ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Optimized Extract Preparation Methods and Reaction Conditions for Improved Yeast Cell-Free Protein Synthesis

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ABSTRACT: Cell-free protein synthesis (CFPS) has emerged as a powerful platform technology to help satisfy the growing demand for simple, affordable, and efficient protein production. In this article, we describe a novel CFPS platform derived from the popular bio-manufacturing organism *Saccharomyces cerevisiae*. By developing a streamlined crude extract preparation protocol and optimizing the CFPS reaction conditions we were able to achieve active firefly luciferase synthesis yields of $7.7 \pm 0.5\ \mu g\,mL^{-1}$ with batch reactions lasting up to 2 h. This duration of synthesis is the longest ever reported for a yeast CFPS batch reaction. Furthermore, by removing extraneous processing steps and eliminating expensive reagents from the cell-free reaction, we have increased relative product yield (μg protein synthesized per $ reagent cost) over an alternative commonly used method up to 2000-fold from $\sim 2 \times 10^{-4}$ to $\sim 4 \times 10^{-1}\ \mu g\,\$^{-1}$, which now puts the yeast CPFS platform on par with other eukaryotic CFPS platforms commercially available. Our results set the stage for developing a yeast CFPS platform that provides for high-yielding and cost-effective expression of a variety of protein therapeutics and protein libraries.

Biotechnol. Bioeng. 2013;110: 2643–2654.

KEYWORDS: cell-free protein synthesis; in vitro translation; cell-free biology; *Saccharomyces cerevisiae*; protein expression

Introduction

There is a growing demand for simple, inexpensive, and efficient protein production technologies. This is because of (i) rapidly increasing markets for protein therapeutics (Leader et al., 2008), (ii) protein biomanufacturing bottlenecks (Dove, 2002), and (iii) the increasing discrepancy between genome sequence data and their translation products. The Sargasso Sea expedition alone, for example, generated 1.2 new million genes, many with unknown function (Venter et al., 2004).

Over the past decade, cell-free protein synthesis (CFPS) platforms have emerged as a powerful technology to help satisfy the current need for protein expression. Prominent applications include the production of pharmaceutical proteins and vaccines that are difficult to produce in vivo (Goerke and Swartz, 2008; Kanter et al., 2007; Stech et al., 2012; Yang et al., 2005); the synthesis of membrane proteins (Kubick et al., 2009); and high-throughput production of protein libraries for protein evolution, functional genomics, and structural studies (Madin et al., 2000; Takai et al., 2010). In addition, bacterial CFPS has been adapted to the manufacturing scale with yields approaching $g\,L^{-1}$ quantities. Strikingly, these reactions demonstrate linear scalability from μL to 5 L reactions of aglycosylated antibodies (Yin et al., 2012) and from μL to 100 L reactions of recombinant human granulocyte macrophage colony stimulating factor (rhGM-CFS) (Zawada et al., 2011). Despite this success, and in particular the rapid growth of the prokaryotic *Escherichia coli* extract based cell-free system (see Carlson et al., 2012 for a review), there is still a strong need to develop an integrated eukaryotic platform with similar batch productivity, scalability, protein folding capability, and cost-effectiveness that could be used for both biotechnology and synthetic biology. In this article, we initiate the development of a yeast based CFPS platform to achieve this goal.

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Contract grant sponsor: Northwestern University
Received 13 December 2012; Revision received 27 March 2013; Accepted 15 April 2013
Accepted manuscript online 23 April 2013;
Article first published online 7 July 2013 in Wiley Online Library (http://onlinelibrary.wiley.com/doi/10.1002/bit.24942/abstract).
DOI 10.1002/bit.24942

The major eukaryotic CFPS platforms previously developed include systems made from wheat germ extract (WGE) (Goshima et al., 2008; Hoffmann et al., 2004; Takai et al., 2010), rabbit reticulocyte lysate (RRL) (Jackson and Hunt, 1983), insect cell extract (ICE) (Ezure et al., 2010; Kubick et al., 2009; Tarui et al., 2001), *Leishmania tarentolae* extract (Kovtun et al., 2010; Mureev et al., 2009), and HeLa and hybridoma cell extract (Mikami et al., 2010) (Table I). Compared to the *E. coli* system, these methods have advantages for producing some types of complex proteins and can achieve post-translational modifications not found in bacteria (Chang et al., 2005). Insect cell-extract systems, for example, have demonstrated acetylation and N-myristoylation (Suzuki et al., 2006), isoprenylation (Suzuki et al., 2007), ubiquitination, (Suzuki et al., 2010), core glycosylation (Merk et al., 2012; Tarui et al., 2001), disulfide bond formation in single chain antibody fragments (Stech et al., 2012), and significant advances in expression and modification of membrane bound proteins (Kubick et al., 2009). However, eukaryotic cell-free platforms often have limited batch protein yields (Carlson et al., 2012), or depend on costly and inefficient continuous exchange reactions that do not scale commercially (Zawada et al., 2011). Furthermore, eukaryotic CFPS systems are generally limited by laborious and expensive extract preparation methods. For example, WGE (the most common eukaryotic system) requires lengthy preparation steps that include grinding, sieving, extensive washing, and eye selection of the embryo to ensure the embryo is in the proper stage of development (Takai et al., 2010). An additional challenge of this approach is that approximately 5 mL of active extract is produced from 5 to 6 kg of starting material after 4–5 days of processing (Takai et al., 2010). In contrast, *E. coli* can be processed quickly and under precise growth conditions to develop a highly active and robust CFPS platform, where 60 g of cells (wet weight) can be converted to 120 mL of extract in only 4–6 h of preparation (Liu et al., 2005). The above limitations motivate the need for a new eukaryotic CFPS platform that is robust, easy to prepare, highly active, and amenable to economical scale-up.

S. cerevisiae is a natural fit for CFPS because like *E. coli*, it is microbial and can be grown quickly and inexpensively under precise conditions in either a bioreactor or shake flasks. Furthermore, due to its eukaryotic nature it is suited to fold eukaryotic proteins and has previously shown some ability for post-translational modifications in vitro, such as glycosylation (Rothblatt and Meyer, 1986). Because it is a model organism, *S. cerevisiae* is well understood at the biochemical level, has a wealth of documented "omics" that can prove useful when trying to characterize a cell-free system, and genetic tools are readily available for facile changes to the host strain (Nielsen and Jewett, 2008). *S. cerevisiae* is also an important bio-manufacturing production platform and accounted for 18.5% of all FDA and EMA

Table I. Comparison of CFPS platforms.

