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Streamlined Extract Preparation for *E. coli-B*ased Cell-Free Protein Synthesis and Rapid Site-Specific Incorporation of Unnatural Amino Acids in Proteins

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A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Master of Science

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ABSTRACT

Streamlined Extract Preparation for *E. coli*-Based Cell-Free Protein Synthesis and Rapid Site-Specific Incorporation of Unnatural Amino Acids in Proteins

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Master of Science

This thesis reports the viability of *E. coli* cell extracts prepared using equipment that is both common to biotechnology laboratories and able to process small volume samples and expression of proteins containing unnatural amino acids (UAAs) at higher level using PCR amplified linear DNA templates (LETs) in cell-free protein synthesis (CFPS) system.

E. coli-based cell extracts are a vital component of inexpensive and high-yielding CFPS reactions. However, effective preparation of *E. coli* cell extract is limited to high-pressure homogenizers (French press style or impinge-style) or bead mill homogenizers, which all require a significant capital investment. This work specifically assessed the following capital cost lysis techniques: (1) sonication, (2) bead vortex mixing, (3) freeze-thaw cycling, and (4) lysozyme incubation to prepare *E. coli* cell extract for CFPS. In this work, simple shake flask fermentation with a commercially available *E. coli* strain was used. Additionally, the RNA polymerase was over expressed in the *E. coli* cells prior to lysis which eliminated the need to add independently purified RNA polymerase to the CFPS reaction. As a result, high yielding *E. coli*-based cell extract was prepared using equipment requiring reduced capital investment and common to biotechnology laboratories. To our knowledge, this is the first successful prokaryote-based CFPS reaction to be carried out with extract prepared by sonication or bead vortex mixing.

LETs are an attractive alternative to plasmids for site-specific incorporation of unnatural amino acids in proteins in the CFPS system because of their short preparation time and ease of production. However, major limitations associated with LETs are: (1) their degradation by RecBCD enzyme present in the cell-extract used for CFPS and (2) high CFPS energy costs. In this work, we report the optimization of LET-based CFPS for improved protein yield by inhibiting the RecBCD enzyme with small inhibitor molecules resulting in three fold increment in yield of protein containing UAA. We also assessed alternative energy sources such as glucose, fructose-1,6-bisphospate, creatine phosphate/creatine kinase, and high glutamate salt for cost reduction. This work could be important for high-throughput applications based on linear expression templates.

This work demonstrates simple *E. coli* extract preparation and improved yield with linear expression templates for further advancements of cell-free protein synthesis system.

Keywords: Prashanta Shrestha, cell-free protein synthesis, *in vitro* protein synthesis, unnatural amino acids, *para*-proparglyoxyphenylalanine (pPa), cell lysis, cell extract, bead milling, sonication, RecBCD, exonucleases, small molecules, linear expression templates, linear DNA, PCR, transcription, translation

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1 INTRODUCTION

Cell-free protein synthesis (CFPS) refers to *in vitro* protein synthesis using a crude cell lysate that contains components necessary for transcription and translation. This technique is different from the traditional cell based technique of protein production since the proteins are not translated in a living cell. In this approach, translational machineries present in the cell extract are used for protein synthesis outside the living cells. CFPS is an open transcription and translation system as it is devoid of any kind of membrane barrier. This gives an unprecedented level of control over the system for its optimization at different levels allowing direct access and superior control over the protein synthesis broth.

Since CFPS is an open system it allows control over messenger RNA (mRNA) [1-3], synthetases [4-8], ribosomes [9-12], chaperones [11, 13, 14], energy sources [15-20] and other cellular machinery which enables protein modification for protein engineering [4, 21, 22] and synthetic biology application [23-25]. CFPS enables direct control over amino acids, buffers, nucleoside triphosphates, reaction pH, synthesis protein redox potential and various cofactor concentrations [15, 18, 26-28]. Various attempts have been made to make the process economical and scalable in light of its wide range of applications for *in vitro* evolution and protein engineering [29, 30], protein translation and folding studies [31], production of functional antibodies [32, 33] and toxic proteins [34, 35], high-throughput functional genomics [36, 37], and pharmaceuticals [38] among others. Other applications of CFPS include protein labeling for microarray assays [39], genetic diagnostics, high-throughput applications [40],

structural proteomics using nuclear magnetic resonance [41] and site-specific incorporation of single or multiple unnatural amino acids in proteins [4, 5, 7, 42].

CFPS efficiently harnesses the biocatalytic machinery for transcription and translation and is versatile in its ability to produce virus-like particles [27, 35, 43], membrane proteins [41, 44, 45] and proteins with unnatural amino acids (UAAs) [4, 7, 46, 47]. This technology has come a long way and has been tested for industrial scale production of therapeutic proteins [48]. Additionally, CFPS compliments other applications including high-throughput functional genomics [36] and screening of industrially relevant enzymes [49], protein evolution [29, 30, 49], structural proteomics and genomics [41, 50], and advancement of synthetic biology [23].

Site-specific incorporation of unnatural amino acids (UAAs) in proteins provides an effective way to engineer proteins with novel biochemical and biophysical properties [4]. They have further applications in pharmacokinetics and protein trafficking studies [4, 51]. Site-specific incorporation of UAAs in proteins provides better control on enzyme orientation for applications like biocatalysis and drug development [4]. Proteins containing UAAs are also used to study pharmacokinetics, protein trafficking, *in vivo* and *in vitro* protein folding, structure, stability, function, protein localization and protein-protein interactions [51, 52]. However, *in vivo* incorporation of UAAs in proteins results in lower protein production yields because of the inherent chemistry of some UAAs, such as cytotoxicity and/or transport limitations across the cell wall [4]. Thus, the cell-free system circumvents the UAA transport limitation problem and gives direct access to UAA by the translational machinery resulting in higher protein yields compared to an *in vivo* system.

In this work, we bridge chemistry, biology and engineering for economical insertion of UAA in protein in *E. coli* crude lysate based CFPS. The work will employ the model system

using *para*-proparglyoxyphenylalanine (pPa) and super folder green fluorescent protein (sfGFP). This system uses an orthogonal pair of *Methanocaldococcus jannaschii* suppressor tyrosyl-aminoacyl-tRNA synthetases ($Mj\text{TyrRS}^{\text{pPa}}$) and *Methanocaldococcus jannaschii* tyrosyl-tRNA with an amber stop anticodon ($Mj\text{tRNA}_{\text{CUA}}^{\text{Tyr}}$). This orthogonal tRNA synthetase acylates the tRNA with pPa and encodes pPa at the amber stop codon (UAG) on the corresponding mRNA as illustrated in Figure 1-1 [4, 53]. Figure 1-1 depicts a model CFPS reaction to incorporating pPa in sfGFP.

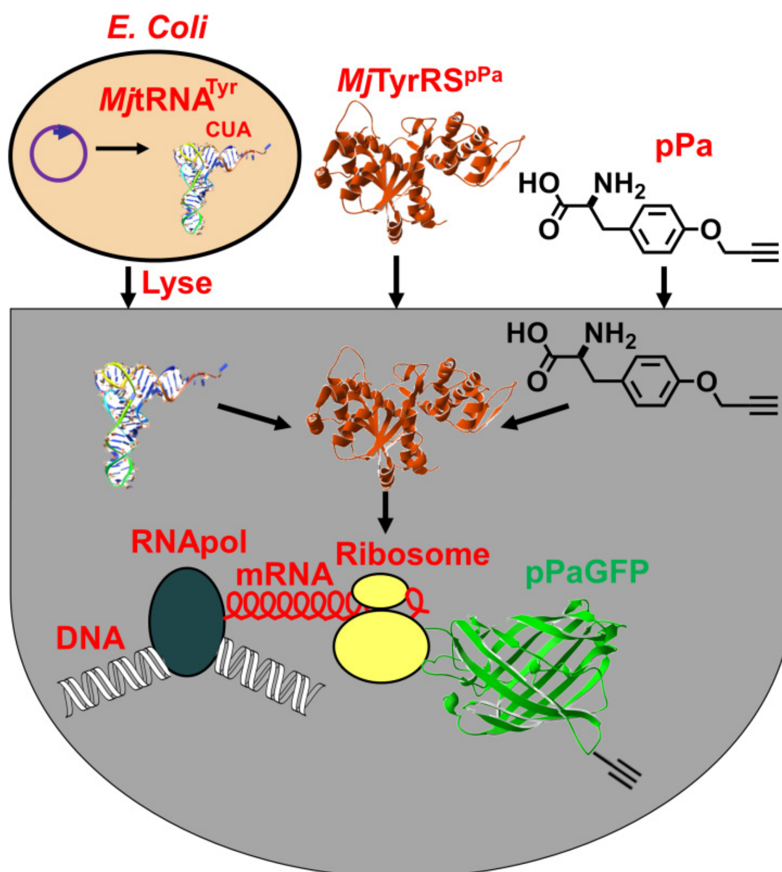


Figure 1-1: A model cell-free protein synthesis reaction. The reaction depicts incorporation of *para*-proparglyoxyphenylalanine in super folder green fluorescent protein [4, 54, 55].

Since the cell-free system is an open system, circular plasmid DNA can be substituted by PCR-generated linear DNA expression templates (LETs) [36, 56, 57]. Compared to circular plasmid DNA, LETs can be constructed in a few hours and be used for screening of random mutagenesis libraries [58]. Plasmids on the other hand take days to construct and can take up to a month or more for some products. Even though lower protein yield with LETs is a challenge, LET-based systems are desirable for high-throughput applications and rapid expression of proteins as it eliminates the traditional time consuming process of DNA cloning [36, 37].

While prior works in CFPS have advanced the technology, only minimal work has been done to eliminate the need for expensive cell lysis equipment or make the technology transferrable to the microbiology, biotechnology, and bioengineering labs. This study addresses this issue by enabling preparation of crude cellular lysate using low capital cost equipment common to most biotechnology research labs. Here we propose to overcome a limitation to widespread laboratory scale use of CFPS by using alternative cell lysis techniques for cell-free viable extract preparation. We also propose to explore alternative energy sources and optimize the use of linear DNA templates for the specific application of high-throughput selection of proteins for optimal positioning of unnatural amino acids.

1.1 Scope of the project

The objectives of this work are as follows:

1. To engineer *E. coli*-based cell extract preparation procedure using equipment common to biotechnology labs.
2. To effectively use the LETs for production of proteins containing UAAs and engineer high expression levels.
3. To reduce the cost of incorporating UAAs in proteins using CFPS.

1.2 Outline

Chapter 2: Background.

This chapter discusses the different modes of CFPS; different energy systems used in CFPS, various methods for cell lysis to prepare cell extract for CFPS and rapid protein expression using LETs.

Chapter 3: Streamlined extract preparation for Escherichia coli-based cell-free protein synthesis by sonication or bead vortex mixing.

This chapter discusses the cell extract preparation techniques for CFPS and then describes the successful utilization of sonication and bead vortexing techniques for preparation of extract for *E. coli*-based CFPS.

This chapter has been adapted from journal paper titled as the chapter name and published in the September 2012 issue of the journal BioTechniques.

Chapter 4: Improved cell-free incorporation of unnatural amino acids in proteins using linear expression templates.

This chapter discusses the importance of linear expression templates in cell-free protein synthesis and discusses the development of higher yielding system for unnatural amino acid incorporation.

Chapter 5: Conclusions and future work.

This chapter provides the summary of the work performed as a part of this thesis and future works that can be done in this area are briefly discussed.

2 BACKGROUND

2.1 Modes of CFPS

Just as there are different modes for operating a reactor in chemical industries, CFPS reactions are also performed in different modes. The main CFPS reaction configurations are (1) batch mode, (2) continuous flow, (3) continuous exchange and (4) bilayer mode as shown in Figure 2-1.

2.1.1 Batch CFPS

In batch reaction configuration, the cell extract, the energy source, and all other required cofactors are mixed together and incubated at the required temperature and time for protein synthesis. This technique is fast and easy to perform. However, because of the rapid depletion of energy sources and accumulation of inorganic phosphate this system is usually performed only for 1 to 8 hours [19, 59, 60].

2.1.2 Continuous flow CFPS

CFPS reaction time can be prolonged by using a continuous flow system designed by Spirin and co-workers. In this system, the reaction mix is constantly replenished with energy and cofactors while the products are continuously taken out. This system is analogous to a continuously stirred tank reactor. With this system, CFPS can be sustained for more than 20 hours although the costs are significantly higher [61].

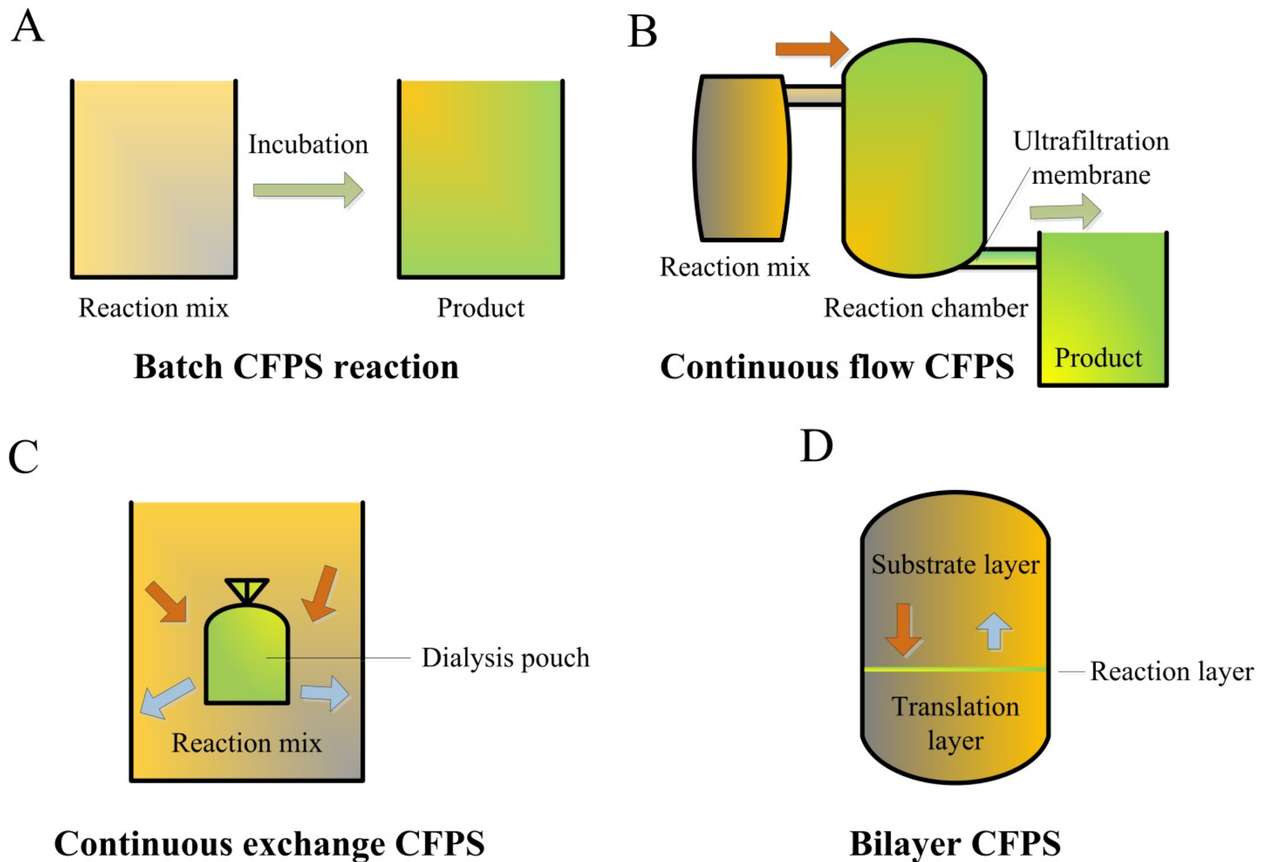


Figure 2-1: Different reaction formats for cell-free protein synthesis. A) Batch reaction, B) Continuous flow, C) Continuous exchange, and D) Bilayer. The orange, green and blue arrows denote the flow of reactant, product and reaction byproducts respectively.

2.1.3 Continuous exchange CFPS

In continuous exchange system, exchange of substrates for CFPS is performed via a dialysis membrane. In this system, a dialysis membrane allows for removal of small molecular byproducts from the reaction chamber and supplies substrate for reaction from the feeding solution. The product is retained in the reaction chamber (represented by dialysis pouch in Figure 2-1 C) in this system. Higher production yields are obtained with this system although at significantly higher costs than the batch reaction [62].

2.1.4 Bilayer CFPS

In the bilayer system, two diffusion layers are needed for cell-free reaction and removal of products. The first layer, called as translation layer, consists of all the requirements of CFPS reaction and is laid and incubated for mRNA synthesis. Next, on top of the translation layer, another layer of CFPS mixture called as substrate layer is laid creating a phase between the two layers. In this system, substrates are continuously supplied to the phase between the two layers and small molecule byproducts are continuously removed from the phase by diffusion [63].

2.1.5 Other formats for CFPS

A recent advancement in CFPS reaction configuration is by Park et al. which uses DNA hydrogels made by cross linking X-shaped DNA adapters with linear DNA templates [64]. Even though functional protein yield with this technique is as high as 5 mg/mL, the high cost associated with this system limits its broad use. From a practical standpoint this configuration is cumbersome and is cost-intensive to perform.

Even though protein yields with the batch reaction mode are lower than with other reaction modes, it is favored over other reaction modes because of its simplicity, practicality, and frugality. Similar to batch reactors in chemical plants, CFPS batch reactions are simpler to design, easier to operate and straightforward to scale-up. Recently, Zawada et al. reported a 100 L CFPS batch reaction to produce multi-sulfide-bonded granulocyte-macrophage colony-stimulating factor (rhGM-CSF), a pharmaceutical grade cytokine protein, with reported yield of 700 mg/L in 10 hours [48]. Continued advancements such as Zawada's successful scale-up continue to support the batch reaction configuration as the most viable CFPS technique for both lab and industrial setting and is therefore employed in this work.

