Biosensor-Assisted Engineering of a High-Yield *Pichia pastoris* Cell-Free Protein Synthesis Platform

Rochelle Aw1,2, and Karen M. Polizzi1,2,\*

1Department of Chemical Engineering, Imperial College London, London, SW7 2AZ, UK

2Imperial College Centre for Synthetic Biology, Imperial College London, SW7 2AZ, UK

\*Corresponding Author

[k.polizzi@imperial.ac.uk](mailto:k.polizzi@imperial.ac.uk)

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**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 3-fold increase in recombinant protein yield compared to 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 μg mL-1. Therefore, this work 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.

**Keywords (3–5 key words or phrases)**

*Pichia pastoris*/*Komagataella phaffi*

Cell-free protein synthesis

*In vitro* transcription translation

Yeast

Synthetic biology

***Introduction***

Despite being used since the 1960s where it was pivotal in unravelling the genetic code ([Nirenberg & Matthaei, 1961](#_ENREF_36)), research on cell-free protein synthesis (CFPS) has recently undergone a resurgence primarily due to advances in synthetic biology ([Carlson, Gan, Hodgman, & Jewett, 2012](#_ENREF_10)). 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](#_ENREF_23)), protein production ([Yang et al., 2005](#_ENREF_55)) and personalized medicine ([Ogonah, Polizzi, & Bracewell, 2017](#_ENREF_37); [Pardee et al., 2016](#_ENREF_41)). Furthermore CFPS is particularly advantageous for the synthesis of proteins with low expression yield, aggregation or toxicity *in vivo* ([Katzen, Chang, & Kudlicki, 2005](#_ENREF_25)). Since 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 (CHO) cells ([Brodel, Sonnabend, & Kubick, 2014](#_ENREF_8)), wheat-germ ([Anderson, Straus, & Dudock, 1983](#_ENREF_2); [Madin, Sawasaki, Ogasawara, & Endo, 2000](#_ENREF_31)), rabbit reticulocytes ([Jackson & Hunt, 1983](#_ENREF_22)) and insect cells ([Stech et al., 2014](#_ENREF_49)), all of which are commercially available. As the choice of host extract can impact the protein folding and post-translational modifications, the development of new CFPS is of interest. In recent years CFPS have been produced using HEK239 ([Bradrick, Nagyal, & Novatt, 2013](#_ENREF_7)), *Streptomyces venezuelae* ([Moore, Lai, Needham, Polizzi, & Freemont, 2017](#_ENREF_34)), *Bacillus* *megaterium* ([Moore et al., 2018](#_ENREF_35)), *BY-2* Tobacco cells ([Buntru, Vogel, Stoff, Spiegel, & Schillberg, 2015](#_ENREF_9))and *Saccharomyces cerevisiae* ([Gan & Jewett, 2014](#_ENREF_15)) 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](#_ENREF_1)). To date over 5000 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](#_ENREF_6)). 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](#_ENREF_11)). 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](#_ENREF_56)). Furthermore, the potential to produce extracts from strains with humanized N-linked glycosylation pathways ([S. R. Hamilton & Gerngross, 2007](#_ENREF_19)) would enable the production of biotherapeutic proteins with the correct post-translational modifications. The nature of CFPS means that novel therapeutics could initially be rapidly screened *in vitro* to test their efficacy and manufacturability prior to undertaking complex and more time consuming *in vivo* production. Furthermore, strategies for cell line engineering to increase 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](#_ENREF_1)). 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](#_ENREF_56)), 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 OD, which as has been previously shown corresponds to a high growth rate ([Rebnegger et al., 2014](#_ENREF_44); [Regenberg et al., 2006](#_ENREF_45)). While supplementing the extract with ribosomes has been shown to result in higher yields of protein ([Panthu et al., 2017](#_ENREF_40)), 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.

***Materials and methods***

**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 μg mL-1 Kanamycin (Sigma Aldrich, Dorset, UK), 25 μg mL-1 Zeocin™ (Thermo Fisher Scientific, Paisley, UK) or 100 μg mL-1 Ampicillin (Sigma). Yeast strains were cultured in a rich YPD medium (2% peptone from casein, 1% yeast extract, 2% dextrose) and with either 100 μg mL-1 Zeocin™ (Thermo Fisher Scientific) or 350 μg mL-1 Geneticin (VWR, Lutterworth, UK) for selection. *Pichia 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.