Cell-free platform	Cell lineage	Source material	Host organism pharmaceutical applications	Extract preparation time	Batch productivity (combined Tx/Tl)	Relative product yield[a]
E. coli extract	Prokaryotic (Gram negative bacteria)	Cell culture	29.8% of Bio-pharmaceuticals[b]	1–2 days	200–500 μg/mL protein[c]	~1.5[c]
HeLa cell extract	Eukaryotic (Human)	Cell culture	None	1–2 days	240 μg/mL active luciferase[d]	~0.5[d]
Insect cell extract (ICE)	Eukaryotic (*Spodoptera frugiperda*)	Cell culture	None	1–2 days	45 μg/mL active firefly luciferase[e]	~0.3[e]
Leishmania tarentolae extract	Eukaryotic (Protozoa)	Cell culture	None	1–2 days	220 μg/mL active enhanced green fluorescent protein[f]	~0.2[f]
Rabbit reticulocyte lysate (RRL)	Eukaryotic (Mammalian/ New Zealand white rabbits)	Rabbit reticulocyte cells	None	4 days to treat rabbit 1 day for extract prep.	1–10 μg/mL active firefly luciferase[g]	~0.03[g]
Wheat germ extract (WGE)	Eukaryotic (Plant)	Wheat germ	None	4–5 days[h]	1–10 μg/ml active firefly luciferase[i]	~0.03[i]
Yeast extract	Eukaryotic (Yeast/*S. cerevisiae*)	Cell culture	18.5% of Bio-pharmaceuticals[b]	1–2 days	8 μg/mL active firefly luciferase (this study)	~0.4[j]

[a]Relative product yield is defined as μg protein synthesized per $ reagent cost.
[b]Ferrer-Miralles et al. (2009).
[c]Protein yield and cost estimation based on Promega S30 T7 High-Yield Expression Kit.
[d]Protein yield and cost estimation based on ThermoScientific 1-Step Human Coupled IVT Kit—DNA.
[e]Protein yield and cost estimation based on Promega TnT® T7 Insect Cell Extract Protein Expression System.
[f]Protein yield and cost estimation based on Jena Biosciences LEXSY in vitro Translation Kit.
[g]Protein yield and cost estimation based on Promega TnT® T7 Coupled Reticulocyte Lysate System.
[h]Takai et al. (2010).
[i]Protein yield and cost estimation based on Promega TnT® T7 Coupled Wheat Germ Extract System.
[j]Cost estimation based off a process model designed using SuperPro Designer (Intelligen, Inc., Scotch Plains, NJ) for yeast extract preparation and Sigma pricing for individual chemical components as of November 2012.

licensed recombinant protein biopharmaceuticals as of January 2009 (Ferrer-Miralles et al., 2009).

Despite these attractive features, there has been limited development of yeast based CFPS systems as a protein synthesis platform since their origin in the 1970s and early 1980s (Gasior et al., 1979a,b; Sissons, 1974; Tuite et al., 1980). Instead, the majority of research involving yeast cell-free translation systems has focused on investigating translation from a fundamental perspective, such as elucidating cap-dependent translation (Iizuka et al., 1994; Iizuka and Sarnow, 1997) and characterizing translation initiation factors (Algire et al., 2002; Hinnebusch et al., 2007; Kurata et al., 2010; Saini et al., 2009; Thompson et al., 2001). Despite a focus on fundamental science, some recent work has shown the potential to use yeast CFPS for making proteins of interest, such as replication and recombination of *Tomato Busy Stunt Virus* (Pogany and Nagy, 2008) as well as virus-like particle synthesis (Wang et al., 2008, 2010); although the extract preparation method developed in the latter example depends on expensive and non-scalable processing steps such as protoplast generation and syringe-lysis.

Here we aim to develop a cost-effective, high-yielding crude extract based yeast CFPS platform by following several guiding principles that were essential to the development of a commercially viable *E. coli* based CFPS platform (Carlson et al., 2012; Swartz, 2006). First, the extract preparation method must be streamlined to remove extraneous processing steps in order to reduce associated time and cost, while simplifying the overall procedure (Liu et al., 2005). Second, the CFPS reaction must be optimized to maximize protein synthesis and reduce substrate cost (Calhoun and Swartz, 2005; Jewett and Swartz, 2004; Swartz, 2006; Zawada et al., 2011). By addressing these challenges, our new yeast CFPS platform realized an improvement in both yield and cost-effectiveness.

Materials and Methods

Plasmid Construction

Plasmid pET23c-GFP-cyc3 was kindly provided by Dr. Markus Pech at the Max Planck Institute for Molecular Genetics (Iskakova et al., 2006). We elected to insert the gene encoding for firefly luciferase (60,755 Da) in place of GFP-cyc3 into the plasmid backbone as our reporter protein. The luciferase gene was inserted between the T7 promoter and T7 terminator sequences using Nde1 and Xho1 restriction digest enzymes using the luciferase sense primer 5′-GGTGGTCA-T**ATG**GAAGACGCCAAAAACAT-3′ and luciferase anti-sense primer 5′-GGTGGTCTCGAGTTTTTTTTTTTTTTT-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTttac-aatttggactttccgc-3′. Encoded in the anti-sense primer was the addition of a 90-mer poly(A) tail to 3′ end of the coding sequence. Plasmids were harbored in *E. coli* DH5α competent cells and purified using E.Z.N.A. Omega Plasmid DNA Maxi Kits (Omega Bio-Tek, Norcross, GA). For combined transcription/translation (Tx/Tl) reactions, the Ω sequence from tobacco mosaic virus was inserted in the 5′-UTR to remove the dependence of the 5′-cap for translation initiation to construct the pET23ΩLucA plasmid (Gan and Jewett, in preparation). For CFPS reactions, the coding sequence—containing the Ω sequence, protein of interest, and polyA tail—was PCR amplified with backbone sense primer 5′-GTGATT-CATTCTGCTAACCAG-3′ and anti-sense primer 5′-CCC-CAAGGGGTTATGCTAGT-3′ using Phusion High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA). The PCR product was purified using the Qiagen PCR Purification Kit (Qiagen).

In Vitro Transcription

In vitro transcription and mRNA capping was performed with the Ambion mMessage mMachine T7 Kit (Life Technologies). After in vitro transcription, template DNA was digested with DNase (supplied by the kit). 5′ 7mGppG-LucA mRNA was further purified through phenol:chloroform extraction. Subsequently, the mRNA was precipitated with ethanol plus 2.5 mM ammonium acetate. Poly(A) mRNA was isolated using Dynabeads Oligo$(dT)_{25}$ magnetic beads (Life Technologies).

