BIO Web of Conferences **59**, 01002 (2023) https://doi.org/10.1051/bioconf/20235901002
ICBB 2023

Applications for Cell-free Synthetic Biology

Yewei Sun*

University College London, London, United Kingdom

Abstract. Cell-free synthetic biology is a flexible and powerful technology that engineers biological systems and parts without using living cells. Their open nature allows reactions to occur in a well-controlled and characterized environment. This review will discuss examples of applications of cell-free synthetic biology as well as their limitations and prospects.

1. Introduction

Synthetic biology is a field that manipulates and redesigns biological systems by engineering biological circuits [1]. Since its emergence, there has been substantial development in the field using the whole cell. However, the use of the whole cell system has limited its potential. For example, to harvest the cell with the desired feature, there is a requirement for genetic encoding in the lab, which is a time-consuming and functionally limited process [2]. Furthermore, concerns on the ethical issue have restricted the research on living cells.

On the other hand, cell-free synthetic biology provides a well-controlled environment which is similar to whole cell conditions, which assists in the design of synthetic circuits and accelerates the development of synthetic systems design beyond the limitations of living organisms [3]. This review describes applications of cell-free synthetic biology in different fields of study, including toxic protein synthesis, generation of biomaterial and nucleotide synthesis. By introducing these applications, this review aims to emphasise the considerable potential that synthetic biology holds and advance researchers' studies.

2. Application of Cell-free System

2.1 Cell free toxic protein synthesis

Antibiotic-resistant bacteria have drawn dramatic attention in recent years since they cause a serious threat to society [4]. Bacteriocin is an antimicrobial peptides/proteins subgroup that is considered a possible alternative to antibiotics [5]. It has several characteristics which make it suitable for treatment. Firstly, it shows the high activity of killing pathogens. Besides, it can be easily engineered and produced by probiotic bacteria. Furthermore, it shows low oral toxicity to the host [6]. Antimicrobials can be synthesized either biologically or chemically [7]. Cell-free protein synthesis (CFPS) offers several advantages in producing toxic proteins compared with cell-based production. CFPS, as a protein production platform, eliminates the demand for maintaining cell viability because machinery for transcription and translation has been extracted from lysed cells [8]. Furthermore, the CFPS platform provides an easily optimized and controlled open reaction environment [9]. Also, more complex cell-free reactions can be created by combining cell extract biotransformation reactions with cell-free protein synthesis [10].

Cell lysate is extracted and filtered to produce the toxic protein with CFPS to synthesize colicins. Colicins are bacteriocins produced by E. coli that carry colicinogenic plasmids responsible for colicin synthesis, release, and immunity [11]. They form pores in the inner membrane of the non-host E. coli and degrade nucleic acids and inhibit cell-wall synthesis [11].

In this study, the author produced several types of colicins with various functions. Colicin M inhibits peptidoglycan synthesis, colicin Ia and E1 that form pores, and nuclease colicin E2 [8]. Firstly, there is an amplification of colicin genes from r colicin plasmid with PCR. During this process, 5'-3'-untranslated regions that include the T7 promoter and terminator were added [8]. Then CFPS reactions are conducted by mixing cell-free extract and CFPS reagents with the PCR product. After incorporating radioactive 14C-Leu, the results in figure 1 show that colicin E1, E2 and Ia are almost 100% soluble with a yield of around 300ug/ml which is 10-fold higher than the production of colicin in vivo (24–28 ug/ml) [12].

* marvin.sun.20@ucl.ac.uk

© The Authors, published by EDP Sciences. This is an open access article distributed under the terms of the Creative Commons Attribution License 4.0 (https://creativecommons.org/licenses/by/4.0/).

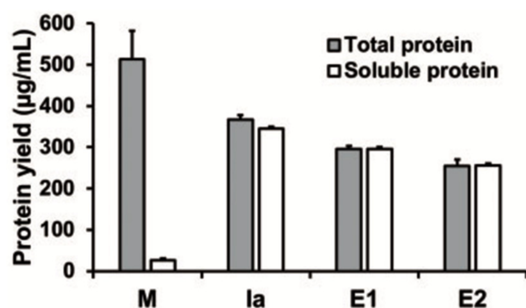

Fig. 1. Soluble and total yield of colicins produced with cell-free synthesis that quantified by 14C-Leu [13].

