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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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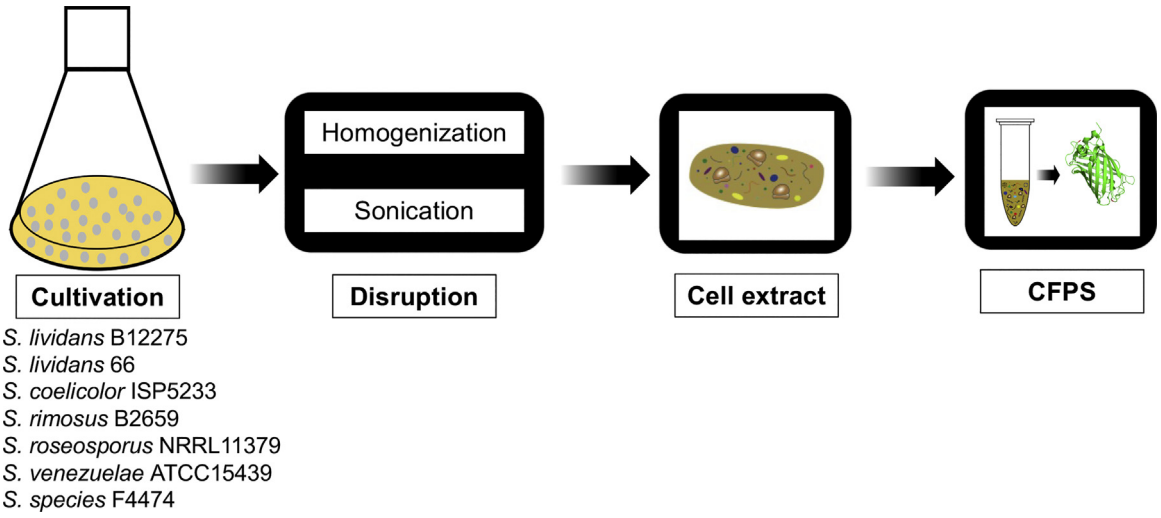

Fig. 1. A flow-chart schematic for the development of multiple *Streptomyces*-based CFPS systems.

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-

tor (Qsonica, Newtown, CT) with the input energy of around 2000 J (45 s On/60 s Off, 3 mm diameter probe, 50% of amplitude). After cell disruption, the lysate was centrifuged at 16,000*g* and 4 °C for 30 min. The resultant supernatant was collected and immediately flash frozen in liquid nitrogen and stored at −80 °C until use.

2.3. Cell-free protein synthesis and EGFP determination

Standard CFPS reactions were carried out as previously described [15]. For investigation of cell extract concentration on the protein yield, 4, 5, 6, 7, and 8 μL of cell extract was added to the 15 μL reaction, respectively. All CFPS reactions were incubated at 23 °C for 3 h [15]. The protein (EGFP) yield was determined by conversion of the fluorescence to concentration (μg/mL) according to a linear standard curve made in house [15]. All measurements were performed at least in triplicate.

3. Results and discussion

3.1. Effect of cell extract concentration on EGFP yield

Recently, we developed a *S. lividans*-based CFPS system for expressing GC-rich genes [15]. After systematic optimization, our *Streptomyces* CFPS system was able to synthesize approximately 50 μg/mL of enhanced green fluorescent protein (EGFP) in a batch reaction format. However, this yield is still lower than the protein yields achieved with *E. coli*-based CFPS systems (>1000 μg/mL), which has been optimized over the past 20 years [10–14]. Since protein synthesis depends on combined mRNA transcription and protein translation, each of the two steps could be limiting, as well as substrates needed to support the chemical reactions (*e.g.,* ATP). Our previous results indicated that substrates indeed were a limitation, but in this work we wondered if transcription or translation might limit the system during the early part of the reaction when reaction substrates are plentiful. Previously, we noted that the EGFP yields reached a similar level (~50 μg/mL) with varying plasmid concentrations [15]. Increasing T7 RNA polymerase concentrations similarly did not enhance yields [15]. Based on these results, we hypothesized that mRNA transcription did not limit protein expression yields.

