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Alternative fermentation conditions for improved *Escherichia coli*-based cell-free protein synthesis for proteins requiring supplemental components for proper synthesis

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ABSTRACT

Escherichia coli-based cell-free protein synthesis is a powerful emerging tool for protein engineering due to the open, accessible nature of the reaction and its straightforward, economical potential for many diverse applications. One critical limitation of this system is the inability to express some complex, eukaryotic, and/or unnatural proteins at high expression yields. A potential solution is a synthetic-biology-like approach where cell-free reactions are supplemented by expressing the required supplemental components in the *E. coli* cells during the fermentation, which cells are used to prepare the extract for cell-free protein synthesis. Here we report adjustments to the fermentation conditions that increase yields of complex proteins upwards of 150% over standard conditions. We consider extracts containing GroEL/ES protein folding chaperones and extracts containing orthogonal tRNA/tRNA synthetase pairs for noncanonical amino acid incorporation. In contrast to standard cell-free synthesis, delaying the harvest of supplemented fermentations lead to increased and more consistent yields of proteins that required supplemental components. Protein yields enhanced by buffering the fermentation media pH lead to an average 52% decrease in yield cost, while costs for cases unchanged or negatively affected by buffering increased an average 14%. An apparent balance is required between the supplemental components and general extract protein profile.

1. Introduction

The *in vitro* protein production system known as cell-free protein synthesis (CFPS) is a propitious system for protein production when direct access to the reaction environment is desired [1,2]. Compared to *in vivo* expression, CFPS maintains many advantages such as improved monitoring and control, reduced reaction volumes, virtually silenced background expression, simplified purification, and removed effect of many toxins [1,3–5]. These traits make it quite versatile for applications in protein engineering such as the development of pharmaceutical proteins [6], toxic proteins [7,8], vaccines [9,10], bioimaging techniques [11], proteomic studies [12] and high-throughput protein engineering [2,13,14]. In addition, cell-free systems are increasingly being exploited for the direct combination of biomachinery from different organisms to create synthetic pathways and products which has resulted in the emergence of cell-free synthetic biology [4,5,15–19].

One prevalent, straightforward, and enduring cell-free system is based on crude extracts prepared from *Escherichia coli* (*E. coli*) [1,20]. Over the last 50 years, *E. coli*-based CFPS (eCFPS) methods have been modified to reduce cost and labor [21–24], decrease background gene expression [23,25], and increase protein production to levels to exceed 1 mg/mL [16,25,26]. While these adjustments have made eCFPS more widely accessible, economic, high-yielding, and applicable than many other CFPS systems, the range of proteins that can be correctly produced is restricted by the inherent limitations of its prokaryotic-based biomachinery [1]. For example, unmodified eCFPS cannot produce active [FeFe]-hydrogenases, cannot correctly fold some complex eukaryotic proteins, and cannot incorporate noncanonical amino acids site-specifically. However, the scenarios mentioned can be and have been accomplished in eCFPS through synthetic pathways by adding necessary purified exogenous components to the *in vitro* reaction and/or by heterologous expression of the necessary components during the *E. coli* fermentation used to prepare the extract eCFPS [13,27–29]. Systems based on purified machinery become more labor intensive and monetarily expensive with each additional component, as epitomized by the P.U.R.E. system where

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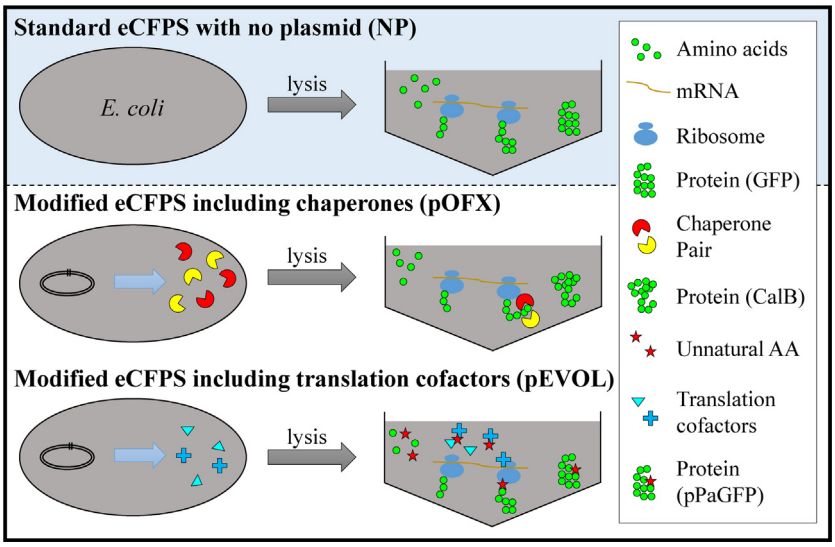

Scheme 1. Standard and modified eCFPS systems employed in this work.

every component is purified and then reconstituted for eCFPS [30], making it greater than 100 times more expensive than crude extract systems [31]. To counter the expense of such purification and increase the accessibility and efficacy of eCFPS systems requiring supplemental components, here we report the effects of plasmid-based heterologous gene expression on cell fermentation and cell extract viability, and demonstrate optimal conditions for such systems. Specifically, we optimized the fermentation conditions for cells heterologously expressing supplemental components in efforts to increase functional protein yields and broaden the potential of protein engineering and synthetic biology applications with eCFPS systems.

