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Short communication

Expanding the palette of *Streptomyces*-based cell-free protein synthesis systems with enhanced yields

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ABSTRACT

Cell-free protein synthesis (CFPS) has emerged as a powerful approach to recombinant protein biosynthesis for applications in biochemical engineering and synthetic biology. To date, CFPS systems have been most commonly derived from a variety of organism sources including microbes (*e.g., Escherichia coli* and yeast), plants (*e.g.,* wheat germ and tobacco), and eukaryotic cells (*e.g.,* rabbit reticulocytes and Chinese Hamster Ovary cells), each with their own advantages and opportunities. To expand the palette of CFPS platforms, we recently established a *Streptomyces lividans*-based cell-free system for the expression, especially, of high GC-content genes that are involved in the biosynthesis of natural products. Unfortunately, batch protein expression yields were limited to ~50 μg/mL of a model enhanced green fluorescent protein (EGFP). Here, we sought to address this limitation and improve protein biosynthesis yields. By increasing the total extract protein concentration in the CFPS reaction, which increases the concentration of catalyst proteins available for protein biosynthesis and energy regeneration, and modifying our extract preparation procedure, we enhanced batch protein biosynthesis yields of EGFP more than 2-fold, to 116.9 ± 8.2 μg/mL. Then, we demonstrated that our simple and robust approach could be applied to six other *Streptomyces* strains. Looking forward, we expect that our more highly productive and efficient *Streptomyces* CFPS systems can be used to synthesize, study, and discover natural product biosynthesis pathways *in vitro.*

1. Introduction

The genus *Streptomyces* is a Gram-positive mycelial bacteria with high GC-content genomes (>70% GC) that produces more than 50% of all known antibiotics of microbial origin, as well as other classes of biologically active secondary metabolites [1]. With the emergence of next-generation sequencing and genome mining technologies, more and more potential secondary metabolite gene clusters have been identified from the genome data of *Streptomyces* microorganisms [2,3]. However, identifying novel secondary metabolites from these data derived clusters remains a significant challenge because overexpression of gene clusters necessary to synthesize natural products remains difficult. Utilization of the native *Streptomyces* producers for production of bioactive natural products is often hampered by their slow growth rate, low productivity, difficulty in activating clusters in laboratory growth conditions, and a lack of versatile genetic tools to engineer the strains for enhanced production [4,5]. Overexpression in heterologous hosts can also be a challenge. For example, to overcome the problems of low expression levels and limited solubility of biosynthetic gene clusters in *E. coli*, it is often necessary to employ low-temperature expression, codon optimization, promoter engineering and chaperone coexpression in combination with major strain engineering and process optimization efforts. Even then, yields of most compounds may not be satisfactory [6]. Collectively, these challenges motivate an opportunity to develop new approaches for rapid biosynthesis of natural product gene clusters from *Streptomyces* for discovering and developing natural products.

Cell-free protein synthesis (CFPS) offers an alternative protein expression platform with potential advantages for expressing natural product biosynthetic enzymes. For example, the cell-free environment allows for design-build-test iterations to be performed without the need to reengineer organisms, DNA for pathway enzymes is directly input, and substrates and cofactors needed for secondary metabolism can be controlled and maintained at defined concentrations. In addition, CFPS systems have

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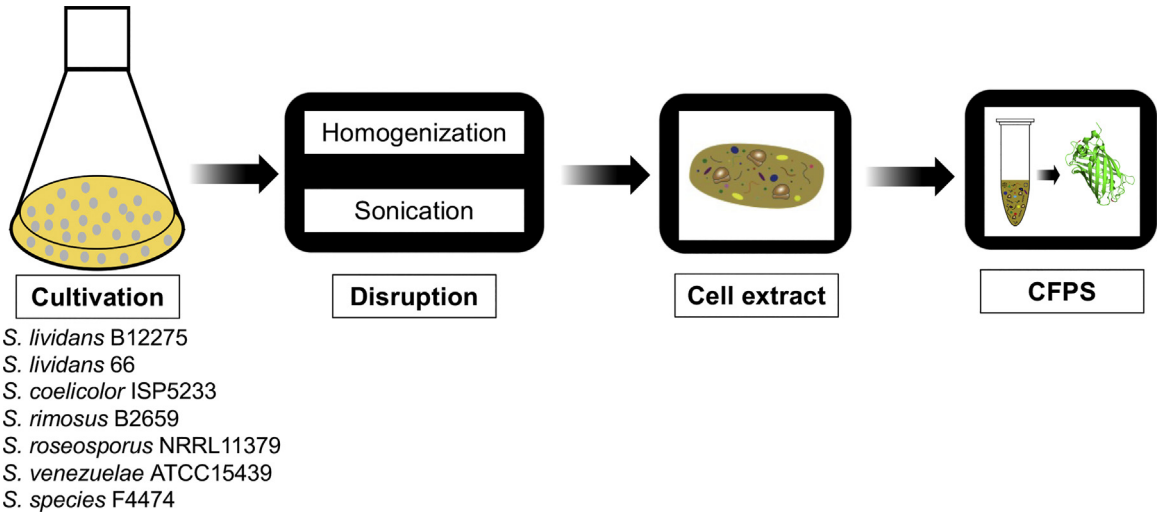