Crude Extract Preparation

For optimal extract preparation, *S. cerevisiae* strain MBS (a generous gift from Dr. Sunnie R. Thompson at the University of Alabama at Birmingham (Thompson et al., 2001)) was grown in either the BIOSTAT Cplus 10 L bioreactor (Sartorius Stedim Biotech S.A., Aubagne Cedex, France), or 1 L of culture in 2.5 L Tunair (Sigma–Aldrich, St. Louis, MO) shake flasks in YPAD media, pH 5.5 supplemented with 50 mM potassium phosphate to 12 OD_{600}. Variations of this method are indicated in the main text. When the OD_{600} reached 12, the yeast culture was cooled quickly to between 4 and 8°C by either harvesting through a stainless steel coil immersed in an ice bath (when grown in the 10 L bioreactor) or by adding 1 L of ice per L of yeast culture (when grown in the shake flasks). To pellet the cells, the cell suspension was centrifuged for 10 min at 3,000*g* and 4°C. The cell pellet was washed with 60 mL of Mannitol Buffer A (30 mM HEPES, pH 7.4 w/5 M KOH, 100 mM potassium acetate, 2 mM magnesium acetate, 2 mM dithiolthretol, 8.5% (w/v) mannitol) per liter of starting culture followed by centrifugation for 5 min at 3,000*g* and 4°C. This step was repeated three times with 20 mL of Mannitol Buffer A per L yeast culture, with the final wash centrifuged for 5 min at 4,000*g*. Potassium and magnesium glutamate were used in place of potassium and magnesium acetate as denoted in the text. Extra buffer was removed by placing the centrifugation bottle upside down and tapping against a paper towel. The cell pellet was weighed, flash-frozen on liquid N_2, and stored at −80°C. Alternatively, the extract preparation can be continued with cellular lysis.

Cell lysis was performed using one of two methods, either with 0.5 mm glass beads (Sigma–Aldrich) or high-pressure

homogenization. For glass beads lysis, the method developed by (Iizuka et al., 1994) was used. Briefly, 5–6 g of wet cell mass was combined with 1.5 mL of cold Lysis Buffer A (Mannitol Buffer A + 0.5 mM PMSF) per 1 g of wet cell mass in a 50 mL falcon tube and the suspension was thawed on ice. Note, PMSF was first dissolved in 100% ethanol and was added fresh before each use. In the cold room, the cells were lysed in capped 50 mL falcon tubes by five 1-min cycles of hand shaking (2 Hz) over a 50 cm hand path, with 1 min cooling on ice water between cycles.

For high-pressure homogenization lysis, a minimum of 3 g of wet yeast cell pellet was lysed at one time. Again, 1.5 mL of cold Lysis Buffer A per 1 g of wet cell mass was added to the cell pellet and the suspension was thawed on ice. Immediately after cell thawing was completed, the cells were lysed by passing through an EmulsiFlex-C5 Homogenizer (Avestin, Ottawa, ON, Canada) at 30,000 psi and a flow rate of approximately 1–3 mL per min. The sample was collected through a cooling coil immediately upon exit that was submerged in ice water.

After cell disruption, the lysate was centrifuged at 4°C and 25,000*g* for 5 min. Immediately, the supernatant was transferred with a pipette into a clean Nalgene spherical bottom high-speed centrifuge bottle for the second centrifugation at 4°C 25,000*g* for 5 min. The aqueous fraction was carefully removed by avoiding crude cell debris at the bottom.

The lysate subsequently underwent buffer exchange through either dialysis or FPLC. For dialysis, the extract was dialyzed against four exchanges of 200-volumes of Buffer A/PMSF (Lysis Buffer A without the addition of mannitol) for 30 min each at 4°C using Slide-A-Lyzer Dialysis Cassettes (2,000 Da MWCO; Thermo Fisher Scientific, Waltham, MA). The dialyzed extract was centrifuged at 12,000*g* for 20 min at 4°C to remove any degraded proteins.

For FPLC, the extract was loaded onto a Sephadex G-25 Superfine (GE Healthcare Biosciences, Pittsburgh, PA) column at 25% of the bed volume using the BioLogic DuoFlow FPLC (Bio-Rad, Hercules, CA). The extract was exchanged against Buffer A/PMSF with a flow rate of 0.65 mL per minute at 4°C. Fractions were collected in 0.5 mL volumes. All fractions with an A_{260} reading >0.45 after 200-fold dilution were pooled together.

After buffer exchange, the extract was immediately aliquoted into 50, 100, and 200 μL samples as desired. The aliquots were rapidly frozen in liquid nitrogen and stored at −80°C for long-term storage. No decrease in activity was seen after several months of storage at −80°C or after up to four freeze–thaw cycles.

Micrococcal Nuclease Pre-Treatment

For Micrococcal Nuclease (MNase) pre-treatment, 1 μL of 50 mM $CaCl_2$ and 0.72 μL of 25 U μL^{-1} Micrococcal Nuclease from *Staphylococcus aureus* (Sigma–Aldrich; final concentration of 0.5 mM $CaCl_2$ and 0.18 U μL^{-1} MNase) were added to 100 μL of crude extract on ice. The solution was mixed by pipetting up and down and the reaction was incubated for 5 min at room temperature. To quench the reaction, 1 μL of 250 mM EGTA was added to the reaction to a final concentration of 2 mM. The reaction was promptly mixed by pipetting up and down and the treated crude extract was placed back on ice and used for downstream CFPS reactions.

Cell-Free Protein Synthesis

CFPS reactions were carried out in 1.5 mL Eppendorf tubes at 21°C in a temperature-controlled water bath in 15 μL reactions. The cell-free reaction mixture was assembled on ice from stock solutions to the following working concentrations for translation only reactions: 22 mM HEPES-KOH pH 7.4, 120 mM potassium glutamate, 2 mM magnesium glutamate, 0.75 mM adenosine triphosphate (ATP), 0.1 mM guanosine triphosphate (GTP), 0.04 mM of each of 20 amino acids, 25 mM creatine phosphate, 1.7 mM DTT, 1 mM putrescine, 0.5 mM spermidine, 0.27 mg mL^{-1} creatine phosphokinase (from rabbit muscle; Sigma–Aldrich), 26.7 U mL^{-1} RNase Inhibitor (Qiagen), 600 ng in vitro transcribed mRNA, and 50% (v/v) yeast extract. For combined transcription and translation reactions the working concentrations varied slightly to: 22 mM HEPES-KOH pH 7.4, 120 mM potassium glutamate (unless otherwise noted), 5 mM magnesium glutamate (unless otherwise noted), 1.5 mM of each ATP, GTP, CTP, and UTP, 0.08 mM of each of 20 amino acids, 25 mM creatine phosphate, 1.7 mM DTT, 1 mM putrescine, 0.5 mM spermidine, 0.27 mg mL^{-1} creatine phosphokinase (from rabbit muscle, Sigma–Aldrich), 26.7 U mL^{-1} RNase Inhibitor (Qiagen), 250 ng ΩLucA PCR amplified DNA, 0.027 mg mL^{-1} T7 RNA polymerase (made in house following the protocol developed by Swartz et al. (2004), and 50% (v/v) yeast extract. The final concentration of yeast extract proteins was 25.7 ± 1.0 mg mL^{-1}, as determined by Bradford Assay using commercially available assay reagents (Bio-Rad) compared to a bovine serum albumin protein standard. All other reagents were purchased from Sigma–Aldrich unless otherwise noted.

