FREIE UNIVERSITÄT BERLIN
Fachbereich Biologie, Chemie, Pharmazie
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Institutsteil Bionanalytik und Bioprozesse

A versatile Cell-free Protein Production Platform for the Synthesis of multifunctional Enzymes

Inaugural-Dissertation
to obtain the academic degree
Doctor rerum naturalium (Dr. rer. nat.)

submitted to the Department of Biology, Chemistry, Pharmacy
of Freie Universität Berlin

by

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Berlin, 2023

This thesis was composed between 01.04.2019 and 24.06.2023 at the Fraunhofer Institute for Cell Therapy and Immunology, Branch Bioanalytics and Bioprocesses (IZI-BB) and has been supervised by Dr. Stefan Kubick.

1st Reviewer: Dr. Stefan Kubick

2nd reviewer: Prof. Dr. Markus Wahl

Date of thesis defense (Disputation): 16.02.2024

Acknowledgment

I would like to thank Dr. Stefan Kubick and Prof. Dr. Markus Wahl for their supervision throughout this work. Additionally, I would like to thank Dr. Stefan Kubick for his project and team management, as well as his valuable feedback on the progress and content of the scientific research.

I would also like to express my sincere appreciation to the professors and staff members of the research groups at Freie Universität, as well as my teachers in school. Their dedication to teaching has provided me with the essential skills needed for scientific work and writing this dissertation.

I am also grateful to the employees at Fraunhofer IZI-BB for their cooperation and assistance, particularly Dr. Anne Zemella, whose experience and availability were invaluable in resolving various challenges.

I would like to express my gratitude to my former master's student, Stephanie Hahn, for her highly productive collaboration and support in the PZ-Syn project.

I would like to thank the cooperation partners in the PZ-Syn project from BTU Cottbus-Senftenberg and TU Dresden for their excellent collaboration.

I want to acknowledge my family for their support in my day-to-day life during the completion of this work.

Lastly, I would like to thank David, Lukas, Anna, Sarah, Niklas and Ruben for their constant motivation.

Declaration of Independence

Herewith I certify that I have prepared and written my thesis independently and that I have not used any sources and aids other than those indicated by me. I further declare that I have not previously submitted this dissertation, either in its entirety or in any modified form, to any other academic institution for assessment.

_____ Berlin, 24.06.2023

Felix Knauer

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Summary

Enzymes are indispensable proteins for all types of organisms, capable of fulfilling various functions by catalyzing diverse chemical reactions. This makes them important tools not only for fundamental research in biochemistry but also for applied research in pharmacology and organic chemistry. However, the recombinant production of enzymes is not trivial, and conventional isolations and expressions from cells are often unsuitable for many applications due to decreased enzyme activity. Cell-free protein synthesis can simplify the synthesis of some enzymes that were previously difficult to produce. Representatives of various enzyme classes were synthesized and subsequently analyzed. Modifications to the established synthesis platforms based on translationally active CHO and *Sf*21 lysates were made. These modifications allowed for the production of a soluble enzyme, a representative of the hydrolases, GH78 from *Xylaria polymorpha*. Kinetic analyses after synthesis optimization confirmed the kinetic similarity to the naturally synthesized enzyme. In particular, temperature and synthesis condition adjustments, as well as DNA template modifications, were found to increase enzyme activity. Efforts to adapt a cell-free synthesis platform for the production of further active fungal enzymes, mainly comprising unspecific peroxygenases in active CHO lysates have not yet yielded successful results. However, progress has been made in laying the foundation for future advancements, including the generation of templates, successful synthesis, and the establishment of activity assays. To provide a mammalian enzyme alternative to the unspecific peroxygenases, difficult-to-express monooxygenases comprising members of the cytochrome P450 enzyme family (CYP), the membrane-bound enzymes CYP1A2, CYP2B6, and CYP3A4 from *Homo Sapiens*, were produced in a eukaryotic translationally active cell lysate. By genetically modifying the CHO cells used for lysate production, an increased amount of CPR, the coenzyme of CYPs, was integrated into the endogenous membrane vesicles of the lysate, thus increasing the activity of the cell-free produced CYPs. Further adaptations of the synthesis, such as synthesis temperature and supplementation with heme, led to further increases in CYP activity in the endogenous membrane vesicles. This allowed for exemplary screening experiments with known CYP substrates.