2.2 Energy requirements in CFPS

Protein synthesis is a highly energy intensive process in growing cells. As expected, CFPS is also coupled to the same energy need although energy can be directed solely towards the protein production. Energy for CFPS is provided by sources like glucose, phosphoenolpyruvate, amino acids and nucleoside triphosphates (NTPs). Maintaining an adequate and steady supply of energy is one of the biggest challenges while performing CFPS. Batch reactions can only be sustained for typically 1 to 8 hours because of the energy depletion. Between transcription and translation, energy requirement for transcription is negligible when compared to energy requirement for translation because mRNAs are repeatedly used during transcription while 4 to 5 ATP per amino acid addition is required for translation. The schematics below show the energy requirements during coupled transcription and translation [65].

Transcription: $NTP \rightarrow NP(mRNA) + PP_i$

Translation:

Charging tRNA: $AA + ATP + tRNA \rightarrow tRNA\text{-}AA + AMP + PP_i$

Polypeptide synthesis: $AA_{p,n} + tRNA\text{-}AA + (2\text{-}3)\ GTP \rightarrow AA_{p,\ n+1} + (2\text{-}3)\ GDP + (2\text{-}3)\ PP_i$

Historically, compounds with high energy phosphate bonds such as phosphoenolpyruvate (PEP) [66] and creatine phosphate [61] were used for ATP regeneration. In one study by Kim et al., the energy cost was estimated at 50% of the total CFPS cost when phosphoenolpyruvate was used the energy source [67]. With increased understanding of complex metabolism in cell-free reactions, it has been possible to energize CFPS with other energy sources. These various energy sources are glucose [15, 16], pyruvate and glutamate [18], fructose-1,6-bishphosphate [67], 3-phosphoglycerate [19], maltodextrin [20], and starch [17] among others.

Traditional energy sources used for CFPS (such as PEP) are expensive relative to the energy source of cells (i.e. Glucose). This cost factor coupled to enormous energy requirement is one of the limiting factors for widespread use of CFPS. These energy sources carrying high energy phosphate bonds phosphorylate ADP for regeneration of ATP leading to accumulation of inorganic phosphate hindering CFPS by magnesium ions sequestration [68]. Therefore, work has been directed towards engineering systems that are economical for large scale use and also circumvent the inorganic phosphate accumulation. One of the systems activates metabolic pathways in ways similar to living cells through multistep reaction pathways like glycolysis and oxidative phosphorylation. This system, engineered to mimic the cytosolic environment, is aptly named as "cytomim" [18]. This system regenerates ATP by oxidative phosphorylation at inverted inner membrane vesicles (IMVs). Some of the widely used energy systems are described below with their merits and draw backs.

2.2.1 PANOxSP

The PANOxSP system gets its name from its main constituents where P stands for phosphoenolpyruvate (PEP), A for amino acid, N for nicotinamide adenine dinucleotide (NAD), O for oxalic acid, S for spermidine and P for putrescine. This system uses PEP supplemented by NAD, oxalate and Coenzyme A (CoA) for one step adenosine diphosphate (ADP) phosphorylation for adenosine triphosphate (ATP) regeneration [4]. An optimal magnesium concentration has been shown to have a strong correlation to translation such that optimization of magnesium is necessary for higher production yields. However, PEP sequesters magnesium ions by accumulation of inorganic phosphates resulting in detrimental effect on the CFPS. In addition, these energy sources are expensive for large scale and widespread use [18, 68].

2.2.2 Fructose-1,6-bisphosphate system

A study from Kim et al. has shown that among other glycolytic intermediates, fructose-1,6-bisphosphate results in higher protein yield [67]. As mentioned before, cofactors and phosphate energy sources add significant cost to the cell-free system. Compared to 1 ATP per PEP molecule, fructose-1,6 bisphosphate generates 4 ATP per molecule as it is incorporated in glycolysis [67]. This is only considering the anaerobic respiration. However, Jewett et al. have shown that due to inverted membrane vesicles it is possible to generate ATP via aerobic pathways (oxidative phosphorylation and tricarboxylic acid cycle) generating more ATP as shown in Table 2-1 [69].

2.2.3 Creatine phosphate

Creatine phosphate is also a high energy phosphate source which can be used for ATP regeneration. In addition, this energy source is supplemented by glucose and creatine kinase for further reduction in cost. Creatine kinase dephosphorylates creatine phosphate with simultaneous addition of phosphate to ADP forming ATP required for CFPS. This reaction is reversible in nature, but the high ATP need in CFPS drives the reaction to form ATP [61, 68].

2.2.4 Glucose

Glucose is the least expensive energy source that can be used in CFPS. Chemically it is non-phosphorous and its metabolism in CFPS doesn't result in accumulation of inorganic phosphate. It enters the glycolytic pathways and generates ATP via glycolysis. Calhoun et al. have used glucose as energy system along with nucleoside monophosphates for reduction in CFPS cost [15, 16]. Further reduction in costs can be achieved in glucose system by removing

the use of expensive cofactors like NAD and CoA. The main drawback associated with glucose is its lower yield.

For improving protein yields, glucose can be supplemented by creatine phosphate. Creatine phosphate is not a natural metabolite in *E. coli* [68]. Hence, it cannot be directly consumed by reactions which are not related to protein synthesis. With glucose, the ATP concentration is low at the initial stage of cell-free reaction. Therefore, creatine phosphate is used to prime the cell-free reaction at the beginning and the inorganic phosphate accumulated is used to drive the metabolism of glucose for further ATP generation [68].

2.2.5 Other energy sources

Other energizing source like starch [17], 3-phosphoglycerate [19], and slow metabolizing maltodextrin [20] have also been used for energizing cell-free systems.

Table 2-1: Different energy sources and their theoretical ATP yields.

Energy source	**Number of carbon atoms**	**Theoretical ATP yields (mol ATP/mol energy source)**
Glucose	6	38
Fructose-1,6-bisphosphate	6	40
Creatine phosphate	4	1
Phosphoenolpyruvate	3	16
Cellulose	Polymer of glucose	38/glucose
Starch	Polymer of glucose	38/glucose

2.2.6 Complex carbohydrates

Complex carbohydrates are polymers of the sugars linked by glycosidic bonds. They are used by plants and animals for energy storage and structural rigidity. For example, starch is preferred for energy storage in plants while glycogen is the preferred form in animals. In addition, cellulose forms the cell wall in plants and is available in abundance. They can be hydrolyzed by enzymes and broken into glucose for incorporation in cell-free reactions. Table 2-2 lists some polysaccharides with their corresponding hydrolyzing enzymes. Although, not currently used to energize CFPS there is future potential for these energy sources.

Table 2-2: Different polysaccharides and enzymes responsible for their degradation [70]

Polysaccharide	Hydrolyzing enzyme	Monosaccharide
Cellulose	Cellulase	Glucose
Glycogen	Glycogen phosphorylase	Glucose
Starch	Starch phoshorylase	Glucose

2.3 Cell lysis techniques for cell extract preparation

The widespread use of cell-free technology is limited because of specialized equipment required for cell growth and extract preparation. Our lab and others have recently demonstrated the effective use of inexpensive shake flasks and common incubator shakers for cell extract preparation, as opposed to traditional high density fermenters [35, 71]. In this work, we also seek to make the technology transferable by evaluating extract lysis techniques with equipment common to biotechnology labs. Currently, cell extracts for cell-free work are made using

expensive homogenizers which cost ~$10,000 to $30,000 (Quotation from Avestin, Ottawa, Canada) and impede initial implementation of the CFPS in academia and start-up companies. Also, it is impractical to use a homogenizer when large numbers of samples in smaller volumes are to be processed to determine the optimal cell strain for preparing extract for a given cell-free application. As the engineering of a different cell lysis technique for productive CFPS could be transformative to the field, other research groups have attempted it to some extent. One research group reported that attempts to prepare *E. coli* cell extract using sonication was unsuccessful and unreliable although the actual data was not provided, nor was the method reported [72]. In another report, freeze-thaw lysis was successfully reported but only with insect cells and not the more economical and efficient *E. coli* cells [73].

For this study, the following cell lysis techniques were used to engineer a simpler cell extract preparation for CFPS: (1) Chemical lysis, (2) Vortexing with bead mill, (3) Freeze-thaw and (4) Sonication. Different *E. coli* cell lysis techniques are summarized in Table 2-3 followed by their brief description. The price list obtained in Table 2-3 are referenced as follows: [1]Quotation from Avestin, Ottawa, Canada, [2]Quotation from Omni International, GA and Biospec products catalogue, OK, [3]Product catalogue from Quasar Instruments, LLC CO and Alkali Scientific, IA, [4]Catalogue from Sonics and Materials, Inc. CT, [5]Price from Central Chemistry Stockroom at BYU, UT and [6]Catalogue price from Sigma-Aldrich, MO.

Table 2-3: Summary of common cell lysis techniques explored in this work.

Cell-lysis method	Description	Equipment/Reagent	Equipment/Reagent Cost [$]
Homogenization	Cells are forced through a narrow slit in high pressure setting	French press Impinge homogenizer	10,000 to 30,000[1]
Bead mill	Cells are ground with glass beads	Glass beads and bead mill	5,000 to 10,000[2]
Bead vortexing	Cells are vortexed with 0.1 mm glass beads	Table top vortex and glass beads	200 to 500[3]
Sonication	Cells are disrupted by high pressure sound	Sonicator	2,600 to 15,000[4]
Freeze-thaw	Cells are subjected to repeated cycle of freezing and thawing	Dry ice or liquid nitrogen	$1.44/lb. of dry ice $1.34/L of liquid nitrogen[5]
Enzymatic	By hydrolysis of glycosidic bond in peptidoglycans	Hen egg white lysozyme for *E. coli*	$ 42.8/ gm[6]

2.3.1 Homogenization

In this lysis method, cells are disrupted by forcing them through a narrow orifice at high pressure. It is a widely used cell lysis method practiced by multiple research groups [35, 59, 71, 74]. One common type of homogenizer is the French press. In this system, cells are forced through a narrow orifice at high pressure, creating shear on the cell wall, and then released to a chamber of low pressure. The shear and the sudden change in pressure disrupt cells. In another type of homogenizer called the impinge homogenizer, cells at high pressure are smashed on a hard impact ring. This homogenizer is the gold standard for *E. coli* lysis, but its associated high cost limits its wider adoption in CFPS platform.

2.3.2 Bead mill

In this method, cells are suspended with small glass beads or ceramic beads in a closed container and then subjected to intense agitation using bead beater (for eg. Bead Ruptor by Omni International, GA) for disruption. This technique is being used by Protein Research Group, RIKEN Genomic Sciences Center [72].

2.3.3 Bead vortexing

When *E. coli* cells are vortexed with glass beads on the order of 0.1 mm diameter, their cell walls are disrupted. The efficiency of this method depends on the cell to bead ratio, the vortexing frequency and the vortexing time. This technique simplifies the bead mill and employs a table top vortexer and is aptly named as bead vortexing. This is a novel technique developed and tested in this work.

2.3.4 Sonication

In sonication, high frequency periodic sound waves agitate the cell buffer mixture. Sonication forms microscopic bubbles which burst and send shockwaves in the cell mixture causing its disruption. For efficient lysis of *E. coli* cells, 20 kHz frequency ultrasound is needed. There are different types of probes available for sonicators for different applications. They are: (1) Cup horn, (2) Microplate horn, (3) Spiral, (4) Dual horn, and (5) Probe sonicators with different probe designs to transfer sound waves [75]. This work will utilize probe sonicator for effective *E. coli* disruption.

2.3.5 Freeze-thaw

This technique involves freezing of cell buffer mixture in a freezing substance (usually dry ice or liquid nitrogen) and thawing them at room temperature. In this process, cells swell during the thawing process and contract during freezing process. One or more cycles of freezing and thawing is required for efficient cell lysis. The stress on the cell wall during freeze and thaw cycle causes cells to rupture. Ezure et al. have efficiently lysed *Trichoplusia ni* (HighFive) insect cells using one cycle of freeze-thaw for extract preparation [73]. They have reported higher protein yields with extract prepared from freeze-thaw than with extract prepared from a dounce homogenizer. This technique is also used to lyse cells for microbial DNA and polysomes extraction [76, 77].

2.3.6 Enzymatic lysis

In this lysis method, enzymes disrupt the chemical integrity of the cell wall. Lysozyme is commonly used for enzymatic lysis. Lysozyme is a natural antibacterial agent found in egg whites and tears and was one of the first enzymes whose 3 dimensional protein structure was determined. Hen egg white lysozyme is an enzyme with 129 amino acid residues and is 14.3 kDa in size. It hydrolyses peptidoglycans, a carbohydrate found in many bacterial cell walls. It cleaves the $\beta 1 \rightarrow 4$ glycosidic C-O bond between the N-acetlymuramic acid and N-acetlyglucosamine destroying the integrity of the cell wall and releasing the cellular translational machinery. Figure 2-2 and Figure 2-3 depicts lysozyme and its hydrolysis mechanism respectively. While effective at *E. coli* lysis, the use of lysozyme could cause the destruction of inverted membrane vesicles which putatively form during cell lysis and enable oxidative phosphorylation [69].

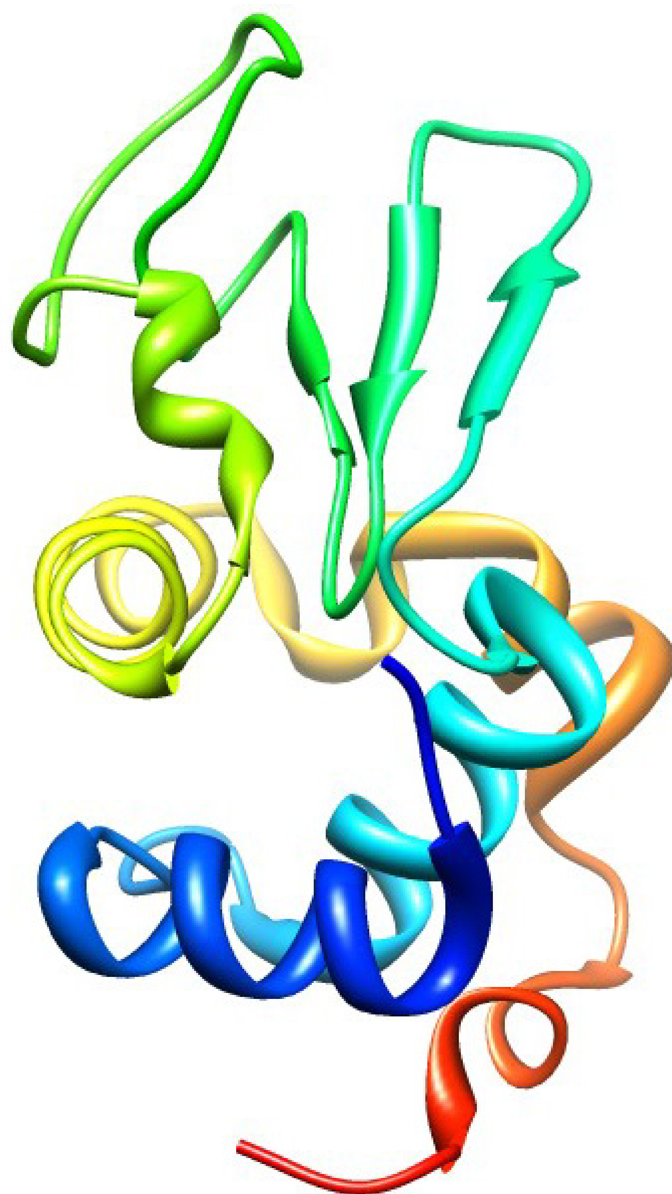


Figure 2-2: Ribbon diagram of hen egg white lysozyme (PDB ID: 1DPX) [55, 78]

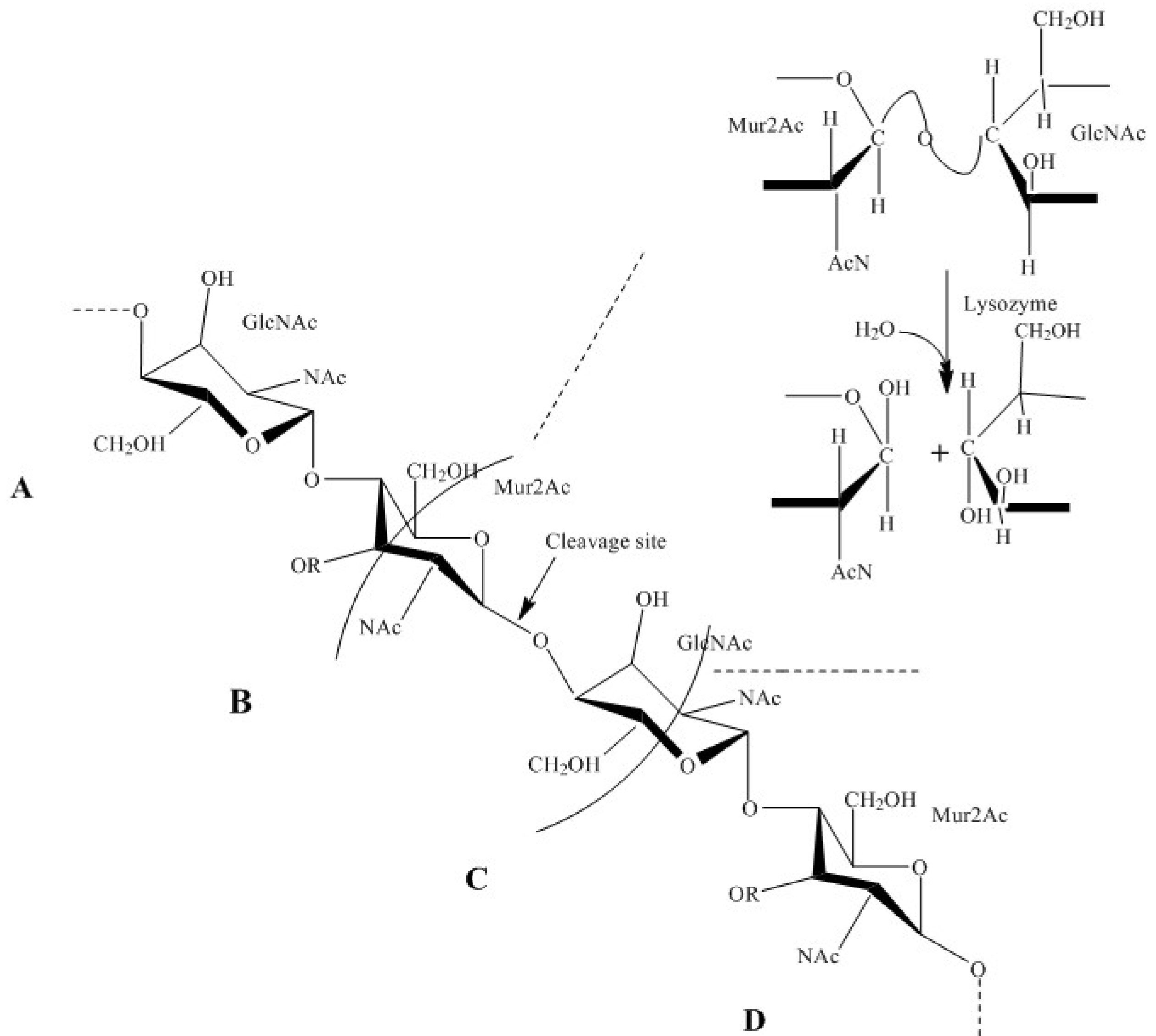


Figure 2-3: Hydrolytic mechanism of lysozyme. The glycosidic C—O bond between sugar residues bound to sites B and C is cleaved, as indicated by the arrow. The hydrolytic reaction is also shown. Mur2Ac is N-acetylmuramic acid; GlcNAc, N-acetylglucosamine. RO— represents a lactyl (lactic acid) group; —NAc and AcN—, an N-acetyl group.