**Strains**

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

**Plasmid and strain construction**

All vectors were made using the Gibson DNA assembly method as described previously ([Gibson et al., 2009](#_ENREF_17)). The luciferase gene was amplified from pGL2 (Promega, Southampton, UK). The human serum albumin gene was amplified from pPICZα-HSA ([Aw, Barton, & Leak, 2017](#_ENREF_3)). The GPR1 and CrPV 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, USA) before the assembly reaction. After 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 XhoI and NotI (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](#_ENREF_33)) and amplified from genomic DNA extracted from *P. pastoris* X33 by the DNeasy® Plant Mini Prep kit (Qiagen). The ribosome biosensors used the pPICZ vector as a backbone (Thermo Fisher Scientific) and the sfGFP sequence was amplified from pPICz-GFP ([Aw & Polizzi, 2016](#_ENREF_4)) and fused to the promoter sequences of genes involved in ribosome biosynthesis and maturation. For the 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](#_ENREF_30))) 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).

**Lysis methods**

Each of the lysis methods was performed on 50 mL of overnight culture (OD600 ~18). Lysis efficiency was analysed by serial dilution and colony counting, and protein concentration was measured using the DC protein assay kit (BioRad, Hertfordshire, UK) according to the manufacturer’s protocol.

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

*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 Zymloyase (Zymo Research Corporation) and 600 U lyticase (Sigma Aldrich) were independently tested. Samples were left for 1 hour at 37°C and after the final centrifugation at 750 xg 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).

*Sonication* Cells were pelleted at 1500 xg at 4°C for 5 minutes and resuspended in 10 mL of lysis buffer A. Cells were sonicated at an amplitude of 50% for 5 minutes with 20 seconds on, and 20 seconds off. Up to three cycles were tested.

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

*High pressure cell disruption* Cells were pelleted at 3000 xg for 10 minutes at 4°C and washed with 20 mL lysis buffer A. Cells were centrifuged again for 5 minutes at 3000 xg at 4°C and 1 mL of lysis buffer A was added per 1g of wet cell weight. The cells were then passed through a Constant Systems disrupter (Daventry, United Kingdom) at 30 KPSIG for one, two, three or four cycles.

**Flow cytometry**

*P. pastoris* cells were collected, diluted in PBS, and fluorescence was measured using an AttuneTM NxT flow cytometer (ThermoFisher 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). Data was analysed with the FlowJo™ software (Ashland, Oregon, USA).

**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 OD600 of 0.1. Cells were grown until an OD600 of 18-20 at 30°C, 250 rpm. Once the target OD600 had been reached, the cells were incubated on ice before centrifugation for 10 minutes at 3000 xg at 4°C. 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, cells were blotted onto 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 prior to 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, 2mM DTT, 0.5 mM PMSF) per 1 g of wet cell weight. Cells were lysed using a high-pressure homogenizer with a one-shot head adapter (Constant Systems, Daventry, United Kingdom). 10 mL of culture was passed through twice at 30 KPSIG.

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

**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 (GTP), 2 mM cytidine triphosphate (CTP), 2 mM uridine triphosphate (UTP), 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), 200 U mL–1 RNase Inhibitor (NEB), 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.

**Luciferase assay**

A luciferase assay mix (20 mM tricine, 100 μM EDTA, 1.07 mM MgCO3, 250 μM luciferin, 250 μM ATP, 2.67 mM MgSO4, 17 mM DTT) ([Siebring-van Olst et al., 2013](#_ENREF_48)) was made fresh prior to the assay. Every 30 minutes, 5 μL of cell-free reaction was added to 30 μL of the luciferase assay mix and luminescence was measured using the POLARstar ® Omega plate reader (BMG Labtech Ltd, Aylesbury, UK). Average readings were taken over 20 minutes as previously reported ([Gan & Jewett, 2014](#_ENREF_15)). Error bars were calculated taking into consideration error propagation ([Gerards, 1998](#_ENREF_16)).

**Human serum albumin assay**

Human serum albumin (HSA) was quantified using the Albumin Blue Fluorescent assay kit (Active Motif, La Hulpe, Belgium) according to the manufacturer’s protocol. Error was calculated using a Taylor series expansion, which takes into consideration the error of the standard curve in addition to the deviation between samples ([Gerards, 1998](#_ENREF_16)).