We therefore considered catalyst concentration of the protein biosynthesis machinery and hypothesized that increasing catalyst concentration would increase protein synthesis yields. To test this hypothesis, we varied the volumes of cell extract from 4 (26.7%, v/v) to 8 (53.3%, v/v) μL per 15 μL CFPS reaction. Our previous optimized system with 5 μL of cell extract was used as a control. We found that the EGFP yields were significantly enhanced with addition of more cell extract. The final yield reached around 90 μg/mL by adding 8 μL cell extract (the maximum we could add to the reaction along with the necessary substrates). This yield is >2 times higher than the yield of 37.6 ± 7.4 μg/mL with addition of 4 μL cell extract (Fig. 2). The increase of EGFP yields suggests that cell extract concentration in the CFPS reaction is indeed a limiting factor for protein synthesis. Our finding is also in agreement with a reported *Bacillus subtilis*-based CFPS system, perhaps hinting at a general strategy for the improvement of CFPS activity [16].

3.2. Enhancing EGFP yield with cell extract prepared by sonication

After identifying total *Streptomyces* protein concentration as limiting, we next aimed to alter our extract preparation strategy to increase the overall total protein concentration of the extract. We focused on the cell lysis step. In general, there are multiple methods to lyse cells for the preparation of cell extracts, including,

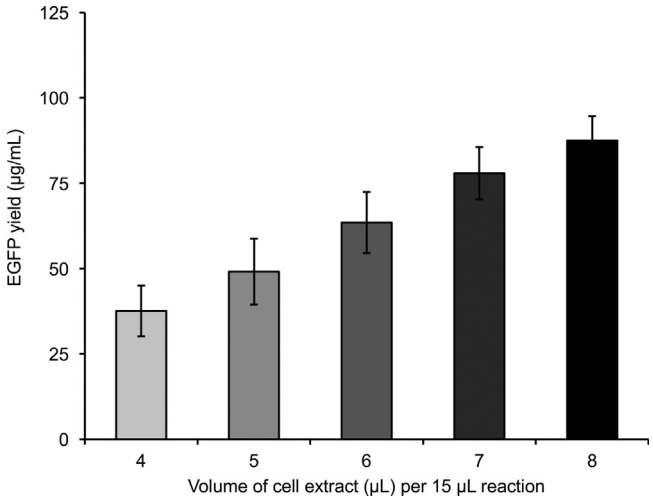

Fig. 2. Enhancement of EGFP yields by adding more cell extract per cell-free reaction. The cell extract was prepared from the strain *S. lividans* B12275 by the homogenization lysis. Values show means with error bars representing standard deviations (s.d.) of at least 3 independent experiments.

but not limited to, high-pressure homogenization [17], sonication [18], glass bead grinding [19], and freeze-thaw cycling [20]. During the initial development of the *Streptomyces*-based CFPS system, we lysed cells by the homogenization method. To do this, we resuspended one gram of wet cell pellet in 2.5 mL cell lysis buffer. After cell disruption and clarification, we determined the total *Streptomyces* cellular protein concentration of the extract to be 17.9 ± 0.3 mg/mL. This total cellular protein yield is lower than that of the *E. coli* cell extract (~40 mg/mL total *E. coli* protein) [18], and is consistent with the idea that adding more *Streptomyces* cell extract to the CFPS reaction increases protein biosynthesis yields (Fig. 2). By making more concentrated cell extract at the initial stage of extract preparation, we hypothesized that we could further improve CFPS yields.