Zusammenfassung

Enzyme sind unverzichtbare Proteine für alle Arten von Organismen, die durch Katalyse verschiedener chemischer Reaktionen verschiedene Funktionen erfüllen können. Dies macht sie nicht nur für die Grundlagenforschung in der Biochemie, sondern auch für die angewandte Forschung in der Pharmakologie und organischen Chemie zu wichtigen Werkzeugen. Die rekombinante Produktion von Enzymen ist jedoch nicht trivial, und herkömmliche Isolations- und Expressionsmethoden aus Zellen sind oft für viele Anwendungen aufgrund verringerte Enzymaktivität ungeeignet. Die zellfreie Proteinsynthese kann die Synthese einiger zuvor schwer herstellbarer Enzyme vereinfachen. Vertreter verschiedener Enzymklassen wurden synthetisiert und anschließend analysiert. Modifikationen an den etablierten Syntheseplattformen basierend auf translationsaktiven CHO- und *Sf*21-Lysaten wurden vorgenommen. Diese Modifikationen ermöglichten die Produktion eines löslichen Enzyms, eines Vertreters der Hydrolasen, GH78 von *Xylaria polymorpha*. Kinetische Analysen nach Optimierung der Synthese bestätigten die kinetische Ähnlichkeit zum natürlichen Enzym. Insbesondere Temperatur- und Synthesebedingungsanpassungen sowie Modifikationen der DNS-Vorlage wurden durchgeführt, um die Enzymaktivität zu erhöhen. Die Anpassung einer zellfreien Syntheseplattform zur Produktion weiterer aktiver Pilzenzyme, hauptsächlich von unspezifischen Peroxygenasen in translationsaktiven CHO-Lysaten, hat bisher keine erfolgreichen Ergebnisse erbracht. Es wurden allerdings bereits die ersten Schritte, insbesondere die Generierung von DNS-Template, erfolgreicher erste Synthesen und der Etablierung von Aktivitätsassays durchgeführt. Als Alternative zu den unspezifischen Peroxygenasen, wurden schwer herstellbare Monooxygenasen der Cytochrom-P450-Enzymfamilie (CYP), in einem eukaryotischen translationsaktiven Zelllysat produziert. Durch genetische Modifikation, der für die Lysatproduktion verwendeten CHO-Zellen wurde, eine erhöhte Menge an CPR, dem Coenzym der CYPs, in die endogenen Membranvesikel des Lysats integriert, was die Aktivität der CYPs erhöhte. Weitere Anpassungen der Synthese, wie z.B. die Synthesetemperatur und die Zugabe von Häm, führten zu weiteren Steigerungen der CYP-Aktivität in den endogenen Membranvesikeln. Dies ermöglichte exemplarische Screening-Experimente mit bekannten CYP-Substraten.

1. Introduction

1.1. Scope of the Thesis

This thesis mainly focuses on two different groups of application-related enzymes: fungal hydrolases, which are particularly relevant in the food industry, and cytochrome P-450 monooxygenases, which are important in the production and approval of biopharmaceuticals. In this context cell-free protein synthesis (CFPS) served as a production platform for the synthesis of said enzymes. CFPS a protein synthesis method that has developed over the past few decades and is already being used advantageously in some areas of biotechnology. The question arises as to whether CFPS can also contribute to enzymological findings or simply to the more efficient synthesis of certain enzymes in the field of enzymology. The following is a brief overview of the research priorities in this project:

At the beginning of these studies, a relatively stable enzyme, a glycoside hydrolase from the fungus *Xylaria polymorpha*, was examined. The glycoside hydrolase 78 from *Xylaria polymorpha* was primarily used as a model enzyme that could be used to establish a workflow for establishing cell-free synthesis of certain classes of enzymes and to identify potential challenges early on.

In a next step, more complex enzymes from fungi were examined, namely unspecific peroxygenases (UPOs). The recombinant production of these monooxygenases is significantly more challenging than the production of the hydrolase. As these enzymes have great potential in the production of fine chemicals, their efficient recombinant production is of outstanding interest.

Finally, a more detailed investigation was conducted on the cell-free recombinant synthesis of other monooxygenases, including human cytochrome P-450 enzymes. These enzymes are critical in the metabolism of toxins in higher organisms and hold significant importance in pharmaceutical research. Due to their membrane-bound nature, their synthesis and isolation pose significant challenges.

1.2. Relevance of Enzymes in Biochemistry and Biotechnology

Biocatalysis is a crucial topic in biochemistry as enzymes are involved in almost all cellular processes, from protein biosynthesis to metabolization. Organisms rely heavily on catalysis due to the narrow temperature range prevailing in their natural

environments. Biocatalysis enables reactions that would otherwise only occur at hostile high temperatures to take place at 37 °C. In biochemistry, the functionality of enzymes is not only studied in detail, but enzymes are also utilized as tools in various biochemical applications. Examples of such applications include the use of glycosidases, restriction enzymes, peroxidases, ligases, polymerases, and numerous others (Robinson 2015). In biotechnology, there is a growing interest in using these proteins for technical purposes, as they can catalyze biologically relevant reactions at room temperature (Schmid et al. 2002), which would otherwise require high-temperature chemical processes that are time-consuming and expensive. Enzymes are used in many everyday applications, including environmentally friendly detergents, paper and textile production, biofuel production, and the production of food, beverages, animal feed, and pharmaceutical products (Katsimpouras und Stephanopoulos 2021; Meghwanshi et al. 2020).

1.3. Extracellular fungal enzymes

Fungi play a crucial role in the carbon cycle as important decomposers, obtaining nutrients by digesting complex organic materials (Thormann 2006). To decompose plant remains, the chemical breakdown of their cell walls is necessary, which is achieved by fungi through the secretion of extracellular hydrolases and oxidoreductases. These enzymes catalyze the hydrolysis of sugar-based polymers, such as cellulose, xylan, and pectin, as well as the oxidation of lignin and derived aromatic substances (Hatakka 1994; Hofrichter und Steinbüchel 2001; Hofrichter 2002; Arnau et al. 2019).