***Results and discussion***

**Optimal conditions for harvest and lysis**

It has previously been reported that high ribosome concentration is essential for successful CFPS ([Zawada & Swartz, 2006](#_ENREF_56)) and as ribosome content is correlated to growth rate, we began by investigating the ideal optical density at which to harvest *P. pastoris* cultures for generating the cell extract. For *S. cerevisiae* the optimal harvest point was determined to be mid-logarithmic phase (OD600 10-12) ([Gan & Jewett, 2014](#_ENREF_15)). However, since *P. pastoris* can reach higher cell densities, we hypothesized that cells harvested a higher OD600 would still be in mid-logarithmic phase, allowing us to maximize the quantity of extract produced per liter of culture. To monitor the dynamics of ribosome content across OD600, we developed four ribosome biosensors (Fig 1A), based on 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](#_ENREF_42)). These genes represent different factors in ribosome biosynthesis and therefore together should provide a representation of changes in transcription of 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 (Fig 1A) and colony PCR was used to determine successful integration (data not shown). To determine optimum harvest time, we monitored fluorescence using flow cytometry and plotted this against OD600 (Fig 1B) and exponential growth rate (Supplementary Fig 1).

The highest ribosome biosensor fluorescence occurred at a much lower OD600 than anticipated, *RPP0* at 1.2, *RPS0A* at 1.2, and *YTM1* at 5.4, which corresponded to growth rates of 0.418 h-1± 0.027, 0.312 h-1 ± 0.041 and 0.381 h-1 ± 0.046, respectively. Although higher growth rates were observed for these strains, these were close to μ*max*, which corresponds to previous observations that growth rate has been shown to correlate with high ribosome content ([Rebnegger et al., 2014](#_ENREF_44); [Regenberg et al., 2006](#_ENREF_45)). 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 OD600 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 previous work, *S. cerevisiae* cultures for cell extract generation were harvested from mid-exponential phase ([Gan & Jewett, 2014](#_ENREF_15)), and as the signal from all four biosensors suggested sustained expression of the ribosome-related genes up to an OD600 of ~20, we chose to harvest cells at OD600 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](#_ENREF_13)). As *P. pastoris* is difficult to lyse ([Kim, Wu, Kim, Kim, & Shin, 2013](#_ENREF_26); [Stowers & Boczko, 2007](#_ENREF_50)), 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 lysis efficiency, cultures were grown to an OD600 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 (Fig 2). The most efficient lysis was achieved by first spheroplasting with Zymolyase or lyticase followed by chemical lysis using YPER. However, protein yields were low (7.5 mg mL‑1) and it is possible that the detergents used in YPER could inhibit the CFPS reaction. Furthermore, spheroplasting requires incubation at 37°C for one hour and as CFPS relies on keeping the extract cold to preserve protein functionality, this may impede 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.

**Overexpressing ribosome related genes**

Since ribosome biosensor signals peaked at a much lower OD600 and growth rate than expected, we aimed to engineer *P. pastoris* strains with higher ribosome content.Pathu et al, 2017 previously demonstrated that 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 overexpression of genes related to ribosome biosynthesis could increase protein production in CFPS without the need for supplementation of purified ribosomes. Eleven genes encoding transcription factors or global regulators of ribosome biosynthesis (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 relatively high fluorescence (Fig 1B) and low population variability in previous experiments (Supplementary Fig 2). To control for differences in behavior due to clonal variation, three independent colonies of each strain were selected for analysis ([Aw et al., 2017](#_ENREF_3); [Schwarzhans et al., 2016](#_ENREF_47)). Cells were grown in YPD medium for 18 hours from a starting OD600 of 0.1, and the fluorescence of the *YTM1* biosensor measured using flow cytometry (Fig 3). Additionally, a time-course measurement of cell density and biosensor fluorescence was carried out over 15 hours (Supplementary Figs 3, 4 and 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 rRNA, ribosome and ribosomal biogenesis genes ([van Riggelen, Yetil, & Felsher, 2010](#_ENREF_53)), but also have various other roles in controlling transcriptional responses in yeast, on their own or as part of heterodimers ([Robinson & Lopes, 2000](#_ENREF_46)). Rap1 in *S. cerevisiae* has been shown to recruit additional transcription factors, includingFhl1, Hmo1, Ifh1 and Sfp1 to help control transcription ([Warner, 1999](#_ENREF_54)) 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 exponential growth ([Lieb, Liu, Botstein, & Brown, 2001](#_ENREF_29)). Therefore, it is possible that the multi-functional nature of these proteins causes additional changes that have a negative effect on ribosome production.