In order to make more concentrated cell extract, we attempted to lyse cells by using sonication instead of the homogenization method because we could more readily add less re-suspension buffer to dilute the cellular proteins less during lysis. Sonication has an additional benefit that avoids the use of expensive equipment, which we anticipate will make *Streptomyces*-based CFPS more accessible to research labs. With our new sonication approach, the resultant cell extract contained 24.4 ± 0.4 mg/mL of total cellular protein, which is 1.4-fold higher than that of the homogenization prepared cell extract. With this more concentrated cell extract, we then evaluated its activity in CFPS. As shown in Fig. 3, under the condition with 5 μL of cell extract per cell-free reaction, the EGFP yield was increased by 1.5 times with the sonication prepared cell extract compared to the homogenization prepared extract. We also investigated the effect of different volumes of cell extract on protein yields as performed before. The results indicated that the EGFP yield was notably improved from 48.5 ± 2.1 μg/mL (adding 4 μL of cell extract) to 116.9 ± 8.2 μg/mL (adding 6 μL of cell extract). However, further increase of cell extract (>7 μL) in the reaction reduced the protein yields, perhaps as a result of introducing more negative factors (*e.g.*, proteases).

In total, our simple sonication method generates highly active *Streptomyces* cell extracts, which should be easily adopted by other laboratories given the easy to access lysis equipment. Of note, ~120 μg/mL EGFP is the highest protein yield reported, to our knowledge, in a batch *Streptomyces*-based CFPS reaction. Although a semi-continuous reaction could produce more protein (~280 μg/mL EGFP) as reported previously [15], our current improved system is more efficient with a synthesis

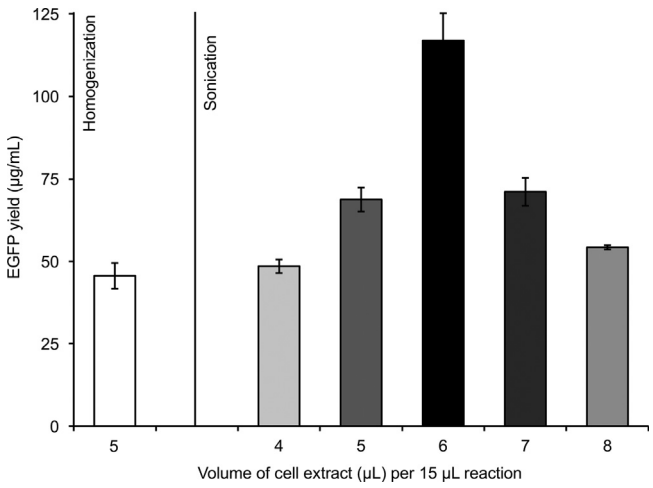

Fig. 3. Boosting EGFP yields with *S. lividans* B12275 cell extract prepared by the sonication lysis. Values show means with error bars representing standard deviations (s.d.) of at least 3 independent experiments.

rate of ~40 μg/mL/h than the semi-continuous reaction format (~6 μg/mL/h). This result is promising because (i) the batch reaction significantly shortens the reaction time to reach a similar level of protein yield; (ii) the batch reaction saves reagents (no substantial dialysis buffer needed); and (iii) the batch reaction will provide an easy and simple way to express complex natural product biosynthetic gene clusters in the future. While the *Streptomyces*-based CFPS yield is lower than that of *E. coli*-based CFPS systems, we believe further efforts will improve the overall protein yields through physicochemical optimization, bioprocess engineering, and strain engineering strategies.

3.3. *Expanding* Streptomyces*-based CFPS systems with high protein yields*

After improving the *S. lividans* B12275 based CFPS system, we sought to apply our new cell extract preparation procedure (*i.e.,* sonication) and cell-free reaction methods to other *Streptomyces* strains. This objective could demonstrate generality of our approach and open the opportunity to explore many *Streptomyces*-based systems, each with their own advantages. To this end, we chose six different *Streptomyces* strains including *S. lividans* 66, *S. coelicolor* ISP5233, *S. rimosus* B2659, *S. roseosporus* NRRL11379, *S. venezuelae* ATCC15439, and *S. species* F4474. For all six strains, the procedures of cell growth, extract preparation, and CFPS reaction were performed the same as *S. lividans* B12275. The EGFP yields were determined after 3 h CFPS reaction carried out at 23 °C.

