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Cell-Free Protein Synthesis with Technical Additives – Expanding the Parameter Space of *In Vitro* Gene Expression

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Abstract

Biocatalysis has established itself as a successful tool in organic synthesis. A particularly fast technique for screening enzymes is the *in vitro* expression or cell-free protein synthesis (CFPS). The system is based on the transcription and translation system of an extract donating organism to which substrates like nucleotides and amino acids, as well as energy molecules, salts, buffer, etc. have to be added. After successful protein synthesis, further substrates can be added for an enzyme activity assay. Although mimicking of cell like conditions is one approach for optimization, the physical and chemical properties of CFPS are not well described yet. To date, mainly standard conditions have been used for CFPS, with little systematic testing of whether conditions closer to intracellular conditions with regards to viscosity, macromolecules,

inorganic ions, osmolarity, or water content are advantageous. Also, no non-physiological conditions have been tested to date that would expand the parameter space in which CFPS can be performed. In this study, properties of an *Escherichia coli* extract based CFPS system are evaluated, and the parameter space is expanded to high viscosities, concentrations of inorganic ions and osmolarity using ten different technical additives including organic solvents, polymers, and salts. It is shown that the synthesis of two model proteins, namely superfolder GFP (sfGFP) and the enzyme truncated human cyclic GMP-AMP synthase fused to sfGFP (t*hsc*GAS-sfGFP), is very robust against most of the tested additives.

Keywords

Cell-free protein synthesis; *Escherichia coli* cell-free extract; TXTL; sfGFP; cGAS

Introduction

Apart from further applications like biomanufacturing or biosensing, cell-free protein synthesis (CFPS) of enzymes has established itself as a tool for rapid screening of biocatalysts [1,2]. The open environment makes the protein synthesis easy to manipulate [3] and allows to follow up the enzyme synthesis with an enzyme activity assay, e.g. for substrate screening [4,5]. The CFPS system is advantageous for proteins that are challenging to express in a viable host cell e.g. due to toxic effects on the metabolism [6]. Furthermore, the protein synthesis needs only a few hours [7], making the process very fast compared to heterologous expression. CFPS relies on the transcription and translation (TX-TL) system of the donating organism [8]. In addition, the reaction solution contains the DNA-template coding for the target protein,

amino acids and nucleoside triphosphates as substrates, an energy regeneration system and further supplements like polyethylene glycol (PEG) [9].

Although CFPS has been used and improved since the 1960s, there are challenges in its application such as low production volume, batch-to-batch reproducibility, and reliable kinetic modeling of the system [10,11]. Furthermore, the transferability from CFPS screening results to the cells is limited but important as the *in vivo* production is often necessary for preparative scale applications [11,12]. So far, the description of CFPS systems focusses mainly on single components: the energy regeneration system, the cell extract itself, or individual buffer components [13,14]. At the most pH and ion strength are general variables that are looked at [13]. Since the main influences of the intracellular environment affecting the way of function and cellular behavior of proteins are composition, viscosity, and macromolecular crowding [15], these parameters could have a strong effect on CFPS performance. The variable composition of CFPS systems with a high number of ingredients and possible reaction conditions [13] opens hence a large parameter space. In addition, to date, non-physiological conditions that would further expand this parameter space in which CFPS can be performed have not been tested. This expansion, however, would be highly desirable for a coupled CFPS and enzyme assay in which e.g. an organic solvent is used to solubilize hardly water-soluble substrates for the enzyme.

In this study, we therefore aim to fill some of the gaps in the consideration of the general physical properties and potential effects on the *Escherichia coli* based CFPS performance. We use technical additives like water-soluble macromolecular polymers and salts that are usually used as deep eutectic solvents (DES) and expand the properties beyond physiological ranges. In addition, we tested several organic solvents that are miscible and un-miscible with water. For the experiments, two model proteins, namely superfolder GFP (sfGFP) and the enzyme human cyclic GMP-AMP synthase-

sfGFP, were used that differ in their size (sfGFP: 27 kDa, t*hs*cGAS-sfGFP: 84 kDa [2,16]) and fractional yield obtained for *in vitro* expression (sfGFP: 58%; t*hs*cGAS-sfGFP: 9% [12]).

Results and Discussion

Effects of additives on fluid properties in CFPS

The fluid properties of the cytoplasm of *E. coli*, the CFPS system and additives were determined to subsequently assess their influence on the synthesis performance of CFPS. Polymers, DES, and organic solvents were considered to modify the fluid properties.

Polymers and deep eutectic solvents (DES) as additives in CFPS

Polymers (PEG, methylcellulose (MC) and carboxymethylcellulose (CMC)) and DES (choline chloride/urea, betaine/ethylene glycol (EG), choline chloride/glycerol) have been selected as additives to vary the viscosity, ion concentration, amount of macromolecules, and osmolarity in CFPS. The calculated values for the properties of the CFPS system with polymers and DES added at different concentrations are shown in Table 1 in comparison with the cytoplasm of *E. coli* and water.

Table 1: Properties of cytoplasm, water, CFPS solution without additives, CFPS with polymers, and CFPS with DES (25 °C, 1 bar). Some values were taken from the literature as indicated. All other values were calculated (Supporting Information). The line marked with the black box is the standard composition of the in-house CFPS system and serves as reference. PEG, MC, CMC: % ≙ % w/v. Other: % ≙ % v/v.

Additive to CFPS	Concentration [%]	Viscosity [mPa·s]	Macromolecules [g/L]	Inorganic ions [mM]	Osmolarity [mosM]	Water content [% v/v]
E. coli cytoplasm	n.a.	3-9.7 [15,17]	300-500 [15]	300 [15]	600[a] [15]	70 [18]
Water	n.a.	0.9 [19]	-	-	-	100
PEG-8000	2[b]	1.4 [20]	167-265	140	405	92
	5	2.2	197-295	140	405	89
	10	8.9 [21]	247-345	140	405	84
Methylcellulose	0.5	3.5 [22]	172-270	140	405	92
	0.75	4.7	174-272	140	405	91
	1	6.0 [22]	177-275	140	405	91
	2	12-18[c,d]	187-285	140	405	90
Carboxymethylcellulose	0.5	17.8	172-270	140	405	92
	0.75	71.3	174-272	140	405	91
	1	142.5	177-275	140	405	91
	2	1000-1500[c]	187-285	140	405	90
Choline Chloride/Urea (1:2)	2	1.5	167-265	232	590	90
	5	1.5	167-265	371	867	87
	10	1.7	167-265	602	1329	82
Choline Chloride/Glycerol (1:2)	2	1.4	167-265	214	553	90
	5	1.5	167-265	325	775	87
	10	1.6	167-265	511	1146	82
Betaine/Ethylene Glycol (1:3)	2	1.4	167-265	140	405	90
	5	1.5	167-265	140	405	87
	10	1.6	167-265	140	405	82

[a]sum of inorganic ions and combined metabolites; [b]standard composition of in-house CFPS system and reference; [c]manufacturer specification; [d]at 20 °C; n.a. - not applicable.