The industry has leveraged the adaptability of reactions catalyzed by fungal enzymes. Currently, approximately 50% of all commercially-used enzymes are derived from fungi. (McKelvey und Murphy 2018). The majority of these enzymes are hydrolases, which are primarily employed in the wine and dairy industries, as well as in the broader food industry. (Parameswaran et al. 2019). Amylases and rhamnosidases are commonly employed in fermentation processes, but they are also used in medical contexts, particularly in the production of specific metabolites. Some of these metabolites possess beneficial properties, such as anti-cancer effects (e.g., ginsenoside-Rh1) (Yu et al. 2002), antibiotic activity (e.g., chlorosporin C), anti-inflammatory effects (e.g., quercetin, prunin), and a variety of mechanisms for combating DNA and RNA viruses (e.g., prunin) (Norouzian et al. 1999).

Furthermore, the production of steroid drugs, like progesterone are performed with the help of rhamnosidases (rewieved in (Yadav et al. 2010)). The range of potential new enzymes is long and new ones are constantly being discovered and synthesized *de novo* (Hofrichter et al. 2020). Therefore, a rapid synthesis system for new enzymes is highly desirable.

1.3.1. Glycoside hydrolase 78 of Xylaria polymorpha

Xylaria polymorpha (XPO) is a fungus that belongs to the phylum Ascomycota. Unlike certain Basidiomycota fungi, XPO does not possess high-redox potential peroxidases, such as manganese and lignin peroxidases, for lignin degradation (Jang et al. 2009). Instead, XPO possesses laccases and several extracellular hydrolases that enable the fungus to employ an alternative pathway for lignocellulose conversion (Liers et al. 2011). This pathway includes the use of a unique glycoside hydrolase 78 (GH78) enzyme, which has four domains and exhibits α-L-rhamnosidase and feruloyl esterase activities. The enzyme was first described by Nghi et al. (2012), who produced it using the wild-type fungus, characterized its substrate spectrum, and determined its enzyme kinetic data (Nghi et al. 2012). It was discovered that GH78 is capable of hydrolyzing ester linkages between lignin and polysaccharide moieties within the plant cell wall. Moreover, GH78 exhibits potent α-L-rhamnosidase activity, which is crucial for the breakdown of hemicellulose and pectin chains.

Despite being a secretory protein that digests polymeric substrates in the extracellular environment of fungal hyphae, GH78 does not possess an N-terminal signal sequence required for translocation. Nevertheless, the glycosylation pattern of the protein indicates that it is translocated into the endoplasmic reticulum (ER) via an alternative pathway, despite the absence of a signal sequence (Nghi et al. 2012). A straightforward approach for measuring GH78's rhamnosidase activity involves using the p-nitrophenyl α-L-rhamnopyranoside (NPRP) assay, which tracks the production of the colored fission product p-nitrophenol. (*Figure 1*) (Romero et al. 1985).

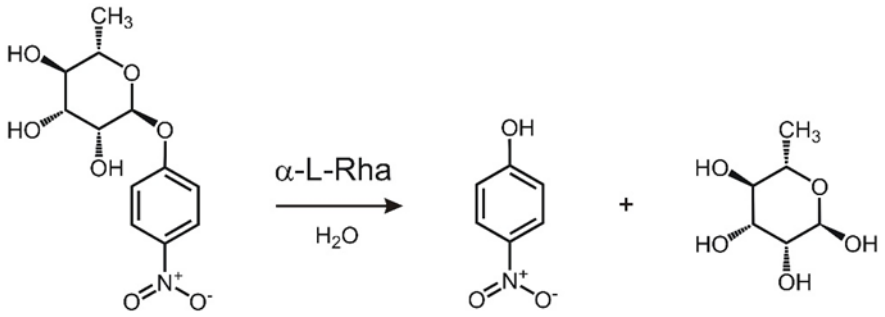


Figure 1: Reaction scheme for GH78 activity detection. GH78 catalyzes the hydrolysis of *p*-nitrophenyl α-L-rhamnopyranoside into rhamnose and *p*-nitrophenol. The latter shows strong absorption at 405 nm at alkaline pH and can be colorimetrically detected. Adapted from (Knauer et al. 2022).

Cultivating *Xylaria polymorpha* and extracting GH78 is a time-consuming process that can take up to 8 weeks (Nghi et al. 2012). Therefore, this synthesis method has limited suitability for applications that require modified forms of the enzyme. However, CFPS provides a simple method for rapidly producing modified proteins for enzymatic characterization within a few hours. GH78 is an excellent candidate for CFPS of fungal enzymes due to its inherent stability and straightforward characterization. Once the methods for cell-free synthesis of specific enzymes have been established, CFPS can be used for high-throughput screening and enzyme engineering. This process involves testing templates that may result in improved or altered enzyme functionality (Dong et al. 2012).

