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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 is 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.

Key Words:

- cell-free protein synthesis
- *in vitro* translation
- extract preparation
- heterologous expression
- noncanonical amino acids
- *Candida antarctica* lipase B

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

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.

MATERIALS AND METHODS

E. coli cell extract growths

All extracts were prepared using the *Escherichia 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 ^{o}C and 280 RPM. Overnight cultures in 5mL LB media were inoculated into 100mL 2xYT media in baffled intermediate flasks and grown until an OD_{600} of 2. Approximately 90 mL from the intermediate flasks were inoculated into 1L volumes of 2xYT media in 2.5L shake flasks. For cells grown with MOPS buffer, the 2.5L shake flasks contained 1L 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 1mM isopropyl-β-d-thiogalactopyranoside and the pEVOL-harboring fermentations were additionally induced with 0.22g L-

arabinose per liter fermentation. At 3, 4.5 and 6 hours after induction 300mL 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].

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 Dong-Myung 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 ^{o}C and 37 ^{o}C for 3 and 8 hours. Four replicates of each reaction were performed. As specified in the Results and Discussion, some reactions with the pY71-sfGFPT216Amber had purified *Methanocaldococcus 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].

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].

RESULTS AND DISCUSSION

The dogmatic approach to eCFPS relies on using extract prepared from cells harvested at mid- to late-log phase [25]. However, when extracts are prepared from cells grown with plasmids heterologously expressing supplemental components to aid in the synthesis of properly produced proteins, harvesting at mid- to late- log phase may not achieve an optimal balance of native and supplemental machinery to enable efficacious production of fully functional protein. To identify potentially more favorable

conditions for cell fermentations used to produce extracts for eCFPS reactions, the following parameters were explored: **1)** delayed harvest times (3, 4.5, or 6 hours post induction) and **2)** buffered growth media with or without MOPS. MOPS was added to adjust against pH deviations typically observed during extended cell growth.

Under these growth parameters, we examined a standard eCFPS system with extract from cells containing no plasmid (NP) and two nonstandard plasmid-bearing systems: 1) extract from cells harboring the plasmid pOFX-GroEL/ES (pOFX), and 2) extract from cells harboring the plasmid pEVOL-*pPrF* (pEVOL). The NP system is defined as the standard eCFPS that uses extract from BL21Star™ DE3 *E. coli* cells without additional plasmids to and is commonly used to produce proteins that do not require additional machinery or synthetic pathways. The pOFX system uses extracts from BL21Star™ DE3 *E. coli* cells harboring the pOFX plasmid and thus includes the folding chaperone proteins GroEL and GroES. The pEVOL system uses extracts from BL21Star™ DE3 *E. coli* cells harboring the pEVOL plasmid such that it includes an *E. coli*-orthogonal tRNA/tRNA synthetase (RS) pair. Thus the pEVOL system allows for the incorporation of a noncanonical amino acid when the amber stop codon is successfully suppressed. This tRNA/RS pair is essential for full length translation in proteins containing internal amber codons.

To test these cases, we expressed green fluorescent protein (GFP), *Candida antarctica* Lipase B (CalB), and a mutant GFP (pPaGFP). The protein GFP requires only native machinery for expression and therefore was chosen as a control across all cases. CalB is a large and complex eukaryotic protein that is only produced at low soluble yields without the aid of folding chaperones [14, 28]. Therefore, CalB was expressed in the pOFX system as well as the standard system. pPaGFP contains a T216 to amber codon mutation that requires the foreign transcription machinery found on pEVOL to produce full-length pPaGFP and therefore was only expressed with the pEVOL system [27].

Growth Conditions and Outcomes

Cells used to prepare eCFPS extract were fermented in 1L cultures using the simple and inexpensive baffled shake flasks, as previously reported [22, 24, 40]. This simple preparation technique allows for the eCFPS technology to be readily adapted by labs without specialized fermenters and may be combined with simple cost-effective lysis technologies, effectively lowering the entrance cost of the technology further [40].

