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ARTICLE

Biosensor-assisted engineering of a high-yield *Pichia pastoris* cell-free protein synthesis platform

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EP/K038648/1; Biotechnology and Biological
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Number: BB/L013770/1**Abstract**

Cell-free protein synthesis (CFPS) has recently undergone a resurgence partly due to the proliferation of synthetic biology. The variety of hosts used for cell-free extract production has increased, which harnesses the diversity of cellular biosynthetic, protein folding, and posttranslational modification capabilities available. Here we describe a CFPS platform derived from *Pichia pastoris*, a popular recombinant protein expression host both in academia and the biopharmaceutical industry. A novel ribosome biosensor was developed to optimize the cell extract harvest time. Using this biosensor, we identified a potential bottleneck in ribosome content. Therefore, we undertook strain engineering to overexpress global regulators of ribosome biogenesis to increase in vitro protein production. CFPS extracts from the strain overexpressing *FHL1* had a three-fold increase in recombinant protein yield compared with those from the wild-type X33 strain. Furthermore, our novel CFPS platform can produce complex therapeutic proteins, as exemplified by the production of human serum albumin to a final yield of 48.1 $\mu\text{g ml}^{-1}$. Therefore, this study not only adds to the growing number of CFPS systems from diverse organisms but also provides a blueprint for rapidly engineering new strains with increased productivity in vitro that could be applied to other organisms.

KEYWORDScell-free protein synthesis (CFPS), in vitro transcription translation, *Pichia pastoris*/*Komagataella phaffii*, synthetic biology, yeast

1 | INTRODUCTION

Despite being used since the 1960s where it was pivotal in unraveling the genetic code (Nirenberg & Matthaei, 1961), research on cell-free protein synthesis (CFPS) has recently undergone a resurgence primarily due to advances in synthetic biology (Carlson, Gan, Hodgman, & Jewett, 2012). The interest in CFPS stems largely from the ability to generate proteins in a short time, making it ideal for pathway design (Jung & Stephanopoulos, 2004), protein production (Yang et al., 2005), and

personalized medicine (Ogonah, Polizzi, & Bracewell, 2017; Pardee et al., 2016). Furthermore, CFPS is particularly advantageous for the synthesis of proteins with low expression yield, aggregation, or toxicity in vivo (Katzen, Chang, & Kudlicki, 2005). Because it is an open reaction system, CFPS can be easily optimized for different products, in a faster and more facile manner than engineering in vivo expression systems.

Theoretically, the cell extract from any organism can be used as the basis for CFPS, although the most common systems include *Escherichia coli*, Chinese hamster ovary cells (Brodell, Sonnabend, & Kubick, 2014),

Abbreviations: ATP, adenosine triphosphate; CFPS, cell-free protein synthesis; CrPV, cricket paralysis virus; HSA, human serum albumin; OD₆₀₀, optical density; sfGFP, superfolder green fluorescent protein; YPD, yeast peptone dextrose.

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wheat-germ (Anderson, Straus, & Dudock, 1983; Madin, Sawasaki, Ogasawara, & Endo, 2000), rabbit reticulocytes (Jackson & Hunt, 1983), and insect cells (Stech et al., 2014), all of which are commercially available. As the choice of host extract can impact the protein folding and posttranslational modifications, the development of new CFPS is of interest. In recent years, CFPS have been produced using HEK239 (Bradrick, Nagyal, & Novatt, 2013), *Streptomyces venezuelae* (Moore, Lai, Needham, Polizzi, & Freemont, 2017), *Bacillus megaterium* (Moore et al., 2018), BY-2 tobacco cells (Buntru, Vogel, Stoff, Spiegel, & Schillberg, 2015), and *Saccharomyces cerevisiae* (Gan & Jewett, 2014) expanding the potential options for in vitro protein production.

Pichia pastoris (syn. *Komagataella* spp.) has been used as an industrial host for the production of recombinant proteins for the past 30 years (Ahmad, Hirz, Pichler, & Schwab, 2014). To date, over 5,000 different proteins have successfully been produced, and it has recently been noted that *P. pastoris* is the most commonly used eukaryotic expression system for research (Bill, 2014). One of the key advantages of *P. pastoris* is the ability to reach high cell densities, which results in high volumetric productivity (Darby, Cartwright, Dilworth, & Bill, 2012). Growth to high cell densities could be particularly beneficial in producing cell extracts for CFPS as the concentration of cellular machinery is a critical factor in achieving high yields (Zawada & Swartz, 2006). Furthermore, the potential to produce extracts from strains with humanized N-linked glycosylation pathways (Hamilton & Gerngross, 2007) would enable the production of biotherapeutic proteins with the correct posttranslational modifications. The nature of CFPS means that novel therapeutics could initially be rapidly screened in vitro to test their efficacy and manufacturability before undertaking complex and more time-consuming in vivo production. Furthermore, strategies for cell line engineering to increase the production are often specific to the particular recombinant protein being expressed and require empirical determination of the optimum for any given target (Ahmad et al., 2014). Thus, there is the potential to use CFPS as a rapid prototyping platform for strain selection on a small scale.

As ribosome content has been identified as a critical attribute for CFPS (Zawada & Swartz, 2006), we first developed biosensors to measure ribosome dynamics over time to identify the optimal harvest point. The results suggested that peak ribosome content occurs at a low optical density (OD), which has been previously shown to correspond to a high growth rate (Rebnegger et al., 2014; Regenberg et al., 2006). While supplementing the extract with ribosomes has been shown to result in higher yields of protein (Panthu et al., 2017), the process of ribosome isolation is not feasible on the large scale. Therefore, we aimed to engineer a *P. pastoris* strain with enhanced ribosome content to generate a high-yielding CFPS platform.