1.3.2. Cell-free protein synthesis for the production of fungal enzymes

Eukaryotic CFPS systems have been developed as a means to enhance the production of "difficult-to-express proteins"(Thoring et al. 2017; Stech et al. 2014; Zemella et al. 2015). The principle of CFPS is schematically depicted in Figure 2. Translationally active lysates serve as the basis for this process, exclusively derived from eukaryotic cells. In multi-step processing, these lysates are made suitable for use in the procedure. This involves, among other things, digestion of endogenous mRNA from the lysate by a nuclease. The synthesis reaction can be initiated by adding RNA polymerase, energy components, amino acids, and a DNA template of the target protein and an energy regeneration system (Carlson et al. 2012). Additionally, the addition of other components, such as cofactors of the protein to be synthesized, is possible. Moreover, some eucaryotic cell lysates contain endogenous microsomes that enable the translocation of membrane proteins and all endoplasmic reticulum-

associated post-translational modifications, such as partial N-glycosylation and disulfide bridges (Sachse et al. 2014).

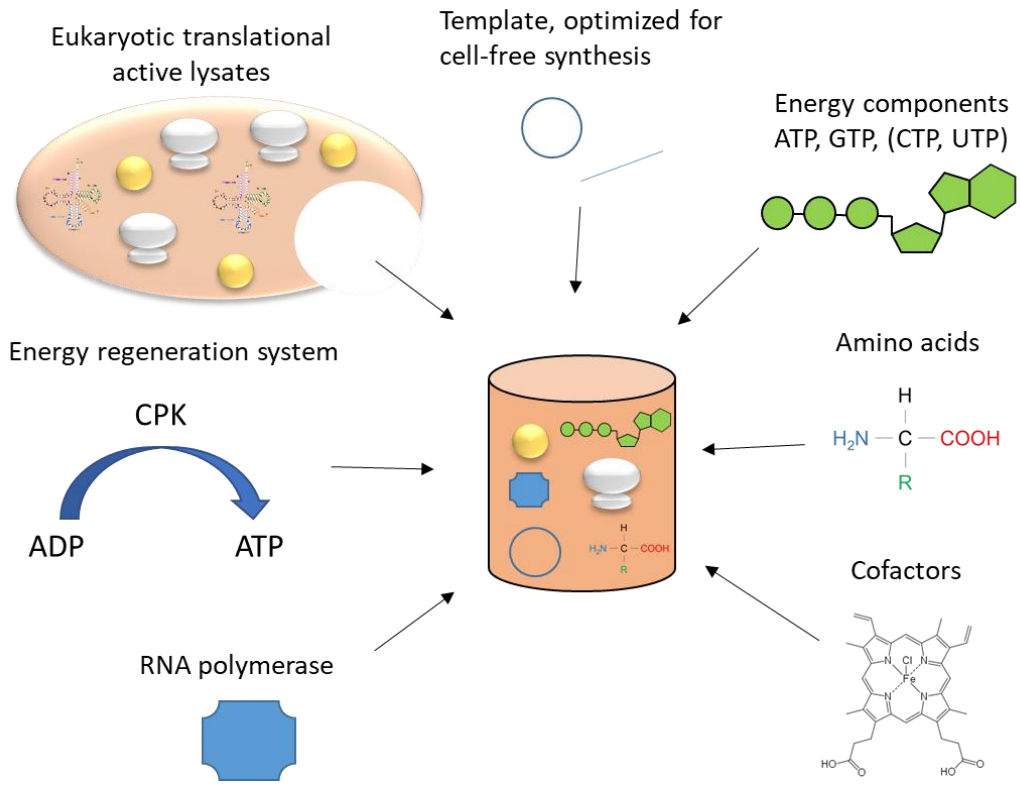


Figure 2: Schematic representation of a classical cell-free reaction for the synthesis of proteins in eukaryotic cell lysates in batch format. The translational active lysate comprises ribosomes, tRNAs and endogenous microsomes that allow translocation of secretory- or membrane located proteins.

Notable advantages of cell-free systems are the significant reduction of production time (Liu et al. 2005) and the possibility to perform high throughput screenings of various templates and synthesis conditions in a cost and time efficient way (He et al. 2008). Circular vectors, mRNA as well as linear PCR products can be used as template (Kirill A. Martemyanov et al. 1997). For protein identification and yield quantification, radioactively labeled amino acids can be added, which are statistically incorporated into the *de novo* synthesized protein (Stech et al. 2014). Enzymatic activity assays can be conducted post protein synthesis or even during the process, utilizing 96-well plate formats, thereby rendering CFPS an indispensable tool for enzymology. Additionally, affinity tags can be incorporated into proteins via diverse methods. In addition to N- or C-terminal fusion tags, which have been previously

described (Puri et al. 2010), it is possible to introduce affinity tags into the produced protein using modified non-canonical amino acids in a site-specific manner. (Bundy und Swartz 2010).