As shown in Figure 1A, NP fermentations exhibited a definitive log phase with a transition near or shortly after 2 hours post induction to a clear stationary phase. Although MOPS clearly buffered the growth media, no apparent or statistical difference appeared between the growth profiles of buffered or unbuffered NP (Figure 1A). Cells harboring plasmids displayed a similar log phase to the NP growths and began to transition out of log phase near or shortly after 2 hours post induction. However, the transitioning period between log and stationary phase took ~6 times longer for plasmid-harboring cells, resulting in higher overall OD_{600} and growth rate at harvest compared to the NP growths (Figure 1A). These results are consistent with previous reports that growth rates and maximum densities of cells harboring plasmids are altered by the presence of the plasmids [33, 44].

The addition of the plasmid expressing heterologous elements during cell growth has the potential to increase the overall metabolic load and in turn change the proteomic profile of the cell (Figure S4 – Supplemental Information) [45]. These effects could be exaggerated by factors such as plasmid copy number, gene expression level, and gene function [44, 45]. Both plasmids employed (pOFX and pEVOL) are high-copy number plasmids and should express the promoted genes at a high level The consistent deviation of the pOFX and pEVOL fermentation profiles from the plasmid-free cells suggests that similar fermentations will also likely result in altered growth profiles

As expected, the pH of media for cells grown with MOPS fluctuated less. Furthermore, all unbuffered growths exhibited a similar pH trend and all buffered growths exhibited a similar trend (Figure 1B).

However, the pOFX growth trends were shifted approximately one half pH unit lower than the NP and pEVOL growths (Figure 1B). The lower pH profile of pOFX growth is attributed to the mechanism of spectinomycin antibiotic resistance, which results in the release of pyrophosphoric acids [46].

To analyze the impact of growth conditions and harvest time on extract protein profiles, SDS PAGE and densitometry were performed on pEVOL and pOFX extracts (Figure S4 – Supplemental Information). The heterologous synthetase and GroEL/ES were overexpressed in pEVOL and pOFX, respectively, depicted in Figure S4. The amount of supplemental components as well as other extract proteins exhibited extract to extract variation and do not appear clearly correlated with harvest time. The changes throughout the extract profiles indicated that supplemental component concentration was not a singly important trait and the overall balance of the endogenous and supplemental components in the extract likely plays a more significant role in extract viability. This is not entirely surprising, considering the many enzymes and small molecules involved in protein synthesis.

eCFPS Yields

After growth, cells were prepared for eCFPS reactions by harvesting, lysis, and clarification as reported previously [40]. Cell extracts were then used with eCFPS to express the test proteins under the following traditionally optional conditions: **1)** eCFPS reaction time (3 or 8 hours) and **2)** eCFPS reaction temperature (30 or 37 ^{o}C) with n=4 replicates (n=2 extract preparations, n=2 eCFPS reactions per extract). To simplify the analysis of parameter impact on yields and cross-case comparisons, yields were fit to a simple linear regression, described in Figure 2. Individual reaction results can be found in Supplement Information (Figure S1).

Harvest Time

The NP system for eCFPS expressing GFP behaved consistent with the expectation that mid- to late-log phase harvesting produces highest yields, with highest yields occurring while using extract harvested nearest the log phase. For GFP, the average yield decreased by approximately 43%-45% for each 1.5

hours delay in harvest. The NP case expressing CalB also produced highest yields (total and soluble) when harvested at 3 hours and, as expected, the average solubility of CalB expressed with NP was low, at approximately 30%.

Although GFP does not require supplemental components, GFP expressed in pOFX and pEVOL extracts yielded 15-30% more protein than the standard NP case and appeared unaffected by harvest time (Figure 2). A possible explanation for the increase in yields is that cells harboring plasmids exhibited a delayed onset of stationary phase during cell growths, likely resulting in a distinct proteomic profile in comparison to the NP case. The lack of impact of harvest time on GFP in pOFX and pEVOL extracts may be due to the similar growth rates observed across all harvest times.

For pOFX extract expressing CalB, longer harvest times results in higher total and soluble CalB yields (Figure 2). These yields were upwards of 3 times higher than previously reported CalB yields with similar CFPS systems [14, 28]. CalB expressed in pOFX extract was more than twice as soluble as CalB in NP (Figure S2 – Supplemental Information). The increase of CalB solubility was expected, as the addition of the GroE chaperone complex should aid in the proper folding of the protein after translation [14, 28]. Curiously, CalB total yield was also more than twice as large in pOFX as NP. The increase in total yields is likely due to the overall increase of yields in pOFX compared to NP. This could also be attributed in part to a reduction of insoluble CalB, since aggregation of CalB may lead to coprecipitation of essential transcription and translation machinery. For reference, a selection of CFPS-produced CalB samples were assayed for lipase activity and achieved an average activity of 70% of a commercially acquired CalB standard (Figure S3 – Supplemental Information).