2.4 Rapid protein production

Use of linear expression templates (LETs) has made the process of protein expression using CFPS rapid by eliminating the need for the time consuming process of DNA cloning [79]. LETs speed up the process of elucidating gene function with rapid synthesis of proteins and demonstrate their efficacy when multiple proteins are to be expressed in parallel. Furthermore, they can be easily integrated in automated systems [80]. Generally, a drop in protein yield has been observed when LETs are used instead of circular DNA plasmids. However, the time efficiency gained with LETs more than compensates for the drop in protein yield.

The endogenous nucleases present in the cell extract used for CFPS degrade LETs. Lee et al. reported the stabilization of functional mRNAs from LETs by use of polyguanine chain at the 3' end of the mRNA [81]. Another attempt at improving protein yields with LETs was directed at eliminating the factors that had negative effect on the functional mRNA in the cell-free reaction mix. This was achieved by the use of cell strain containing mutation to inhibit the mRNA degradation activity of the RNase E [82, 83]. Recently, small molecule inhibitors of RecBCD have been discovered and were employed to improve the protein yield in our CFPS system [84].

3 STREAMLINED EXTRACT PREPARATION FOR *ESCHERICHIA COLI*-BASED CELL-FREE PROTEIN SYNTHESIS BY SONICATION OR BEAD VORTEX MIXING

3.1 Introduction

Cell-free protein synthesis (CFPS) enables direct control and optimization of protein synthesis by performing the reaction in a test tube wherein the transcription, translation, and protein folding machinery provided by cell extract are combined with energy sources to catalyze the synthesis of only the target protein. Hence, viable cell extract is a vital constituent of effective cell-free reactions and cell lysis is a key unit operation in cell extract preparation. Due to the superior control and direct engineering that CFPS affords over protein synthesis, many independent researchers have developed, simplified and optimized CFPS reactions and cell-extract preparation procedures [9, 59, 71-73, 85-89]. However, methods for high yielding *E. coli*-based CFPS still require specialized cell lysis equipment resulting in a significant capital investment. In this work, we assess the use of cell lysis techniques with common biotechnology equipment requiring a smaller capital investment to prepare viable *E. coli*-based CFPS extract.

CFPS is an open system devoid of a membrane barrier and thus allows for manifold manipulations of the system, including adjustment of energy, cofactors, and genetic template concentrations, as well as the cell extract itself. For example, different energy sources such as phosphoenolpyruvate [66], phosphocreatine [61, 90], glucose [15, 16], and fructose-1,6 bisphosphate [67] have been successfully incorporated into CFPS and *E. coli* central metabolism and oxidative phosphorylation have been activated [18, 69]. Additionally, polymerase chain

reaction-generated linear DNA templates have been incorporated in CFPS [36, 56, 57]. To enable more scientist and engineers to reap the benefits offered by CFPS, a simple, robust, convenient, and high-yielding cell extract preparation method is needed.

The *E. coli*-based system is the least expensive, the highest yielding, and the most time efficient CFPS system [91]. *coli* extract preparation protocol for CFPS dates back to a protocol from Nirenberg in 1963 [89] which was further modified by Zubay [86] and Pratt [87]. More recently, Kigawa et al. [72], Liu et al. [9], Kim et al. [59], and Yang et al. [71] have sought to streamline the extract preparation protocol. Kim et al. [59] eliminated unnecessary steps and reduced the reagent cost and processing time for extract preparation by 80% when compared to the protocol established by Pratt [87]. In addition, Kigawa et al. [72] and Yang et al. [71] have reported the use of shake flask fermentation to simplify the cell growth. Kim et al. [59] also reported the use of the commercial BL21 (DE3) strain (Invitrogen, Carlsbad, CA) to overexpress the T7 RNA polymerase during cell extract preparation and eliminate the need to add independently purified T7 RNA polymerase to the CFPS reaction as required by other protocols [9, 71, 72]. More recently, the same research lab reported the use of a BL21 Star™ (DE3) (Invitrogen) containing mRNA stabilizing mutation and is used in this work [92]. Figure 3-1 provides an overview of these developments. As shown in Figure 3-1, all of the aforementioned protocols use a specialized bead mill or high pressure homogenizer for cell disruption, requiring a significant capital investment before research labs can assess the efficacy of *E. coli*-based CFPS for their protein of interest or application.

Most of the results and analysis reported in this chapter are published in the September 2012 issue of the peer-reviewed journal Biotechniques [74].

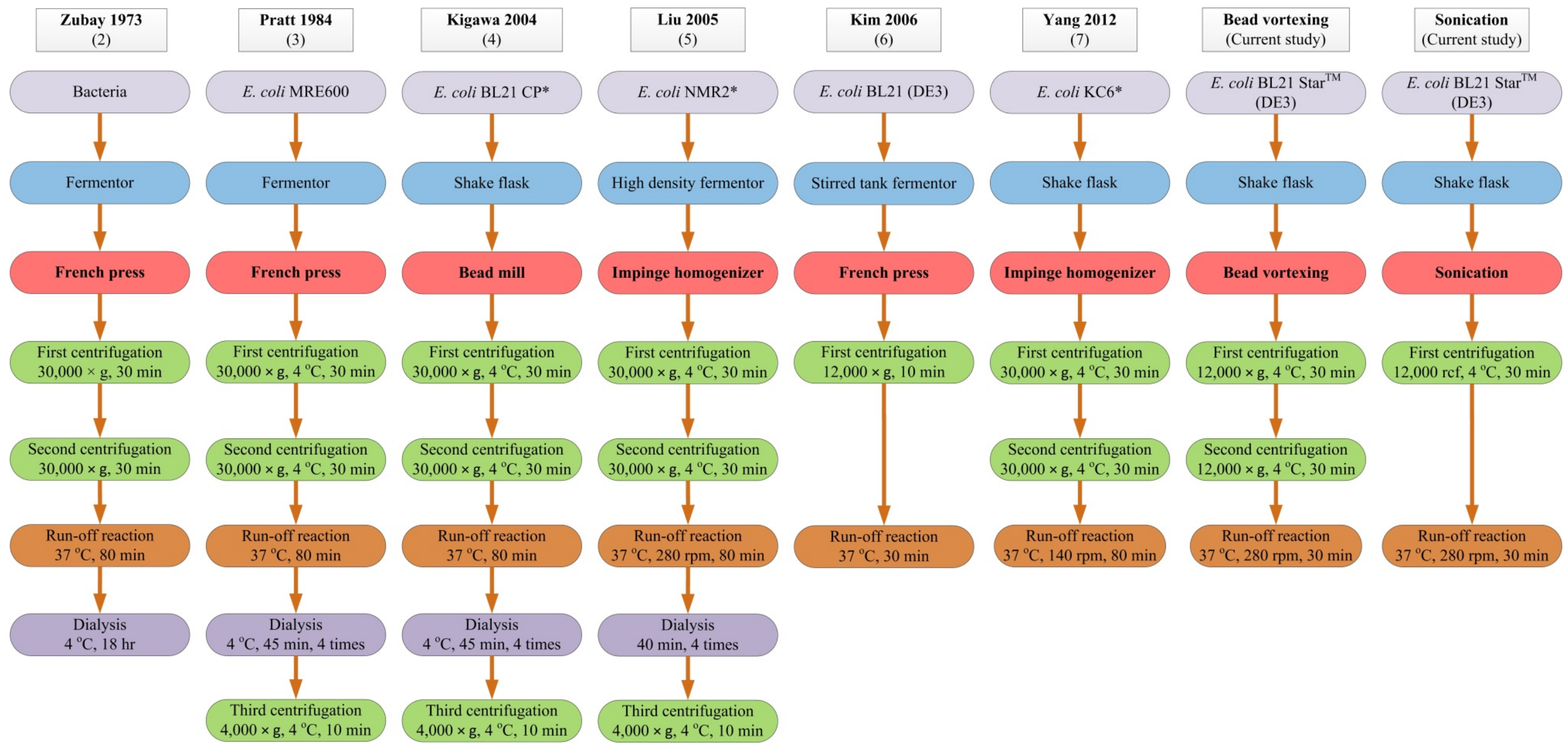


Figure 3-1: Schematic comparison of different extract preparation methods. These methods were developed over the last 50 years. Methods with a star by the cell strain require the addition of independently purified RNA polymerase to the final CFPS reaction. The bead vortexing and sonication methods are reported in this paper.

3.2 Materials and methods

3.2.1 Shake flask cell culture

Cell growth for extract preparation was performed using BL21 Star™ (DE3) cells harboring the pEVOL-*p*PrF plasmid [93] in a 2.5 L baffled tunair flasks (IBI Scientific, Peosta, IA). Cells were cultured at 37 ^{o}C with 280 rpm in an Innova™ 4300 Incubator Shaker (New Burnswick Scientific, Enfield, CT). The fermentation was performed with and without the presence of 100 mM 3-morpholinopropanesulfonic acid (MOPS) in 2x yeast extract and tryptone growth media (2xYT). The fermentations were induced with 1 mM isopropyl β-D-1-thiogalactopyranoside and 0.02 % (w/v) L-arabinose at an optical density (OD_{600}) of 0.6. Cells were harvested at mid to late logarithmic growth phase at an OD_{600} of 2.7 to 3.8, 4 h after the induction by centrifugation at 8000 rpm in Sorvall RC® 6 plus Superspeed centrifuge (Thermo Scientific, Waltham, MA) at 4 ^{o}C for 30 min. Cells were then washed by suspending in 10 mL ice-cold buffer A (10 mM Tris base, 14 mM magnesium acetate, 60 mM potassium glutamate, and 1 mM dithiothreitol) per gram of cell and centrifuged at 6000 rpm in Sorvall RC® 6 plus Superspeed centrifuge at 4 °C for 30 min and subsequently resuspended in 1 mL ice-cold buffer A per gram of cell in preparation for cell lysis. Finally, the cell suspension was flash frozen in liquid nitrogen and stored at -80 ^{o}C prior to lysis.

3.2.2 Cell lysis and extract preparation

High pressure homogenization: Thawed cell suspensions were lysed with 3 passes through an Avestin Emulsiflex-B15 French press-style high pressure homogenizer (Avestin, Inc. Ottawa, ON, Canada) at 24,000 psi with sample cooling for a 1 min in an ice-water bath after the second pass. The lysate was centrifuged at 12,000 g for 10 min at 4 ^{o}C and the pellet was

discarded. The supernatant was carried forward for a run-off reaction by incubating at 37 ^oC with 280 rpm agitation for 30 min. The run-off reaction destroys endogenous mRNAs in the extract and allows the translation of mRNAs transcribed from the DNA used for the reaction. The extract was flash frozen in liquid nitrogen and stored at -80 ^oC until use.

Sonication: Thawed cell suspensions were lysed using a Vibra-cell VCX 400 probe sonicator with a CV 26 probe (tip diameter of 3 mm) (Sonics and Materials, Inc., Newtown, CT) at a frequency of 20 kHz and power intensity of 0.7 W/cm^2. The sample vial was kept in an ice-water bath to prevent significant heating in the sample during sonication. The lysate was centrifuged for 30 min at 12,000 g and 4 ^oC and the run-off reaction was performed by incubating the supernatant at 37 ^oC with 280 rpm agitation for 30 min. The extract was flash frozen in liquid nitrogen and stored at -80 ^oC until use. Sonication was performed by cycling at the sonication and cooling intervals as shown in Table 3-1.

Table 3-1: Sonication time and cooling time intervals for cell lysis.

Sonication burst time	**Cooling time**	**Sonication burst time**	**Cooling time**
10 s	30 s	3 min	2 min
20 s	60 s	10 min	2 min
60 s	90 s	20 min	2 min

Bead vortexing: Glass beads of 0.1 mm diameter (Scientific Industries, Inc., Bohemia, NY) at 10%, 20%, 50% or 80% w/v beads to cell suspension ratio were used for cell lysis in 1.5 mL or 2 mL microcentrifuge tubes. The cell and bead suspension were vortexed on a table top vortexer Fisher Vortex Genie 2 (Scientific Industries, Inc.) at 3200 rpm for different time

intervals with a 1 min cooling period between each vortexing. The lysate was centrifuged twice at 12,000 g at 4 ^{o}C for 30 min with the supernatant retained each time. The run-off reaction was performed at 37 ^{o}C and 280 rpm shaking for 30 min. The extract was flash frozen in liquid nitrogen and stored at -80 ^{o}C until use.

Lysozyme incubation: Hen egg white lysozyme (Sigma-Aldrich, St. Louis, MO) was added to the thawed cell suspension at a concentration of 0.1 mg/mL and incubated at 37 ^{o}C with gentle shaking (80 rpm) for 3 h similar to as reported previously [94]. The lysate was centrifuged at 12,000 g and 4 ^{o}C for 10 min. The run-off reaction was performed by incubating the supernatant at 37 ^{o}C and 280 rpm shaking for 30 min. The extract was flash frozen in liquid nitrogen and stored at -80 ^{o}C until use.

Freeze-thaw cycling: Thawed cell suspensions of 500 µL in 1.5 mL microcentrifuge tubes were subjected to 3 freeze/thaw cycles by freezing the cell suspensions on liquid nitrogen or dry ice for 15 min and thawing for 15 min in a water bath at 25 ^{o}C similar to previously reported methods [95, 96]. The lysate was centrifuged at 12,000 g and 4 ^{o}C for 10 min. The run-off reaction was performed by incubating the supernatant at 37 ^{o}C and 280 rpm shaking for 30 min. The extract was flash frozen in liquid nitrogen and stored at -80 ^{o}C until use.

All run-off reactions were performed in Innova™ 4300 Incubator Shaker (New Brunswick Scientific).

3.2.3 Cell lysis efficiency determination

The concentration of the *E. coli* cells used for extract preparation was determined using the wet-weight as measured following cell harvest (1 trillion *E. coli* cells per gram wet weight). Following lysis and before clarification by centrifugation, the lysate was diluted 50, 2500, 120,000 and 24,000,000 times. 20 µL from the dilutions were plated on sterile Luria-Bertani agar

petri dishes (without antibiotics) and incubated overnight at 37 ^{o}C. The lysis efficiency was determined by comparing the numbers of colonies on each plate to the total number of cells that would be present in the corresponding same volume and dilution if the cells were not lysed.

3.2.4 Cell-free protein synthesis reaction

PANOxSP energized cell-free reactions were performed on a flat bottomed 96-well black microtitter plate (Thermo Scientific, Waltham, MA) with reaction volume of 15 μL at 37 ^{o}C for 3 h with the following modifications as reported by Bundy et al. [43]: (1) radiolabeled Leucine was not added to the reaction mix, (2) cell extract was used at 24-25 % w/v concentration, and (3) BL21 Star™ DE3 *E. coli* cell strain was used for extract preparation which eliminated the need for addition of purified T7 RNA polymerase. Phosphoenolpyruvate and *E. coli* tRNA mixture were obtained from Roche Molecular Biochemicals (Indianapolis, IN). All other reagents were obtained from Sigma Aldrich. The super-folder green fluorescent protein (sfGFP) expression plasmid used in this work has been reported previously [4]. The protein yield was determined by diluting the reaction volume to 60 μL with Ultrapure water from Barnstead E-pure Ultrapure Water Purification Systems (Thermo Scientific) in flat-bottomed 96-well black microtiter plates and measuring the fluorescence with a SYNERGY MX microplate reader (BioTek Instruments, Winooski, VT) at a sensitivity setting of 50 and excitation/emission wavelengths of 485 and 510 nm, respectively. A calibration curve was used to determine sfGFP concentration (Figure 3-2).

3.2.5 Protein concentration calibration curve

The standards of sfGFP were prepared by performing CFPS reactions with PANOxSP energy system with 5.25 µM L-[U-14C] Leucine [43]. The reaction was incubated at 37 °C for 3 hr. After incubation, the reaction mixture was diluted 20, 50, and 100 times in Ultrapure water (Thermo Scientific). Total sfGFP concentration in the original reaction mixture and the three diluted reaction mixtures was determined using Trichloroacetic acid insoluble radioactivity assay in conjunction with Ecolume™ liquid scintillation cocktail (MP Biomedicals, Solon, OH) and Beckman Coulter LS 6500 Multipurpose Scintillation Counter (Beckman Coulter, Brea, CA) according to a previously described protocol [97]. Corresponding fluorescence of the diluted samples was measured as described in section 3.2.4. A linear correlation between fluorescence and sfGFP concentration was obtained. The red circles represent the experimental data, while the black line represents the linear regression (Figure 3-2).