The amount of active firefly luciferase produced was determined by adding 12 μL of CFPS sample to 30 μL of ONE-Glo Luciferase Assay System (Promega) in a white 96-well plate. The total luminescence was read every 2 min over a 20-min interval using a BioTek (Winooski, VT) Synergy 2 plate reader. The maximum amount of relative light units (RLUs) was recorded for each cell-free reaction. RLUs were then compared to a linear standard curve of recombinant luciferase (Promega) added directly to the ONE-Glo reaction mixture.

For the pre-incubation experiments, all soluble components of the cell-free reaction were initially assembled on ice except the crude extract, T7 polymerase (T7 Pol), and creatine phosphokinase (CK). For each individual reaction, the T7 Pol and CK were added to the cell-free reaction immediately before the extract had finished "pre-incubating". After the pre-incubated extract was added to the reaction mixture, the CFPS reaction proceeded for an additional 2 h. In order to assay all of the samples simultaneously, the

cell-free reactions were quenched by flash freezing in liquid nitrogen, stored at −20°C and thawed simultaneously before being assayed for active luciferase yield.

Autoradiography

Autoradiography was used to determine the size of protein synthesized. 1.7 μL of ^{35}S-Methionine (~18 μCu) (PerkinElmer, San Jose, CA) was added to each 15 μL CFPS reaction. Following 3 h incubation, the CFPS reaction was loaded onto a NuPAGE 4–12% Bis–Tris Gel (Life Technologies) following the manufacturer's instructions. The NuPAGE gels were stained with SimplyBlue SafeStain (Life Technologies) and all proteins present in the cell-free reaction were visualized using the Gel Doc XR+ (Bio-Rad). The gels were dried and exposed overnight on a Storage Phosphor Screen (GE Healthcare Biosciences) and imaged with the Storm 860 Phosphoimager (GE Healthcare Biosciences). This image was digitally compared to the SimplyBlue stained image that included a protein standard ladder to determine the length of synthesized proteins.

Results

Optimizing the Extract Preparation Protocol

Because CFPS exploits an ensemble of catalytic proteins prepared from the crude lysate of cells, the cell extract (whose composition is sensitive to growth media, lysis method, and processing conditions) is the most critical component of extract-based CFPS reactions. In recent years, systematic optimization of each step in extract preparation for *E. coli* CFPS has led to more robust and productive extracts (Carlson et al., 2012). Based on these successes, we first chose to vary yeast extract preparation conditions in search of parameters that would reduce time and cost, improve reproducibility between extract preps., increase the level of protein synthesized, and allow for potential downstream scalability.

There are two previously reported methods for yeast cell-free translation (Iizuka and Sarnow, 1997; Wang et al., 2008). Because the method of Wang et al. (2008) uses protoplast formation, expensive lyticase treatment, and lysis with a 25-gauge needle, it is restricted to bench scale. Thus, with the long-term goal of scalability, we selected the Iizuki et al. (1997) method as our starting point for development since it did require protoplast formation or lyticase treatment. In addition, we also chose to start with the Iizuki et al. method because it is the most commonly used technique for making extracts for yeast cell-free translation. This basis "canonical" yeast CFPS procedure is shown in Figure 1A (Iizuka et al., 1994; Iizuka and Sarnow, 1997). In our method (Fig. 1B), we used a bioreactor for cellular growth, high-pressure homogenization for mechanical lysis of the cells, and dialysis for buffer exchange. Although tangential flow filtration would be more applicable for a large-scale process (Zawada et al., 2011), dialysis was chosen in part for ease of use at the laboratory scale. The crude extracts prepared by these two methods were evaluated by assessing the total active luciferase yield from batch cell-free translation only reactions with capped in vitro transcribed luciferase RNA. Translation of luciferase was carried out in a 15 μL batch reaction for 2 h at 21°C. Strikingly, extract from our new method synthesized nearly an order or magnitude more active luciferase (380.9 ± 2.2 ng mL^{-1}) relative to previous methods (53.7 ± 0.7 ng mL^{-1}) (Fig. 1C).

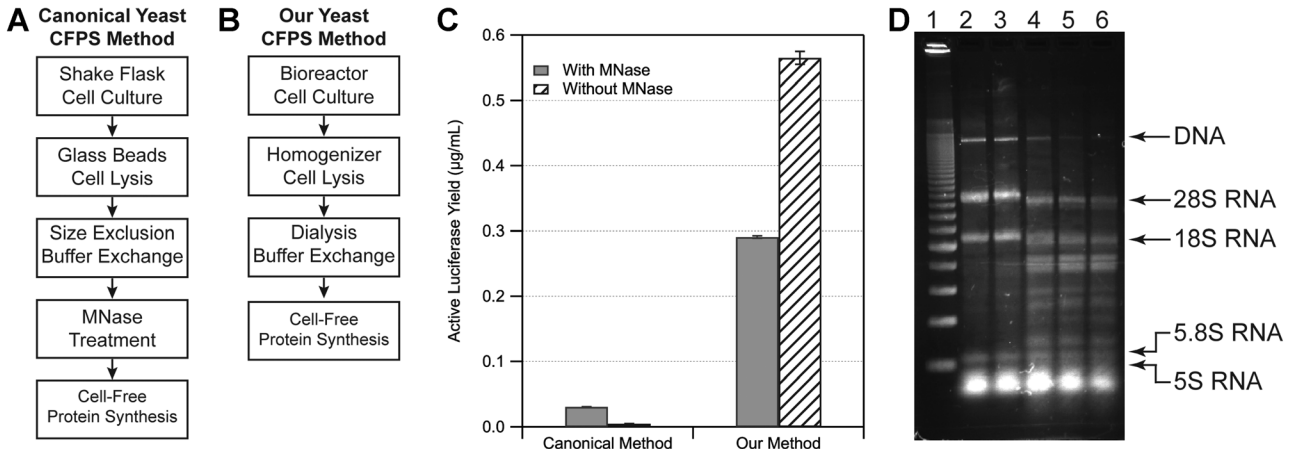

Figure 1. Extract preparation and CFPS flow diagrams and synthesis yields. **A**: A cartoon schematic of the canonical method for preparing yeast extract and CFPS is depicted (Iizuka et al., 1994; Iizuka and Sarnow, 1997). **B**: A cartoon schematic of our yeast CFPS system, which is a combination of both the canonical method and standard *E. coli* crude extract preparation methods (Liu et al., 2005; Zawada et al., 2011). **C**: Active luciferase yield from cell-free translation only reactions comparing the two extract preparation methods with and without addition of Micrococcal Nuclease (MNase) pre-treatment. Values show means with error bars representing standard deviations (s.d.) of at least three independent experiments. **D**: MNase pre-treatment degrades ribosomal RNA (rRNA), as shown by the disappearance of the original rRNA bands. Lane 1: 200 bp DNA step ladder (Promega), lane 2: Crude extract with no MNase pre-treatment, lanes 3–6: Crude extract after MNase pre-treatment for 0, 10, 20, and 30 min, respectively.