The pEVOL case expressing pPaGFP (GFP modified for noncanonical amino acid pPa incorporation) performed consistently better with delayed harvest time (Figure 2). The full-length pPaGFP production yields with the PEVOL extract were originally very low (less than 4% of the standard GFP yields),

causing us to hypothesize there may not be sufficient foreign tRNA-synthetase or tRNA. Indeed while we performed this work, Albayrak and Swartz reported that the synthetase is likely the primary limiting factor in this relationship [47]. We therefore performed reactions with exogenously purified tRNA-synthetase supplementing the pEVOL extract (pEVOL+RS, Figure 2). The ensuing yields increased by over 300%, confirming that the synthetase had been limiting (Figure S1 – Supplementary Information). Despite the limitation of plasmid-expressed synthetase, there remained a significant positive trend in yields with regards to delayed harvest times, suggesting that more plasmid-expressed tRNA may be available at later harvest times, resulting in an overall average increase in yields of greater than 150% by harvesting the pEVOL extract well after log phase (Figure S1 – Supplementary Information).

Delaying harvest was universally beneficial for pEVOL extracts and improved the efficacy of pOFX extracts, as well. The strong benefit in pEVOL is likely due to the essential nature of the transcription machinery expressed in that system. In essence, the pEVOL machinery must compete with endogenous release factor 1 for the amber stop codon and is therefore more heavily affected by concentration. Thus, a delayed harvest is particularly important when there is competition between the exogenous and endogenous machinery.

pH Buffer

Extracts from cells fermented in MOPS-buffered media typically performed equally as well or better than their unbuffered counterparts. Buffering the media during growth likely decreases the burden on cellular mechanisms that adjust against suboptimal pH environments and thus may protect against shifts in the proteome that could be deleterious to extract viability [48]. An exception from this general trend was the expression of soluble CalB in pOFX extracts where MOPS buffering was generally detrimental to yields. The exception is not all together surprising due to the pH-reducing effect of the antibiotic resistance mechanism used by the pOFX plasmid. Indeed, this effect can be observed in Figure 1B, where at harvest

times of 3, 4.5, and 6hrs the pH of the unbuffered pOFX fermentation was closer to the MOPS-buffered NP and pEVOL fermentations than the unbuffered NP and pEVOL fermentations.

Closer inspection of the pH profiles and the respective yields suggests that maintaining the extracellular pH in the range 7.2-7.5 is preferred for viability of supplemented extracts. High or low deviations from this range appear to be deleterious to extract viability. This relationship is expected, as previous reports show that *E. coli* is sensitive to pH, particularly during active cellular reproduction [48, 49].

Augmenting the growth media with MOPs adds a further cost to the extract preparation and raises the concern that addition of this chemical reduces the economy of the eCFPS. Yield improvements due to buffering more than offset the cost of the buffering agent in half of the reactions, with an average decrease in cost of 52% (data not shown) [31]. The reactions costs not offset by yield improvements increased the average cost of reaction by 14%. The cases with decreased reaction costs generally corresponded to the cases benefitting from MOPs buffering, as seen in Figure 2.

eCFPS Time and Temperature

Universally, all proteins expressed in eCFPS reactions for 8 hours outperformed 3 hour reactions, with the exception of GFP in NP and soluble CalB yields. Generally, longer reaction time should allow for higher overall conversion. The inconsequential effect of reaction time on GFP in NP may imply that the majority of the protein is synthesized within the first three hours and that most of the energy is exhausted, as has been previously reported [21]. Previous works have also reported similar asymptotic maximums in protein yield between 3-8 hours [27].

The reduced solubility of CalB in 8 hour reactions compared to 3 hour reactions indicates increased aggregation of CalB. The complexity, disulfide bonds, and overall bulk of CalB likely contribute to higher aggregation rates than production rate. As expected, the presence of GroE chaperones reduces the negative impact of longer reaction time on solubility.