To calculate protein yields from CFPS reactions using the calibration curve, 15 µL of CFPS reaction product was diluted four fold by adding 45 µL Ultrapure water (Thermo Scientific) and the fluorescent reading was taken as described in the materials and methods section. The linear calibration curve was then used to correlate the fluorescence value to the active sfGFP concentration.

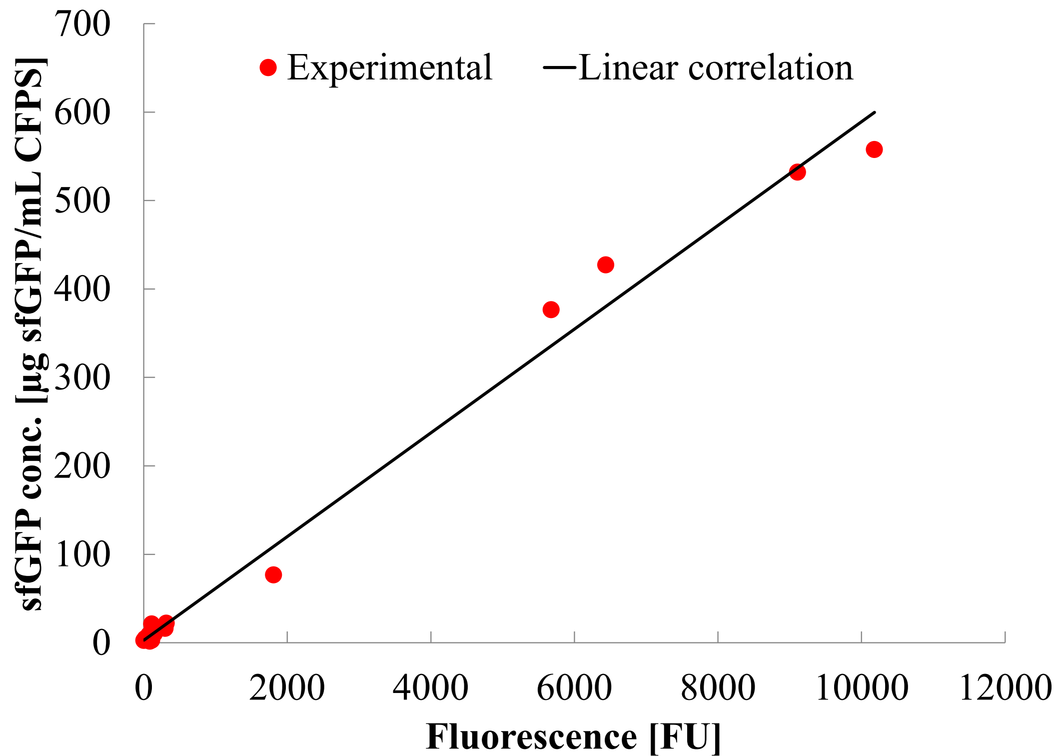


Figure 3-2: Protein calibration curve. The x-axis represents the absolute fluorescence units and the y-axis represents the protein concentration as measured by Trichloroacetic acid insoluble radioactivity assay. The slope and the intercept of the line are 0.0586 μg/mL and 2.748 μg/mL respectively. The coefficient of determination (R^2) was 0.9894.

3.3 Results and discussion

3.3.1 Shake flask cell culture

The use of shake flask fermentation and a common commercial *E. coli* strain, BL21 Star™ (DE3) for extract preparation simplifies the fermentation and eliminates the need to add independently purified RNA polymerase to the reaction or the acquisition of a specialized cell strain. While effective at producing *E. coli* cells viable for producing CFPS extract [71], the nutrient concentrations and pH are not directly controlled during shake flask fermentation. To

assess the effect of the pH change during fermentation on extract viability, *E. coli* cells were fermented with or without the presence of 100 mM MOPS. Although, over the course of the fermentation, the pH exhibited a smaller change with MOPS (Figure 3-3A), the average sfGFP production yields from the extract prepared from cells grown in MOPS was within a standard deviation of the extract prepared from cells grown without MOPS (Figure 3-3B). Thus the inclusion of MOPS in the fermentation did not significantly affect extract performance.

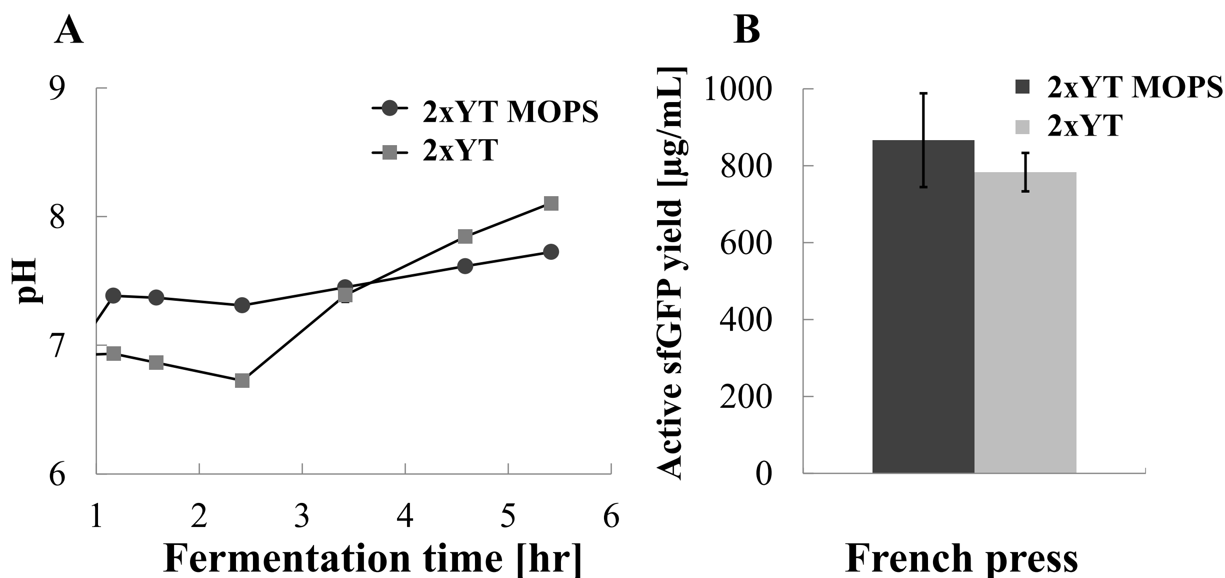


Figure 3-3: Effect of MOPS buffer in cell growth and extract performance. A) Change of pH in 2xYT growth media with or without MOPS buffer during shake flask fermentation. The error bar on data points represents the standard deviation of duplicate growth experiments. Since the maximum deviation was less than 1%, the error bars are not visible. B) sfGFP yields with extract prepared from cells grown in media with or without MOPS. The extracts were prepared using a French press (Avestin, Inc.). Error bars represent the standard deviation of three cell-free reactions.

3.3.2 Performance of extract prepared with sonication

The purpose of using sonication for cell lysis as opposed to the previously reported lysis by bead mill and high pressure homogenizers is to reduce the capital cost and enable researchers without access to such specialized equipment the opportunity to assess and use CFPS for their desired application. The capital cost of the bead mill or high pressure homogenizers used in this work and reported by others to produce CFPS extract is approximately $10,000 to $40,000 compared to the commonly available sonicator which costs about $4,000 (Table A 1). In Table A 1, the price of sonicator represents the price of a newer version of the sonicator employed in this work as sold by the same manufacturer.

For cell lysis, the sonication burst periods and 4 ^{o}C cooling periods were initially selected based upon commonly reported protocols for cell lysis which use 10 s to 60 s sonication burst periods and totaling 3-10 cycles as shown in Table 3-1 [98-101]. Table 3-1 shows the highest obtained sfGFP yield with corresponding standard deviation (n = 3). High variability in the table is defined as variation of greater than 25% in protein production yields whereas low variability is defined as variation less than 25% in protein production yields when identical conditions for preparing the CFPS extract were repeated. However, following these sonication protocols for cell lysis, the resulting CFPS extract produced protein at yields less than 25% of that achieved using a high pressure homogenizer (Figure 3-4).

Also, different from the simplified Kim et al. [59] protocol using a French press-style high pressure homogenizer (Figure 3-1) centrifugation for 30 min was needed to clarify the extract to appear similar to the one obtained by high pressure homogenization and 10 min of centrifugation. Although the sonication produced extract was less productive than that produced by high pressure homogenization, continuing to repeat the sonication burst/cooling cycle for

more cycles and thus longer total sonication times did in most cases result in a more productive CFPS extract (Figure 3-4). However, a significant limitation was the variation in extract productivity observed among replicates (Table 3-2 and Figure 3-4). This result was not surprising given that Kigawa et al. [72] claimed that in their tests sonication was not suitable for *E. coli*-based CFPS extract preparation. Although Kigawa et al. did not report the method employed or the actual data from testing sonication; it is likely they also employed common sonication protocols for *E. coli* cell lysis.

Based upon the trend of higher yields from both longer sonication burst periods and longer total sonication time (Table 3-2 and Figure 3-4), we increased the sonication burst period to 10 min or 20 min with a 2 min 4 ^{o}C cooling period. For a direct comparison to the traditional extract preparation protocol with a high pressure homogenizer, cell aliquots from the same shake flask fermentations (with or without MOPS) reported in Figure 3-3 were used with sonication. The protein production yields obtained with CFPS extracts prepared using the 10 min or 20 min sonication burst and a 2 min cooling period are shown in Figure 3-5A and Figure 3-5B. Extracts prepared with cell culture with MOPS or without MOPS and lysed by sonication performed similar to the extract prepared by French press type high pressure homogenizer lysis (Figure 3-3B). Also, continuing the sonication burst/cooling cycle beyond the first sonication burst/cooling cycle was not necessary to obtain a CFPS extract as productive as that obtained from high pressure homogenization (Figure 3-5A and Figure 3-5B). In addition, the CFPS extract productivity remained fairly constant over 100 min of total sonication time (10 cycles for the 10 min sonication burst, and 5 cycles for the 20 min sonication burst) (Figure 3-5A and Figure 3-5B).

Table 3-2: sfGFP yield from extract prepared using sonication. The yields reported in this table are the highest obtained yields for the condition. *Data obtained beyond 100 min of total sonication time was not included in the analysis

Sonication burst time	Cooling time	sfGFP yield [μg/mL]	Variability
10 s	30 s	587 ± 178	High
20 s	60 s	588 ± 66	High
60 s	90 s	731 ± 66	High
3 min	2 min	761 ± 208	High
10 min	2 min	1004 ± 82	Low*
20 min	2 min	1010 ± 194	Low*

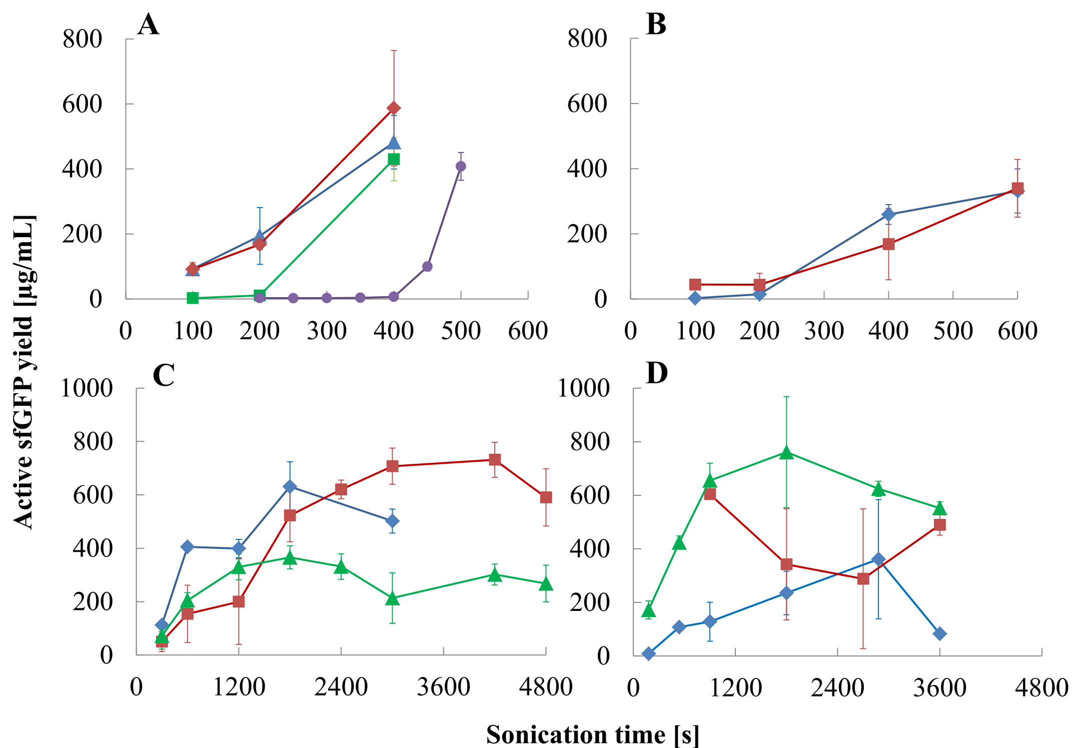


Figure 3-4: Extract to extract variability with extract prepared using sonicator. Each square, diamond, or triangle represents one CFPS extract created from *E. coli*-cells lysed after the total sonication time represented on the x-axis. Lines on each graph represent CFPS extracts created from the same *E. coli*-cell aliquot on the same day. Error bars at each data point represents the standard deviation of sfGFP production from three CFPS experiments using the extract. A) Lysis occurred by the repeated sonication cycle of 10 s sonication burst and 30 s of cooling in ice-water. B) Lysis occurred by the repeated sonication cycle of 20 s sonication burst and 60 s of cooling in ice-water. C) Lysis occurred by the repeated sonication cycle of 60 s sonication burst and 90 s of cooling in ice-water. D) Lysis occurred by the repeated sonication cycle of 180 s sonication burst and 120 s of cooling in ice-water. The x-axis represents the total time of sonication and does not include the time the sample spent cooling in ice-water.

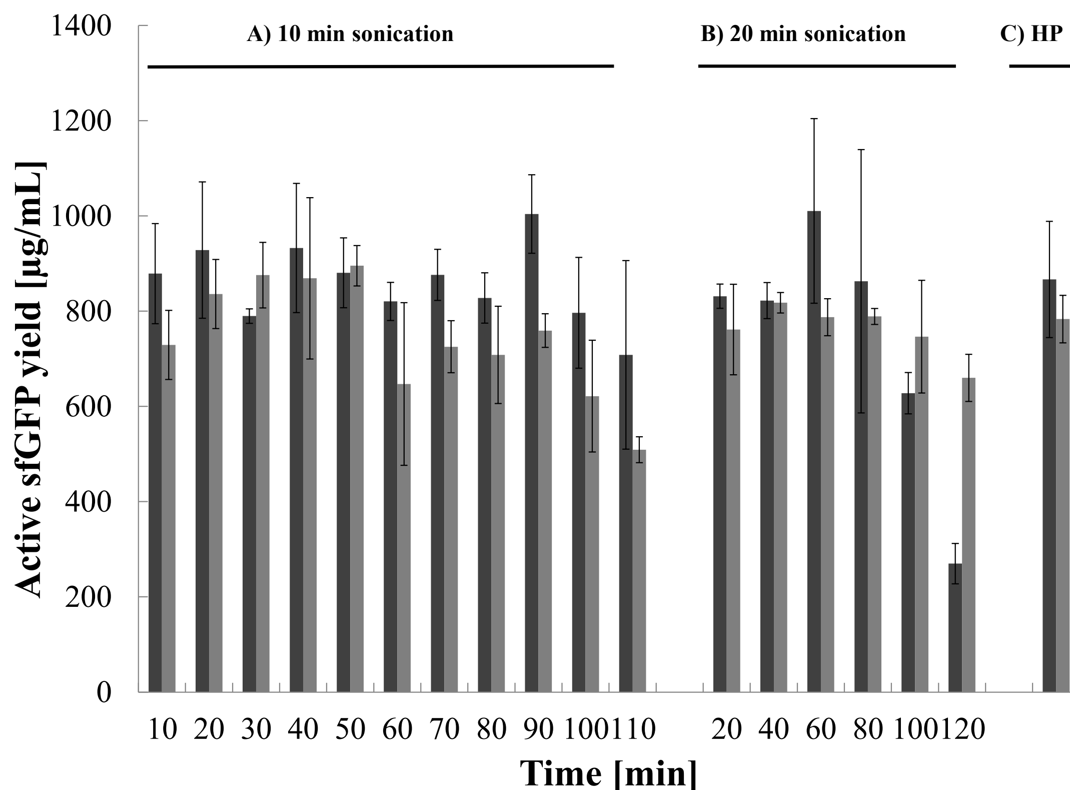


Figure 3-5: sfGFP yields from extracts prepared by longer sonication. A) 10 min sonication burst/2 min 4 °C cooling cycles. B) 20 min sonication burst/2 min 4 °C cooling cycles. C) French press-style high pressure homogenization (HP). The dark and the light bars represent the protein yields from CFPS reactions using extract prepared from cells grown in fermentations with and without MOPS respectively. Error bars represent the standard deviation from triplicate CFPS reactions performed from the extract prepared. The x-axis represents the total amount of sonication time or bead vortexing time applied to the cells for lysis and excludes the time spent cooling. Each time point effectively represents an additional lysis and cooling cycle.

3.3.3 Performance of cell extract prepared using bead vortexing

With simplifying the extract preparation as our ultimate goal, we also sought to simplify the bead milling process for extract preparation by using a table top vortexer with 0.1 mm diameter glass beads. This method is significantly economical than the commercial bead mill method (approximately $10,000-$40,000 for a commercial bead mill (Table A 1) compared to about $350 for the commonly available table top vortexer (Table A 1). Commercial bead milling

equipment has been regularly used for protein purification [102, 103], DNA extraction [104], cell-free extract preparation [72, 105], and lipid extraction [106].