2 | MATERIALS AND METHODS

2.1 | Media and growth conditions

Bacterial strains were cultured in Lennox lysogeny broth (LB) medium (1% peptone from casein, 0.5% yeast extract, 0.5% NaCl)

supplemented with either 37 $\mu\text{g ml}^{-1}$ Kanamycin (Sigma-Aldrich, Dorset, UK), 25 $\mu\text{g ml}^{-1}$ Zeocin™ (Thermo Fisher Scientific, Paisley, UK), or 100 $\mu\text{g ml}^{-1}$ Ampicillin (Sigma-Aldrich). Yeast strains were cultured in a rich yeast peptone dextrose (YPD) medium (2% peptone from casein, 1% yeast extract, and 2% dextrose) and with either 100 $\mu\text{g ml}^{-1}$ Zeocin™ (Thermo Fisher Scientific) or 350 $\mu\text{g ml}^{-1}$ Geneticin (VWR, Lutterworth, UK) for the selection. *P. pastoris* strains were cultured in baffled glass flasks or in 50 ml Falcon tubes in a maximum volume of 5 ml starting from an OD₆₀₀ of 0.1.

2.2 | Strains

Bacterial recombinant DNA manipulation was carried out in *E. coli* strain NEB 5- α (New England Biolabs, Hertfordshire, UK). *P. pastoris* (syn. *Komagataella phaffii*) X33 was obtained from Invitrogen (Carlsbad, CA).

2.3 | Plasmid and strain construction

All vectors were made using the Gibson DNA assembly method as described previously (Gibson et al., 2009). The luciferase gene was amplified from pGL2 (Promega, Southampton, UK). The human serum albumin (HSA) gene was amplified from pPICZ α -HSA (Aw, Barton, & Leak, 2017). The GPR1 and cricket paralysis virus (CrPV) internal ribosome entry site (IRES) sequences were synthesized by Integrated DNA Technologies (Leuven, Belgium). For the cell-free protein synthesis plasmids, the pET-28b vector (Merck UK Ltd, Hertfordshire, UK) was used as a backbone. To create the expression constructs, the desired fragments were amplified with 30 bp of homology using primers purchased from Thermo Fisher Scientific and Phusion® High Fidelity DNA polymerase (New England Biolabs). For each of the coding sequences a Kozak sequence (GAAACG) was included in the primers. This sequence was chosen as it has previously been shown to be effective for the production of recombinant proteins in *P. pastoris* and is recommended in the ThermoFisher PichiaPink™ Expression System manual. The PCR fragments were gel extracted using the Zymoclean™ Gel DNA Recovery kit (Zymo Research Corporation, Irvine, CA) before the assembly reaction. After the correct plasmid assembly was confirmed, a synthetic 50 bp polyA tail was generated using annealed primers and inserted into the vector by restriction cloning using *Xho*I and *Not*I (New England Biolabs). Plasmids for cell-free protein synthesis reactions were extracted using the Qiagen Midi Prep Kit (Crawley, UK).

Targets of interest for both the biosensor and overexpression strains were identified by bioinformatics using Pichiagenome.org (Mattanovich et al., 2009) and amplified from genomic DNA extracted from *P. pastoris* X33 by the DNeasy® Plant Mini Prep kit (Qiagen, Manchester, UK). The ribosome biosensors used the pPICZ vector as a backbone (Thermo Fisher Scientific) and the superfolder green fluorescent protein (sfGFP) sequence was amplified from pPICZ-GFP (Aw & Polizzi, 2016) and fused to the promoter sequences of the genes involved in ribosome biosynthesis and

maturation. For strains overexpressing genes involved in ribosome biogenesis, target genes were cloned into the pKANB vector (a kind gift from Geoff and Joan Lin-Cereghino, University of the Pacific [Lin-Cereghino et al., 2008]) and the AOX1 promoter was replaced with the GAP promoter for constitutive expression using Gibson DNA assembly. *P. pastoris* transformations were carried out using the method described in the *Pichia* Expression manual (Thermo Fisher Scientific).

2.4 | Lysis methods

Each of the lysis methods was performed on 50 ml of overnight culture ($OD_{600} \sim 18$). Lysis efficiency was analyzed by serial dilution and colony counting, and the protein concentration was measured using the DC protein assay kit (BioRad, Hertfordshire, UK) according to the manufacturer's protocol.

2.4.1 | Y-PER

For cultures lysed with Y-PER™ the manufacturer's protocol was followed (Thermo Fisher Scientific).

2.4.2 | Spheroplasting

The spheroplasting protocol was adapted from the *Pichia* Expression Manual (Thermo Fisher Scientific) with the following modifications: volumes were scaled down to reflect the smaller starting culture, both 30 U Zymoyase (Zymo Research Corporation) and 600 U lyticase (Sigma-Aldrich) were independently tested. The samples were left for 1 hr at 37°C and after the final centrifugation at 750g at 4°C the pellets were resuspended in lysis buffer A (20 mM HEPES, pH 7.4, 100 mM potassium acetate, 2 mM magnesium acetate).

2.4.3 | Sonication

The cells were pelleted at 1,500g at 4°C for 5 min and resuspended in 10 ml of lysis buffer A. The cells were sonicated at an amplitude of 50% for 5 min with 20 s on, and 20 s off. Up to three cycles were tested.

2.4.4 | Dounce homogenization

The cells were pelleted at 3000g for 10 min at 4°C and washed with 20 ml lysis buffer A. The cells were centrifuged again for 5 min at 3000g at 4°C and 1 ml of lysis buffer A was added per 1 g of wet cell weight. A tight-fitting Dounce homogenizer (GPE Scientific Ltd, Bedfordshire, UK) was used for 10–15 strokes.