Removal of Non-Essential Processing Steps

We next evaluated the merits of extraneous processing steps, specifically Micrococcal Nuclease (MNase) treatment and a separate in vitro transcription reaction. Both of these steps include costly reagents and were not obviously beneficial to the overall extract preparation design and CFPS reaction. MNase was originally introduced to the extract preparation protocol as a means of digesting endogenous mRNA and mitigating unwanted competition with the gene of interest, as it preferentially digests single stranded nucleic acids (Heins et al., 1967). When we performed cell-free translation using our extract preparation method with the removal of MNase pre-treatment we found that our cell-free translation only reaction had an increase in active luciferase yield from 380.9 ± 2.2 to 681.2 ± 10.2 ng mL^{-1} (Fig. 1C). We hypothesized that the MNase decreased activity in the extract by non-productively degrading other RNA species that were beneficial for CFPS, specifically ribosomal RNA. To explore this hypothesis we prepared RNA samples from extracts generated with and without MNase pre-treatment. Our results suggest that in addition to digesting endogenous mRNA as expected, MNase also digests ribosomal RNA as an undesirable consequence (Fig. 1D). We therefore elected to remove MNase pre-treatment for all future CFPS reactions.

To further remove unnecessary and costly processing steps, we sought to activate combined transcription and translation (Tx/Tl) in a one-pot reaction. We therefore developed, in a parallel effort to our work reported here, a strategy to leverage the Ω sequence from tobacco mosaic virus (TMV) to initiate translation (Gan and Jewett, in preparation). In brief, combining Tx/Tl (i) eliminated an extraneous processing step (in vitro transcription) (Fig. 1A), (ii) removed the dependence of the reaction on the costly and potentially inhibitory m^{7}G(5')ppp(5')G RNA cap structure analog, (iii) eliminated inconsistency issues with the capping reaction, a known problem for eukaryotic CFPS reactions (Takai et al., 2010), and (iv) improved our overall yields ~2-fold over cell-free translation only reactions when using a linear DNA template. We therefore used a combined Tx/Tl system for all further CFPS reactions.

Optimizing the Growth Conditions

The composition of the cellular machinery at the time of harvest directly affects the CFPS potential of the crude extract. Historically, yeast cells used for cell-free translation experiments have been harvested in early exponential phase (1.5 OD_{600} (Tarun and Sachs, 1995; Wu et al., 2007) or 3-5 OD_{600} (Iizuka et al., 1994)). We were interested in determining if harvesting in mid-exponential phase (as done for *E. coli* CFPS) would provide benefits for translation. Since the cells are rapidly dividing in this phase, they are expected to have highly active translation machinery. Moreover, from a scaling standpoint, the ability to harvest at a later optical density would allow for larger cell mass recovery per fermentation, leading to a larger volume of total crude extract prepared per fermentation for improved overall system economics. Typically, 1 L of cell culture yields around 6 g of wet cell mass when harvested at 12 OD_{600} compared to ~1.5 g of wet cell mass when harvest at 3 OD_{600}. Subsequently, 1 g of wet cell mass leads to ~2 mL of crude extract.

Accordingly, we grew yeast cells to 3, 6, 9, 12, 15, and 18 OD_{600} and prepared individual batches of crude extract originating from each of these fermentations. To compare these extracts, we carried out combined transcription and translation reactions, where DNA harboring the TMV Ω sequence was used as a template. This is in contrast to cell-free translation only reactions shown in Figure 1, which used capped and purified mRNA as the template. Also in contrast to cell-free translation only reactions, combined transcription and translation required all NTPs in order to generate mRNA (Gan and Jewett, in preparation). As nucleotide concentration has a strong buffering effect on the concentration of free magnesium, increasing the total pool of NTPs from 0.85 mM (cell-free translation only) to 6 mM (combined transcription and translation), also required higher amounts of magnesium (Gan and Jewett, in preparation). Using 6 mM magnesium, combined transcription and translation was carried out in 15 μL batch reactions with extracts from different harvest ODs for 2 h. The most active extracts were obtained from yeast culture harvested at mid-exponential phase: 1.34 ± 0.25 μg mL^{-1} for OD_{600} 6–12 compared to 0.32 ± 0.05 μg mL^{-1} for OD_{600} of 3 and 0.49 ± 0.08 μg mL^{-1} for OD_{600} 15–18 (Fig. 2). Beyond assessing the impact of harvest OD_{600}, we also evaluated the addition of inorganic phosphate to the growth media and saw a 21% increase in protein synthesis capability for extracts generated from cells grown with 50 mM potassium phosphate (Supplementary Fig. 1). A similar effect was seen while developing the *E. coli* cell-free platform that showed the addition of phosphate to the growth media reduces phosphatase expression in the cell, thus stabilizing nucleotide triphosphate (NTP) concentrations during the cell-free reaction (Kim and Choi, 2000).

Optimizing CFPS Reaction Conditions

We then carried out a systematic optimization of the CFPS reaction conditions using extracts harvested at 12 OD_{600} from cells grown on 50 mM potassium phosphate, starting with magnesium concentration, which has been previously shown to be a critical component of yeast CFPS reactions (Wang et al., 2008). Re-optimization of magnesium was necessary because in Figure 2 we selected a magnesium concentration that worked best with extracts harvested from six different ODs. Here, we observed the optimal magnesium concentration for a 2 h combined Tx/Tl reaction from extracts harvested at 12 OD_{600} to be 7 mM (Fig. 3A). Next, we optimized the cell-free reaction temperature and found that active luciferase yield was optimal at 21°C (Fig. 3B).