Initial attempts with bead vortexing were performed with 10%, 20%, 50%, and 80% w/v bead. Of the different combinations, only the bead concentration recommended by the manufacturer (80% w/v bead to cell-buffer ratio) produced a significant amount of protein (results not shown), and was therefore chosen for further experiments. Also, cell lysates obtained by bead vortexing were observed to have a higher viscosity and 2 centrifugation steps of 30 min each were required to adequately clarify the lysate. The protein yield obtained with extract prepared by cycling between bead vortexing and cooling in ice-water resulted in lower protein production yields and higher extract-to-extract variability (Figure 3-6 and Figure 3-7) as compared to protein production yields from extracts prepared by high pressure homogenization and 10+ min of sonication.

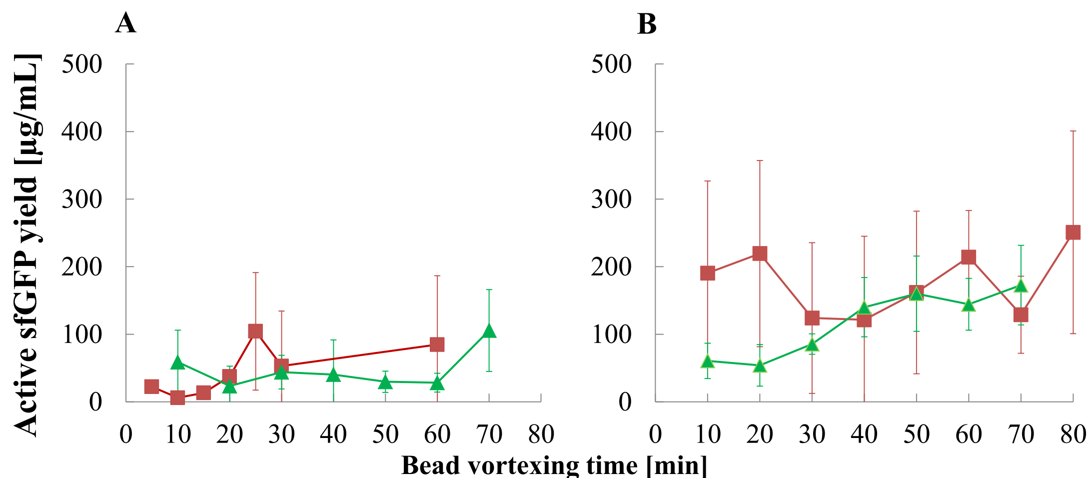


Figure 3-6: Variability of extract prepared using bead vortexing. A) Bead vortexing was performed by cycling 1 min of vortexing and 1 min cooling in an ice-water bath. B) Bead vortexing was performed by cycling 10 min of continuous vortexing and 1 min of cooling in an ice-water bath. Lines on each graph represent CFPS extracts created from the same *E. coli*-cell aliquot on the same day. The error bars represent standard deviation of sfGFP production from triplicate CFPS experiments using the extract.

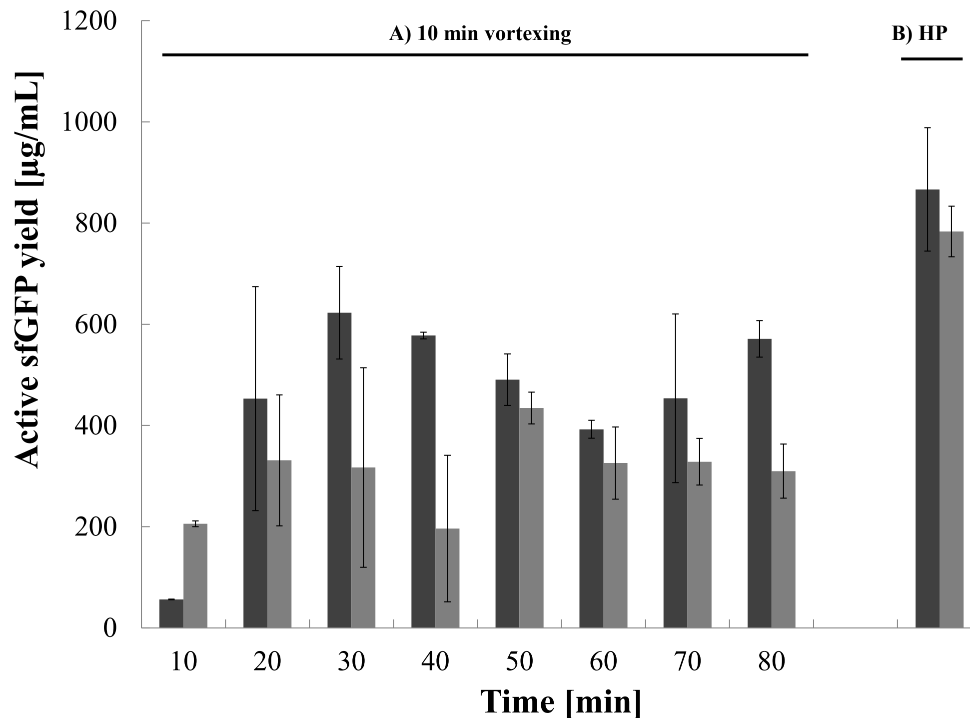


Figure 3-7: sfGFP yields from extracts prepared by bead vortexing. Cells were lysed by 10 min bead vortexing/1 min 4 ^{o}C cooling cycles. The cell culture used for this experiment and the one reported on Figure 3-6 are different. HP in the figure represents the French press-style high pressure homogenization. The dark and the light bars represent the protein yields from CFPS reactions using extract prepared from cells grown in fermentations with or without MOPS respectively. Error bars represent the standard deviation from triplicate CFPS reactions performed from the extract prepared. The x-axis represents the total amount of sonication time or bead vortexing time applied to the cells for lysis and excludes the time spent cooling. Each time point effectively represents an additional lysis and cooling cycle.

3.3.4 Sample heating during cell lysis by sonication and bead vortexing

One concern of lysis by sonication is sample heating and Kigawa et al. [72] postulated that sample heating is a possible reason why in their tests sonication was not suitable for preparing CFPS extract. The temperature of cell samples were measured over 120 min with continuous cycling between the 10 min or 20 min sonication burst and 2 min cooling. The temperature of 3 independent experiments was measured by K-type mini thermocouple

(ThermoWorks, Lindon, UT) and the average remained below 15 °C throughout the 120 min run (Triangle and square data in Figure 3-8).

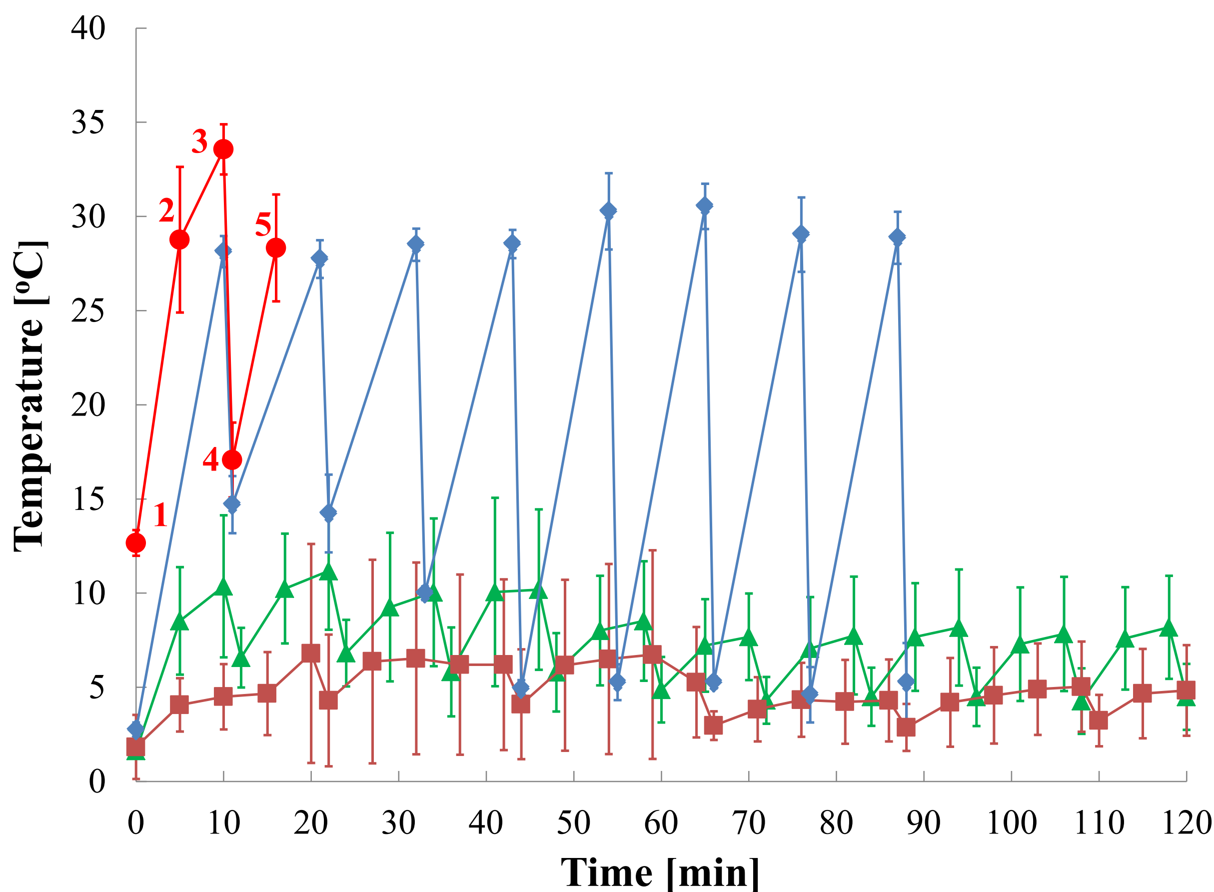


Figure 3-8: Temperature of sample during sonication and bead vortexing. Blue diamonds represent 10 min continuous bead vortexing with a 1 min cooling in ice-water bath. Green triangles and red squares represent continuous sonication for 10 min and 20 min with 2 min cooling time in between each burst. Sonication and cooling were performed in ice-water bath. Red dots represent the temperature of the sample at different stages during French press-style high pressure homogenization with the order of pass above or beside the dot. Legend for high pressure homogenization: 1) Temperature of the sample at the beginning, 2) Temperature of the lysate after the first pass, 3) Temperature of the sample after the second pass, 4) Temperature of the sample after cooling for a 1 min in a steel beaker in ice-water bath and 5) Temperature of the lysate after the third pass. The error bar with corresponding color represents the standard deviation of triplicate temperature measurements.

Since sonication generates heat, the ice-water mixture was manually stirred for water circulation around the microfuge tube for better cooling effect. The ice-water bath was stirred more frequently during 20 min sonication burst than 10 min sonication burst which resulted in better cooling and hence lower temperature for 20 min sonication burst than the 10 min sonication burst. In comparison, the average temperature of the cell samples processed by high pressure homogenization (Circular data in Figure 3-8) was much higher with the maximum approaching 34 ^{o}C. Also, sonicated CFPS extract productivity did not significantly decrease over 100 min of sonication suggesting the extract was not damaged by heat.

During bead vortexing large temperature swings were observed by cycling between vortexing and cooling, although temperatures higher than that obtained with the high pressure homogenizer were not observed (Diamond data point in Figure 3-8) Although there are challenges associated with this method, yields up to 600 µg/mL sfGFP production were obtained from CFPS extract prepared with bead vortexing.

3.3.5 Cell lysis efficiency

The efficiency of lysis by sonication and bead vortexing was also assessed relative to a French press-style high pressure homogenizer by plating dilutions of lysate on LB broth agar petri dishes. Lysis efficiency increased with total sonication time with 10 cycles of 10 s sonication resulting in 98.898% lysis, 40 cycles of 10 s sonication resulting in 99.789% lysis, and 10 min of continuous sonication resulting in 99.988% lysis. The high pressure homogenizer had the highest efficiency at 99.9996%. By comparing lysis efficiency alone, it appears that very high lysis efficiency (>99.98%) is needed for the consistent preparation of productive *E. coli*

extract for CFPS. Although, other factors also play a role, such as the formation of soluble inverted membrane vesicles to facilitate oxidative phosphorylation CFPS as reported with extracts prepared using a high pressure homogenizer [69, 107]. Lysis efficiency following 2 cycles and 5 cycles of 10 min bead vortexing was 99.378% and 99.479% which is lower than that observed with the high pressure homogenization and 10 min of sonication. The bead vortexing lysis efficiency observed is similar to that observed at lower sonication times which also resulted in CFPS extract with lower protein yields and higher variability between replicate extract preparations. Increasing the vortexing time beyond 80 min seemed unreasonable for a streamlined extract preparation method.

3.3.6 Performance of cell extract prepared by freeze-thaw and lysozyme incubation

As part of this work, the simple cell lysis methods of freeze-thaw and enzymatic lysis by lysozyme were also assessed for preparing CFPS extract. However, the extracts prepared using these techniques did not produce measurable amounts of protein in CFPS reactions (results not shown). Lysis efficiencies of 99.609% to 99.976% for freeze-thaw and 99.991% for lysozyme were observed. These efficiencies are comparable to the lysis efficiency obtained after 10 min of sonication (99.988%) suggesting insufficient lysis was not the reason for the unviable extract. In contrast to lysis by a high pressure homogenization or sonication, the freeze-thaw and lysozyme incubation lysis methods do not involve mechanical shearing which may be important for viable *E. coli* cell extract [94, 108]. However, freeze-thaw lysis has been reported as a viable method to produce insect cell extract for CFPS [73] which suggests that with further engineering such a method could be developed for *E. coli* cell extract preparation.

3.3.7 Performance of cells grown in small volume cultures

As the main aim of this work was to make the cell-free viable extract preparation accessible, we tested the performance of the extracts grown in erlenmeyer flasks and culture tubes in CFPS. Cell cultures in those devices require minimum use of growth media and saves time required for the cell culture when performing fewer proof of concept experiments, and these devices are readily available in research labs.

Cell culture in Erlenmeyer flasks: Three baffled erlenmeyer flasks with 25 mL 2xYT media were used to perform cell culture. Cell culture was performed with three different variations as tabulated below in Table 3-3.

Figure 3-9 shows the growth pattern of the various cultures. They resemble typical bacterial growth pattern with lag and exponential growth phase. However, the yield with extract prepared from cells grown in smaller culture did not result in higher yield in all of the three variations. The lysis was performed by sonicating the sample either for one 10 min sonication burst or three 10 min sonication bursts with 2 min cooling time in between the bursts. The sfGFP yields are summarized below in Table 3-3. The culture without seed culture whose growth curve is shown in Figure 3-9C had a maligned growth. Hence, it was excluded from extract preparation. IPTG-induction is necessary for T7 RNA polymerase expression which is necessary for transcription in CFPS. Hence, cell extract from culture A was not successful in production of sfGFP. Culture B did produce viable extract, albeit at about 20% of the viability of extract prepared from 1 L cultures. The lower protein production could be because of lower harvest OD_{600}.

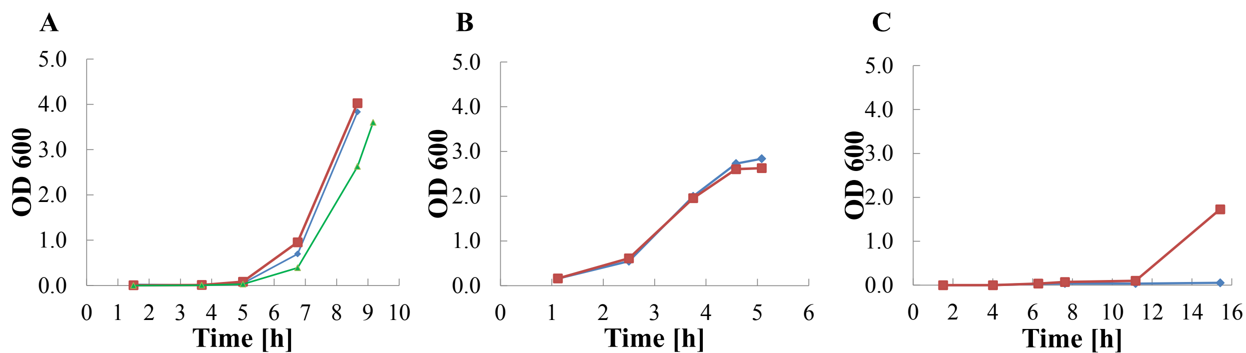


Figure 3-9: Growth curve of cell culture in Erlenmeyer flasks. Graph A, B and C represents the culture as described in Table 3-3.

Table 3-3: sfGFP yield with cells cultured in Erlenmeyer flasks. The error associated with the yields represents the standard deviation of three experiments. *ND: Not determined.

Culture	Seed culture	Induction with IPTG and L-arabinose	sfGFP Yield [μg/mL]
A	Yes	No	6.07 ± 1.49 3.24 ± 0.53 8.83 ± 3.10
B	Yes	Yes	171.19 ± 33.41 172.37 ± 97.05
C	No	Yes	ND*

Cell culture in culture tubes: Cells were cultured in 5 mL 2xYT media in culture tube. 4 tubes were combined during harvesting to get enough cells for cell lysis. Cells were cultured with different variations as tabulated below in Table 3-4. In all of the cases, the sfGFP yield was low compared to the extract prepared from cells grown in baffled tunair flasks. The growth curves of the cultures are shown in Figure 3-10 and the sfGFP yields are tabulated in Table 3-4. Similar to the culture without IPTG-induction in Figure 3-9A, the culture without IPTG-

induction in Figure 3-10A did not result in significant sfGFP expression and likely due to lower harvest OD_{600} the culture in Figure 3-10B did not result in higher protein production yield.