2.4.5 | High-pressure cell disruption

The cells were pelleted at 3000g for 10 min at 4°C and washed with 20 ml lysis buffer A. The cells were centrifuged again for 5 min at 3000g at 4°C and 1 ml of lysis buffer A was added per 1 g of wet cell weight. The cells were then passed through a Constant Systems

disrupter (Daventry, UK) at 30 KPSIG for one, two, three, or four cycles.

2.5 | Flow cytometry

P. pastoris cells were collected, diluted in PBS, and the fluorescence was measured using an Attune™ NxT flow cytometer (Thermo Fisher Scientific). Recorded data from flow cytometry consisted of 10,000 events (cells) and GFP fluorescence was measured using excitation with a 488 nm laser and a 510/10 nm emission filter (BL1). The data were analyzed with the FlowJo™ software (Ashland, OR).

2.6 | Crude extract preparation

An overnight culture of *P. pastoris* was grown in 5 ml of YPD medium and used to inoculate 200 ml of YPD medium to an OD_{600} of 0.1. The cells were grown until an OD_{600} of 18–20 at 30°C, 250 rpm. Once the target OD_{600} had been reached, the cells were incubated on ice before centrifugation for 10 min at 3000g at 4°C. The cells were washed three times in buffer A (20 mM HEPES, pH 7.4, 100 mM potassium acetate, 2 mM magnesium acetate) and centrifuged as above. After the final centrifugation, the cells were blotted onto the paper to remove excess buffer and either flash frozen in a methanol-dry ice bath or lysed immediately.

Flash frozen cells were thawed on ice before use. The cells were resuspended in 1 ml of Lysis Buffer A (20 mM HEPES, pH 7.4, 100 mM potassium acetate, 2 mM magnesium acetate, and 2 mM DTT, 0.5 mM PMSF) per 1 g of wet cell weight. The cells were lysed using a high-pressure homogenizer with a one-shot head adapter (Constant Systems). Ten millilitres of culture was passed through twice at 30 KPSIG.

After lysis, the extract was centrifuged for 30 min at 18,000g at 4°C. The supernatant was transferred to a fresh tube and centrifuged again for 30 min at 18,000g at 4°C. The supernatant was then loaded onto a hydrated 3.5 K MWCO Slide-A-Lyzer™ G2 dialysis cassette (Thermo Fisher Scientific) and dialyzed against 50-volumes of Lysis Buffer A for a total of 4 times for 30 mins each. Once dialysis was completed, the supernatant was transferred to a fresh tube and centrifuged for 1 hr at 18,000g at 4°C. The cell extract was then aliquoted and flash frozen in a methanol-dry ice bath.

2.7 | Coupled cell-free protein synthesis

Coupled in vitro transcription translation reactions were set up on ice and contained 40 nM DNA, 25 mM HEPES–KOH pH 7.4, 120 mM potassium glutamate, 6 mM magnesium glutamate, 1.5 mM adenosine triphosphate (ATP), 2 mM guanosine triphosphate, 2 mM cytidine triphosphate, 2 mM uridine triphosphate, 0.6 mM of each of 19 amino acids with the exception of leucine at 0.5 mM (biotechrabbit GmbH, Hennigsdorf, Germany), 25 mM creatine phosphate, 2 mM DTT, 0.54 mg ml⁻¹ creatine phosphokinase (C3755-1KU, Sigma-Aldrich), 200 U ml⁻¹ RNase Inhibitor (New England Biolabs), 100 U T7 polymerase (Thermo Fisher Scientific), and 50% (v/v) extract. CFPS reactions were run at room temperature in 50 µl reactions without shaking.

2.8 | Luciferase assay

A luciferase assay mix (20 mM tricine, 100 μ M EDTA, 1.07 mM $MgCO_3$, 250 μ M luciferin, 250 μ M ATP, 2.67 mM $MgSO_4$, and 17 mM DTT; Siebring-van Olst et al., 2013) was made fresh before the assay. Every 30 min, 5 μ l of the cell-free reaction was added to 30 μ l of the luciferase assay mix and the luminescence was measured using the POLARstar[®] Omega plate reader (BMG Labtech Ltd, Aylesbury, UK). Average readings were taken over 20 min as previously reported (Gan & Jewett, 2014). The error bars were calculated taking into consideration error propagation (Gerards, 1998).

2.9 | Human serum albumin assay

HSA was quantified using the Albumin Blue Fluorescent assay kit (Active Motif, La Hulpe, Belgium) according to the manufacturer's protocol. The error was calculated using a Taylor series expansion, which takes into consideration the error of the standard curve in addition to the deviation between the samples (Gerards, 1998).