The comparison of the fluid properties of the natural cytoplasm in *E. coli* [15,17,18] with those calculated for our standard CFPS system shows that although the values are not the same, they do not differ by orders of magnitude. The CPFS system has a lower viscosity compared to that of the *E. coli* cytoplasm where the transcription-translation system is naturally operating [23]. The quantity of macromolecules is 167-265 g/L on average only slightly lower in the CFPS system than in a living cell. The

calculation is based on the estimated macromolecular concentration in the cell extract, tRNA, plasmid and PEG. Although PEG-8000 does not exactly fit the definition for macromolecules of having a molecular weight of at least about 10,000 g/mol [24] it was considered in the calculation of macromolecules as it is known as artificial crowding agent [23]. Inorganic ions in CFPS refer to magnesium and potassium glutamate in the system adding up to 140 mM but do not reach half of the concentration of what is given for the cellular environment. The osmolarity of the cytoplasm of about 600 mosM [15] is 50% higher than what is calculated for CFPS. The water content, which considers all defined components in the CFPS, is 22% higher than in the cytoplasm, as expected for a diluted system.

The fluid properties of the CFPS can be changed by adding various additives. The viscosity of the CFPS can be increased by adding polymers. Different concentrations of PEG-8000, methylcellulose (MC) and carboxymethylcellulose (CMC) cover a wide range of viscosities including that of the cytoplasm up to a very viscous mixture. The methylcellulose and carboxymethylcellulose concentrations are limited to 2% as the viscosity would have become too high. Simultaneously polymers contribute to the concentration of macromolecules. PEG, a water-soluble, macromolecular polymer, is a commonly used crowding agent to mimic cellular environment *in vitro* [23]. The standard composition of the in-house CFPS system contains 2% PEG-8000, resulting in a lower viscosity of the liquid system. By adding up to 10% PEG-8000, the viscosity as well as the amount of macromolecules of the CFPS system reach in the physiological range.

In order to increase the concentration of inorganic ions, DES have been added to the CFPS solution. Even though the viscosities of the pure DES are relatively high (choline chloride/urea (1:2): 1200 mPa·s [25], choline chloride/glycerol (1:2): 300 mPa·s [25],

betaine/ethylene glycol (1:3): 65 mPa·s [26]) the impact on the viscosity of the CFPS system is almost negligible with an addition of 2-10%. The choline chloride containing DES as additives increases the concentration of inorganic ions to the range of cytoplasmic concentration of about 300 mM and up to twice as much. The osmolarity is enhanced by the increasing salt concentrations as well. With the addition of choline chloride DES the osmolarity of the CFPS system is up to 1329 mosM for 10% of choline chloride/urea. Betaine/ethylene glycol (EG) is considered as an environmentally friendly natural deep eutectic solvent (NADES) [27] and has been tested because betaine-based DES are widely used and have been successfully applied with proteins [28]. As it does not consist of any ions there is only a slight increase in the viscosity, but no changes for the other parameters are assumed. Except for the water content which decreases by the percentage of added substance as for all the additives. The lowest value is a water content of 82%, which is still more than 10% above the cytosolic water content of 70% [18], but 10% below the standard conditions of our CFPS system.

Solvents as additives in CFPS

For some applications, the usage of solvents in CFPS might be beneficial. Organic solvents as additives do not contribute to more cell-like conditions in CFPS systems but might enable the usage of poorly soluble substrates when tolerated.

The impacts of organic solvents on the properties of the CFPS system are different to that of polymers and DES. Some fluid properties of the pure solvents and calculated viscosity of the CFPS system with different concentrations of water-soluble solvents are displayed in Table 2. MTBE and *n*-hexane have a low solubility in water and formed a second phase on top of the aqueous CFPS solution. To avoid evaporation of the solvent in the headspace of the reaction vessel due to the high vapor pressures, the size of the vessel has been decreased and the volume of the reaction has been

increased to 100 µL for the experiments with *n*-hexane and MTBE. In contrast to the standard volumetric ratio of 20 µL in a 1.5 mL microreaction tube, a visible gradient in the concentration of sfGFP occurred under these conditions. Therefore, shaking at 700 rpm was established for sufficient mixing. DMSO and methanol are highly soluble in water, which makes the handling easier. Their influence on the viscosity of the CFPS system is neglectable as can be seen in Table 2. The polarities of the different solvents cover a wide range to show the impact on CFPS and give various options for soluble substances.

Table 2: Properties of water and added solvents (25 °C, 1 bar). Given values are for pure substances. Viscosities of water-soluble solvents (DMSO and methanol) are additionally calculated for solutions with CFPS kit at displayed concentrations.

	Water	DMSO	Methanol	MTBE	*n*-hexane
Molecular weight [g/mol]	18.02 [19]	78.14 [29]	32.04 [19]	88.15 [30]	86.18 [19]
Density [g/L]	997 [19]	1100[a] [31]	786 [19]	741 [30]	655 [19]
Solubility in water [g/L]	-	1000[b] [29]	1000 [32]	26[a] [33]	0.009 [34]
Vapor pressure [mm Hg]	23.8 [35]	0.6 [29]	127 [32]	245 [30]	153 [34]
Viscosity [µPa·s]	890 [19]	2140[a] [31] 2%: 1409 5%: 1414 10%: 1423	544 [19] 2%: 1411 5%: 1420 10%: 1434	370[c] [36]	298 [19]
Polarity [D]	2.9 [37]	3.96 [38]	2.61 [39]	1.25 [40]	1.08[b] [41]
log P [-]	-	-1.35[b] [29]	-0.77[b] [32]	0.94[b] [30]	3.9[b] [34]

[a]at 20 °C; [b]Temperature unknown; [c]at 15 °C

Effects of technical additives on the CFPS performance

In vitro sfGFP production with additives

The *in vitro* expression of sfGFP, or GFP variants in general, is well established and is often used as a model system for optimization (e.g. with active learning workflows [42])

and performance evaluation. This is convenient as product formation and even concentration can be easily quantified measuring the fluorescence intensity. To take advantage of this, fusion proteins with sfGFP can be constructed for CFPS performance evaluation [43].