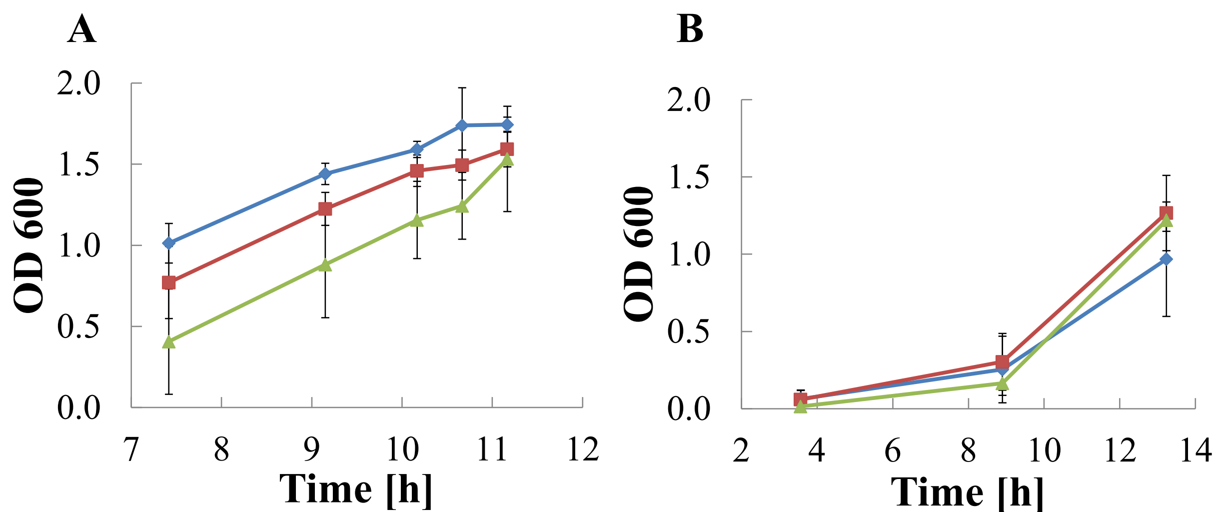


Figure 3-10: Growth curve of cell culture in culture tube. Graph A and B represents the culture as described in Table 3-4. Error bars represent the standard deviation of OD_{600} of four cultures that were combined prior to lysis.

Table 3-4: sfGFP yield with cells cultured in 5 mL glass culture tubes. There are only two sfGFP yields for culture set B as the culture represented by the green line was added to the culture represented by the maroon and the blue line prior sonication. The error associated with the yields represents the standard deviation of three experiments.

Culture	OD_{600}	Induction with IPTG and L-arabinose	sfGFP Yield [µg/mL]
A	Monitored	No	3.71 ± 0.49 5.05 ± 2.01 2.63 ± 1.02
B	Monitored	Yes	55.94 ± 29.68* 10.94 ± 2.55*
C	Not monitored	No	7.93 ± 1.05 215.49 ± 38.29 40.17 ± 3.48

In this work, the extracts prepared from cells grown in culture tubes and erlenmeyer flasks did not resulted in higher protein yields as compared to the extract prepared using cells grown in baffled tunair flasks. Future work using a more nutrient rich media such as Terrific broth may improve cell density which may be necessary to achieve high protein production yields.

3.4 Conclusion

In this work, we simplified the cell extract preparation method by lysing cells using equipment common to biotechnology labs, thus eliminating the need for specialized growth and lysis equipment. This was accomplished by performing cell growth in shake flasks and assessing alternative *E. coli* disruption techniques of: (1) sonication, (2) bead vortexing, (3) enzymatic lysis, and (4) freeze-thaw cycling. Using sonication, we produced high-yielding CFPS extract and, in terms of capital equipment cost, sonication is approximately 60% to 90% less expensive than the developed techniques shown in Figure 3-1. Also sonication is well suited for labs with access to a sonicator but not a bead mill or a high pressure homogenizer. Another benefit of sonication is that sample volumes as low as 150 μL can be processed and 96-well plate sonicators are available for high-throughput applications (Table A 1). Extracts produced by sonication could also be engineered for other cell-free configurations such as continuous exchange CFPS which enables extended reaction durations and higher productivities. For researchers without access to a sonicator, bead vortexing can also be used for extract preparation, although the CFPS yields obtained through this lysis method are lower than those obtained using sonication or high pressure homogenization with higher extract-to-extract variability. In conclusion, the combined shake flask cell culture, sonication or bead vortexing cell lysis technique, and streamlined extract preparation protocol, described in this work can significantly

reduce the time, effort, and capital cost expended in initial proof of concept experiments with CFPS. We believe this simple extract preparation technique could become an economically sound milestone for extract preparation and will enable more scientists and engineers to test CFPS for their desired application.

4 IMPROVING CELL-FREE INCORPORATION OF UNNATURAL AMINO ACIDS IN PROTEINS WITH LINEAR EXPRESSION TEMPLATES

4.1 Introduction

Cell-free protein synthesis (CFPS) efficiently harnesses the innate ability of the cellular machinery to transcribe and translate while simultaneously allowing superior control over the synthesis environment. CFPS can be subjected to direct control and engineering and allows firsthand access to the protein synthesis environment. This openness enables the use of PCR amplified linear DNA for high-throughput expression of engineered protein variants and permits the use of different cost-effective energy sources. An elegant application of CFPS technology is the incorporation of unnatural amino acids that may not readily transport across membranes [4]. While prior CFPS technology research has independently focused on (1) incorporating UAAs site-specifically [4, 5, 109], (2) exploring more cost-effective energy sources [16, 18, 68], and (3) using Linear DNA expression templates (LETs) for CFPS [7, 19, 37, 92], these areas of research have yet to be combined. In addition, each individual development has commonly resulted in lower production yields and thus it is necessary to mitigate this challenge through engineering. In this study, we seek to address this issue by engineering *E. coli*-based CFPS for incorporation of UAAs by optimizing the energy system and by protecting the LETs.

The site-specific incorporation of unnatural amino acids (UAAs) in proteins is of particular interest because it provides a method for engineering proteins with novel biochemical and biophysical properties [110-112]. Modification of proteins using UAAs provides a robust

tool for functional and structural proteomics [113-115]. Insertion of UAAs in proteins provides a platform to expand the chemistry of life by enabling site-specific biomolecule conjugation [4, 5, 113]. In addition, UAA modifications in proteins are notably useful for studying pharmacokinetics and protein trafficking [51, 116]. Site-specific immunogenic UAA modification of proteins has been used for development of therapeutic vaccines against self-proteins associated with cancer or inflammation [113, 117, 118]. A cell-free approach to site-specific incorporation of UAA is not limited by its transport into the cell and thus enables direct engineering and optimization of the synthesis environment, such that a less-soluble and putatively transport-limited UAA can be incorporated at higher yields using a cell-free approach [4].

Using LETs with CFPS eliminates the need for DNA cloning [79] and enables rapid high-throughput screening [3, 58, 119]. Rapid expression of genomic libraries and high-throughput genomic analysis can be performed with LET templated CFPS [36, 37]. Although LETs simplify DNA preparation, there remains the challenge of lower production yields from LETs compared to plasmid DNA. The major contributor to the lower yields has been attributed to LET degradation by nucleases present in the cell extract [19, 120, 121]. For improved protein yields, cell extract deficient in mRNA degradation activity of the RNase E enzyme [56, 82, 83] or deficient in polynucleotide phosphorylate (PNPase) have been used [122] so that even if the LET is degraded the corresponding mRNAs will be functional for longer time in the cell-free reaction. Furthermore, Lee et al. reported higher yields by stabilizing functional mRNAs from LETs by including polyguanine chain at the 3’ end of mRNA [81]. The polyguanine chain protects mRNA by providing strong resistance against the PNPase [123]. Concurrent use of cell extract deficient in mRNA degradation activity of the RNase E enzyme and hairpin loop at the 3’

end of the mRNA also resulted in improved protein yields [92]. Additionally, GamS protein from the lambda phage has been shown to inhibit the RecBCD complex and stabilize LETs, and thus improve the protein yield. In order to improve protein yields with LETs, this chapter assesses the concurrent impact of recently discovered multiple RecBCD inhibitors and extract deficient in the mRNA degradation activity of RNase E in *E. coli*-based CFPS.

One of the most important but expensive component of CFPS is the energy source [15]. Traditionally, compounds with high energy phosphate bonds such as phosphoenolpyruvate [66] or creatine phosphate [61] were used for CFPS resulting in high energy cost per mg protein produced. In one study by Kim et al., the energy cost was estimated to be 50% of the total CFPS cost when phosphoenolpyruvate was used as the energy source [67]. Hence, inexpensive glycolytic intermediates have been assessed to reduce the energy. Simple carbohydrates like glucose [15, 16], metabolic intermediates such as fructose-1,6-bishphosphate [67] and 3-phosphopoglycerate [19], complex carbohydrates such as maltodextrin [20] and starch [17], combined energy source of creatine phosphate and glucose [68] and pyruvate and glutamate [18, 124] have been developed. For further cost reduction, expensive nucleoside triphosphates (NTPs) are replaced by nucleoside monophosphates (NMPs) [15, 67] which are converted to NTPs in cell-free reaction by kinases present in the cell extract [15]. These alternative energy sources have reduced the cost of energizing CFPS and paved a way to make CFPS commercially viable.

The work described in this chapter utilizes *E. coli*-orthogonal pair of tyrosyl-aminoacyl-tRNA synthetase ($Mj\text{TyrRS}^{\text{pPa}}$) and tyrosyl-tRNA with an amber stop anticodon ($Mj\text{tRNA}^{\text{Tyr}}_{\text{CUA}}$) to incorporate pPa at the location encoded by the amber stop codon (UAG) on the corresponding mRNA [53]. This chapter also assesses alternative energy sources for production of protein with

site-specific incorporation of less-soluble UAA and also reports the enhanced production of the same protein by LET catalyzed CFPS in presence of RecBCD inhibiting reagents. These combinations allows for rapid and inexpensive expression of proteins with site-specifically incorporated UAA for various high-throughput applications.

4.2 Materials and methods

4.2.1 Preparation of cell extract and tRNA synthetase

Cell extract for this work was prepared using BL21 Star™ (DE3) cells (Invitrogen, Carlsbad, CA) harboring the pEVOL-pPrF plasmid [93]. The cells were grown in shake flask fermentations, and at 0.6 OD_{600} the growth was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside and 0.02% (w/v) L-arabinose to express T7 RNA polymerase and tRNA synthetase ($Mj\text{TyrRS}^{\text{pPa}}$) respectivey. Cells were harvested at late exponential phase (OD_{600} 4 to 5.4). Cell extract was prepared by lysing cell using a French Press (Avestin, ON, Canada) as described previously in section 3.2.2.

The tRNA synthetase ($Mj\text{TyrRS}^{\text{pPa}}$) was expressed in BL21 Star™ (DE3) strain with plasmid and purified using HisTrap™ HP columns (GE Healthcare, WI) [4, 125].

4.2.2 Preparation of linear expression templates (LETs)

LETs were generated using two step PCR. The first PCR amplified the gene of interest from the plasmid using gene specific primers. The amplified genes were then advanced to second PCR with ultramers to include the ribosome binding site, T7 promoter and T7 terminator with sequences based on those optimized by Ahn et al. [92, 126]. The final PCR product was purified using QIAquick® PCR purification kit following the manufacturer's instruction (Qiagen,

Valencia, CA). The PCR program and primer sequences used in this work are tabulated in Table 4-1 and Table 4-2 respectively.

Table 4-1: PCR program used for generation of LETs. Both step I and step II PCR were performed using this program.

PCR stage	**Temperature [^{o}C]**	**Time [min:s]**	**Number of cycles**
Initial denaturation	95	00:30	1
Denaturation	30	00:30	30
Annealing	52	1:00	30
Elongation	72	2:00	30
Final elongation	72	10:00	1

Table 4-2: Primers used in this study. All primers were synthesized by Integrated DNA Technologies (San Jose, CA) [92, 126].

sfGFP amplification forward primer	5'-GTT TAA CTT TAA GAA GGA GAT ATA CAT ATG AGC AAA GGT GAA GAA CTG-3'
sfGFP amplification reverse primer	5'-GGT TAT ATG TCG ACC TCG AGT TAT TAT TTT TCG AAC TGC GGA TGG CTC-3'
5' untranslated region forward primer	5'-TCG ATC CCG CGA AAT TAA TAC GAC TCA CTA TAG GGA GAC CAC AAC GGT TTC CCT CTA GAA ATA ATT TTG TTT AAC TTT AAG AAG GAG ATA TAC ATA TG-3'
3' untranslated region reverse primer	5'-CAA AAA ACC CCT CAA GAC CCG TTT AGA GGC CCC AAG GGG TTA TAT GTC GAC CTC GAG TTA-3'

4.2.3 Reaction mix and protein yield determination

Phosphoenolpyruvate (PEP), *E. coli* tRNA mixture, creatine phosphate (CP), and creatine kinase (CK) were purchased from Roche Molecular Biochemicals (Indianapolis, IN) and L-[U-14C] Leucine was purchased from PerkinElmer Inc. (Waltham, MA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO). All reagents were used without further purification. *para*-propargyloxyphenylalanine (pPa) was synthesized and characterized as described previously [4]. The following energy systems were used in this work: (1) PANOxSP [127], (2) simplified PANOx (PANOx*) [128, 129], (3) creatine phosphate (CP) [68], glucose [15], (4) fructose 1,6-bisphosphate (F1,6BP) [67], and (5) high glutamate salt. The detailed reaction compositions at the beginning of the reaction are listed in Table A 2 in the appendix section. Plasmid based reactions were performed with sfGFP plasmid encoding for super folder green fluorescent protein (sfGFP) and sfGFP-T216UAA plasmid encoding for sfGFP with pPa incorporated at position 216 (pPaGFP) [4].

Protein yield was determined using a linearly correlated calibration curve between fluorescence measurement and protein concentration as described in the section 3.2.5.

4.2.4 Pre-CFPS amino acylation of tRNA

Prior to the CFPS reaction setup, $Mj\mathrm{tRNA}^{\mathrm{Tyr}}_{\mathrm{CUA}}$ was purified from the cell extract used for catalyzing CFPS using Direct-zol™ RNA Miniprep (Zymo Research Corporation, Irvine, CA) or TRIzol RNA extraction method (Invitrogen, Carlsbad, CA). The purified tRNA was aminoacylated in a 6x $Mj\mathrm{tRNA}^{\mathrm{Tyr}}_{\mathrm{CUA}}$ solution with the following components: 6x purified $Mj\mathrm{tRNA}^{\mathrm{Tyr}}_{\mathrm{CUA}}$ (e.g. 40 ng/µL for CFPS requiring 6.67 ng/µL), 12 mg/mL $Mj\mathrm{TyrRS}^{\mathrm{pPa}}$, 0.5 mM pPa, 10 mM magnesium glutamate, 30 mM potassium glutamate, 0.5 mM dithiothreitol, and 8

mM ATP [130, 131]. The reactions were incubated at 47 ^{o}C or 80 ^{o}C for 30 min to allow amino acylation of the $Mj\text{tRNA}^{\text{Tyr}}_{\text{CUA}}$ with pPa. Following this incubation period, CFPS reactions were performed with the appropriate amount of pre-charged $Mj\text{tRNA}^{\text{Tyr}}_{\text{CUA}}$ solution using the PANOxSP energy system described previously in section 4.2.3.

4.3 Results and discussion

In this work, we capitalize on the open environment provided by CFPS and optimize the reaction for site-specific incorporation of pPa in sfGFP. Additionally, we have shown that addition of *in vitro* amino acylated tRNA in CFPS results in a higher yield of proteins containing UAA. These two external additives also manifest the benefits of openness provided by cell-free system. Furthermore, protein with UAA was produced at a lower cost with different alternative energy sources. Using this setup, we compare the traditional ATP regeneration system PANOxSP [127] with alternative energy sources such as glucose [15, 16], simplified PANOx* [128, 129], creatine phosphate [68], high glutamate salt system, and fructose 1,6-bisphosphate [67] without the use of expensive cofactors like nucleoside triphosphates. Furthermore, we have demonstrated the use of LETs for rapid and improved yield for production of proteins with site-specifically inserted UAA.

4.3.1 Optimization of cell-free reaction for pPa incorporation

This work transfers the technology developed by Schultz and coworkers to the CFPS system and site-specifically incorporates pPa in sfGFP [53, 132]. In this method, suppressor tRNAs are amino acylated with the desired UAA and expressed in CFPS system with the DNA of interest. Since the reaction is dependent on the tRNA and the synthetase, optimization of the tRNA and the synthetase concentration is necessary for effective reaction.

4.3.2 Dependence on tRNA and synthetase

Since the synthetase charges the tRNA with pPa, the efficiency of pPa incorporation should be dependent on their relative concentration. For efficient pPa incorporation, the concentration of pPa charged $Mj\text{tRNA}_{\text{CUA}}^{\text{Tyr}}$ must be sufficient in quantity to compete with release factor 1 (RF1) for the amber stop codon [133]. Since the CFPS system is an open system, it enables relatively fast optimization and precise control of the tRNA and the synthetase concentration. Therefore, the effect of $Mj\text{tRNA}_{\text{CUA}}^{\text{Tyr}}$ and $Mj\text{TyrRS}^{\text{pPa}}$concentration on pPa incorporation was determined. Without additional $Mj\text{tRNA}_{\text{CUA}}^{\text{Tyr}}$ and $Mj\text{TyrRS}^{\text{pPa}}$, *in vitro* incorporation of pPa was modest with total yield at 39 μg/mL. Therefore, additional $Mj\text{tRNA}_{\text{CUA}}^{\text{Tyr}}$ and $Mj\text{TyrRS}^{\text{pPa}}$ were purified and added to the CFPS reaction to determine if higher concentrations of $Mj\text{tRNA}_{\text{CUA}}^{\text{Tyr}}$ and $Mj\text{TyrRS}^{\text{pPa}}$ would result in higher incorporation of pPa. As shown in Figure 4-1, when $Mj\text{TyrRS}^{\text{pPa}}$ was increased without additional $Mj\text{tRNA}_{\text{CUA}}^{\text{Tyr}}$ (at 0 ng/μL) increase in pPaGFP yield was observed. This increment was observed only to a certain extent after which inhibitory effect was seen. Similar increment in pPaGFP yield was also observed at moderate level (10 ng/μL) and higher (20 ng/μL) level of $Mj\text{tRNA}_{\text{CUA}}^{\text{Tyr}}$. With two-factor ANOVA test, it was concluded that additional $Mj\text{TyrRS}^{\text{pPa}}$ had a statistically significant

effect on the pPa incorporation (P-value = 0.000303, criteria P-value < 0.05) while the effect of additional tRNA was statistically insignificant (P-value = 0.316, criteria P-value > 0.05). Therefore, tRNA was not added for further experiments in this work while $Mj\text{TyrRS}^{\text{pPa}}$ was purified and added.