The gene expression profile of bacterium changes according to the growth rate and phase of the cell [32,33]. Standard eCFPS dogmatically states that cells should be harvested during the mid- to late-log phase in order to achieve maximum protein yields [25,34]. The basis of this tenet is part empirical and part logical. Cells growing the most rapidly contain an efficient balance of transcription/translation machinery to maintain the steady pace of cellular division [32]. In our experience, the heuristic of harvesting during mid- to late-log phase is highly accurate when standard, simple and stable proteins are being produced. To our knowledge, there have been no other reports exploring harvesting cells for eCFPS extracts outside of the mid- to late-log phase. However, Seo, Bailey and others demonstrated that maximum growth rates seen during log phase correspond to minimum plasmid copy number *in vivo* [33,35]. Others have demonstrated the positive correlation between plasmid copy number and expression of plasmid-borne genes [36–38]. Based on these findings, we hypothesized that a delayed harvest time following the log phase would yield higher levels of supplemental components and achieve more favorable ratios of endogenous to supplemental machinery. To test the effects of delaying harvest time after log phase on extract viability of eCFPS, we explored three distinct cases: (1) the standard case of *E. coli* without plasmids as a control, (2) the modified case of *E. coli* containing a plasmid with genes for protein-folding chaperone complex GroEL/GroES (GroE), and (3) the modified case of *E. coli* containing plasmid with tRNA/tRNA-synthetase (RS) pair capable of incorporating a noncanonical amino acid at the amber codon (UAG) using a cell-free synthetic biological pathway (Scheme 1).

The modifications described in this report are applied to two distinct eCFPS cases that demonstrate a conserved optimum harvest condition, suggesting that similar conditions could benefit other eCFPS systems using heterologous expression of essential supplemental components. These techniques enhance the feasibility for using eCFPS as a high-throughput, economical, and efficient protein engineering and synthetic biology tool.

2. Materials and methods

2.1. *E. coli* cell extract growths

All extracts were prepared using the *E. coli* strain BL21Star™ DE3 (Invitrogen, Carlsbad, CA) as follows: (1) containing no plasmid (NP), (2) containing pEVOL-*pPrF* plasmid (pEVOL), and (3) containing pOFX-GroEL/ES plasmid (pOFX). The pEVOL-*pPrF* plasmid, a kind gift from Dr. Peter Schultz (Scripps Research Institute), expresses chloramphenicol antibiotic resistance protein as well as a *Methanocaldococcus jannaschii* tyrosyl-aminoacyl-tRNA synthetase/tRNA pair [39]. The pOFX-GroEL/ES plasmid, a kind gift from Dr. Dong-Myung Kim (Chungnam National University), expresses spectinomycin antibiotic resistance protein as well as the chaperone proteins GroEL and GroES [28].

Each extract was grown with appropriate antibiotic and using sterile technique. All tubes and flasks were incubated at 37 °C and 280 RPM. Overnight cultures in 5 mL LB media were inoculated into 100 mL 2xYT media in baffled intermediate flasks and grown until an OD_{600} of 2. Approximately 90 mL from the intermediate flasks were inoculated into 1 L volumes of 2xYT media in 2.5 L shake flasks. For cells grown with MOPS buffer, the 2.5 L shake flasks contained 1 L of 0.1 M MOPS in 2xYT media. At a cell density between OD_{600} 0.5 and 0.7 in the flasks, all fermentations were induced with 1 mM isopropyl-β-D-thiogalactopyranoside and the pEVOL-harboring fermentations were additionally induced with 0.22 g L-arabinose per liter fermentation. At 3, 4.5 and 6 h after induction 300 mL of extract were harvested. Two replicates were performed for each extract condition. Harvested cells were pelleted, homogenized using an Avestin Emulsiflex-B15 Homogenizer, and prepared as previously reported [40].

2.2. Cell free protein synthesis reactions

Cell-free protein synthesis reactions were performed with the following plasmids: (1) pY71-sfGFP (2) pY71-sfGFPT216Amber [27] and (3) pk7-CalB, a generous gift from Kim [28]. Reactions were run as previously described [41] with the following modifications. Reactions of 15 μL were conducted in flat bottom 96-well plates covered with plate sealing covers. Reactions were performed at 30 °C and 37 °C for 3 and 8 h. Four replicates of each reaction were performed. As specified in Section 3, some reactions with the pY71-sfGFPT216Amber had purified *M. jannaschii* aminoacyl-tRNA synthetase added at 12 μg per mL, as previously described [41]. All reactions with the pk7-CalB plasmid contained a 4:1 molar ratio of oxidized:reduced glutathione to stimulate disulfide bond formation [28,42].

2.3. Protein yield assays

GFP standard curve was assessed by comparing radioactivity and fluorescence yields, as previously described [40]. Protein yields for CalB were measured by adding C^{14}-leucine to the eCFPS reactions and scintillation counting the TCA-precipitated reaction product, as reported previously [43].