3 | RESULTS AND DISCUSSION

3.1 | Optimal conditions for harvest and lysis

It has previously been reported that a high ribosome concentration is essential for successful CFPS (Zawada & Swartz, 2006) and as ribosome content is correlated to the growth rate, we began by investigating the ideal OD_{600} to harvest *P. pastoris* cultures for generating the cell extract. For *S. cerevisiae* the optimal harvest point was determined to be the mid-logarithmic phase (OD_{600} of 10–12; Gan & Jewett, 2014). Because *P. pastoris* can reach higher cell densities, we hypothesized that the cells that harvested a higher OD_{600} would still be in the mid-logarithmic phase, allowing us to maximize the quantity of the extract produced per liter of the culture. To monitor the dynamics of ribosome content across OD_{600} , we developed four ribosome biosensors (Figure 1a), on the basis of the promoters driving the expression of ribosomal subunits (RPP0, PAS_chr1-3_0068; RPL19, PAS_chr3_0091; RPS0A, PAS_chr1-4_0471) and proteins involved in maturation (YTM1, PAS_chr1-3_0188), whose expression had been previously shown to vary under different growth conditions (Prielhofer et al., 2015). These genes represent different factors in ribosome biosynthesis and therefore together should provide a representation of changes in the transcription of the ribosome-related genes over time. The promoter regions were amplified from the genome of *P. pastoris* X33, fused to a sfGFP coding sequence, and subsequently cloned into the pPICZ backbone. Each vector was individually integrated into the *P. pastoris* genome through homologous recombination into the native locus (Figure 1a), colony PCR was used to determine the successful integration (data not shown). To determine optimum harvest time, we monitored fluorescence using flow cytometry and plotted this against OD_{600} (Figure 1b) and exponential growth rate (Supporting Information Figure 1).

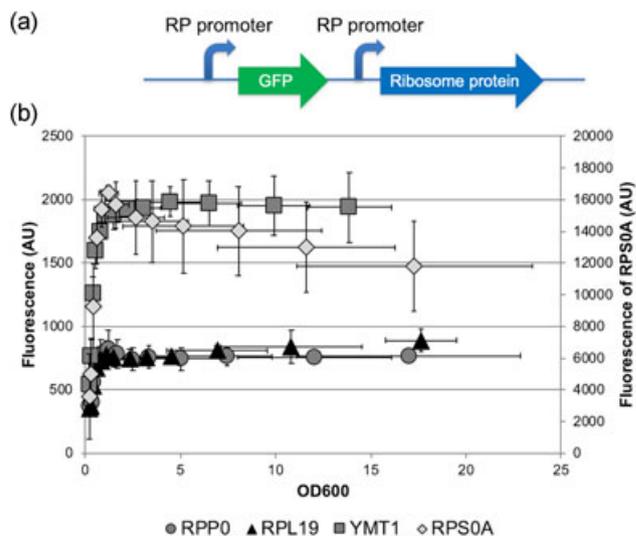
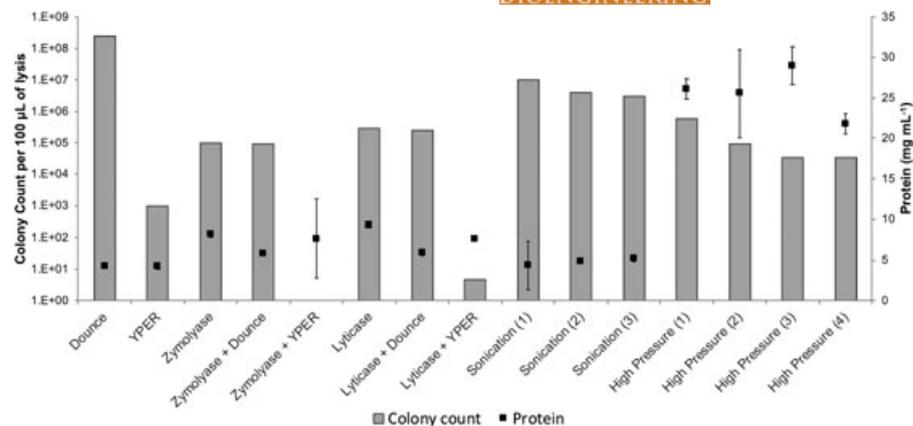


FIGURE 1 Ribosome biosensors. Ribosome biosensors were generated to evaluate ribosome expression dynamics. (a) Schematic representation of the biosensor. (b) Growth and GFP fluorescence profiles (average geometric mean) of biosensor strains. Fluorescence was determined by flow cytometry and the OD_{600} measured at the same time. Four biosensor strains were investigated on the basis of the promoters of the *RPP0*, *RPL19*, *YTM1*, and *RPS0A* genes. Error bars represent the standard deviation of the mean of three biological repeats. GFP: green fluorescent protein

The highest ribosome biosensor fluorescence occurred at a much lower OD_{600} than anticipated, *RPP0* at 1.2, *RPS0A* at 1.2, and *YTM1* at 5.4, which corresponded to the growth rates of $0.418 \text{ hr}^{-1} \pm 0.027$, $0.312 \text{ hr}^{-1} \pm 0.041$, and $0.381 \text{ hr}^{-1} \pm 0.046$, respectively. Although higher growth rates were observed for these strains, these were close to μ_{\max} , which corresponds to previous observations that the growth rate has been shown to correlate with high ribosome content (Rebnegger et al., 2014; Regenberget al., 2006). The exception was the signal from the *RPL19* biosensor, which continued to show an increase in fluorescence over time, albeit with little variation after an OD_{600} of 1.1. The maximum growth rate of the *RPL19* biosensor strain was 0.385 ± 0.02 , which was the lowest maximum growth rate observed from all the strains. In the previous work, *S. cerevisiae* cultures for cell extract generation were harvested from the mid-exponential phase (Gan & Jewett, 2014), and as the signal from all four biosensors suggested sustained expression of the ribosome-related genes up to an OD_{600} of ~20, we chose to harvest cells at OD_{600} of 18–20 when preparing the extracts.