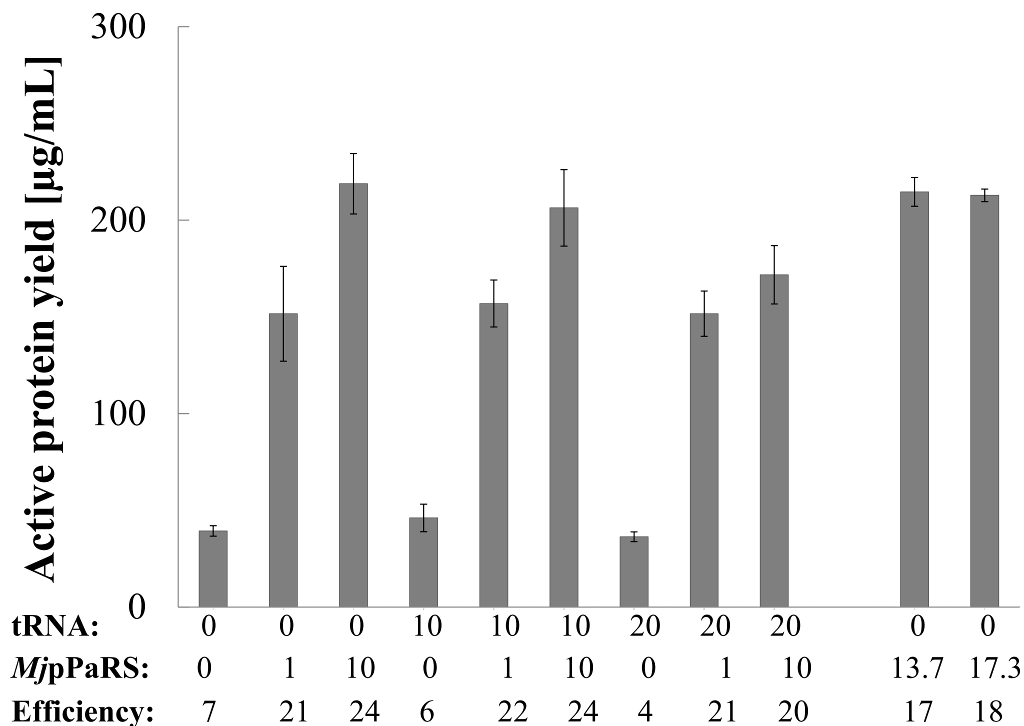


Figure 4-1: pPaGFP production at different tRNA and synthetase concentration. The error bar represents the standard deviation of three replicates of plasmid based reactions. tRNA and synthetase are reported in ng/µL and mg/mL unit. The efficiency represents the percentage ratio of pPaGFP to sfGFP yield at the same condition.

4.3.3 Pre-CFPS amino acylation of tRNA

For site-specifically incorporating pPa in sfGFP, the $Mj\text{tRNA}_{\text{CUA}}^{\text{Tyr}}$ needs to be acylated with pPa. Furthermore, this acylated tRNA must compete with RF1 for incorporation or termination of the peptide at the amber stop codon, respectively. At the beginning of the CFPS reaction, the population of pPa charged $Mj\text{tRNA}_{\text{CUA}}^{\text{Tyr}}$ available is low since pPa is not added to the cell culture during extract preparation. Also, the addition of pPa during extract preparation is unlikely to result in higher incorporation as it has limited transport across the cell wall [4]. Furthermore, the $Mj\text{tRNA}_{\text{CUA}}^{\text{Tyr}}$ is from an extreme thermophilic archea *Methanocaldococcus jannaschii* that grows optimally at 85 ^{o}C [134] while the reaction occurs at 30 ^{o}C, potentially slowing the kinetics of pPa acylation to $Mj\text{tRNA}_{\text{CUA}}^{\text{Tyr}}$in a standard CFPS reaction. To circumvent the problem of insufficient pPa acylated $Mj\text{tRNA}_{\text{CUA}}^{\text{Tyr}}$ availability at the beginning of the reaction, *in vitro* acylation was performed at 47 ^{o}C and 80 ^{o}C. Elevated temperatures were chosen to potentially enhance the activity of the $Mj\text{TyrRS}^{\text{pPa}}$ as it is natively found in an extreme thermophile. In our test, *in vitro* acylation at both 47 ^{o}C and 80 ^{o}C resulted in higher incorporation of pPa in sfGFP. Addition of *in vitro* acylated $Mj\text{tRNA}_{\text{CUA}}^{\text{Tyr}}$ appears to increase the concentration of pPa charged $Mj\text{tRNA}_{\text{CUA}}^{\text{Tyr}}$ at the initiation of transcription and translation, resulting in almost double pPaGFP yields (Figure 4-2). Although the precharging of the $Mj\text{tRNA}_{\text{CUA}}^{\text{Tyr}}$ with pPa increases yields, it comes at high time and labor cost. Producing and purifying the essential tRNA takes multiple days and more specialized chemicals and equipment. Furthermore, the tRNA should be pre-charged directly before the CFPS reaction. This pre-reaction effort diminishes the stream-line potential of the LET system and reduces the potential for high-throughput analysis.

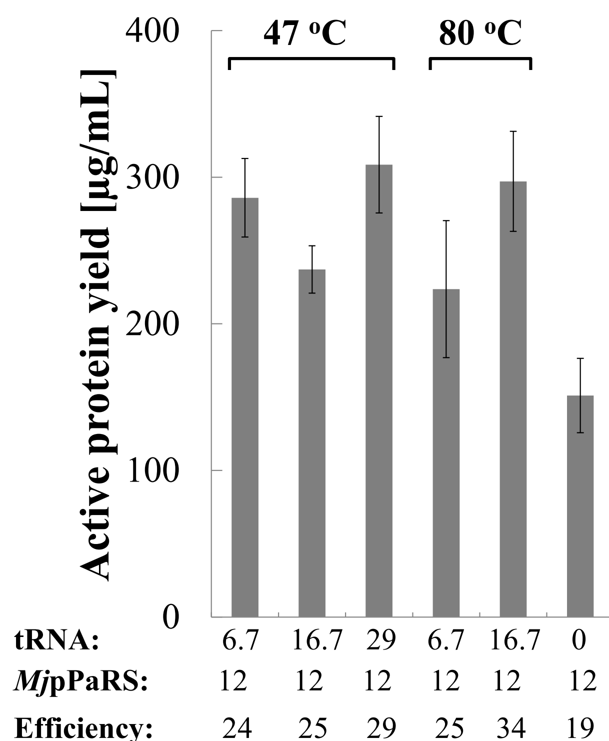
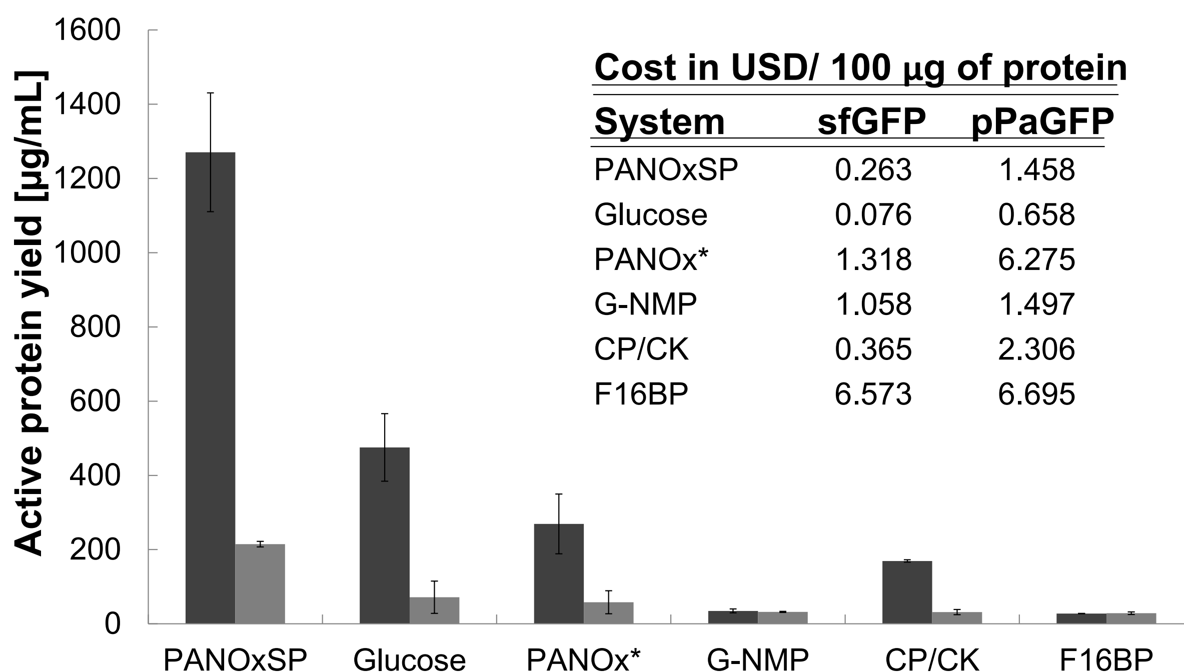


Figure 4-2: Effect of precharging on CFPS yield. The *in vitro* acylation was performed with the specified amount of tRNA, synthetase, 0.08 mM pPa, 1.67 mM magnesium glutamate, 5 mM potassium glutamate, 0.08 mM DTT and 1.33 mM ATP for 30 min at 47 °C and 80 °C prior to addition to the CFPS reaction. The error bar represents the standard deviation of three replicates of plasmid based reactions. tRNA and *Mj*pPaRS are reported in ng/μL and mg/mL unit. The efficiency represents the percentage ratio of pPaGFP to sfGFP yield at the same condition.

4.3.4 Alternative energy sources

An inexpensive and efficient energy source for prolonged energy supply for ATP regeneration is vital for economical incorporation of unnatural amino acid in proteins. Since cell-free system is an open system, it allows flexibility in the use of these energy sources. With that in mind, cell-free reactions were performed with alternative energy sources and without expensive cofactors. These changes reduced the cost of protein synthesis to a fraction of cost compared to the widely used PANOxSP system. Compared to the standard energy system, the glucose system

reduced the cost of sfGFP and pPaGFP production by 81% and 78% respectively. To explore the economic side of the unnatural amino acid incorporation, other energy sources including creatine phosphate [61, 68], glucose [15, 16], fructose 1,6-bisphosphate [67], and high glutamate salt system were used without expensive cofactors such as acetyl coenzyme A, synthetic tRNA, and NTPs. For cost determination, the prices for reagents were obtained from the 2012 online catalogues of Roche Molecular Biochemical and Sigma-Aldrich. The cost determination excludes the cost of human labor. Figure 4-3 shows the protein yields with different energy sources and the cost of production of 100 microgram of active sfGFP and pPaGFP respectively.



System	sfGFP	pPaGFP
PANOxSP	0.263	1.458
Glucose	0.076	0.658
PANOx*	1.318	6.275
G-NMP	1.058	1.497
CP/CK	0.365	2.306
F16BP	6.573	6.695

Figure 4-3: Protein production with different energy systems. The darker shade represents the sfGFP whereas the lighter shade represents the pPaGFP yields. The table in the inset represents the cost of producing mg of sfGFP and pPaGFP using the different energy system. The error bars represent the standard deviation of three replicates of the plasmid based reactions.

The total protein yield with glucose is lower than with the standard system. However, owing to the fact that glucose is incomparably inexpensive than PEP, it is more economical when cost per gram of protein produced is determined. The cost of energy source for the standard PANOxSP system is around 52% of the total cost whereas for the glucose system, the energy cost is only 0.23%. Cost breakdown of different components in PANOxSP and glucose system is shown in Figure 4-4. From the figure, it can be observed that for pPa incorporation, the major cost of PANOxSP system is the energy while the major expense in glucose system is the plasmid preparation.

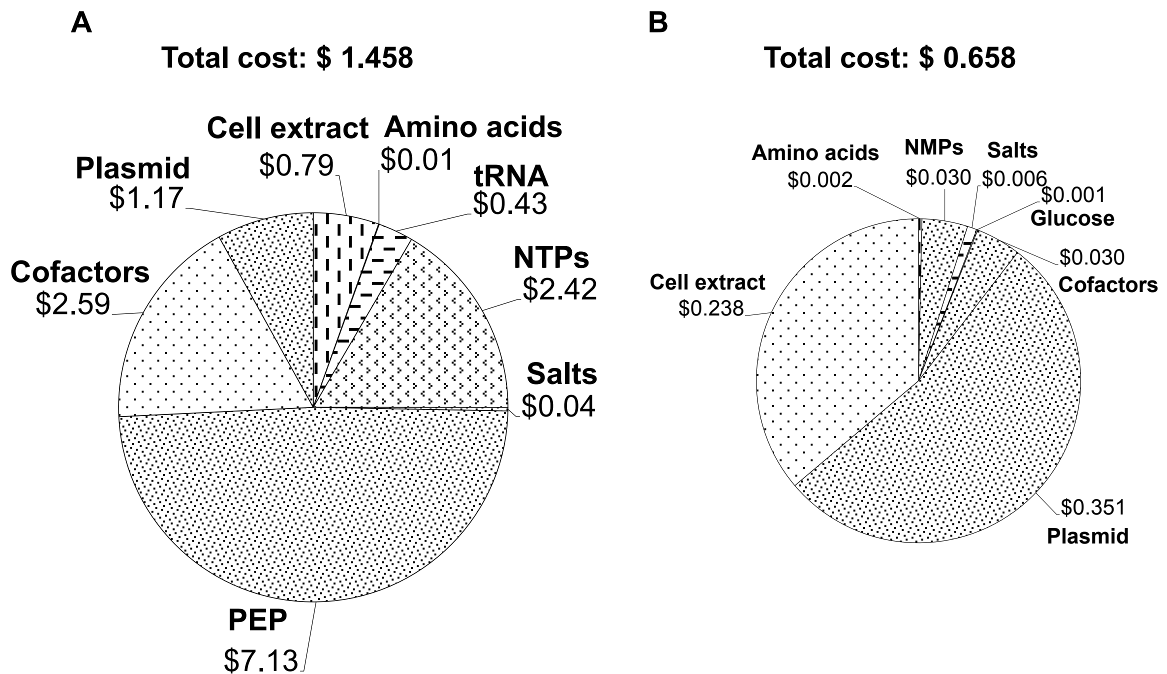


Figure 4-4: Cost break down of plasmid catalyzed CFPS. The pie chart represents A) PANOxSP and B) glucose energized CFPS to produce 100 µg of pPaGFP. The total CFPS cost is provided at the top of the figures. The sizes of the graphs are normalized to its absolute cost.

4.3.5 Linear expression templates

Although the use of linear expression templates (LET) eliminates the need for time consuming and labor intensive DNA cloning; a drop in protein yields has typically been observed when employing linear DNA in lieu of circular plasmids. However, there are cases where comparable or even greater yields have been observed [92]. Even when a drop in production yield is observed, the time efficiency and high-throughput capability compensate for the loss in yield. The drop in yield has been attributed to the deterioration of LET by exonucleases present in the cell extract. One major class of exonuclease that degrades LET is RecBCD [120, 121].

Recently, Amundsen et al. have reported the screening of small RecBCD enzyme inhibiting molecules [84]. Among different molecules screened, molecules CID 697851 (IC_{50} of 33 μM) and CID 1517823 (IC_{50} of 5.1 μM) from chemical classes cyanothiophene and pyrimidopyridone were used for this work. The compounds were first dissolved in DMSO and appropriate dilutions for CFPS reactions were made in DI-water. CFPS reaction was performed with or without the inhibiting reagents. Presence of these compounds improved production yields as much as 220% for pPaGFP produced with LET (Figure 4-5). A corresponding yield enhancement was not observed when expressing sfGFP using LETs (Figure A 1 in the appendix). While studying the kinetics of synthesis of these two proteins, ~80% of total sfGFP was synthesized in the first three hours while only half of pPaGFP was synthesized in the first three hours (Figure 4-7A). This could have led to the pronounced effect on pPaGFP yield by the inhibitor molecules as they could potentially inhibit RecBCD effectively for longer time.

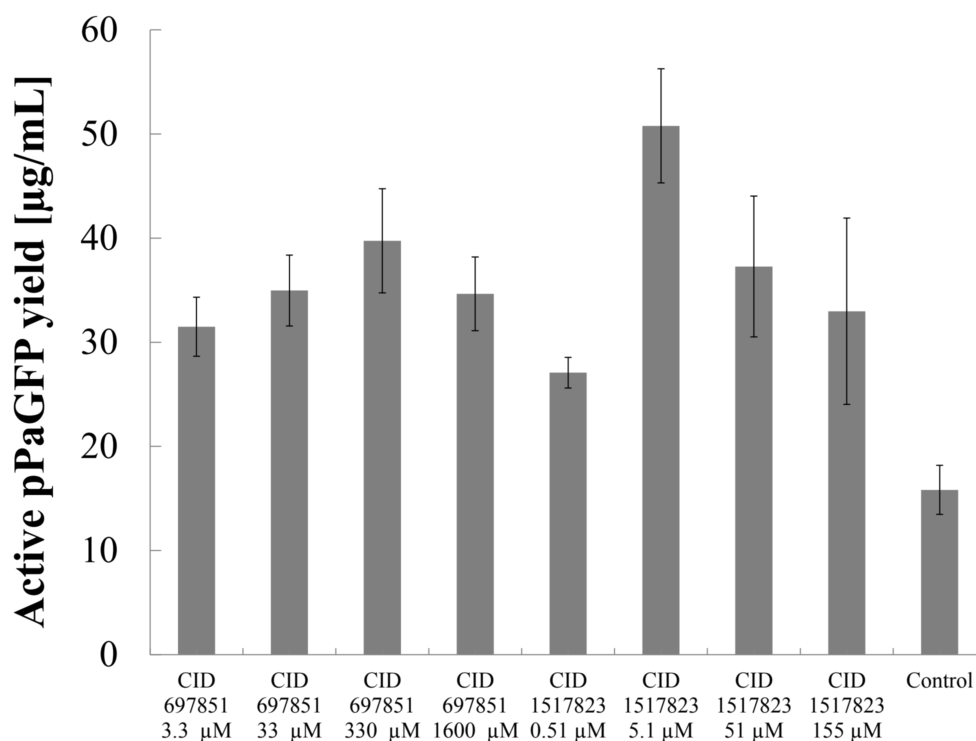


Figure 4-5: LET based pPaGFP yield with RecBCD inhibiting molecules. The control reaction represents reaction without any addition of inhibitory molecules. The error bars represent the standard deviation of three experiments.