CFPS enables the user to address a broad spectrum of different applications for protein characterization. Therefore, it is crucial to carefully evaluate the requirements of each specific application to select the most suitable cell-free system and reaction conditions. The available cell-free systems include translationally active lysates derived from various sources, such as *E.coli*, yeast, *Leishmania tarentolae*, wheat germ, *Sf*21, CHO, rabbit and cultured human cells (Gregorio et al. 2019; Zemella et al. 2015). Each system has its distinct features, advantages, and limitations (Dondapati et al. 2020).

To this day, a major limitation of CFPS is the practical limitations in upscaling and relatively low target protein yields which makes this method unsuitable for mass production of proteins. The latter is often associated with the production of inhibitory by-products that stop the cell-free synthesis reaction after a certain time (around 3 hours). To overcome this issue, a continuous exchange cell-free system (CECF) has been developed, in which the by-products can diffuse out of the reaction chamber through a semipermeable membrane, thus allowing for significantly higher protein yields to be obtained (Stech et al. 2014).

1.3.3. Template modification

The design of the template greatly affects efficient translation initiation during cell-free protein synthesis. In eukaryotic systems, various translation initiation strategies can be utilized (Sorokin et al. 2021). Cap-induced translation initiation, similar to in vivo protein expression, is possible, but requires at least twelve different active co-factors in the lysate (Hinnebusch und Lorsch 2012). To overcome the dependence on these initiation co-factors, viral translation initiation sequences can be incorporated into the template sequence. Among the most frequently inserted translation initiation sequences for cell-free protein synthesis are internal ribosome entry sites (IRES) (Marques et al. 2022). The conventional mechanism of translation initiation involves the recognition of a 5' cap structure by the small ribosomal subunit, which then scans the mRNA until it reaches the start codon (usually AUG) for protein synthesis. However, in the presence of an IRES, translation can initiate internally enabling the ribosome to directly access the coding region of the mRNA without

scanning from the 5' end. The process of translation initiation at an IRES involves several steps. Initially, certain cellular proteins known as IRES trans-acting factors (ITAFs) bind to the IRES sequence within the mRNA. These ITAFs recruit initiation factors and ribosomal subunits to the mRNA. Subsequently, the ITAFs and initiation factors assist in the assembly of the large ribosomal subunit and initiator tRNA at the IRES region, positioning them for translation initiation (Komar und Hatzoglou 2011).

The IRES of Cricket Paralysis virus (CrPV-IRES) is commonly integrated into the gene sequence for CHO and *Sf*21 based CFPS (Brödel et al. 2013). Other IRES sequences have been published for translation initiation in CFPS, including Encephalomyocarditis virus IRES, *Rhopalosiphum padi* virus IRES, and Tobacco mosaic virus IRES (Roberts und Paterson 1973). For additional strategies, disordered 5' untranslated region (UTR) sequences modeled after those found in poxviruses have been suggested for translation initiation that is not species-specific. (Mureev et al. 2009). Multiple trans acting factors have been discovered to support the translation initiation via different IRES (Komar und Hatzoglou 2011). While the integration of IRES has led to an improvement in CFPS, there have been few studies on how the sequence context downstream of the IRES affects synthesis performance (Davies und Kaufman 1992). Possible modifications to the sequence context include the insertion of an N-terminal fusion peptide or tag, the optimization of codon usage, or truncation of the target protein, all with the aim of improving the translation efficiency of the target protein (*Figure 3*). Of particular interest is the region in close proximity to the IRES, where the secondary structure of the mRNA can potentially influence the efficiency of translation initiation by the IRES.