The efficiency of cell lysis is essential to achieve both high concentration extracts and subsequently high yields of in vitro CFPS. It has previously been reported that protein yields above 20 mg ml^{-1} are required for productive CFPS (Fujiwara & Doi, 2016). As *P. pastoris* is difficult to lyse (Kim, Wu, Kim, Kim, & Shin, 2013; Stowers & Boczeko, 2007), we investigated a variety of methods to determine their suitability for extract preparation. The optimal method would lead to a low number of unlysed, viable cells, without requiring unnecessary dilution (i.e. with buffers), which would lower the final protein concentration. To estimate the lysis efficiency,

FIGURE 2 Evaluation of lysis methods. Solutions obtained from each lysis method were subjected to colony counting to determine the number of unlysed cells and the concentration of protein in the extract after centrifugation was determined. The data have been standardized to the same volume of lysis solution. Error bars represent the standard deviation of three technical repeats



cultures were grown to an OD_{600} of 18, lysed using different methods, and the number of intact viable cells remaining after each treatment was estimated by serial dilution and colony counting. In addition, the DC™ protein assay was used to quantify protein concentration of the resulting extracts (Figure 2). The most efficient lysis was achieved by first spheroplasting with zymolyase or lyticase followed by chemical lysis using Y-PER. However, the concentration of protein in the lysate was low (7.5 mg mL^{-1}) and it is possible that the detergents used in Y-PER could inhibit the CFPS reaction. Furthermore, spheroplasting requires incubation at 37°C for 1 hr and as CFPS relies on keeping the extract cold to preserve the protein functionality, this may impede the protein synthesis. Therefore, we chose high-pressure cell disruption as the lysis method for further extract preparation, even though the cultures treated by this method were not fully lysed.

3.2 | Overexpressing ribosome-related genes

Because ribosome biosensor signals peaked at a much lower OD_{600} and growth rate than expected, we aimed to engineer *P. pastoris* strains with a higher ribosome content. Panthu et al. (2017) previously demonstrated that the ribosomes can be purified separately and added to the CFPS reaction. However, the process of isolating ribosomes requires ultracentrifugation, which is laborious and not compatible with large-scale production of extracts. Instead, our hypothesis was that the overexpression of the genes related to ribosome biosynthesis could increase protein production in CFPS without the need for supplementation of purified ribosomes. A total of 11 genes encoding transcription factors or global regulators of ribosome biosynthesis (Supporting Information Table S1) were selected for overexpression. The coding regions were fused to the constitutive *GAP* promoter in the pKANB vector and transformed into the *YTM1* biosensor strain for initial screening as this biosensor showed a relatively high fluorescence (Figure 1b) and low population variability in previous experiments (Supporting Information Figure 2). To control for differences in behavior due to clonal variation, three independent colonies of each strain were selected for the analysis (Aw et al., 2017; Schwarzans et al., 2016). The cells were grown in

YPD medium for 18 hr from a starting OD_{600} of 0.1, and the fluorescence of the *YTM1* biosensor measured using flow cytometry (Figure 3). In addition, a time-course measurement of the cell density and biosensor fluorescence was carried out over 15 hr (Supporting Information Figure 3, 4 and Supporting Information Table S2). Overexpression of the coding region *PAS_chr3_0712* (a basic helix-loop-helix transcription factor with homology to the myc-family of transcription factors) or *Rap1* (*PAS_chr1-3_0252*) decreased the biosensor signal, suggesting a decrease in ribosome content. The myc-family transcription factors directly regulate transcription of ribosomal RNA, ribosome, and ribosomal biogenesis genes (van Riggelen, Yetil, & Felsher, 2010), but also have various other roles in controlling transcriptional responses in yeast, on their own or as part of heterodimers (Robinson & Lopes, 2000). *Rap1* in *S. cerevisiae* has been shown to recruit additional transcription factors, including *Fhl1*, *Hmo1*, *lfh1*, and *Sfp1* to help control the transcription (Warner, 1999) and also has a role in telomere maintenance. In fact, one study estimated that *Rap1* plays a role in as much as 37% of all RNA polymerase II initiation events in yeast during the exponential growth (Lieb, Liu, Botstein, & Brown, 2001). Therefore, it is possible that the multifunctional nature of these proteins causes additional changes that have a negative effect on ribosome production.

Conversely, overexpression of *Fhl1* (*PAS_chr4_0980*) or *Hmo1* (*PAS_chr2-2_0488*) resulted in an increase in GFP fluorescence from the *YTM1* biosensor, suggesting an increase in the ribosome content. *Fhl1* is a forkhead DNA binding protein that is thought to exclusively bind at ribosomal protein gene loci and recruit *lfh1*, which is a coactivator of the transcription (Fermi, Bosio, & Dieci, 2016). *Hmo1* is a transcription factor that recruits RNA polymerase to the initiation complex (Gadal, Labarre, Boschiero, & Thuriaux, 2002). As both proteins have a much more targeted impact on ribosomal gene expression, they may represent limiting factors in the production of ribosomes without having additional negative global impacts. Further investigations would be necessary to determine the precise functionality of these proteins in *P. pastoris*. It would also be of interest to determine whether coexpressing *lfh1* (which alone had no effect) alongside *Fhl1* would have a synergistic effect on ribosome biosynthesis. To determine the optimum harvest point for the strains