Universally, all extracts performed equally well or better at 30 ^{o}C than 37 ^{o}C. Although 37 ^{o}C is a traditional eCFPS reaction temperature, our results are consistent with multiple previous reports that advise performing reactions at 30 ^{o}C to achieve higher yields, particularly when nonstandard components are employed [27, 50].

Here we have considered alternative fermentation conditions for three fundamentally different cases of eCFPS: 1) a standard extract - NP, 2) an extract supplemented with nonessential components - pOFX, and 3) an extract supplement with essential components for successful translation – pEVOL. The traditional method of harvesting during or toward the end of log phase was shown to be best for the NP case compared to harvesting at later time points. However, a clear benefit to delayed harvesting of cells for extract preparation was observed when supplemental components were plasmid-expressed during fermentation. In addition, buffering the fermentation with MOPS was shown to be typically cost-reducing when beneficial to yields, although this effect can be complicated by factors such as antibiotic resistance mechanisms that affect the pH. To further reduce cost, other buffering agents might serve as possible inexpensive alternatives. Overall, the conservation of positive benefit at delayed harvest time and the positive effects of MOPS buffering intimates that the principles discussed here would apply to eCFPS systems requiring other supplemental components and the emerging field of cell-free synthetic biology [4, 15, 16]. This potentially expands the range of proteins that could be produced in eCFPS and enhances the potency of protein engineering and synthetic biology applications with eCFPS.

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SUPPLEMENTAL INFORMATION

Supplemental information contains individual eCFPS yields (Figure S1), graphical analysis of CalB solubility (Figure S2), lipase activity of CFPS-produced CalB compared to commercially available CalB (Figure S3), and example densitometry analysis of pEVOL and pOFX extracts on SDS PAGE (Figure S4).

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FIGURE LEGENDS

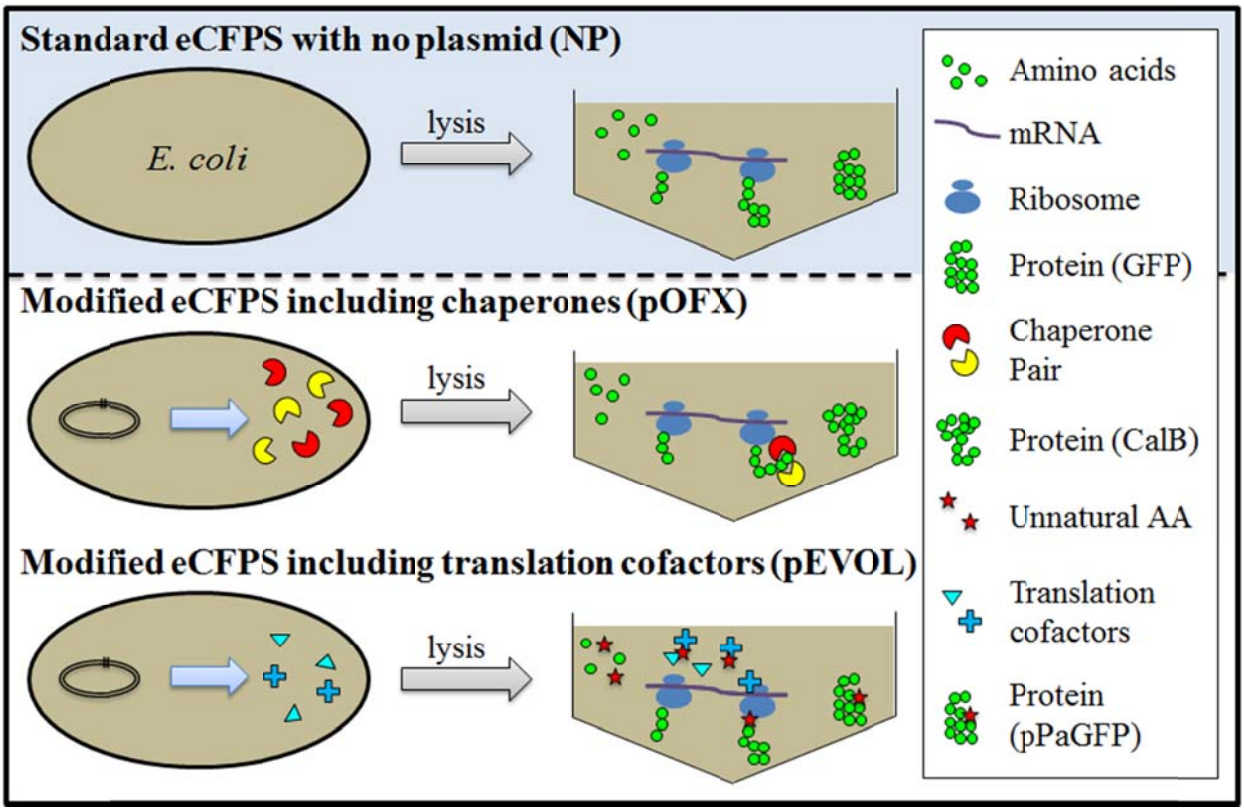
Scheme 1 – Standard and Modified eCFPS Systems Employed in this Work.