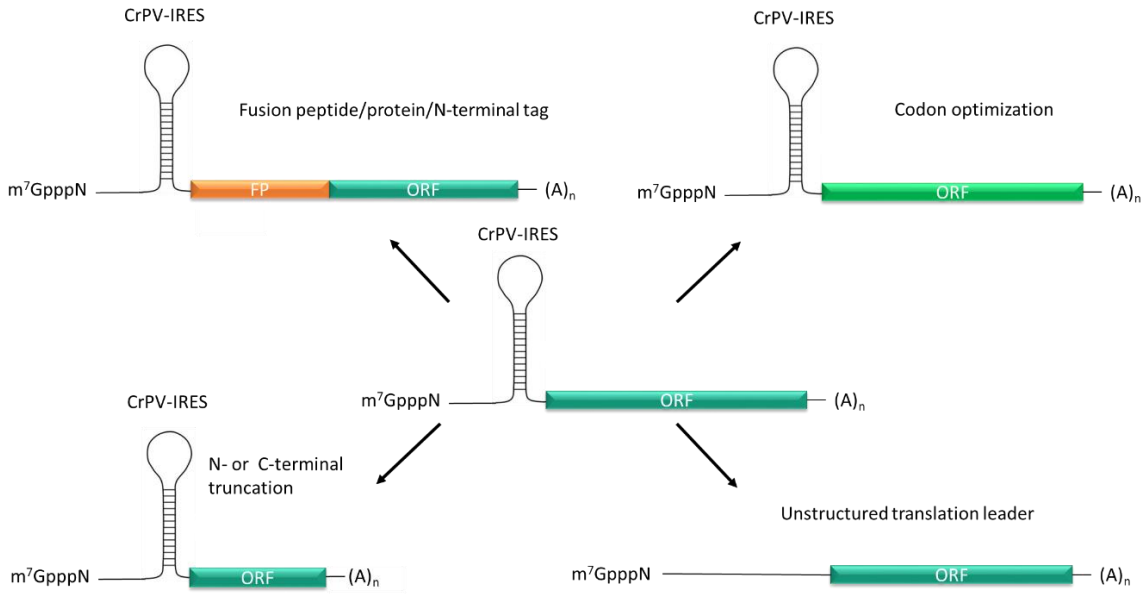


Figure 3: Scheme of possible template modifications for increased translation efficiency after IRES mediated translation initiation. ORF = open reading frame, FP = Region coding for a fusion peptide

In addition to codon changes and truncations, the introduction of fusion affinity tags offers a means to modify the sequence context of the N-terminus of the coding sequence. Furthermore, affinity tags facilitate the immobilization and purification of the produced proteins.

1.3.4. Immobilization of proteins in a one-pot batch system

The resource-efficient and sustainable utilization of biomolecules can significantly reduce resource consumption and expenditure. Hence, the development of simple, rapid, and cost-effective methods for enzyme synthesis and substrate turnover is imperative. Over the past few years, there has been extensive research on enzyme immobilization (protein binding), which has demonstrated a proven track record of success (reviewed in (Mohamad et al. 2015) (Datta et al. 2013)). Immobilization offers a significant advantage in terms of enhancing the operational stability of enzymes, thereby promoting their reusability. Among various immobilization techniques, affinity-tag immobilization is considered to be one of the most straightforward methods (Silvana Andreescu und Bogdan Bucur, and Jean-Louis Marty 2006) and can serve as a foundation for optimizing and refining reaction conditions. Through the application of CFPS, the stages of protein production, enzyme immobilization, and substrate turnover can be accomplished within a single

batch-based synthesis system. This approach significantly enhances the appeal of enzyme immobilization for industrial applications. A schematic of this principle is presented in *Figure 4*. Such one-pot synthesis, immobilization, and turnover configurations are only conceivable using CFPS.

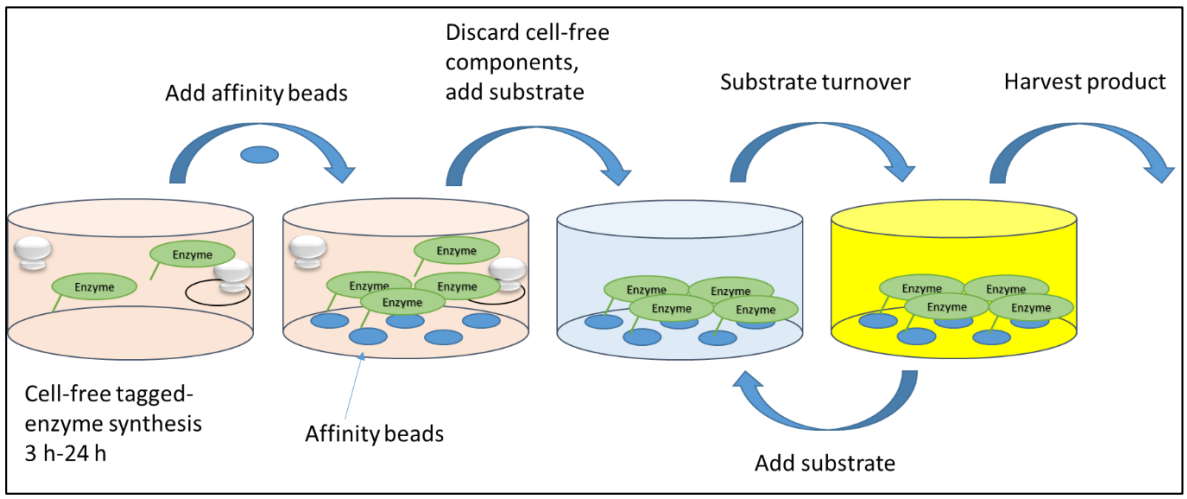


Figure 4: In vitro model of enzyme synthesis and immobilization (enzyme reactor) that can be executed within a single tube using CFPS. The protein synthesis machinery is represented by ribosomes in white, while the affinity beads used for immobilization are depicted in blue. The enzymes are depicted in green. Adapted from (Knauer et al. 2022).

1.3.5. Cell-free synthesis of unspecific Peroxygenases (UPOs)

Fungi are essential for the carbon cycle, as they break down dead organic material, including dead plants. To do this, fungi require the chemical lysis of cell walls, which can be achieved through the secretion of enzymes such as hydrolases and peroxygenases. Fungal peroxygenases are able to perform monooxygenation on a wide variety of substrates, making them valuable in many synthesis processes in the chemical and pharmaceutical industry, as well as for the degradation of pollutants (Nghi et al. 2012; Kinne et al. 2009). They offer valuable applications in pharmaceutical synthesis, where they can selectively oxygenate drug precursors, yielding desired intermediates or active pharmaceutical ingredients (Wang et al. 2017). In fine chemical synthesis, fungal peroxygenases play a role in the production of flavors, fragrances, and dyes by introducing oxygen functional groups at specific positions (Monterrey et al. 2023). Their ability to degrade environmental pollutants makes them valuable in bioremediation efforts, as they can oxidize contaminants like polycyclic aromatic hydrocarbons (PAHs), pesticides, and industrial pollutants (Hofrichter und Ullrich 2014; Hofrichter et al. 2022; Karich et al. 2017).