Therefore, sfGFP was used to establish a reference CFPS synthesis under standard conditions containing 2% PEG-8000. A concentration of 1.77 mg/mL sfGFP was obtained after 4 hours. The calculated fractional yield of 114% based on the amino acid concentration added is higher than expected, which can be explained either by deviations in the measurement or by the undefined addition of amino acids through the cell-free extract. Irrespective of this, the high sfGFP concentration achieved shows the high level of optimization of the synthesis. It is therefore not expected that the addition of technical additives will increase the synthesis yield further; instead, the negative influence of all additives will be examined. In Figure 1, the results of sfGFP synthesis with different technical additives are presented. All values were normalized in relation to the fluorescence intensity of the reference with 2% PEG-8000.

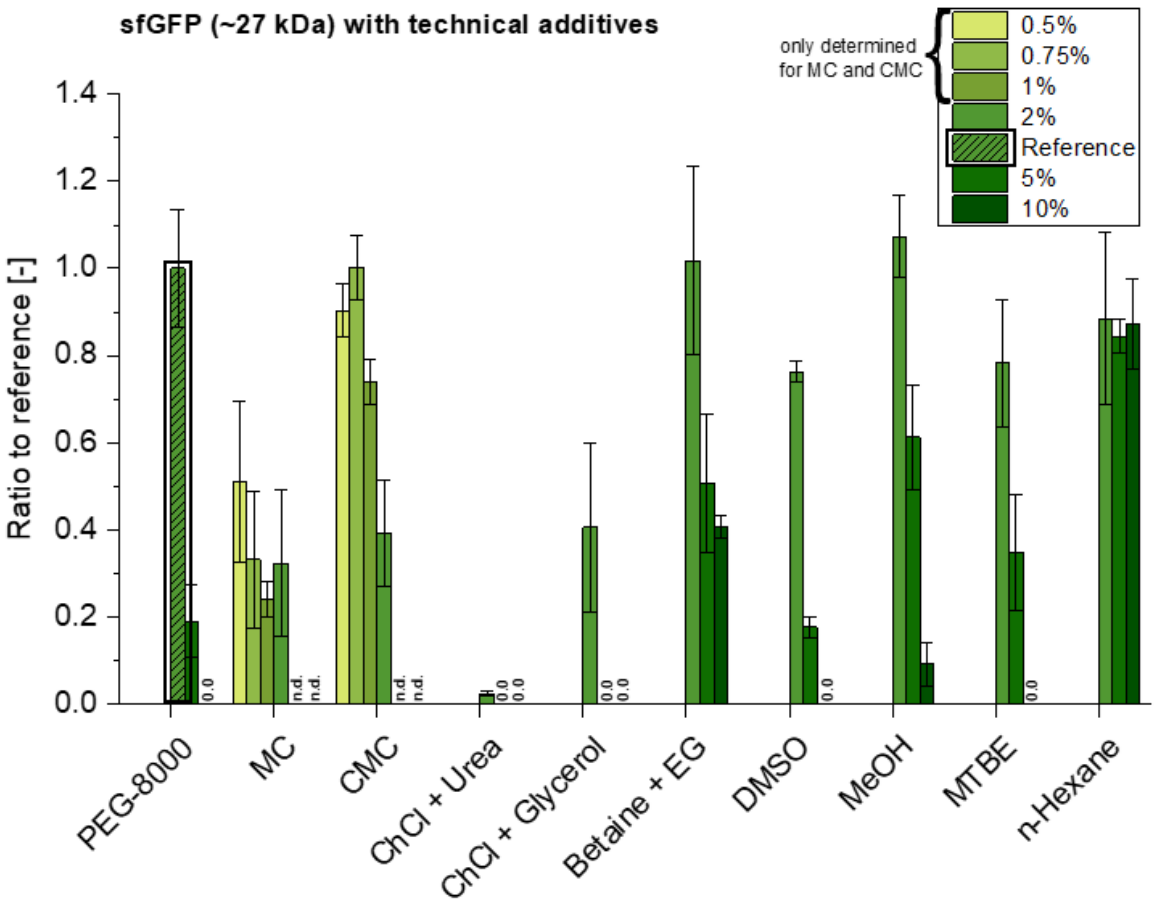

Figure 1*:* CFPS of sfGFP with different technical additives at various concentrations. 2% PEG-8000 serves as reference, equal to 1.77 ± 0.24 mg/mL. Measurements in triplicates. 0.5-1% only for MC and CMC. PEG, MC, CMC: % ≙ % w/v. Other: % ≙ % v/v.
n.d. – not determined; 0.0 – no detectable amount; PEG – polyethylene glycol; MC – methylcellulose; CMC – carboxymethylcellulose; ChCl – choline chloride; EG – ethylene glycol; DMSO – dimethyl sulfoxide; MeOH – methanol; MTBE – methyl tert-butyl ether.

Macromolecular crowding is a known mechanism that positively influences CFPS reactions [23]. Interestingly, the increase in PEG-8000 concentrations, and thus the increase in the viscosity and macromolecule concentration of the CFPS solution towards the properties of the cytoplasm, has a negative effect on the sfGFP synthesis. This is consistent with results on co-optimization of PEG with phosphoenolpyruvate, which indicate an optimal concentration of 4% PEG (7.5 kDa) and a decrease of the activity of the CFPS system at concentrations above 5% [44]. PEG-8000 has been proven to influence transcription and translation contrary. While transcription is stable

up to concentrations of 10%, translation is inhibited already at 1% [45]. If the viscosities are increased with methyl cellulose or carboxymethyl cellulose, no such negative influence on the sfGFP synthesis is observed. This indicates that the impact of the polymer itself is higher than of the viscosity adjusted by its addition. A positive effect on the stability and activity of the model enzyme β-D-glucuronidase through the addition of carboxymethylcellulose has been reported already [46]. However, the effects and states of molecular crowding in the cell are much more complex than what can be mimicked by the sole addition of a polymer. The diffusion of the macromolecules depends on the perceived viscosity in the cell, but this is inhomogeneous and depends on the location in the cell and its growth phase [47,48].