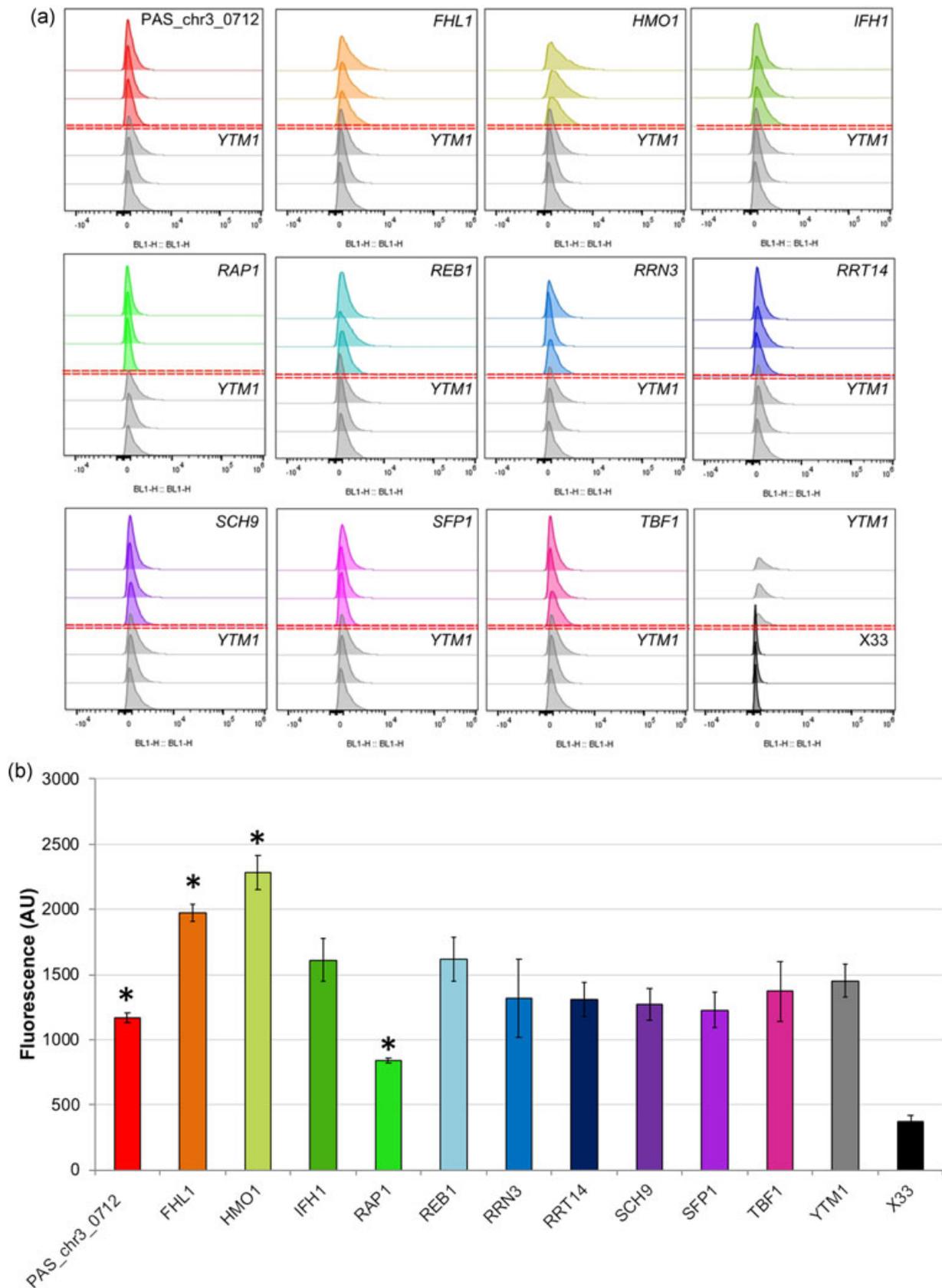


FIGURE 3 Overexpression of ribosome biosynthesis genes. Eleven different overexpression strains were generated in the strain housing the YTM1 biosensor and three independent clones of each were evaluated. The fluorescence was measured by flow cytometry and compared with the YTM1 biosensor background strain (a) Flow cytometry histograms. (b) The geometric mean of GFP fluorescence. Error bars represent the standard deviation of the mean of three independent clones. Asterisks indicate a significant difference compared with the YTM1 biosensor strain, where $p \leq 0.05$

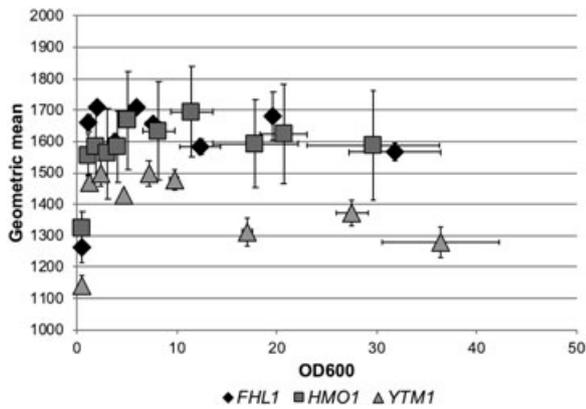


FIGURE 4 Ribosome profiles of *FHL1*, *HMO1*, and *YTM1* strains. Fluorescence was determined by flow cytometry and the OD₆₀₀ measured at the same time. Error bars represent the standard deviation of the mean of three independent clones

overexpressing *FHL1* and *HMO1*, the cells were grown in baffled glass flasks and growth curves and biosensor fluorescence over time were compared with the *YTM1* biosensor strain (Figure 4). Across the experiment, GFP fluorescence was significantly increased in the strains overexpressing *FHL1* and *HMO1* compared with the *YTM1* biosensor background strain ($p = 0.030278$ and $p = 0.00652$, respectively), suggesting an increase in ribosome production. For example, at an OD₆₀₀ of 20, strains overexpressing *FHL1* still showed 98% of their maximal GFP fluorescence compared with 90% in the background strain. The sustained biosensor signal suggests that the ribosome biosynthesis remains elevated at higher OD₆₀₀, which will allow us to take advantage of the high cell densities that *P. pastoris* can grow to when producing the cell extracts. Despite the improved fluorescence, the strains overexpressing *HMO1* grew significantly slower than the other strains (Supporting Information Figure 5), with a maximum growth rate in flasks of $0.359 \pm 0.071 \text{ hr}^{-1}$ compared with $0.428 \pm 0.021 \text{ hr}^{-1}$ for the strain overexpressing *FHL1* and $0.455 \pm 0.079 \text{ hr}^{-1}$ for the *YTM1* biosensor strain. The growth rates of all three strains in flasks are significantly higher than in Falcon tubes, as may be expected due to better aeration; however, the differences between the growth rates were more pronounced than those observed in tubes (Supporting Information Table S2). Overall, the results suggest that the burden of *HMO1* overexpression leads to significant impacts on cell growth.