We then sought to alter the physicochemical environment of the CFPS reaction to better mimic the cytoplasm and

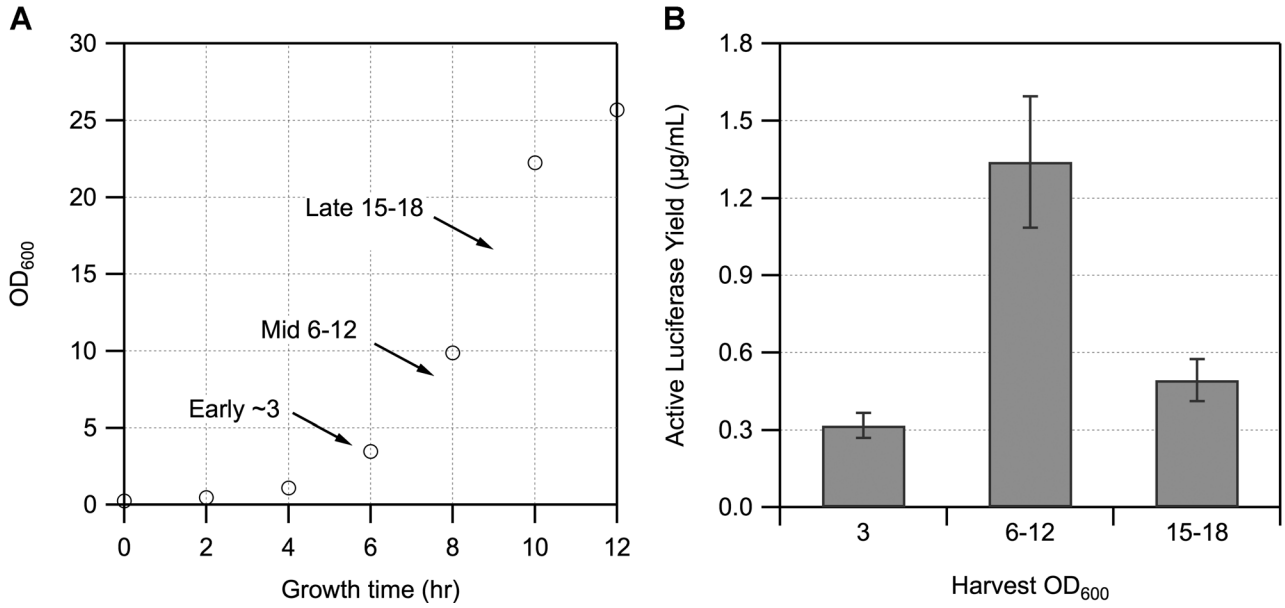

Figure 2. Effects of growth phase on crude extract activity. **A**: Typical growth curve for yeast culture on YPAD media, pH 5.5 at 30°C. **B**: Active luciferase yield in combined transcription and translation reactions with 6 mM magnesium acetate from extracts made with cells harvested at 3, 6, 9, 12, 15, and 18 OD_{600}. Extracts were grouped together according to their protein synthesis activity. The groupings were 3, 6–12, and 15–18 OD_{600} with most active extract at mid-exponential phase harvest. Values show means with error bars representing standard deviations (s.d.) of at least three independent experiments.

improve protein synthesis activity. Such an approach had previously been used to significantly improve *E. coli* CFPS systems (Jewett and Swartz, 2004). As an initial step, we targeted changes in the ionic composition, first seeking to use glutamate as the primary anion instead of acetate. Glutamate, which is the most predominant anion used in the cell, is also the preferred anionic species used in vitro because of its dispersed electron charge density compared to acetate or chloride (Jewett and Swartz, 2004; Record et al., 1998). Substituting glutamate salts for acetate salts, improved active luciferase yield more than twofold from 1.35 ± 0.11 to $3.18 \pm 0.25\ \mu g\ mL^{-1}$ (Fig. 3C). We then sought to add polyamines, specifically spermidine and putrescine, which act to modify the function of and stabilize DNA, RNA, and tRNA (Tabor and Tabor, 1985). Polyamines have been shown to improve bacterial CFPS (Jewett and Swartz, 2004). In our yeast CFPS system, we observed that the addition of polyamines was beneficial (Fig. 3D). The optimal concentrations of putrescine and spermidine for luciferase expression were 1 and 0.5 mM, respectively. In order to account for the increase in positively charged small molecules in the cell-free reaction, magnesium concentration was decreased from 7 to 5 mM (Fig. 3D). Combined these changes improved active luciferase yield to $3.89 \pm 0.17\ \mu g\ mL^{-1}$ (Fig. 3D).

A technical design criteria for our work was to maximize protein synthesis yield, while minimizing reaction cost. Therefore, we next investigated the necessity of adding RNase Inhibitor (Qiagen) to the cell-free reaction. RNase Inhibitor is expensive and may no longer be a productive component during combined Tx/Tl reactions (i.e., mRNA could be continually synthesized with sufficient nucleotides present). Interestingly, we observed that the addition of the storage buffer of the RNase Inhibitor (2 mM KH_2PO_4, 8.0 mM Na_2HPO_4, 3.0 mM KCl, 150 mM NaCl, pH 7.4, and 50% glycerol) had the same effect of adding the RNase Inhibitor itself (Supplementary Fig. 2). Furthermore, the removal of RNase Inhibitor had no obvious effect on overall yield. This result, somewhat serendipitously, led to the discovery that the addition of glycerol (50% of the RNase Inhibitor storage buffer) improved active luciferase yields to $7.69 \pm 0.53\ \mu g\ mL^{-1}$ (Fig. 3E). We hypothesize that glycerol may act as a chemical chaperone and improve protein stability, which has been a previously reported function in *S. cerevisiae* (Burg and Ferraris, 2008; Kai et al., 2013). Overall, our newly designed CFPS system—which includes glutamate salts, NTPs, spermidine, putrescine, and glycerol as well as re-optimized magnesium concentrations—resulted in a sixfold improvement of CFPS yield relative to the non-optimized reaction conditions shown in Figure 2.

Extending the Reaction Lifetime

Figure 4A shows active luciferase yield throughout the duration of the CFPS batch reaction. The final yield of luciferase after a 120-min incubation was $7.69 \pm 0.53\ \mu g\ mL^{-1}$. To the best of our knowledge, this duration of synthesis is the longest ever reported for a yeast CFPS batch reaction. There are several potential reasons the cell-free reaction may stop synthesizing protein after 2 h. This list includes but is not limited to: DNA/mRNA degradation, substrate limitations,