Furthermore, fungal peroxygenases can modify lignin, selectively oxidizing its structures and enabling controlled depolymerization for potential applications in biofuels, biomaterials, and other value-added products (Komisarz et al. 2022). Fungal peroxygenases are also called "unspecific Peroxygenases" (UPOs) due to their unusual substrate variety. Due to their ability to monooxygenize various organic molecules, UPOs may become an important enzyme in the mentioned synthesis processes in the chemical and pharmaceutical industry (Ana Conesa, Peter J. Punt, Cees A.M.J.J. van den Hondel 2002).

Currently, most UPOs are obtained from white, brown, or soft rot fungi. The secreted proteins can be obtained directly from the culture medium after cultivation. However, the gain of a relevant amount of peroxygenases using this method can take up to several weeks and requires several optimizations during fermentation (Poraj-Kobielska et al. 2012). Therefore, few UPOs have been obtained this way in a relevant amount, comprising *Agrocybe aegerita* UPO and Marasmius rotula UPO, for example (Ullrich et al. 2004). The recombinant expression of peroxygenases in living cells as an alternative synthesis method is even more challenging. Heterologous synthesis in a fungal expression system often leads to degradation of the target protein due to the huge number of proteases. Peroxygenases expressed in other eukaryotic systems are often inactive, incorrectly glycosylated, and not soluble due to little incorporation of the necessary heme group. Production of peroxygenases in *E. coli* faces similar problems. However, some approaches have been made by resolubilizing peroxygenases from inclusion bodies and introducing the heme group via solubilization and refolding (Eggenreich et al. 2016). Additionally yeast based expression systems have been proven useful for recombinant UPO expression and enzyme engineering (Molina-Espeja et al. 2015).

Although this method leads to the refolding and reactivation of up to 80% of precipitated protein, the stability of the peroxygenases, especially due to the lack of glycosylation, remains a problem. The stability of fungal-based peroxygenases is one of the main constraints for the industrial application, especially because the peroxygenase substrate hydrogen peroxide causes inactivation of the enzyme in high concentration (Ana Conesa, Peter J. Punt, Cees A.M.J.J. van den Hondel 2002). An adequate method for the cost-efficient production of diverse active and stable fungal peroxygenases is therefore required.

A CFPS system might be the key to achieving this goal. Such a system may enable the production of active and stable UPOs in a cost-efficient manner, allowing for wider use of these versatile enzymes in various industries. Moreover, this system may allow for the engineering of UPOs for improved stability and activity. The potential of CFPS systems for the production of diverse active and stable fungal Peroxygenases has been recognized in recent years (Chiba et al. 2021).

1.4. Cell-free synthesis of membrane bound enzymes

1.4.1. Cytochrome P450 monooxygenases enzyme family

Cytochrome P450 (CYP) are oxidoreductase enzymes that contain heme as a cofactor. The name is derived from their characteristic light absorbance property, which is attributed to the heme group (Werck-Reichhart und Feyereisen 2000). These monooxygenases are present in all forms of life and serve a variety of functions (Esteves et al. 2021). There are 18 mammalian cytochrome P450 (CYP) families identified, consisting of 57 genes in the human genome. Among these families, CYP2, CYP3, and CYP4 exhibit a significantly greater number of genes compared to the remaining 15 families (Nebert et al. 2013). Due to their unique properties, reaction mechanisms, and long evolutionary history, these enzymes have been extensively studied since their discovery (Hrycay und Bandiera 2015). These studies have led to significant practical benefits and have facilitated the use of drugs in medicine today (Ogu und Maxa 2000; Zhao et al. 2021).

1.4.2. Localization and function

Unlike soluble cytochromes that participate in the respiratory chain, CYPs are membrane-bound enzymes (Finnigan et al. 2020). While prokaryotic CYPs are localized in the plasma membrane, eukaryotic CYPs can be found in the membrane of mitochondria and endoplasmic reticulum (Šrejber et al. 2018). Within mitochondria, CYPs are responsible for synthesizing steroid hormones such as sex hormones, glucocorticoids, mineralocorticoids, and vitamin D hormone (Wang et al. 2000). During this process, cholesterol or sterols are hydroxylated multiple times, catalyzed by CYPs. Several different types of CYPs are located in the inner mitochondrial membrane, with their catalytic sites exposed to the matrix to allow for the multi-step synthesis (Omura 2006). A variety of CYPs can be found anchored to the cytosolic-facing membrane of the endoplasmic reticulum in hepatocytes (Šrejber et al. 2018). These CYPs catalyze reactions that are similar to those in mitochondria,