The comparison of the salt concentration reveals that the salt concentration in CFPS is below that in cells. It was therefore increased by the addition of DES that have been used already for biological applications and [49] are considered promising environmentally friendly alternative solvents [50]. The addition of the choline chloride containing DES has obviously a strong negative impact for the *in vitro* synthesis of sfGFP. Even though at a concentration of 2%, the concentration of inorganic ions and osmolarity is about to reach physiological conditions, only 2% resp. 41% of sfGFP were produced compared to the standard composition. This is less than with any other additive. All other parameters are constant which leads to the suggestion that increased amounts of salts have negative effects for CFPS. Usually, higher salt concentrations can cause an increased precipitation of proteins [51], which would decrease the amount of detectable CFPS product. Other publications describe that the solubility of proteins can be improved with increased salt concentration by the addition of NaCl [51]. Testing of other salts at high concentrations is necessary to clear up if the salt concentration or the salt itself is responsible for the low *in vitro* protein production with choline chloride as an additive.

Interestingly, the addition of organic solvents has little influence on the synthesis performance at concentrations up to 2%. Even methanol concentrations of 5% are well tolerated by the system demonstrating a high robustness against these additives. With *n*-hexane the amount of sfGFP is stable at a value of around 85% compared to the reference for all tested concentrations. The reason might be that the influence on the CFPS system does not increase with a higher amount of *n*-hexane due to the low solubility of *n*-hexane in water, a limited interfacial area and evaporation in the headspace. A clear trend for the influence of the polarity or the log P of the added solvents is not visible. Methanol whose dipole moment is relatively close to that of water is better accepted among the water-miscible additives than DMSO.

In vitro t*hs*cGAS-sfGFP production with additives

The used CPFS system or CFPS in general is not further optimized for the production of t*hs*cGAS-sfGFP or other special enzymes. Larger and more complex enzymes are usually more difficult to synthesize with CFPS [53], although there are exceptions, such as the production of nonribosomal peptide synthetases with more than 100 kDa [54]. However, these enzymes are often of great interest for special applications in biomanufacturing. Cyclic GMP-AMP synthase (cGAS) is one of them. cGAS and its biocatalytic product 2'3'-cyclic GMP-AMP (cGAMP) are part of the innate immune response in higher eukaryotes [55]. cGAMP is therefore a promising candidate for pharmaceutical applications [56]. Successful synthesis of the fusion protein of truncated human cGAS and superfolder GFP (t*hs*cGAS-sfGFP) with the in-house *Escherichia coli* (*E. coli*) based CFPS system was already shown before under standard conditions [16]. We have now repeated this experiment and tested the synthesis of t*hs*cGAS-sfGFP with the addition of additives.

The average production of t*hsc*GAS-sfGFP under reference conditions with the in-house CFPS system was 0.13 mg/mL, which is comparable to published data [12]. The fractional yield for t*hsc*GAS-sfGFP is about 10.5% and has therefore potential for optimization. In Figure 2, the results for the production of t*hsc*GAS-sfGFP are summed up. In general, the trend of the obtained protein concentrations is consistent with what was observed for sfGFP. Production levels correspond to or are below the reference value of 2% PEG-8000. Within the additives, 0.75% of carboxymethylcellulose and 2% of betaine/EG, methanol and *n*-hexane are best with 76 - 93% compared to the reference. The addition of carboxymethylcellulose results at 75% for sfGFP and t*hsc*GAS-sfGFP.

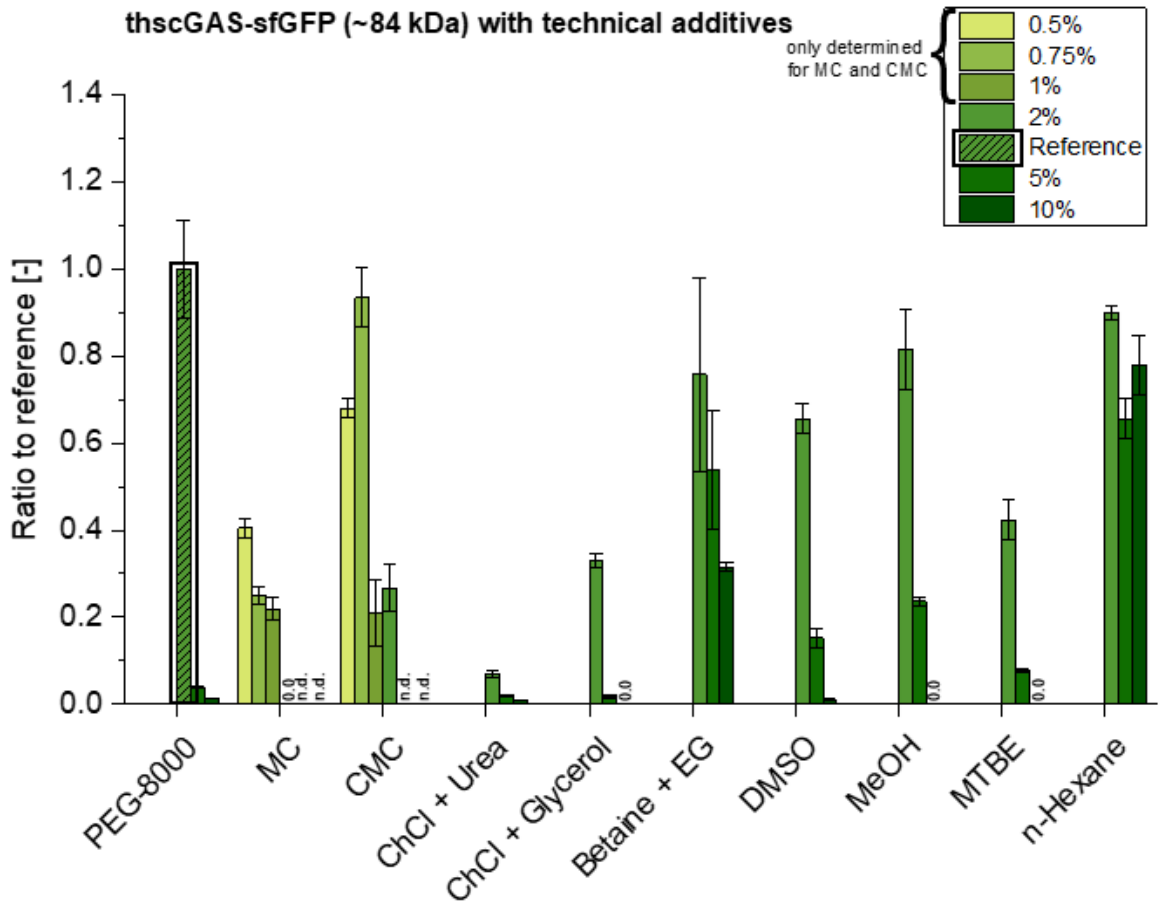

Figure 2: CFPS of t*hsc*GAS-sfGFP with different technical additives at various concentrations. 2% PEG-8000 serves as reference, equal to 0.13 ± 0.02 mg/mL. Measurements in triplicates. 0.5-1% only for MC and CMC. PEG, MC, CMC: % ≙ % w/v. Other: % ≙ % v/v.n.d. – not determined; 0.0 – no detectable amount; PEG – polyethylene glycol; MC – methylcellulose; CMC – carboxymethylcellulose; ChCl –

choline chloride; EG – ethylene glycol; DMSO – dimethyl sulfoxide; MeOH – methanol; MTBE – methyl tert-butyl ether.