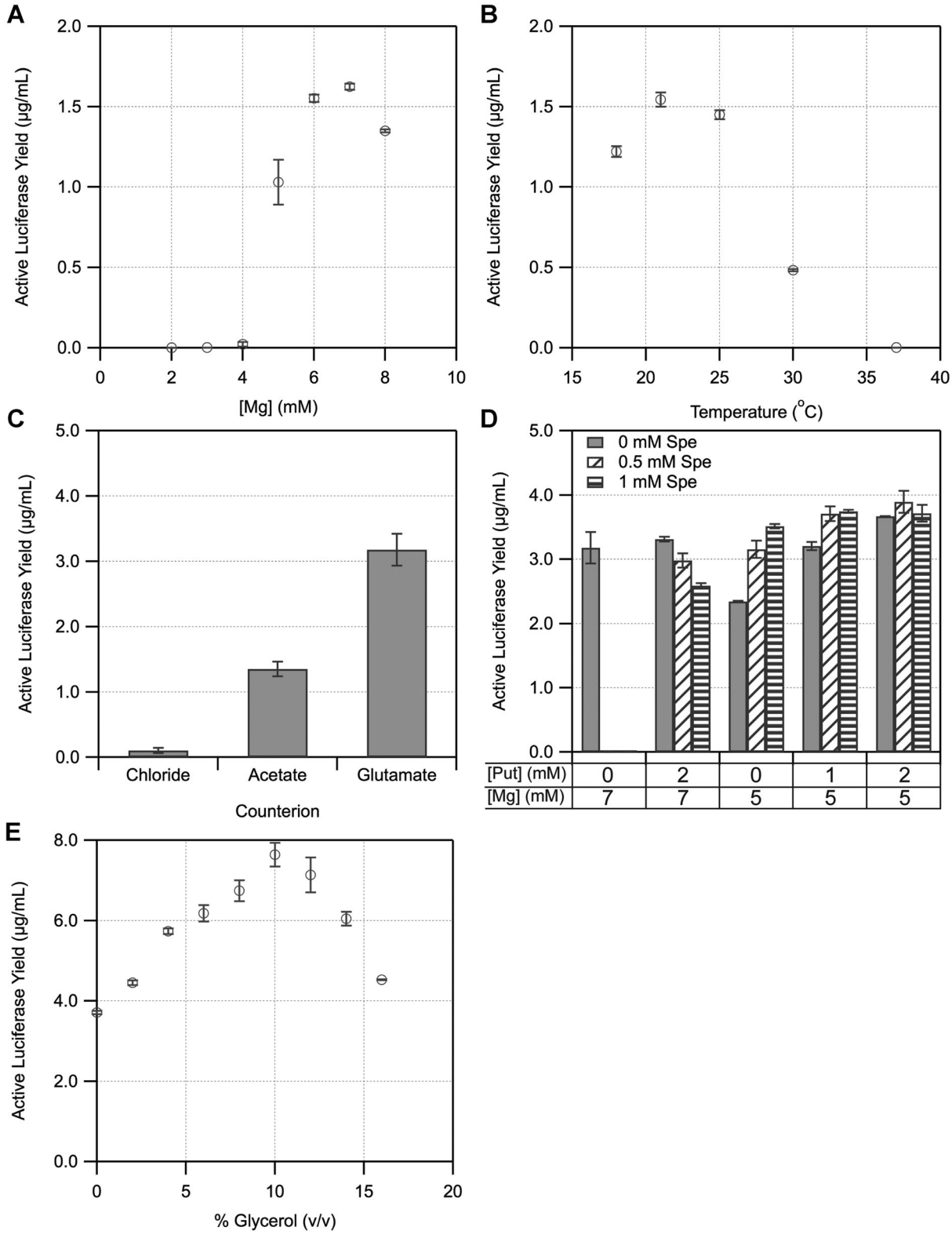

Figure 3. CFPS reaction optimization. The physicochemical environment of the CFPS reaction was optimized by altering (**A**) magnesium concentration, (**B**) temperature, (**C**) anions used with potassium and magnesium salts (chloride, acetate, and glutamate as shown), (**D**) polyamine concentration (putrescine and spermidine), and (**E**), glycerol concentration. Values show means with error bars representing standard deviations (s.d.) of at least three independent experiments.

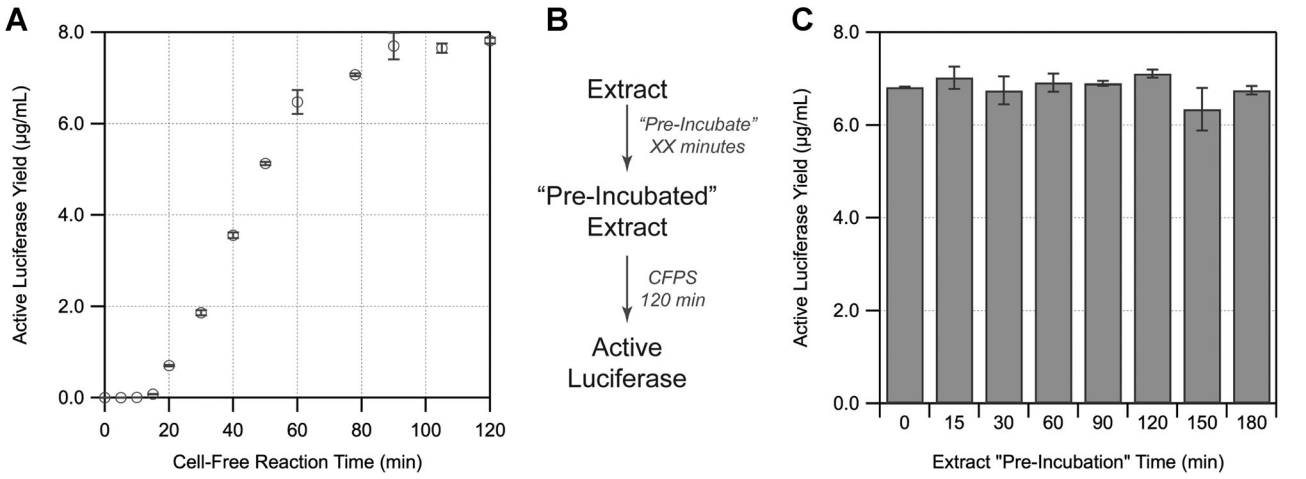

Figure 4. Synthesis of active luciferase over time and pre-incubation experiments. **A**: Protein synthesis over the course of a batch reaction. Fifteen microliters of batch reactions were prepared in different tubes for each time point and sampled for active luciferase yield. **B**: Experimental design schematic of pre-incubation experiments. **C**: Active luciferase yield from extracts pre-incubated for the specified time. Values show means with error bars representing standard deviations (s.d.) of at least three independent experiments.

toxic molecule accumulation, and loss of crude extract activity. Activity loss of crude extract proteins would be particularly detrimental. To test the validity of this concern, we designed a set of experiments directed toward unveiling if degradation of factors in the extract alone could be responsible for reaction termination. To do this, we "pre-incubated" the extract at 21°C for 0, 15, 30, 60, 90, 120, 150, and 180 min and then used the pre-incubated extract for the 2 h batch CFPS assay (Fig. 4B,C). Strikingly, we found no change in final CFPS luciferase yield after up to three hours of pre-incubation. These data suggest that catalyst activity is not responsible for reaction termination.