3.3 | Cell-free protein synthesis using *P. pastoris* cell extract

On the basis of the experiments above, the cells were harvested at an OD₆₀₀ of 18–20 and lysed using high-pressure disruption. The reaction mix was based on the protocol for *S. cerevisiae* described in Gan and Jewett (2014) with some modifications. The amino acids concentrations were increased to 0.6 mM (except leucine at 0.5 mM), the concentration of creatine phosphokinase was increased to 0.54 mg ml^{-1} and 100 U of T7 RNA polymerase were used per 50 μl reaction.

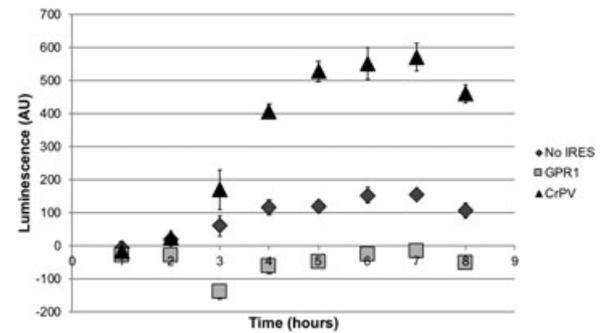


FIGURE 5 Initial protein production by CFPS. Luciferase production was measured over 8 hr with sampling every hour. The vectors without an IRES (Kozak only) and containing the GPR1 or CrPV IRES were all evaluated. Error bars represent the standard deviation of the mean of three biological repeats and are calculated using error propagation. CFPS: cell-free protein synthesis; CrPV: cricket paralysis virus; IRES: internal ribosome entry site

The functionality of the CFPS reaction was tested using luciferase as a reporter. To facilitate cap-independent translation an IRES sequence was included upstream of the luciferase gene. As the GPR1 IRES has successfully been used in vivo in *P. pastoris* (Liang, Lin, Li, & Ye, 2012) this was chosen alongside the CrPV IRES, which shows a broad host range (Brodell et al., 2014; Hodgman & Jewett, 2014; Reavy & Moore, 1981; Thoring, Dondapati, Stech, Wüstenhagen, & Kubick, 2017). In addition, a vector with no IRES was included as a benchmark. All three vectors contained a yeast Kozak sequence (GAAACG) upstream of the initiation codon (Supporting Information Figure 6). Initially, reactions using the extracts from *P. pastoris* X33 were used to test the three vectors in 50 μl static reactions for 8 hr at room temperature (Figure 5).

The CFPS reaction using *P. pastoris* extract has a significant lag phase, with luciferase expression not detectable until after 2 hr and at its maximum at 7 hr. Protein synthesis occurs when using the vectors containing the CrPV or without an IRES, but not with the GPR1 IRES. Therefore, this vector was excluded from future experiments. This result is unexpected, as GPR1 has been shown to function in vivo in *P. pastoris*. However, it is possible that additional proteins such as IRES trans-acting factors (Liang et al., 2012; Pacheco & Martinez-Salas, 2010) are required for its function. Alternatively, the ionic strength of the reaction mix may need to be optimized to allow the correct folding of GPR1. Our results in vitro suggest that it may be worth testing the CrPV IRES for the multigene expression in vivo in *P. pastoris* where it has not yet been used.

Using the CrPV IRES resulted in a three-fold increase in luciferase expression compared with no IRES (108 and $34 \mu\text{g ml}^{-1}$, respectively), a weaker effect than initially anticipated. In mammalian CFPS, an IRES is essential for the production of the protein from coupled transcription-translation reactions (Brodell et al., 2014). However, previous CFPS using *S. cerevisiae* did not examine whether an IRES was essential for the translation (Gan & Jewett, 2014; Hodgman & Jewett, 2014), so it is possible that the requirements in yeast may be different than in mammalian cells.

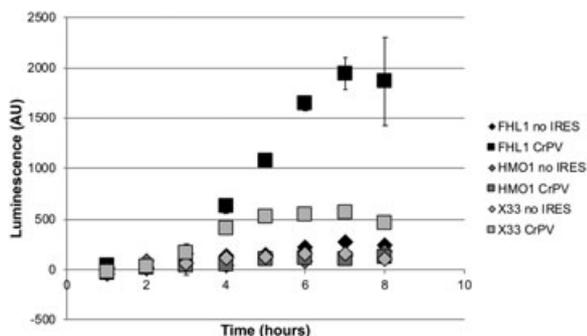


FIGURE 6 Improved CFPS yields with extracts from the strain overexpressing *FHL1*. Luciferase production was measured over 8 hr, with samples taken every hour. The vectors without an IRES (Kozak only) and containing the CrPV IRES were evaluated. Error bars represent the standard deviation of three biological repeats and are calculated using error propagation. CFPS: cell-free protein synthesis; CrPV: cricket paralysis virus; IRES: internal ribosome entry site

As overexpression of *FHL1* or *HMO1* showed an increase in the ribosome biosensor signal, we also prepared cell extracts from these strains to examine the effect on in vitro protein yields. To ensure that there was no additional metabolic burden from producing the GFP reporter, we recreated the strains using wild-type *P. pastoris* X33 as the background. Figure 6 compares the luciferase expression from CFPS reactions using cell extracts of wild-type X33, and the strains overexpressing *FHL1* or *HMO1*. CFPS reactions with extracts made from the cells overexpressing *FHL1* have increased the protein expression compared with CFPS reactions with extracts made from wild-type *P. pastoris* X33. Luciferase expression is increased 1.8-fold in the absence of an IRES and 3.4-fold when using the CrPV IRES. The dynamics of the production remain similar between the two extracts with an initial lag phase of 2 hr, and maximal expression at 7 hr. This implies that overexpressing *FHL1* results in increased protein production initially, but other factors limit the overall productivity of the system. Using a dialysis-based continuous system could overcome these limitations in the future.