Figure 1 –Cell Growth and Media pH Curves for BL21Star™ DE3 with and without Harboring Plasmids.

Data was collected beginning at time of induction ($0.5<OD_{600}<0.7$). Growths were performed with and without 0.1M MOPS Buffer. 1A) OD_{600} profile of the fermentations. 1B) pH profile of the fermentations. The results from 2 separate fermentations (n=2) were averaged for each data point. Error bars represent one standard deviation.

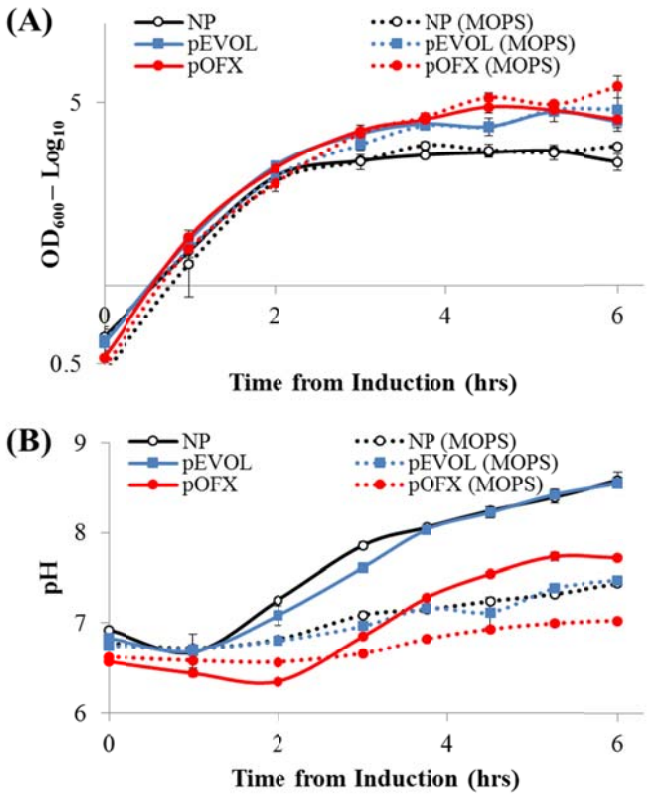
Figure 2 – Normalized Impact of Individual Parameters on eCFPS Yield.

A simple linear regression (Equation A) considering only parameter extremes allows for facile analysis of a given parameter's impact on specific extract/protein combinations. Parameter coefficients (β_i) were normalized within their regression, as described in Equation B. Normalized parameter coefficients ($^{\circ}\beta_i$) are plotted to readily compare relative impact within and between extract/protein conditions. For example, GFP yields expressed in NP extract are heavily affected by harvest time, with this statistically significant parameter coefficient accounting for roughly 55% of the regression weight and favoring a 3hr harvest time point. In contrast, the normalized harvest time parameter coefficients ($^{\circ}\beta_0$) for GFP yields produced in pEVOL or pOFX have no statistical significance to their respective linear regressions. The individual yields for each case are available in Figure S1 (Supplemental Information). Parameters were simplified to high/low as follows: $x_{HarvestTime}$ [3hr = -1; 6hr = 1], x_{MOPS} [No = -1; Yes = 1], $x_{CFPSTime}$ [3hr = -1; 8hr = 1], and $x_{CFPSTemp}$ [30 ^{o}C = -1; 37 ^{o}C = 1]. Error bars represent 95% confidence interval for the regression coefficient. *Designates the null hypothesis p-value of regression coefficient is <0.05. The individual yields for each case are available in Figure S1 (Supplemental Information).