Then the cell-killing activity of unpurified cell-free synthesized colicins is tested. Firstly, the optimum concentration of colicin E1 is determined with a cell viability test. The K361 cell culture is exposed to colicin at 37oC for 1 hour. The serial dilution is applied to quantify the survival cell, followed by plating on LB agar. The results in figure 2A show no surviving cells after exposure to colicin E1 at 250 ng/mol or higher [8]. They also observed that at 750 ng/mol of E1, over 99.9% of cells are inactivated with one minute of exposure (figure 2B). Similar results are observed in colicin E2 and Ia. These results indicate that colicin produced by the CFPS system is active and kills cells with relatively high efficiency in a rich medium [8].

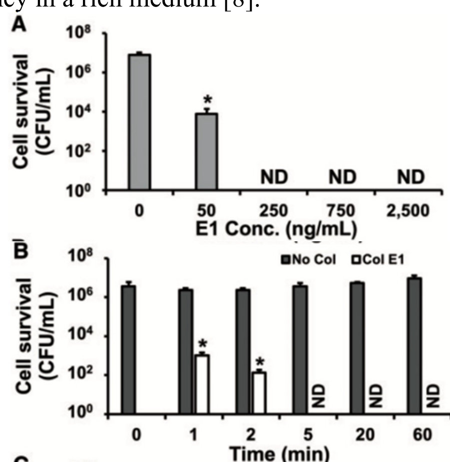

Fig. 2. (A) survival of K361 cells when treated with different concentration of cell-free synthesized colicin E1. (B) Time course cell survival after addition of 750ng/mol of colicin E1 [13]

2.2 Biomaterial Synthesis

We are living in a world that is surrounded by various materials. During the production of these materials, substantial pollutant has been generated, and we continue to approach irreversible climate change [14]. There are more than 5 trillion plastic pieces in the ocean [15], and a high concentration of microplastic is present in the aquatic environment [16]. This may lead to the accumulation of microplastic in marine organisms, eventually leading to the accumulation of microplastic in humans [17]. A possible solution is synthesizing biomaterial with cell-free synthetic biochemistry. The cell-free synthetic biochemistry could give 100% theoretical yields from input sugar because the substantial unwanted side reactions are removed [18]. In this study, the pentose-bifido-glycolysis (PBG) cycle, a new synthetic biochemistry pathway, was developed to convert glucose into acetyl coenzyme A (acetyl-CoA) efficiently.

Firstly, glucokinase converts two glucose molecules into glucose-6-phosphate (G6P) by consuming two ATP (figure 3). Then in the first phase of the cycle, two G6P are oxidized into two xylulose-5-phosphate (X5P) with 4 NAD(P)H are produced (18). In the next phase, two X5P molecules are split into two glyceraldehyde-3-phosphate (G3P) and two acetylphosphate molecules by X5P phosphoketolase (Xfp) from the bifidobacterium shunt cycle (18). After that, G3P is recycled in the third phase, and through the phosphotransacetylase (Pta), Acetylphosphate is converted into acetyl-CoA, which is used as the building block for PHB production (18). In the third phase, fructose bisphosphate aldolase (Fba) is introduced to condense two G3P molecules into fructose-1,6-bisphosphate (FBP). Then phosphofructokinase B (PfkB) is used to reverse the FBP into G6P by producing one ATP [18]. This ATP is used by glucokinase to complete the cycle by allowing another glucose molecule to enter the cycle as G6P.

In order to run the system more efficiently, two dehydrogenase purge valves at Zwf and Gnd steps are added to regulate NAD(P)H level and a metabolite salvage pathway from the Xfp enzyme [18]. After the test of the system by mixing PBG enzyme, PHB biosynthetic pathway with cofactors, and 50nM of glucose. An increase at 600nm is absorbed compared with control, which indicates the production of the PHB. The result shows its potential to become a possible industrial alternative [18].