Conversely, overexpression of Fhl1 (PAS\_chr4\_0980) or HmoI (PAS\_chr2-2\_0488) resulted in an increase in GFP fluorescence from the *YTM1* biosensor, suggesting an increase in ribosome content. Fhl1 is a forkhead DNA binding protein that is thought to exclusively bind at ribosomal protein gene loci and recruit Ifh1, which is a co-activator of transcription ([Fermi, Bosio, & Dieci, 2016](#_ENREF_12)). Hmo1 is a transcription factor that recruits RNA polymerase to the initiation complex ([Gadal, Labarre, Boschiero, & Thuriaux, 2002](#_ENREF_14)).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 co-expressing Ifh1 (which alone had no effect) alongside Fhl1 would have a synergistic effect on ribosome biosynthesis. To determine the optimum harvest point for the strains overexpressing *FHL1* and *HMO1,* cells were grown in baffled glass flasks and growth curves and biosensor fluorescence over time were compared to the *YTM1* biosensor strain (Fig 4). Across the experiment, GFP fluorescence was significantly increased in the strains overexpressing *FHL1* and *HMO1* compared to 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 to 90% in the background strain. The sustained biosensor signal suggests that 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 (Supplementary Fig 5), with a maximum growth rate in flasks of 0.359 h-1 ± 0.071 compared to 0.428 h-1 ± 0.021 for the strain overexpressing *FHL1* and 0.455 h-1 ± 0.079 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 (Table S2). Overall, the results suggest that the burden of *HMO1* overexpression leads to significant impacts on cell growth.

**Cell-free Protein Synthesis using *P. pastoris* cell extract**

Based on the experiments above, 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-1and 100 U of T7 RNA polymerase were used per 50 μL reaction.

The functionality of the CFPS reaction was tested using luciferase as a reporter. To facilitate cap-independent translation an internal ribosome entry site (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](#_ENREF_28)) this was chosen alongside the cricket paralysis virus (CrPV) IRES, which shows broad host range ([Brodel et al., 2014](#_ENREF_8); [Hodgman & Jewett, 2014](#_ENREF_20); [Reavy & Moore, 1981](#_ENREF_43); [Thoring, Dondapati, Stech, Wüstenhagen, & Kubick, 2017](#_ENREF_51)). Additionally, a vector with no IRES was included as a benchmark. All three vectors contained a yeast Kozak sequence (GAAACG) upstream of the initiation codon (Supplementary Fig 6). Initially reactions using extracts from *P. pastoris* X33 were used to test the three vectors in 50 μL static reactions for 8 hours at room temperature (Fig 5).

The CFPS reaction using *P. pastoris* extract has a significant lag phase, with luciferase expression not detectable until after 2 hours and at its maximum at 7 hours. 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 (ITAFs) ([Liang et al., 2012](#_ENREF_28); [Pacheco & Martinez-Salas, 2010](#_ENREF_39)) 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 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 to no IRES (108 μg mL-1 and 34 μg mL-1 respectively), a weaker effect than initially anticipated. In mammalian CFPS, an IRES is essential for the production of protein from coupled transcription-translation reactions ([Brodel et al., 2014](#_ENREF_8)). However, previous CFPS using *S. cerevisiae* did not examine whether an IRES was essential for translation ([Gan & Jewett, 2014](#_ENREF_15); [Hodgman & Jewett, 2014](#_ENREF_20)), so it is possible that the requirements in yeast may be different than in mammalian cells.

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 cells overexpressing *FHL1* have increased protein expression compared to 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 production remain similar between the two extracts with an initial lag phase of 2 hours, and maximal expression at 7 hours. This implies that overexpressing *FHL1* results in increased protein production initially, but that other factors that limit the overall productivity of the system. Using a dialysis-based, continuous system could overcome these limitations in future.

CFPS reactions using extracts from cells overexpressing *HMO1* resulted in decreased protein production compared to wild-type *P. pastoris* X33. Although this result is unexpected based on *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.