Figure 4-6 shows the cost breakdown of reaction components in LET catalyzed CFPS PANOxSP and glucose based CFPS. Even though glucose is significantly inexpensive than PEP, the two systems have similar cost. Compared to pPaGFP yield from PEP catalyzed CFPS reaction, the pPaGFP yield with glucose catalyzed CFPS reaction was very low. Hence, large volume of CFPS reaction requiring large volume of cell extracts needs to be performed with glucose energized CFPS reaction for the same amount of protein production. The cost of cell extract is quite significant and hence increases the cost of glucose energized CFPS.

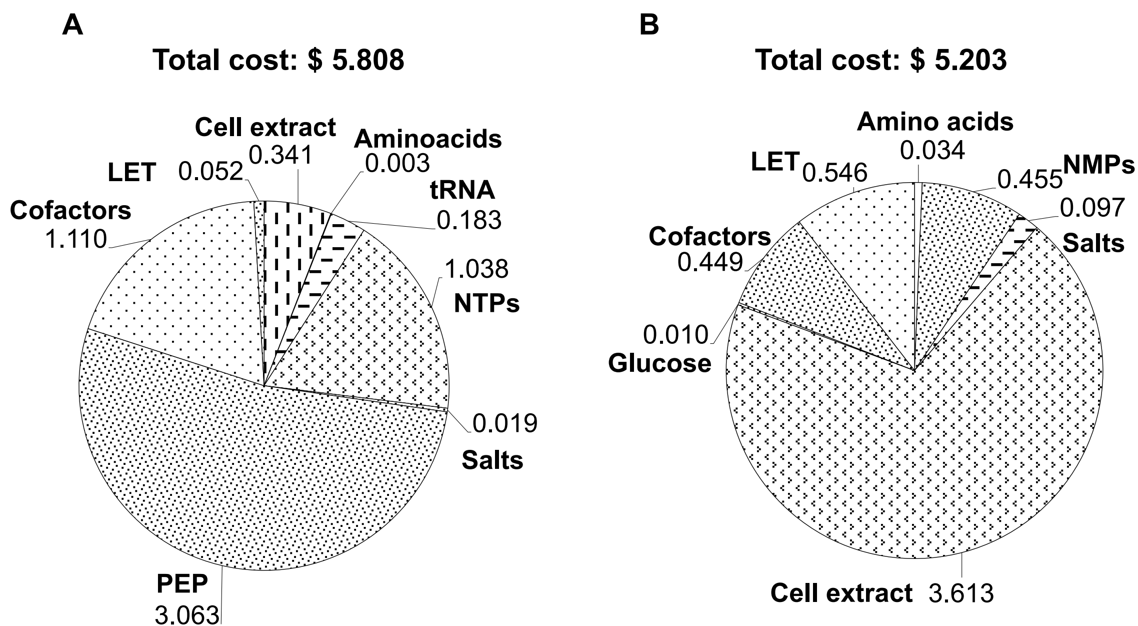


Figure 4-6: Cost break down of LET catalyzed CFPS. The pie chart represents A) PANOxSP and B) glucose energized CFPS to produce 100 µg of pPaGFP. The total CFPS cost is provided at the top of the figures. The cost calculation doesn't include the cost of the inhibiting reagents. The sizes of the graphs are normalized to its absolute cost.

In separate experiments, circular plasmid DNA of both sfGFP and pPaGFP were digested ahead of the transcription site using restriction endonucleases BamHI and EcoRI. When digested full length linear plasmids of sfGFP were used in CFPS, it produced proteins at levels comparable to circular sfGFP plasmid. However, the yield with digested linear pPaGFP plasmid dropped by half (Figure 4-7B). However, with both the genes higher protein yield was obtained with digested plasmids than with LETs. The digested plasmids are as long as the plasmids while the LETs are significantly shorter which might have had effect on the CFPS reaction. The longer length of the digested plasmid might have protected it from exonuclease activity in the cell extract. These results suggest that there are other limitations as well in engineering higher yields with LETs.

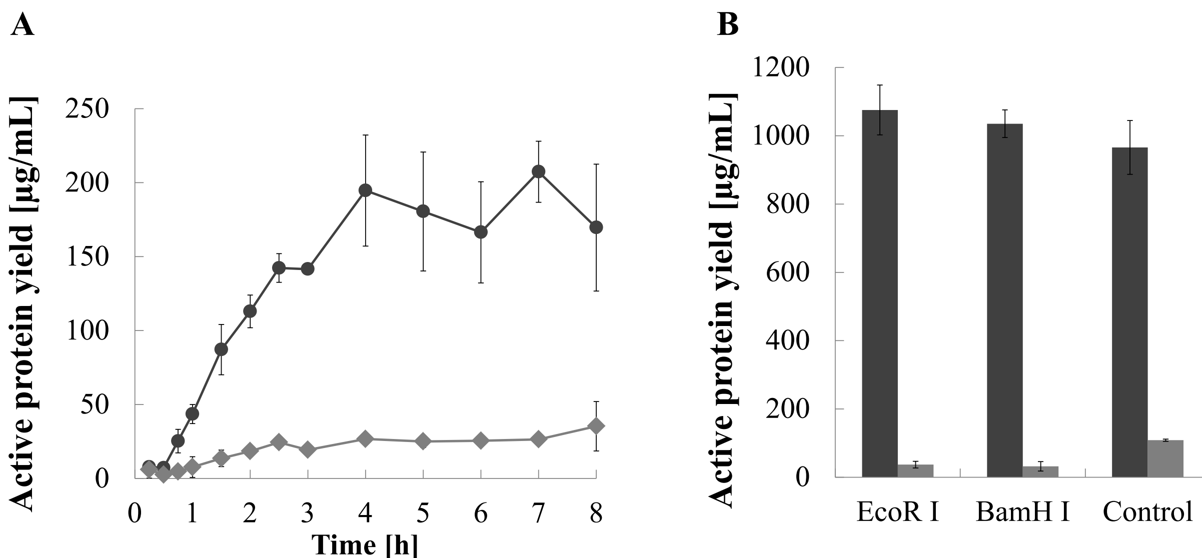


Figure 4-7: CFPS kinetics using LETs and yield from restriction digested plasmids. A) Kinetics of sfGFP and pPaGFP synthesis using LET as genetic template. The circle data points represent the sfGFP while the diamond data points represent the pPaGFP. The error bars represent the standard deviation of duplicate experiments. B) Protein yield from restriction digested plasmids. The dark bar represents the sfGFP while the light bar represents the pPaGFP. The control reaction represents the reaction with circular plasmid. The error bar represents the standard deviation of three experiments.

4.4 Conclusions

Here we have demonstrated for site-specific unnatural amino acid incorporation using LETs and alternative energy sources in a cell-free system. Although LET based yield of protein with UAA dropped to 25% of that obtained with the plasmid based wild-type protein yield, the production yield of 50 µg /mL is comparable to the highest reported yield of pPa incorporation *in vivo* which ranges from 1 µg/mL to 20 µg/mL [4]. Our attempts to improve production yields were met with mixed success and there remains significant potential for improved production yields and lower costs. However, yield of 50 µg /mL is reasonable when considering a high-throughput system to develop a library with the UAA incorporated at different locations. In

addition, LETs can be prepared in 3 - 5 h compared to plasmids which take days to prepare. In this work, we demonstrate increment in the yield of proteins with unnatural amino acid using PCR-amplified linear DNA by inhibition of the RecBCD exonuclease by addition of small molecule inhibitors in the CFPS reaction. Further developments of such a system could significantly impact biocatalysis, pharmaceutical, and medical diagnostic applications.

Most of the results and analysis reported in this chapter and in the appendix are published in the peer-reviewed journal New Biotechnology. Reference: Shrestha, P., M.T. Smith, and B.C. Bundy, Cell-free unnatural amino acid incorporation with alternative energy systems and linear expression templates. New Biotechnology, 2013. http://dx.doi.org/10/1016/ j.nbt.2013.09.002.

5 CONCLUSIONS AND FUTURE WORK

5.1 Conclusions

The objectives of this research were to engineer high-yielding cell-free extracts in an economical way and to improve protein production using PCR amplified linear DNA templates at lowered cost in an *E. coli*-based cell-free system. To achieve that goal, common cell growth and cell lysis techniques were employed as discussed in chapter 3. The chapter discussed different methods and previous research at simplifying the extract preparation procedure. To simplify and make the technology transferrable, cell growth was performed in shake flasks instead of high density fermenters and simpler cell lysis techniques were employed for cell lysis. Among various methods employed in this work, sonication was the most reliable cell lysis technique in terms of the ease, yield and ability to process lower sample volume. In chapter 4, challenges associated with LETs in cell-free were discussed followed by a potential solution to the degradation of LETs by RecBCD. In this work, improved yield of proteins with UAA was achieved by use of RecBCD inhibiting reagents.

Initially, this work focused on developing cell extracts for cell-free research. Preliminary work with different lysis techniques identified sonication and bead vortexing as promising candidates for *E. coli*-based cell extracts. The main challenges associated with using these two techniques were sample heating, extract to extract variation in yield, and lower yields associated with the extract. The initial protocols used to lyse cells using these techniques and variations in extract performance are discussed in chapter 3. To solve the problem of sample heating, the vial

containing cells were immersed in ice-water bath during sonication. During bead vortex mixing, the sample vial was cooled periodically in ice-water bath. Further work with these two techniques helped achieve higher yield with the extracts prepared. Consistently high yields were achieved by longer sonication and vortexing time. This increment in time had greater impact on improving the yield with sonication. The simple growth and lysis technique are economically sound and has great usefulness in making the technology transferable. To highlight the progress, the use of sonicator and vortexer for cell lysis for high yielding extract production can be considered as a technical milestone in the area of extract preparation.

Chapter 4 discussed the advantages of LETs in cell-free work and the current challenges associated with it. In this work, higher yield with protein with UAA in LET catalyzed CFPS was achieved by the addition of *in vitro* pPa acylated tRNAs. The addition of *in vitro* acylated tRNA resulted in double pPaGFP yields. The addition of *in vitro* acylated tRNAs increases the bias of stop codon to act as a codon for pPa incorporation rather than to act as a stop codon and truncate the protein. Additionally, improved yield with LETs for pPa incorporation was achieved using RecBCD inhibiting reagents. Use of these reagents resulted in 220% increment in the pPaGFP yield. Additionally, different energy source were employed in this work to reduce the cost of production of pPaGFP.

This work offers two main advancements in the field of cell-free work. The first advancement is the technical renaissance in cell extract preparation as demonstrated by the use of sonicator and bead vortexer for cell lysis. The second advancement demonstrated by this work is the inhibition of RecBCD enzyme that has deleterious effect in CFPS. These improvements and optimizations can further help in higher protein expression in CFPS.

5.2 Future work

The method employed in this work for cell extract preparation and improved protein yield with LETs can have transformative effect on CFPS as it allows for rapid testing of cell extracts and rapid protein synthesis using LETs. This work was successful in using a sonicator and bead vortexer for cell extract preparation and in achieving higher yield with LET-based CFPS.

Further opportunities in cell extract preparation include optimization of (1) enzymatic lysis and (2) freeze-thaw for cell extract preparation. Additionally, these techniques can be expanded for yeast cell and insect cells as well. The successful implementation of these techniques might require careful optimization of lysis buffer among many other factors. The work with LETs can be furthered optimized for higher yields with proteins with multiple UAA incorporation. This work focused on protein yield with UAA incorporation at one site. UAAs incorporation at multiple sites results in dramatic drop in full length protein yield. This is another challenging problem that requires further work. Cell extract devoid of release factor 1 might be useful for this work. Furthermore, new energy sources can be developed for energizing cell-free reactions. For instance, cellulose which is universally available can be used for energizing cell-free reactions. The use of cellulose will require extract with cellulase or external addition of the enzyme to break down the cellulose.

There have been great advancements in CFPS and it has been used for myriads of applications. The development of economical cell extract preparation techniques and improved yield with LETs as performed in this thesis work will help many researchers reap the benefit of cell-free and establish cell free as a strong platform for laboratory scale protein research.

APPENDIX

Table A 1: Approximate cost of cell lysis equipment discussed in this work.

Equipment	Price [$]	Manufacturer	Source
High pressure homogenizers	10,000 – 35,000	Avestin, Inc.	Personal communication. 04/26/2012
Bead mill	10,000 – 40,000	Yasui Kikai Corp. and Omni International, Inc.	Personal communication, 04/26/2012 and 06/27/2012
Sonicator	3000 - 4500	Sonics and Materials, Inc.	Catalogue, 05/07/2012
Vortex-Genie 2*	350	Scientific Industries, Inc.	Online listing by Scientific Industries at http://www.scientificindustries.com/genie2.html, 06/29/2012

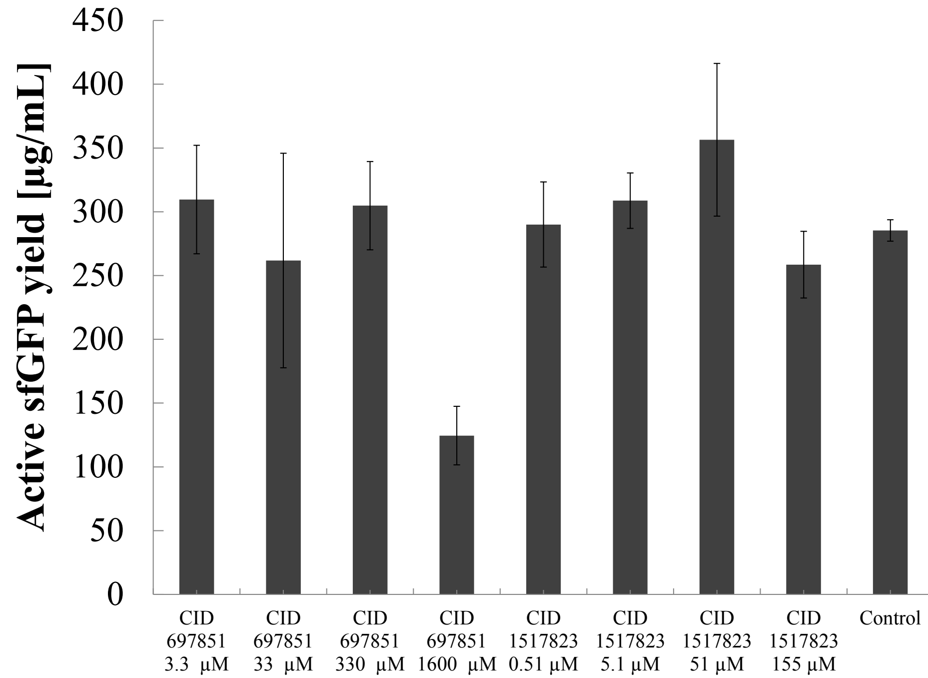


Figure A 1: sfGFP yield with RecBCD inhibiting molecules at different concentration. The control reaction represents reaction without any addition of inhibitory molecules. The error bars represent the standard deviation of three experiments.

Table A 2: Different energy system used in this work.

Constituents	PANOxSP [127]	PANOx* [128, 129]	CP/CK [68]	Glucose [15, 16]	F1,6BP [67]	High glutamate salt	Unit
PEP	**33.33**	**66**	-	-	-	-	mM
Creatine phosphate	-	-	**66**	-	-	-	mM
Creatine kinase	-	-	**3.2**	-	-	-	µg/mL
Glucose	-	-	-	**66**	-	-	mM
Fructose 1,6-bisphosphate	-	-	-	-	**66**	-	mM
Magnesium glutamate	10	-	-	-	-	-	mM
Magnesium acetate	-	16	16	-	8	20	mM
Ammonium acetate	-	80	80	80	80	80	mM
Ammonium glutamate	10	-	-	-	-	-	mM
Potassium Glutamate	175	90	90	90	90	**260**	mM
Potassium Oxalate	2.7	-	-	-	-	2	mM
Potassium phosphate	-	-	-	-	-	32	mM
Diaminobutane	1	-	-	-	-	-	mM
Spermidine Stock	1.5	-	-	-	-	-	mM
NAD	0.33	0.33	0.33	0.33	0.33	0.33	mM
CoA	0.27	-	-	-	-	-	mM
ATP	1.2	-	-	-	-	-	mM
CTP	0.86	-	-	-	-	-	mM
GTP	0.86	-	-	-	-	-	mM
UTP	0.86	-	-	-	-	-	mM
AMP	-	1.2	1.2	1.2	1.2	1.2	mM
CMP	-	0.85	0.85	0.85	0.85	0.85	mM
GMP	-	0.85	0.85	0.85	0.85	0.85	mM
UMP	-	0.85	0.85	0.85	0.85	0.85	mM
Folinic Acid	0.17	0.034	0.034	0.034	0.034	0.034	mg/mL
HEPES buffer	-	10	10	10	10	10	mM
DTT	-	1.7	1.7	1.7	1.7	1.7	mM
PEG-8000	-	1	1	1	1	1	% w/v
tRNA	0.0853	-	-	-	-	-	mg/mL
Amino acid[1]	2	0.5	0.5	2	2	2	mM
Expression plasmid	12	12	12	12	12	12	nM
pPa	2	2	2	2	2	2	mM
Cell extract	25	25	25	25	25	25	% v/v
*Mj*pPaRS^{pPa}	13.7	12	12	12	7	12	mg/mL

[1]With the exception of glutamate.

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