Fig. 1. A flow-chart schematic for the development of multiple *Streptomyces*-based CFPS systems.

been applied in combination with high-throughput microfluidic and lab-on-a-chip systems to study synthetic genetic constructs and molecular interactions that are involved in gene regulation of artificial cells [7,8]. We aim to establish CFPS methods for studying and engineering natural product pathways. As a step towards this goal, we recently demonstrated the use of an *E. coli*-based CFPS platform to biosynthesize a diketopiperazine by coexpression of two large (>120 kDa) nonribosomal peptide synthetases from *Brevibacillus brevis* [9]. This work represented a key proof-of-principle for how one could apply CFPS to synthesize and discover natural products.

Although *E. coli*-based CFPS systems have been developed for more than two decades to express a variety of proteins with enhanced yields [10–14], these systems may not be able to efficiently express high GC-content genes originated from *Streptomyces* due to, for example, codon usage bias, solubility issues, and post-translational modifications. In order to utilize CFPS for natural product discovery and synthesis in the future, we recently developed a *Streptomyces lividans*-based CFPS system for the expression of GC-rich genes [15]. The newly established *Streptomyces* CFPS system notably increases the solubility of high GC genes-encoded biosynthetic enzymes as compared to an *E. coli*-based cell-free system, indicating the *Streptomyces* CFPS is an efficient platform to express complex natural product gene clusters from various *Streptomyces* microorganisms, and eventually for natural product biosynthesis. Unfortunately, batch protein synthesis yields in this platform remain lower than the *E. coli* CFPS system and need to be improved.

The goal of this paper was to improve the productivity of our previous *S. lividans*-based CFPS system and demonstrate robustness of the procedure to other *Streptomyces* species (Fig. 1). To achieve this goal, we focused on increasing catalyst concentration in the CFPS reaction. To synthesize proteins of interest, crude extract based CFPS systems harness an ensemble of catalytic components (*e.g.*, RNA polymerases, ribosomes, aminoacyl-tRNA synthetases, translation initiation and elongation factors, *etc.*) that are essential for protein synthesis. Our reported *Streptomyces*-based CFPS system contained ~6 mg/mL total *Streptomyces* protein, whereas the traditional and more productive *E. coli* system is ~10 mg/mL total *E. coli* protein. Therefore, we hypothesized that increasing total protein concentrations could increase recombinant protein expression yields. Indeed, by increasing total *Streptomyces* protein concentration in the CFPS reaction and also applying bioprocess engineering strategies to modify our extract preparation procedures to keep the extract more concentrated, we showed more than a 200% increase in protein biosynthesis yields of a reporter protein. After improving CFPS yields, we applied our new procedure to six different *Streptomyces* strains and showed that they were all active in protein biosynthesis. It is important to note that our goal in this work was not to apply our CFPS system to natural product pathways, but rather to increase CFPS yields with a robust and simple procedure that expands the palette of *Streptomyces*-based CFPS systems. Our goal was met, which we anticipate can be applied to characterizing, engineering, and discovering natural products in the future.

2. Materials and methods

2.1. Bacterial strains, culture medium, and plasmid

The *Streptomyces* strains *S. lividans* B12275, *S. lividans* 66, *S. coelicolor* ISP5233, *S. rimosus* B2659, *S. roseosporus* NRRL11379, and *S. species* F4474 were obtained from the Agricultural Research Service Culture Collection (Peoria, IL). *S. venezuelae* ATCC15439 was purchased from American Type Culture Collection (Manassas, VA). All *Streptomyces* strains were grown in the liquid yeast extract-malt extract (YEME) medium consisting of (per liter) 3 g yeast extract, 5 g peptone, 3 g malt extract, 10 g glucose, 340 g sucrose and 5 mM $MgCl_2$. The plasmid pJL1-EGFP harboring the enhanced green fluorescent protein (EGFP) gene [15], which is codon optimized for the expression in *Streptomyces*, was used as a template for the CFPS reactions.

2.2. Preparation of cell extracts

The procedures of *Streptomyces* growth, harvest, and wash were performed the same as described in our previous report [15]. After the final wash and centrifugation, the pelleted cells were resuspended in the S30 lysis buffer (50 mM HEPES-KOH pH 7.5, 10 mM $MgCl_2$, 50 mM NH_4Cl, 5 mM β-mercaptoethanol, and 10% (v/v) glycerol). Then, the *Streptomyces* cells were lysed by either homogenization or sonication. The homogenization lysis was performed as reported previously [15]. Briefly, the cell pellets were resuspended in 2.5 mL of lysis buffer per gram of wet weight, followed by disruption through the EmulsiFlex-B15 homogenizer (Avestin, Ottawa, Canada) with single pass at a pressure of 12,000 psig. For the sonication lysis, 6 g of wet cells were resuspended with 6 mL of lysis buffer in a 50 mL falcon tube and placed in an ice-water bath during sonication. Then, the cells were disrupted by using a Q125 Sonica-