CFPS reactions using the extracts from the cells overexpressing *HMO1* resulted in a decreased protein production compared with wild-type *P. pastoris* X33. Although this result is unexpected on the basis of in vivo screening, it is possible that the observed changes in the growth rate of this strain led to changes in the metabolic activity of the extracts that are detrimental to in vitro protein synthesis. Further work would be necessary to determine the precise cause of the reduced CFPS activity.

3.4 | Human serum albumin production

To ensure that the extract can be used to produce more complicated proteins, HSA was tested as a representative biopharmaceutical. HSA has been produced in high yields in *P. pastoris* in vivo, with titers up to 11 g L^{-1} (Mallem et al., 2014; Ohya, Ohyama, & Kobayashi, 2005). HSA is a 66.5 kDa protein with 35 cysteinyl residues forming 17 disulfide bridges plus one free thiolate (Kobayashi, 2006). Due to the high demand globally, research regarding yield optimization is ongoing, for

TABLE 1 Human serum albumin protein yields ($\mu\text{g ml}^{-1}$)

Strain	Yield ($\mu\text{g ml}^{-1}$)	
	HSA	CrPV-HSA
X33	4.7 ± 9.3	20.1 ± 11
FHL1	29.9 ± 10.5	48.1 ± 7.89

Note. CrPV: cricket paralysis virus; HSA: human serum albumin

example, recently codon optimization was explored to improve the production (Zhu et al., 2018). The ability to use a CFPS platform to screen for optimized variants such as these could increase the efficiency of using *P. pastoris* as a production host.

HSA was cloned into the CFPS backbone vectors without an IRES and with the CrPV IRES upstream of the HSA and both vectors were tested with extracts from wild-type X33 and the *P. pastoris* strain overexpressing *FHL1* (Table 1). The reactions were run overnight to allow sufficient time for disulfide bond formation. There was a very low production of HSA in the absence of an IRES when using the extract from wild-type *P. pastoris* X33 in the CFPS reaction. However, the extracts from the strain overexpressing *FHL1* produced $29.9 \mu\text{g ml}^{-1} \pm 10.5 \mu\text{g ml}^{-1}$, a 6.4-fold increase. The maximum titer of HSA produced in CFPS reactions was $48.1 \mu\text{g ml}^{-1} \pm 7.89 \mu\text{g ml}^{-1}$, using the extracts from the strain overexpressing *FHL1* and the CrPV IRES to enable cap-independent translation. Despite the complexity of HSA, the quantities produced are higher than the reported yields of GFP from the *S. cerevisiae* extract ($12.5 \mu\text{g ml}^{-1} \pm 2.5 \mu\text{g ml}^{-1}$; Gan & Jewett, 2014). The Albumin Blue (AB) fluorescence assay only results in a signal if the lipid binding pocket is fully formed (Personal Communication with the manufacturer, Active Motif, S. D., August 23, 2018), suggesting that active protein is produced. It has been shown that AB 580 and HSA interact on a 1:1 basis (Tseng, Chiu, Weng, & Chang, 2001). Furthermore, the binding of the AB 580 molecule induces a conformational change of the HSA protein, which results in a 17-fold increase in fluorescence (Hamilton, 2002).

Final titers of HSA from CFPS reactions are significantly lower than the reported titers for secreted production of HSA in vivo where 11 g l^{-1} (Mallem et al., 2014) has been obtained; however the timescales for in vivo production are longer (e.g. 12 hr glycerol batch phase followed by 395 hr of methanol feeding). There is a significant potential to use the CFPS platform to produce proteins on a small scale to screen for variants or test vaccine targets, and the speed with which it can be accomplished highlights the importance of this tool (Carlson et al., 2012; Kanter et al., 2007). As with standard intracellular expression in vivo, purification of proteins is possible through various chromatography steps or via fused purification tags such as poly-histidine or glutathione-S-transferase tags (Basu, Castellano, Thomas, & Mishra, 2013; Isaksson, Enberg, Neutze, Göran Karlsson & Pedersen, 2012).

4 | CONCLUSIONS

CFPS has evolved into a powerful synthetic biology tool and as a platform for the production of proteins. The generation of a novel

CFPS platform utilizing the industrially relevant yeast *P. pastoris* will allow for the evaluation of proteins on a small scale and could be useful for the screening of novel targets. Furthermore, the increased yields from the extracts of the strain overexpressing *FHL1* indicate that further advancements can be made in developing this platform using strain engineering approaches. Our strategy of engineering strains for higher-yielding extracts by the overexpression of global regulators of ribosome biogenesis could be applicable to a range of hosts, and there is a potential to further engineer the *P. pastoris* extract for additional improvement.

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DATA AVAILABILITY

The data underpinning this study that is not found in the manuscript or its supporting information will be made available on request from the authors and can be used without restriction.

AUTHOR CONTRIBUTION

RA designed and performed the experiments and helped draft the manuscript. KP helped design the experiments and draft the manuscript. Both authors read and approved the final manuscript.

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