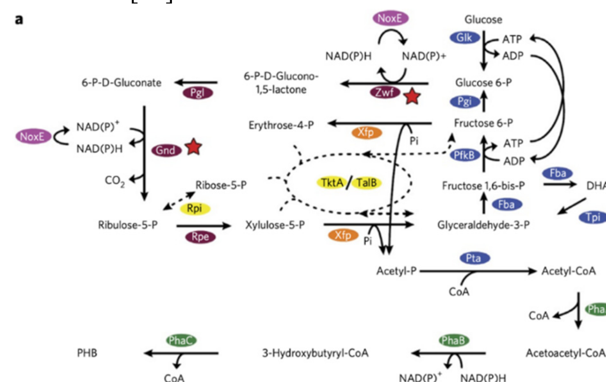

Fig. 3. The PGB pathway is illustrated, components from the pentose phosphate pathway is shown in brown, the Bifidobacterium shunt is shown in orange, and component shown in blue is first half of the glucolytic pathway. Acetyl CoA produce from PBG cycle enters the PHB pathway (Green) and converted into PHB bioplastic [18]

2.3 Nucleotide Synthesis

Modified nucleotides play essential roles in pharmaceutical and molecular biology applications. Nucleoside-5'-triphosphates (NTPs) and their analogs are the crucial building blocks for fluorescent in situ hybridization (FISH), PCR and next-generation sequencing [19]. Traditionally, the synthesis of

nucleotides is largely dependent on chemical methods such as the Ludwig-Eckstein method or the Yoshikawa protocol [20]. These multistep syntheses have a common disadvantage: the formation of different phosphorylation products caused by the limited regioselectivity. Furthermore, there is limited control over the exclusive formation of the triphosphate since the product with higher phosphorylation can be generated [21]. This study developed a modular one-pot four-enzyme cascade with a PEP-based phosphate donor recycling system to produce various NTPs easily and efficiently [21].

The modular enzymatic cascade system is aimed to produce various natural and sugar or base modified NTPs from nucleosides 1-8. In this case, the aim is to access cytidine-5'-triphosphate (1c) and its 2'-deoxy (2c), arabinofuranosyl (3c) and 5-fluoro analogs (4c) as well as adenosine-5'-triphosphate (5c) and its 2'-deoxy (6c), arabinofuranosyl (7c) and 2-fluoro analogs (8c) through a one-pot enzyme cascade reaction [21]. After analyzing reaction samples with HPLC, the final ratio of nucleoside:NMP:NDP:NTP is 0:31:47:22%for 1 and 0:30:53:18% for 3 [21]. The result indicates the successful delivery of desired phosphorylation product from nucleosides through cascade reaction by selected enzymes [21]. The one-pot enzyme reaction efficiently synthesises natural and modified BTPs from substrate nucleosides. This method holds promise for future biocatalytic syntheses of NTPs from nucleosides with high yields [21].