Parameter scope and robustness of CFPS

The results of the synthesis of sfGFP and t*hsc*GAS-sfGFP show a high robustness of CFPS against various additives including cytotoxic solvents. This is important because the composition of reaction media for enzymatic applications especially in the chemical industry has expanded into the field of technical additives [57]. In general, protein synthesis with CFPS is best for low additive concentrations and the optimized production of sfGFP is more robust than that of t*hsc*GAS-sfGFP. Still the production of sfGFP works for most of the additives up to concentrations of 10%. For t*hsc*GAS-sfGFP the results were similar, even though the obtained protein concentrations were generally lower. Nevertheless, only 10% of MTBE or choline chloride/glycerol completely inhibited the synthesis for both tested proteins, for all other conditions detectable amounts of protein have been produced. The robustness of the transcription-translation machinery is astonishing, expanding the parameter scope for CFPS. Successful protein syntheses were observed at very high viscosities, increased concentrations of macromolecules, organic ions, and osmolarity. The values (determined for standard conditions: 25 °C, 1 bar) for tolerated viscosity range from 1.4 to about 1000 mPa·s. At concentrations of inorganic ions of up to 602 mM and an osmolarity of 1329 mosM still tiny amounts of t*hsc*GAS-sfGFP have been detected. The concentration of macromolecules and water content of the standard CFPS system are within the ranges of *E. coli* cytoplasm. We were able to extend them for the CFPS system to values between 167 and 265 g/L respectively 82 to 92% of water content. The effect of *n*-hexane in the experiments might be limited because of the low solubility. For the other tested additives, 5-10% seem to be the limit, but betaine/EG might be accepted in higher concentrations.

Conclusion

This study shows that CFPS is robust against various technical additives. The general trend is a decreased protein concentration obtained with increased concentrations of additives, but still detectable amounts of product were reported with 10% of PEG, choline chloride/urea, betaine/ethylene glycol, DMSO, methanol and *n*-hexane. Results are most promising for betaine/ethylene, methanol and *n*-hexane and open new potential for applications like on site synthesis of enzymes for a following biotransformation. Besides the evaluation of the physical properties of a standard CFPS system, the parameter scope for CFPS was successfully expanded to high values of viscosity, concentrations of inorganic ions, and osmolarity. Herein, carboxymethylcellulose was identified as an interesting alternative crowding agent. This provides a starting point for a multifactorial approach to optimize the synthesis of non-model enzymes.

Experimental

Additives and preparation of deep eutectic solvents

The selected additives are supposed to shift the properties of the reaction solution in more extreme directions or are interesting for other reasons. PEG-8000 (Sigma, Darmstadt, Germany) is a molecular crowder that is used in the CFPS system by default. Carboxymethylcellulose sodium salt (Roth, Karlsruhe, Germany) and methylcellulose (VWR, Darmstadt, Germany) are polymers that expand the viscosity range. Choline chloride (VWR, Darmstadt, Germany) + urea (Roth, Karlsruhe, Germany) (molar ratio 1:2) and choline chloride + glycerol (Roth, Karlsruhe, Germany) (molar ratio 1:2) were chosen to increase the amount of salts in the solution. Betaine

(Sigma, Darmstadt, Germany) + ethylene glycol (Roth, Karlsruhe, Germany) (molar ratio 1:3) was selected as additional common DES. The DES were prepared by weighting out the substances and stirring at up to 100 °C until liquid state was reached [58]. For the organic solvents, DMSO (Roth, Karlsruhe, Germany) and methanol ≥ 99% (Roth, Karlsruhe, Germany) were used as water-soluble, and MTBE (Arcos organics, Schwerte, Germany) and *n*-hexane (Lach:ner, Neratovice, Czech Republic) as non-water soluble solvents. Values for properties were taken from the databases Chemistry WebBook by the National Institute of Standards and Technology (NIST) (https://webbook.nist.gov/) at 1 bar and 25 °C, PubChem by the National Center for Biotechnology and Information (https://pubchem.ncbi.nlm.nih.gov/), and GESTIS-Stoffdatenbank by Institut für Arbeitsschutz der Deutschen Gesetzlichen Unfallversicherung (https://gestis.dguv.de/), datasheets provided by manufacturers and other sources as referenced at the corresponding point. Viscosity of the standard CFPS system was estimated as that of 2% PEG in water. For the other concentrations and polymers, the value was assumed as that for the component with pure water as well, as the contribution of the other components to the viscosity is considered neglectable in comparison to the high viscosity of the polymer-water mixtures. Some of these were inter- or extrapolated from published values. For carboxmethylcellulose values were derived from manufacturer specification with the rule of thumb that doubling the concentration increases the viscosity by a factor of about 8 [59]. As a simple approach according to Arrhenius, the viscosity for mixtures with DES and the water-soluble solvents was calculated with equation (1) [60], as only minor influences are assumed at the concentrations used in this work:

$$\log \eta_S = N_1 \log \eta_1 + N_2 \log \eta_2 \quad (1)$$

The concentration of macromolecules was calculated based on the cytosolic composition and the average OD_{600} at the harvest of the culture for the cell-free extract.

With the derived number of cells, the cellular volume of 4.4 µm³ per *E. coli* cell [61] and the intracellular concentration of macromolecules the range for the total amount of macromolecules extracted from the culture was determined. The volume of buffer, dilution at CFPS assembly and PEG-8000, tRNA and plasmid as further macromolecules were included for the calculation of the macromolecular concentration of the CFPS mix.