**Human serum albumin production**

To ensure that the extract can be used to produce more complicated proteins, human serum albumin (*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](#_ENREF_32); [Ohya, Ohyama, & Kobayashi, 2005](#_ENREF_38)). HSA is a 66.5 kDa protein with 35 cysteinyl residues forming 17 disulphide bridges plus one free thiolate (Cys34) ([Kobayashi, 2006](#_ENREF_27)). Due to the high demand globally, research regarding yield optimisation is ongoing, e.g. recently codon optimisation was explored to improve production ([Zhu et al., 2018](#_ENREF_57)). 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). Reactions were run overnight to allow sufficient time for disulphide bond formation. There was very low production of HSA in the absence of an IRES when using extract from wild-type *P. pastoris* X33 in the CFPS reaction. However, extracts from the strain overexpressing *FHL1* produced 29.9 ±10.5 μg mL-1, a 6.4-fold increase. The maximum titer of HSA produced in CFPS reactions was 48.1 ± 7.89 μg mL-1, using 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 ± 2.5 μg mL-1) ([Gan & Jewett, 2014](#_ENREF_15)). 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), 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](#_ENREF_52)). 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 ([J. A. Hamilton, 2002](#_ENREF_18)).

Final titers of HSA from CFPS reactions are significantly lower than reported titers for secreted production of HSA *in vivo* where 10 g L-1 ([Mallem et al., 2014](#_ENREF_32)) has been obtained; however the time scales for *in vivo* production are longer (e.g. 12 hour glycerol batch phase followed by 395 hours of methanol feeding). There is significant potential to use the CFPS platform to produce proteins on a small scale in order 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](#_ENREF_10); [Kanter et al., 2007](#_ENREF_24)). 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 (GST) tags ([Basu, Castellano, Thomas, & Mishra, 2013](#_ENREF_5); [Isaksson, Enberg, Neutze, Göran K., & Pedersen, 2012](#_ENREF_21)).

**Conclusions**

Cell-free protein synthesis has evolved into a powerful synthetic biology tool and as a platform for the production of proteins. The generation of a novel CFPS platform utilising 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 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 potential to further engineer the *P. pastoris* extract for additional improvement.

***List of abbreviations***

ATP - Adenosine triphosphate

CrPV – cricket paralysis virus

CTP - Cytidine triphosphate

CFPS - Cell-free protein synthesis

GTP – Guanosine triphosphate

HSA – Human serum albumin

OD600 – optical density

UTP - Uridine triphosphate

YPD – yeast peptone dextrose

sfGFP- superfolder Green Fluorescent Protein

***Authors’ 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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***Data availability***

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

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***Figure Captions***

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 OD600­ measured at the same time. Four biosensor strains were investigated based on the promoters of the *RPP0*, *RPL19*, *YTM1* and *RPS0A* genes. Error bars represent the standard deviation of the mean of three biological repeats.

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. Data have been standardized to the same volume of lysis solution. Error bars represent the standard deviation of three technical repeats.

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 to the *YTM1* biosensor background strain A) Flow cytometry histograms. B) Geometric mean of GFP fluorescence. Error bars represent the standard deviation of the mean of three independent clones. Asterisks indicate a significant difference compared to the *YTM1* biosensor strain, where p ≤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.

Figure 5. **Initial protein production by CFPS**. Luciferase production was measured over 8 hours 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.

Figure 6. **Improved CFPS yields with extracts from the strain strain overexpressing *FHL1.*** Luciferase production was measured over 8 hours, 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.

Supplementary figure 1. **Growth curves of ribosome biosensor strains**. Error bars represent the standard deviation of three biological repeats.

Supplementary figure 2. **Population distribution of biosensor signal at peak GFP fluorescence.**  Representative histograms showing single-cell GFP fluorescence of biosensor strains at the OD600 corresponding to the maximum signal.

Supplementary figure 3. **Growth curves of ribosome overexpressing strains.** Error bars represent the standard deviation of three biological repeats

Supplementary figure 4. **Ribosome biosensor profiles of overexpressing 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.

Supplementary figure 5. **Growth curves of strains overexpressing *FHL1* and *HMO1* and the *YTM1* biosensor background strain in shake flasks.** Error bars represent the standard deviation of three biological repeats

Supplementary figure 6. **Graphical representation of vectors.** Three vectors were designed for the expression of luciferase containing either no IRES, CrPV IRES or GPR1 IRES. All vectors had the same backbone and used a T7 promoter and yeast Kozak sequence and containing a 50 bp polyA tail