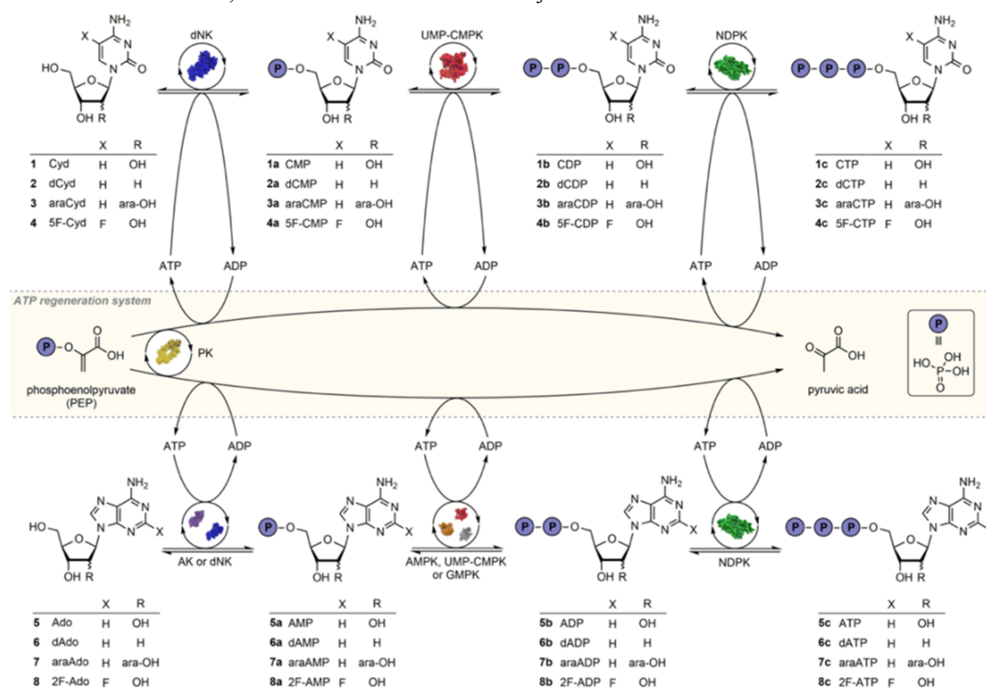

Fig. 4. The phosphorylation of natural and modified nucleosides in a cascade reaction involving multiple enzymes. ATP regeneration system is also included based on phosphoenolpyruvate and pyruvate kinase [21].

3. Conclusion

Cell-free synthetic biology is a promising, highly customizable biotechnology and is beginning to show a positive impact on many fields of study, including but not limited to protein synthesis with CFPS system, biomaterial engineering, nucleotide synthesis and many other prospects that are not mentioned in this review.

Despite the promising features and dramatic improvements that have been achieved in this field, there are also clear challenges ahead. First is protein post-translational modification, including phosphorylation, glycosylation, ubiquitination, methylation, nitrosylation, acetylation, methylation, proteolysis and lipidation [22]. Then there is the reuse of a cell-free system. Furthermore, to reduce the variability of cell-free extract batch and increase synthesis yield, a deeper understanding of the compositions and biochemical activities of processed cell extracts and cell-free reactions [23]. For industrial applications, cell-free biomanufacturing quality control standards, cell-free metrology and scalability need to be improved [24]. In order to tackle these challenges, cell-free synthetic biosystems require more modifications to regulate the translation and transcription through adding exogenous substances and gene editing [22]. Besides, a possible solution for cell-free system reusing is to continuously remove the inhibitory molecules by designing a membrane bioreactor [22].

Cell-free synthetic biology could bring biology and biotechnology to new horizons. It will create creative and unexpected solutions in biologic products for industrial applications and biomanufacturing therapeutics for biomedical applications.

Reference

1. Jeong, D., Klocke, M., Agarwal, S., Kim, J., Choi, S., Franco, E., and Kim, J. (2019) Cell-Free Synthetic Biology Platform for Engineering Synthetic Biological Circuits and Systems. Methods Protoc 2