Magnesium- and potassium-glutamate were considered as the contributing inorganic ions for the reference composition, for the DES corresponding salts were added to the value of 140 mM.

For the osmolarity the concentrations of all defined components were multiplied with their number of dissociated particles, which was assumed as 1 for most components and 2 for magnesium- and potassium-glutamate and combined with the calculated concentration of macromolecules.

For the water content, the amount of all known components was subtracted from the 100% of pure water, additives decreased that value by the percentage of their contribution. Calculations and further details can be found in the supporting information.

Cell-free protein synthesis

CFPS was prepared and performed according to Rolf et al. [16] with the described strains *E. coli* BL21(DE3) pAR1219 for extract preparation and *E. coli* DH5α pETH6sfGFP and *E. coli* DH5α pETSUMOt*hs*cGASGFP for plasmid production. Minor variations are stated in the following. The preculture for extract preparation was grown for 20 h at 200 rpm and 37 °C, centrifugation for cell harvesting and washing was performed for 20 min at 3220 g and storage was at -70 °C. The extract contained 46 – 67 mg/mL protein. It was premixed with the buffer consisting of magnesium and

potassium-glutamate, 20 amino acids, HEPES, ATP, GTP, cytidine triphosphate (CTP), uridine triphosphate (UTP), tRNA, coenzyme A (CoA), nicotinamide adenine dinucleotide (NAD), cyclic adenosine monophosphate (cAMP), folinic acid, spermidine, 3-PGA and PEG-8000 to obtain a master mix. The master mix was assembled with the plasmid encoding for sfGFP respectively t*hs*cGAS-sfGFP and nuclease-free water, which added up the free volume to the final CFPS volume of 20 µL in a 1.5 mL microreaction tube. The final composition of the reaction was 11 – 16 mg/mL protein from extract, 10 mM magnesium glutamate, 130 mM potassium glutamate, 1.5 mM of each of 20 amino acids except for leucine, which is 1.25 mM, 50 mM HEPES, 1.5 mM ATP and GTP, 0.9 mM CTP and UTP, 0.2 mg/mL tRNA, 0.26 mM CoA, 0.33 mM NAD, 0.75 mM cAMP, 0.068 mM folinic acid, 1 mM spermidine, 30 mM 3-PGA, 2% PEG-8000, and 1 nM plasmid DNA. Reactions were incubated for 4 h at 37 °C with no shaking. Resulting fluorescence intensities were measured from 2 µL reaction solution in 98 µL 0.5M HEPES buffer (pH 8.0) in 384-well microplates with a FLUOstar® Omega multi-mode microplate reader (BMG LABTECH, Ortenberg, Germany). The endpoint-measurement was set to a gain of 1390, λ_{Ex} 485 nm and λ_{Em} 520 nm.

For the experiments with organic solvents, the corresponding volume of 2, 5, and 10% v/v was added right before the incubation. To keep the final volume at 20 µL the volume of added water was decreased by the same volume. For the non-water-soluble solvents, the scale was linearly increased to 100 µL in a 200 µL microreaction tube, incubation was with shaking at 700 rpm. DES and PEG were pre-diluted with nuclease-free water for better pipettablity and added to a final concentration of 2, 5 and 10% v/v respectively 2, 5 and 10 % w/v for PEG. Methylcellulose and carboxymethylcellulose were added as solid powders to the master mix in the appropriate amount to set the final concentration in the reaction to 0.5, 0.75, 1 and 2% w/v.

All reactions were prepared in triplicates with an additional negative control without the addition of DNA. For all reactions with additives a triplicate of the standard composition was run at the same time and with the same cell-free extract as a reference.

Correlation of fluorescence intensities and protein concentrations

Plasmids for CFPS were expressed in *E. coli* BL21 (DE3) and purified as described by Rolf et al. [16]. The quantifications of purified proteins and set dilution series were performed with Bradford Assay [62]. Purity of the *in vivo* produced proteins was checked with sodium dodecyl sulfate polyacrylamide gel-electrophoresis (SDS-PAGE) [63]. Impurities were quantified with ImageJ [64] and measured protein concentrations corrected by the results to gain concentrations of pure sfGFP and t*hs*cGAS-sfGFP. Fluorescence was measured with FLUOstar® Omega multi-mode microplate reader (BMG LABTECH, Ortenberg, Germany) under the same conditions as for the *in vitro* produced proteins to determine the correlation between fluorescence intensity and protein concentration for each protein.

Fractional yield

The fractional yield is the ratio between the theoretically achievable protein concentration based on the amount of provided amino acids in a CFPS system and the sequence of the target protein and the experimentally achieved result [12]. Fractional yields in this work have been calculated using the excel sheet provided by Rolf et al. [12] and can be found in the supporting information.

Supporting Information

Supporting Information File 1:

File Name: Calculations for Table 1.xlsx

File Format: Excel spreadsheet

Title: Calculations of viscosity, macromolecules, inorganic ions, osmolarity and water content for CFPS in Table 1

Supporting Information File 2:

File Name: Calculations for Table 2.xlsx

File Format: Excel spreadsheet

Title: Calculations of viscosity for CFPS with water-soluble solvents in Table 2

Supporting Information File 3:

File Name: Fractional Yield sfGFP according to Rolf et al 2023.xlsx

File Format: Excel spreadsheet

Title: Fractional Yield of average sfGFP production with CFPS

Supporting Information File 4:

File Name: Fractional yield t*hsc*GAS-sfGFP according to Rolf et al 2023.xlsx

File Format: Excel spreadsheet

Title: Fractional yield of average t*hsc*GAS-sfGFP production with CFPS

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References

(1) Karim, A. S.; Jewett, M. C. *Cell-Free Gene Expression*, Springer US: New York, NY, 2022; doi:10.1007/978-1-0716-1998-8

(2) Rolf, J.; Rosenthal, K.; Lütz, S. *Catalysts* **2019**, 9(2), 190. doi:10.3390/catal9020190

(3) Jewett, M. C.; Calhoun, K. A.; Voloshin, A.; Wuu, J. J.; Swartz, J. R. *Mol. Syst. Biol.* **2008**, 4:220. doi:10.1038/msb.2008.57

(4) Quertinmont, L.T., Orru, R., Lutz, S. *Chem. Commun.* **2015**, 51, 122-124. doi:10.1039/C4CC08240K

(5) Rolf J., Nerke P., Britner A., Krick S., Lütz S., Rosenthal K. *Catalysts* **2021**, 11(9), 1038. https://doi.org/10.3390/catal11091038