The final EGFP yields of all tested *Streptomyces* strains are shown in Fig. 4. The optimized *S. lividans* B12275 CFPS system was used as a control for comparison. Our data indicated that all six other *Streptomyces* cell extracts were active to produce EGFP without further optimization. Although the observed protein yields were different, four out of the total seven strains produced more than 60 μg/mL of EGFP. Specifically, the *S. rimosus* B2659 CFPS system produced nearly 100 μg/mL of EGFP without any optimization, showing this strain is a good candidate for further development (*e.g.,* physicochemical optimization, strain engineering) to be a highly productive CFPS system. For the *S. lividans* 66 system, the yield of EGFP reached 84.7 ± 4.2 μg/mL, which is around 2 times higher than our previous reported yield (42.7 ± 2.4 μg/mL) [15]. This also demonstrates that the sonication method can make higher active cell extract than the homogenization preparation. In addition, we also tested the *S. venezuelae*-based CFPS system and obtained the reporter protein yield at 45 ± 1.4 μg/mL. Our system produced a higher titer of protein than that of a recent reported yield (36 μg/mL) from another *S. venezuelae* cell-free system [21]. Taken together, our work provides a simple and robust approach to develop multiple *Streptomyces*-based CFPS systems by simply changing the chassis organism.

Not surprisingly, we observed variation in protein synthesis yields from different strains, which was difficult to predict. For example, the engineered strain *S. coelicolor* M1152 with four native gene clusters deleted shows high expression of heterologous gene clusters *in vivo* [1]. However, the *S. coelicolor* M1152 strain based CFPS system showed the lowest productivity of the four tested *Streptomyces* strains in our previous report [15]. Despite not yet being able to predict how different strains perform, we envision that more productive *Streptomyces* cell-free systems will be established in the future for the purpose of expressing GC-rich gene clusters that originated from *Streptomyces* microorganisms for natural product biosynthesis.

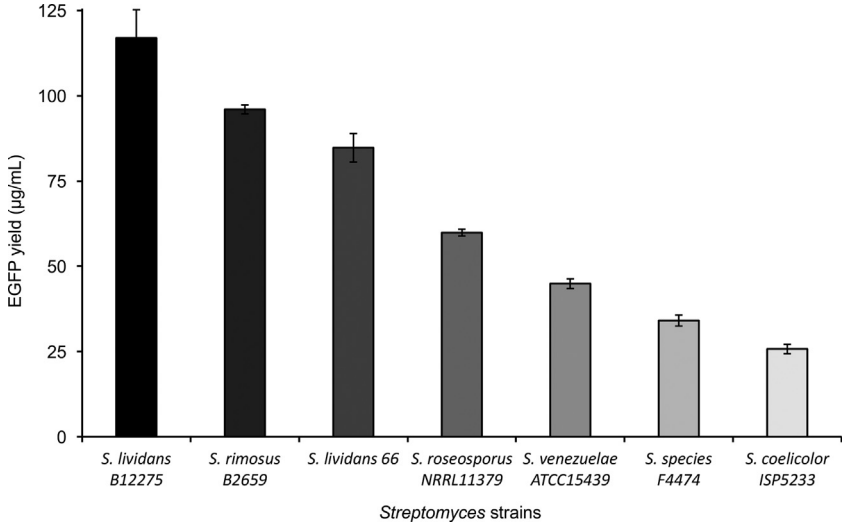

Fig. 4. Expanding the range of *Streptomyces*-based CFPS systems with enhanced protein yields. Values show means with error bars representing standard deviations (s.d.) of at least 3 independent experiments.

4. Conclusions

In this work, we improved the productivity of the *S. lividans*-based CFPS system by increasing the concentration of catalyst per CFPS reaction by two approaches. In one approach, we observed that adding more extract increased yields. In another approach, we found that modifying our process to maintain higher total protein concentration during extract preparation improved protein synthesis yields. The result of our optimization is that a batch mode CFPS reaction derived from *Streptomyces* extracts synthesized EGFP with 116.9 ± 8.2 μg/mL, which is the highest yield reported to date. To demonstrate the versatility of our new system, we expanded our procedure to six other *Streptomyces* organisms. Our results establish new *Streptomyces* cell-free systems as tools for applications in the field of synthetic biology and the increased protein expression yields make possible new opportunities in studying natural product pathways.

Conflict of interest

The authors declare that they have no conflict of interest.

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