Standard eCFPS with no plasmid (NP)
E. coli
lysis
Modified eCFPS including chaperones (pOFX)
lysis
Modified eCFPS including translation cofactors (pEVOL)
lysis
Amino acids
mRNA
Ribosome
Protein (GFP)
Chaperone Pair
Protein (CalB)
Unnatural AA
Translation cofactors
Protein (pPaGFP)

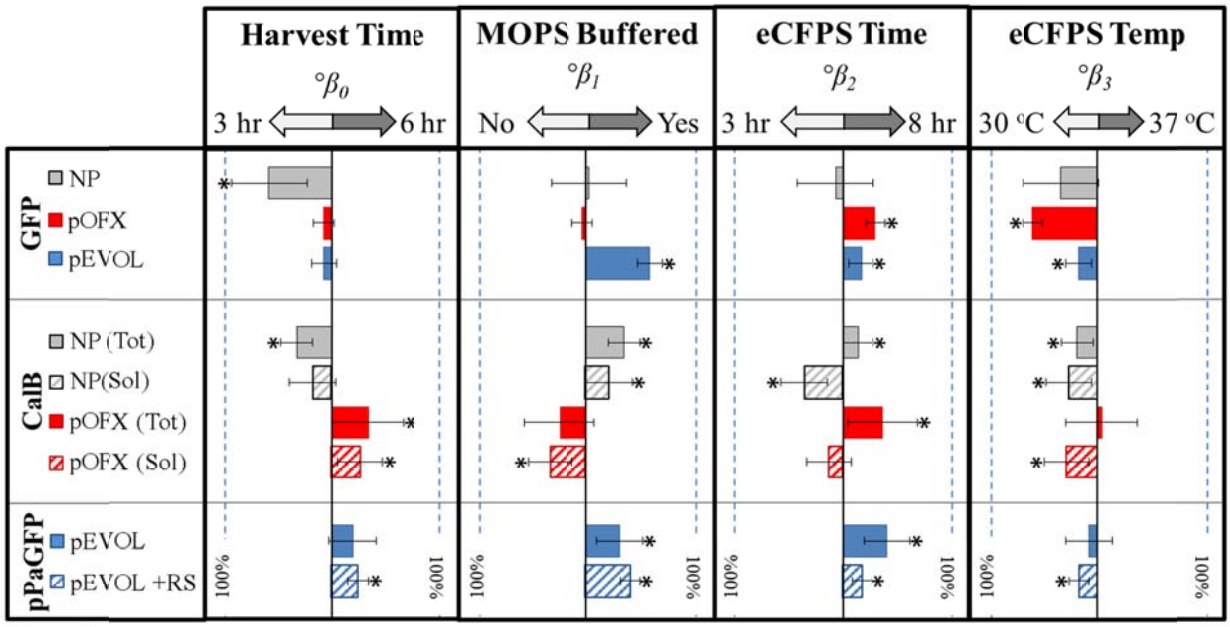
1

2


(A)
NP
pEVOL
pOFX
NP (MOPS)
pEVOL (MOPS)
pOFX (MOPS)
OD600 – Log10
5
0.5
0
2
4
6
Time from Induction (hrs)
(B)
NP
pEVOL
pOFX
NP (MOPS)
pEVOL (MOPS)
pOFX (MOPS)
pH
9
8
7
6
0
2
4
6
Time from Induction (hrs)

1

2



Equation A

$$Yield_{CFPS} = \beta_0 x_{Harvest\ Time} + \beta_1 x_{MOPS} + \beta_2 x_{CFPS\ Time} + \beta_3 x_{CFPSTemp} + \beta_4$$

Equation B

$$\beta_{i\text{-}normalized} = {}^{\circ}\beta_i = \frac{\beta_i}{\sum_{k=0}^{3} |\beta_k|}$$

1