(6) Orth, J. H. C.; Schorch, B.; Boundy, S.; Ffrench-Constant, R.; Kubick, S.; Aktories, K. *Toxicon* **2011**, *57*, 199–207. doi:10.1016/j.toxicon.2010.11.011

(7) Caschera, F.; Noireaux, V. *Biochimie* **2014**, *99*, 162–168. doi:10.1016/j.biochi.2013.11.025

(8) Swartz, J. R.; Jewett, M. C.; Woodrow, K. A. *Methods Mol. Biol.* **2004**, 267, 169-82. doi:10.1385/1-59259-774-2:169

(9) Sun, Z. Z.; Hayes, C. A.; Shin, J.; Caschera, F.; Murray, R. M.; Noireaux, V. *J. Vis. Exp.* **2013**, 79. doi:10.3791/50762

(10) Tissikres, A.; Schlessinger, D.; Gros ; Matthaei, F.; Nirenberg ; Kameyama, M. W.; Novelli ; Kirsch, G. D.; Siekevitz, P.; Palade, G. E.; Mora, P. T.; Merler, E.; Maury ; Anson, P.; Sevag, J.; Lackmann, D. B.; Smolens ; Allfrey, J.; Mirsky, A. E.; Hurwitz, J.; Bresler, A.; Diringer, R.; Stevens, A.; Weiss, S. B.; Nakamoto, T. *Proc. Natl. Acad. Sci.* **1960**; 47(19):1588-1602. doi:10.1073/pnas.47.10.1588

(11) Silverman, A. D.; Karim, A. S.; Jewett, M. C. *Nat Rev Genet* **2020**, *21*, 151–170. doi:10.1038/s41576-019-0186-3

(12) Rolf, J.; Handke, J.; Burzinski, F.; Lütz, S.; Rosenthal, K. *Biotechnol Prog* **2023**, 39(6):e3373. doi:10.1002/btpr.3373

(13) Dopp, B. J. L.; Tamiev, D. D.; Reuel, N. F. *Biotechnol. Adv.* **2019**, 37(1), 246–258. doi:10.1016/j.biotechadv.2018.12.006

(14) Huang, M.; Wang, W.; Guo, T.; Long, X.; Niu, F. Cell-Free Escherichia Coli Synthesis System Based on Crude Cell Extracts: Acquisition of Crude Extracts and Energy Regeneration. *Processes* **2022**, 10(6),112. doi:10.3390/pr10061122

(15) Theillet, F. X.; Binolfi, A.; Frembgen-Kesner, T.; Hingorani, K.; Sarkar, M.; Kyne, C.; Li, C.; Crowley, P. B.; Gierasch, L.; Pielak, G. J.; Elcock, A. H.; Gershenson, A.; Selenko, P. *Chemical Rev.* **2014**, 114(13), 6661–6714. doi:10.1021/cr400695p

(16) Rolf, J.; Siedentop, R.; Lütz, S.; Rosenthal, K. *Int. J. Mol. Sci.* **2020**, 21(1):105. doi:10.3390/ijms21010105

(17) Mullineaux, C. W.; Nenninger, A.; Ray, N.; Robinson, C. *J. Bacteriol.* **2006**, 188, 3442–3448. doi:10.1128/JB.188.10.3442-3448.2006

(18) Luby-Phelps, K. *Int. Rev. Cytol.* **2000**, 192, 189-221. doi:10.1016/s0074-7696(08)60527-6

(19) Lemmon, E., Huber, M. McLinden, M. **2013**, NIST Standard Reference Database 23: Reference Fluid Thermodynamic and Transport Properties-REFPROP, Version 9.1, Natl Std. Ref. Data Series (NIST NSRDS), National Institute of Standards and Technology, Gaithersburg, MD, [online], https://tsapps.nist.gov/publication/get_pdf.cfm?pub_id=912382 (Accessed June 12, 2024)

(20) Syal, V. K.; Chauhan, A.; Chauhan, S. *J. Pure Appl. Ultrason.* **2005**; 27, 61-69.

(21) González-Tello, P.; Camacho, F.; Blázquez, G. *J. Chem. Eng. Data* **1994**; 39, 3, 611-614. doi:10.1021/je00015a050

(22) Pijper, A. *J. Bacteriol.* **1947**, 53(3), 257–269. doi:10.1128/jb.53.3.257-269.1947

(23) Verma, P. K.; Kundu, A.; Ha, J. H.; Cho, M. *J. Am. Chem. Soc.* **2016**, 138, 16081–16088. doi:10.1021/jacs.6b10164

(24) Elias H.G. *Makromoleküle*; Georg Thieme Verlag: Stuttgart, 2009.

(25) Stefanovic, R.; Ludwig, M.; Webber, G. B.; Atkin, R.; Page, A. J. *Phys. Chem. Chem. Phys.* **2017**, 19(4), 3297–3306. doi:10.1039/c6cp07932f

(26) Kučan, K. Z.; Perković, M.; Cmrk, K.; Načinović, D.; Rogošić, M. *ChemistrySelect* **2018**, 3(44), 12582–12590. doi:10.1002/slct.201803251

(27) Mulia, K.; Adam, D.; Zahrina, I.; Krisanti, E. A. *Int. J. Technol.* **2018**, 2, 335–344. doi:10.14716/ijtech.v9i2.1008

(28) Rodrigues, L. A.; Cardeira, M.; Leonardo, I. C.; Gaspar, F. B.; Radojčić Redovniković, I.; Duarte, A. R. C.; Paiva, A.; Matias, A. A. *J. Mol. Liq.* **2021**, 335, 116201. doi:10.1016/j.molliq.2021.116201

(29) National Center for Biotechnology Information. PubChem Compound Summary for CID 679, Dimethyl Sulfoxide

(30) National Center for Biotechnology Information. PubChem Compound Summary for CID 15413, Methyl tert-butyl ether

(31) Institut für Arbeitsschutz der Deutschen Gesetzlichen Unfallversicherung. Eintrag zu Dimethylsulfoxid in der GESTIS-Stoffdatenbank

(32) National Center for Biotechnology Information. PubChem Compound Summary for CID 887, Methanol

(33) Institut für Arbeitsschutz der Deutschen Gesetzlichen Unfallversicherung. Eintrag zu Methyl-tert-butylether in der GESTIS-Stoffdatenbank

(34) National Center for Biotechnology Information. PubChem Compound Summary for CID 8058, n-HEXANE

(35) Lide D.R. *CRC Handbook of Chemistry and Physics*, CRC Press: Boca Raton, 2004.