Discussion/Summary

Our work has shown that optimizing the yeast crude extract preparation method and the physicochemical environment of the in vitro system provides substantial advantages for protein synthesis. Overall, we were able to increase protein synthesis yield ~250-fold to $7.69 \pm 0.53\ \mu g\ mL^{-1}$ and increased relative product yield ~2,000-fold to 0.39 μg protein synthesized per $ reagent cost relative to a canonical yeast extract CFPS method (Fig. 5). To continue to improve *S. cerevisiae* CFPS utility as a platform technology, it will be necessary to increase protein synthesis yield. Our immediate future goal is to have protein synthesis yield exceed $100\ \mu g\ mL^{-1}$ for batch reactions, above or on par with wheat germ extract (Madin et al., 2000), *L. tarentolae* extract (Mureev et al., 2009), and the insect cell extract (Kubick et al., 2009) based systems. Three approaches to improve yields could include the following. First, as was done in the development of *E. coli* CFPS (Calhoun and Swartz, 2006; Jewett et al., 2008; Jewett and Swartz, 2004; Kim and Swartz, 2000; Swartz, 2006), substrate limitations should be identified and alleviated. Second, removal of background protein synthesis of endogenous mRNA will be important as it currently accounts for more than 1/2 of all synthesized proteins during CFPS (Supplementary Fig. 3). Third, DNA stabilization could also extend reaction lifetime if template instability is an issue (Michel-Reydellet et al., 2005). Future

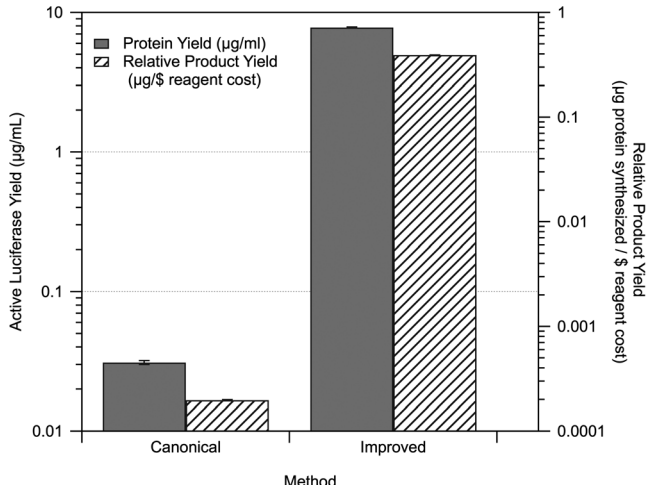

Figure 5. Comparison of our yeast CFPS method to the canonical approach as measured by active protein synthesis yield ($\mu g\ mL^{-1}$; left axis) and relative product yield (μg protein synthesized per $ reagent cost; right axis). Substrate cost includes all substrates used to treat the crude extract, make the genetic template, and assemble the CFPS reaction. Historically CFPS reaction cost is dominated by the energy substrates (Carlson et al., 2012).

Table II. Relative comparison of CFPS methods.

Extract preparation method	MNase	Combined/ separated Tx/Tl	CFPS reaction conditions	Active luciferase yield (μg/mL)	Fold increase relative to the canonical MNase treated Tx/Tl reaction
Canonical	Yes	Separated	Canonical	0.03 ± 0.00	—
Canonical	No	Combined	Improved	1.47 ± 0.24	47
Improved	No	Separated	Improved	2.41 ± 0.03	77
Improved	No	Combined	Improved	7.69 ± 0.53	245

Combined Tx/Tl means that transcription and translation occur together in a one-pot reaction.
Separated Tx/Tl means that transcription of mRNA and capping was performed in a separate reaction prior to cell-free translation.

efforts outside the scope of this work will explore these strategies.

Although we used the method of extract generation described by Iizuka et al. (1994) as our starting point, it should be noted that a previous report by Wang et al. (2008) has shown it possible to synthesize $\sim 50\ \mu g\ mL^{-1}$ of the HPV16 L1 protein with yeast extract. Even though our new approach shows ~250-fold improvement in yield over the Iizuka et al. approach (1994) it has a lower overall yield of a different reporter protein (active luciferase vs. total L1) than the method reported by Wang et al. (2008). Despite the lower overall yield, our approach has a ~4-fold higher relative product yields (μg $ reagent $cost^{-1}$) and longer batch reaction duration. Additional advantages of our method include: the use of a combined transcription and translation system, the use of technically simple and scalable extract processing techniques, and the removal of extra processing steps (e.g., protoplast development, MNase treatment, and in vitro capping/transcription).

Beyond biotechnology objectives, our system improvements have implications for using yeast CFPS as a model to study translation. Both active protein synthesis using (i) extract derived from the canonical "benchtop" protocol (e.g., glass beads lysis) for combined Tx/Tl reactions (Shrestha et al., 2012) and (ii) extract derived from our optimized extract preparation protocol for translation only reactions was improved (Table II). For example, by altering the reaction conditions and taking advantage of the developed Tx/Tl method, we found a 47-fold increase in active protein synthesis over previously published methods. Translation only reactions primed with extract generated with the optimized protocol developed in this report show a similar increase with a 77-fold improvement over previous methods. Furthermore, in both cases the yields crested the $1\ \mu g\ mL^{-1}$ threshold and demonstrate a significant improvement in utility of the cell-free system for future efforts to study translation.

In summary, the system described here provides a novel yeast CFPS platform. With batch yields of $\sim 10\ \mu g$ protein mL^{-1}, our system is quickly approaching yields achieved by the best eukaryotic CFPS platforms (~ 1–$250\ \mu g$ protein mL^{-1} in batch mode), which have been under development for technological applications for decades (Table I). While it is still early in the development of yeast CFPS, our advances, along with the fact that the system is not currently limited in biocatalytic potential, suggest promise for further development, particularly given other cell-free platforms that will serve as a guide (Carlson et al., 2012; Endo and Sawasaki, 2006; Jewett et al., 2008; Mureev et al., 2009; Swartz, 2006; Takai et al., 2010). Because *S. cerevisiae* is a model organism and is already a highly productive bio-manufacturing production platform in vivo, we anticipate that yeast CFPS will become a significant player on the stage with other CFPS technologies in the upcoming years.

We thank Jennifer Schoborg for her advice and discussions regarding the manuscript, Sunnie R. Thompson at the University of Alabama at Birmingham for sharing *S. cerevisiae* strain MBS, Dr. Markus Pech at the Max Planck Institute for Molecular Genetics for sharing plasmid pET23c-GFP-cyc3, and Byung-Sik Shin at the National Institute of Child Health and Human Development for sharing with us the detailed protocol on how to generate yeast extract using canonical methods. The authors gratefully acknowledge funding from Northwestern University.

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Supporting Information

Additional supporting information may be found in the online version of this article.