exogenous (i.e. T7 promoter) transcription and translation mechanisms [30]. In other words, the new system maintained high yield expression capabilities of existing CFPS systems, but also preserve endogenous regulatory mechanisms for metabolic engineering and synthetic biology applications.

Because transcription and translation are both energy-intensive processes, it is critical to develop an inexpensive and durable energy regeneration system to maintain efficient expression of heterologous genes and metabolic pathways. The simplest method is to add high energy source compounds directly to fuel *in vitro* transcription and translation, such as phosphoenolpyruvate (PEP) and creatine phosphate (CP) [26]. Unfortunately, these high energy phosphate bond containing molecules can be degraded rapidly by phosphatase in the cell extract. One strategy is to feed PEP periodically to regenerate adenosine triphosphate (ATP), which extended protein synthesis from ~20 min to at least 80 min and increased the production of recombinant proteins accordingly [31]. However, the accumulation of inorganic phosphate in the reaction mixture inhibited protein synthesis. A new approach for ATP regeneration without the accumulation of inorganic phosphate was developed by introducing *Pediococcus sp.* pyruvate oxidase [32], converting pyruvate to acetyl phosphate. The released acetyl phosphate can be used for ATP regeneration by endogenous acetyl kinase. Later, glucose [33] and glycolytic intermediates (fructose-1,6-bisphosphate [34]) were determined to be cheap and efficient energy sources for ATP regeneration (PANOx system) without the accumulation of inorganic phosphate. In addition, maltose [35], maltodextrin [36], and soluble starch [37] were also found to be good energy sources for CFPS, which could not only allow recycling of inorganic phosphate, but also maintain a relatively homeostatic environment with stable pH.

3. CFPS driven metabolic engineering (CFPS-ME)

With the development of metabolic engineering and synthetic biology, cell-free metabolic engineering (CFME) has been constructed to produce biomaterials, biofuels, and drug precursors [38]. Initially, CFME was developed by assembling a metabolic pathway using purified enzymes (Fig. 2A). For example, fatty acid biosynthetic pathway was reconstituted *in vitro* and used to investigate and engineer fatty acid production quantitatively [39]. However, *in vivo* protein expression and purification of every pathway protein are laborious and time-consuming. To speed up DBT cycles for metabolic engineering, Jewett group [12] constructed a new CFME framework based on crude cell lysate for rapid building and optimizing biosynthetic pathways (Fig. 2B). In this framework, each pathway gene was overexpressed individually in *E. coli*, which was used as the source strain to make crude cell lysate. Then the CFME crude lysate was mixed in a combinatorial manner to initiate DBT cycles, exploring the pathway design space with the highest efficiency (product yield).

Readers of interest are directed to recent reviews on CFME based on purified enzymes or crude cell lysates [40]. This review mainly focuses on CFPS-ME, where pathway genes are directly expressed in CFPS for subsequent biocatalysis. In other words, gene cloning, bacterial transformation, *in vivo* protein expression, cell lysate

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