(36) Landaverde-Cortes, D. C.; Estrada-Baltazar, A.; Iglesias-Silva, G. A.; Hall, K. R. *J. Chem. Eng. Data* **2007**, *52*, 1226–1232. doi:10.1021/je600554h

(37) Badyal, Y. S.; Saboungi, M.-L.; Price D.; Shastri, S. D.; Haeffner, D. R.; Soper, A. K. *J. Chem. Phys.* **2000**, 112(21), 9206-9208. doi:10.1063/1.481541

(38) Nelson, R. D.; Lide, D. R.; Maryott, A. A. *Selected Values of Electric Dipole Moments for Molecules in the Gas Phase*; **1967**. doi:10.6028/nbs.nsrds.10

(39) Jorge, M.; Gomes, J. R. B.; Barrera, M. C. *J. Mol. Liq.* **2022**, *356*. doi:10.1016/j.molliq.2022.119033

(40) Suenram, R. D.; Lovas, F. J.; Pereyra, W.; Fraser, G. T.; Hight Walker, A. R. *J. Mol. Spec.* **1997**; 181.

(41) Cayman Chemicals. CommonSolventsNonpolar https://cdn2.caymanchem.com/cdn/cms/caymanchem/cmsImages/news/502/CommonSolventsNonpolar.pdf (accessed Jan 17, 2024)

(42) Pandi, A.; Diehl, C.; Yazdizadeh Kharrazi, A.; Scholz, S. A.; Bobkova, E.; Faure, L.; Nattermann, M.; Adam, D.; Chapin, N.; Foroughijabbari, Y.; Moritz, C.; Paczia, N.; Cortina, N. S.; Faulon, J. L.; Erb, T. J. *Nat. Commun.* **2022**, 13(1): 3876. doi:10.1038/s41467-022-31245-z

(43) Kahn, T. W.; Beachy, R. N.; Falk, M. M. *Curr. Biol.* **1997**, 7(4), 207-208. doi:10.1016/S0960-9822(06)00100-X

(44) Kim, D. M.; Kigawa, T.; Choi, C. Y.; Yokoyama, S. *Eur. J. Biochem.* **1996**, *239*, 881–886. doi:10.1111/j.1432-1033.1996.0881u.x

(45) Ge, X.; Luo, D.; Xu, J. *PLoS One* **2011**, *6*. doi:10.1371/journal.pone.0028707

(46) Li, J.; Jiang, Z.; Wu, H.; Liang, Y.; Zhang, Y.; Liu, J. *Carbohydr. Polym.* **2010**, *82*, 160–166. doi:10.1016/j.carbpol.2010.04.045

(47) Chai, Q.; Singh, B.; Peisker, K.; Metzendorf, N.; Ge, X.; Dasgupta, S.; Sanyal, S. *J. Biol. Chem.* **2014**, *289*, 11342–11352. doi:10.1074/jbc.M114.557348

(48) Śmigiel, W. M.; Mantovanelli, L.; Linnik, D. S.; Punter, M.; Silberberg, J.; Xiang, L.; Xu, K.; Poolman, B. *Sci. Adv.* **2022**; 8(32). doi:10.1126/sciadv.abo5387

(49) Ribeiro, B. D.; de Carvalho Iff, L.; Coelho, M. A. Z.; Marrucho, I. M. *Curr. Biochem. Eng.* **2019**, 5(1), 57–68. doi:10.2174/2212711906666190710181629

(50) Yadav, A.; Pandey, S. *J. Chem. Eng. Data* **2014**, *59*, 2221–2229. doi:10.1021/je5001796

(51) Shih, Y. -C; Prausnitz, J. M.; Blanch, H. W. *Biotechnol. Bioeng.* **1992**, *40*, 1155–1164. doi:10.1002/bit.260401004

(52) Kress, C.; Sadowski, G.; Brandenbusch, C. *Eur. J. Pharm. Biopharm.* **2017**, *112*, 38–44. doi:10.1016/j.ejpb.2016.11.016

(53) Gregorio, N. E.; Levine, M. Z.; Oza, J. P. A. *Methods Prot.* **2019**, 2(1), 24. doi:10.3390/mps2010024

(54) Goering, A. W.; Li, J.; McClure, R. A.; Thomson, R. J.; Jewett, M. C.; Kelleher, N. L. *ACS Synth. Biol.* **2017**, *6*, 39–44. doi:10.1021/acssynbio.6b00160

(55) Ablasser, A.; Goldeck, M.; Cavlar, T.; Deimling, T.; Witte, G.; Röhl, I.; Hopfner, K. P.; Ludwig, J.; Hornung, V. *Nature* **2013**, *498*, 380–384. doi:10.1038/nature12306

(56) Bartsch, T.; Becker, M.; Rolf, J.; Rosenthal, K.; Lütz, S. *Biotechnol. Bioeng.* **2021**, 119(3), doi:10.1002/bit.28027

(57) Pätzold, M.; Siebenhaller, S.; Kara, S.; Liese, A.; Syldatk, C.; Holtmann, D. *Trends Biotechnol.* **2019**, *37*, 943–959. doi:10.1016/j.tibtech.2019.03.007

(58) Kučan, K. Z.; Perković, M.; Cmrk, K.; Načinović, D.; Rogošić, M. *ChemistrySelect* **2018**, *3*, 12582–12590. doi:10.1002/slct.201803251

(59) Adden, R.; Hübner-Keese, B.; Förtsch, S.; Knarr, M. Cellulosics. In *Handbook of Hydrocolloids*; Phillips G. O.; Williams P. A. Eds.; Elsevier: Cambridge, U.S., 2021; pp 481–508. doi:10.1016/B978-0-12-820104-6.00011-5

(60) Grunberg, L.; Nissan, A. H. *Nature* **1949**, *164*, 799–800. doi:10.1038/164799b0

(61) Volkmer, B.; Heinemann, M. *PLoS One* **2011**, *6*, 1–6. doi:10.1371/journal.pone.0023126

(62) Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248–254. doi:10.1016/0003-2697(76)90527-3

(63) Laemmli, U. K. *Nature* **1970**, *227*, 680–685. doi:10.1038/227680a0

(64) Schneider, C. A.; Rasband, W. S.; Eliceiri, K. W. *Nat. Methods* **2012**, *9*, 671–675. doi:10.1038/nmeth.2089