2. Tinafar, A., Jaenes, K., and Pardee, K. (2019) Synthetic Biology Goes Cell-Free. BMC Biol 17, 64
3. Hong, S. H. (2019) "Cell-Free Synthetic Biology": Synthetic Biology Meets Cell-Free Protein Synthesis. Methods Protoc 2
4. Blair, J. M. A., Webber, M. A., Baylay, A. J., Ogbolu, D. O., and Piddock, L. J. V. (2015) Molecular mechanisms of antibiotic resistance. Nature Reviews Microbiology 13, 42-51
5. Jin, X., Kightlinger, W., and Hong, S. H. (2019) Optimizing Cell-Free Protein Synthesis for Increased Yield and Activity of Colicins. Methods and Protocols 2, 28
6. Cotter, P. D., Ross, R. P., and Hill, C. (2013) Bacteriocins — a viable alternative to antibiotics? Nature Reviews Microbiology 11, 95-105
7. Bahar, A. A., and Ren, D. (2013) Antimicrobial peptides. Pharmaceuticals (Basel) 6, 1543-1575
8. Jin, X., and Hong, S. H. (2018) Cell-free protein synthesis for producing ‘difficult-to-express’ proteins. Biochemical Engineering Journal 138, 156-164
9. Liu, W.-Q., Zhang, L., Chen, M., and Li, J. (2019) Cell-free protein synthesis: Recent advances in bacterial extract sources and expanded applications. Biochemical Engineering Journal 141, 182-189
10. Karim, A. S., and Jewett, M. C. (2018) Chapter Two - Cell-Free Synthetic Biology for Pathway Prototyping. in Methods in Enzymology (Scrutton, N. ed.), Academic Press. pp 31-57
11. Cascales, E., Buchanan, S. K., Duché, D., Kleanthous, C., Lloubès, R., Postle, K., Riley, M., Slatin, S., and Cavard, D. (2007) Colicin Biology. Microbiology and Molecular Biology Reviews 71, 158-229
12. Kienker, P. K., Jakes, K. S., and Finkelstein, A. (2008) Identification of channel-lining amino acid residues in the hydrophobic segment of colicin Ia. J Gen Physiol 132, 693-707
13. Jin, X., Kightlinger, W., Kwon, Y.-C., and Hong, S. H. (2018) Rapid production and characterization of antimicrobial colicins using Escherichia coli-based cell-free protein synthesis. Synthetic Biology 3
14. Lenton, T. M., Rockström, J., Gaffney, O., Rahmstorf, S., Richardson, K., Steffen, W., and Schellnhuber, H. J. (2019) Climate tipping points - too risky to bet against. Nature 575, 592-595
15. Eriksen, M., Lebreton, L. C. M., Carson, H. S., Thiel, M., Moore, C. J., Borerro, J. C., Galgani, F., Ryan, P. G., and Reisser, J. (2014) Plastic Pollution in the World's Oceans: More than 5 Trillion Plastic Pieces Weighing over 250,000 Tons Afloat at Sea. PLOS ONE 9, e111913
16. Xu, S., Ma, J., Ji, R., Pan, K., and Miao, A. J. (2020) Microplastics in aquatic environments: Occurrence, accumulation, and biological effects. Sci Total Environ 703, 134699
17. Vethaak, A. D., and Legler, J. (2021) Microplastics and human health. Science 371, 672-674
18. Opgenorth, P. H., Korman, T. P., and Bowie, J. U. (2016) A synthetic biochemistry module for production of bio-based chemicals from glucose. Nature Chemical Biology 12, 393-395
19. Ni, S., Yao, H., Wang, L., Lu, J., Jiang, F., Lu, A., and Zhang, G. (2017) Chemical Modifications of Nucleic Acid Aptamers for Therapeutic Purposes. International Journal of Molecular Sciences 18, 1683
20. Burgess, K., and Cook, D. (2000) Syntheses of Nucleoside Triphosphates. Chemical Reviews 100, 2047-2060
21. Fehlau, M., Kaspar, F., Hellendahl, K. F., Schollmeyer, J., Neubauer, P., and Wagner, A. (2020) Modular Enzymatic Cascade Synthesis of Nucleotides Using a (d)ATP Regeneration System. Frontiers in Bioengineering and Biotechnology 8
22. Lu, Y. (2017) Cell-free synthetic biology: Engineering in an open world. Synth Syst Biotechnol 2, 23-27
23. Foshag, D., Henrich, E., Hiller, E., Schäfer, M., Kerger, C., Burger-Kentischer, A., Diaz-Moreno, I., García-Mauriño, S. M., Dötsch, V., Rupp, S., and Bernhard, F. (2018) The E. coli S30 lysate proteome: A prototype for cell-free protein production. N Biotechnol 40, 245-260
24. de Lorenzo, V., and Schmidt, M. (2018) Biological standards for the Knowledge-Based BioEconomy: What is at stake. New Biotechnology 40, 170-180