but with a wider range of hydrophobic substrates. Due to their responsibility to catalyze hydroxylation of different non-activated carbon atoms, CYPs exhibit a broad and overlapping substrate specificity, as well as a variety of isoforms (Kobayashi et al. 2002). Endoplasmic reticulum-resident CYPs are primarily involved in the processing of xenobiotics, which are molecules from the environment not naturally produced in the body (Patterson et al. 2010). The majority of xenobiotics are deactivated by CYPs, usually through a three-phase enzymatic modification and transportation process that facilitates their excretion from the body (McDonnell und Dang 2013). CYPs conduct the Phase I reaction, which bioactivates the substrate by oxidation. In Phase II, the xenobiotic may be modified by a transferase, and in Phase III, it may be transported out of the body by a transporter protein (Esteves et al. 2021). CYPs play a critical role in medical research and have been extensively reviewed in the literature (Manikandan und Nagini 2018; McDonnell und Dang 2013; Klein und Zanger 2013). Their interaction with drugs, which are ultimately also xenobiotics, is particularly crucial in drug development. Due to the uniqueness of each individual's proteome, CYPs are also a key factor in the implementation of personalized medicine, particularly with regard to drug dosages (Zanger und Klein 2013). Well-known CYP drug interactions are summarized in the *Drug Interactions Flockhart Table*™. Food items also contain substances that can interact with and potentially inhibit CYP enzymes. A prominent example of this is grapefruit juice, which should be avoided when consuming certain medications (Fujita 2004).

1.4.3. Cofactors, substrates, and reaction cycle

To investigate the synthesis and analysis of CYPs, it is essential to understand the reaction mechanism underlying the monooxygenation reaction. The reactions catalyzed by different CYPs are similar and differ only in the substrate. The catalytic center of the reaction is the heme group, which contains the Fe^{3+} ion necessary for interaction with the oxygen molecule. Only one reaction mechanism has been proposed (see *Figure 5*). Electron transfer to the reaction is facilitated by cytochrome P450 reductase (CPR/POR) (Riddick et al. 2013; Wang et al. 1997). Like in many other biochemical reactions, NADPH serves as an electron donor for the mono-oxygenations. Eukaryotic CYPs are unique and versatile, but they are challenging to produce. When the substrate is close to the enzyme, the Fe^{3+} ion is reduced to Fe^{2+} by an electron derived from NADPH and catalyzed by the CYP cofactor CPR (Riddick et al. 2013).

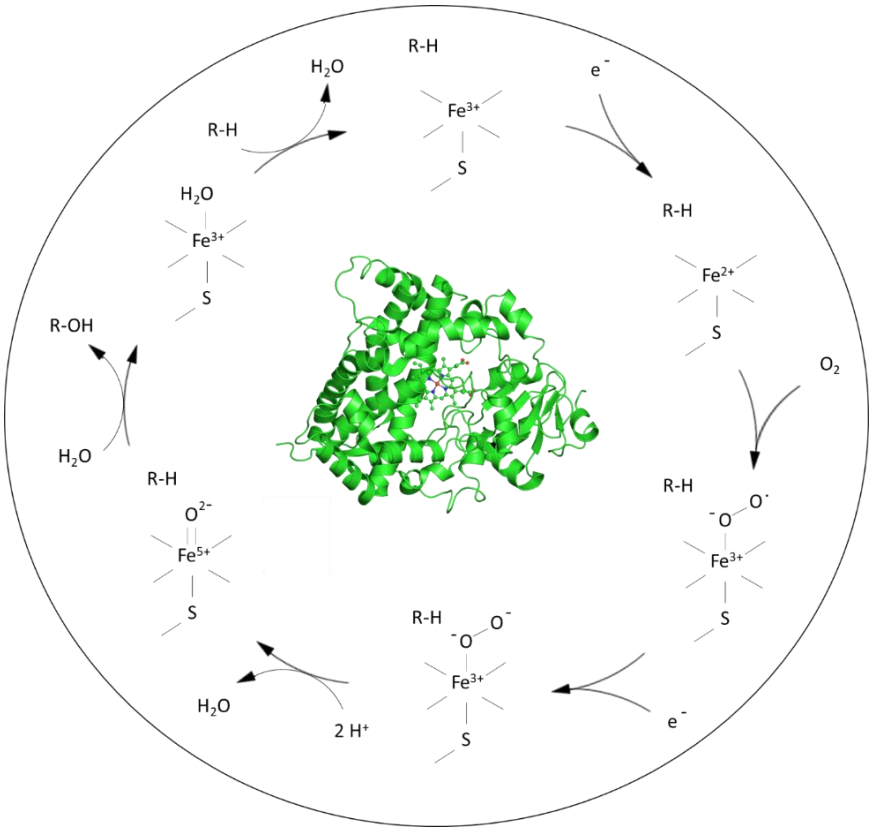


Figure 5: Reaction cycle and structure of CYPs: Schematic representation of a CYP catalyzed hydroxylation of a substrate R-H. In the middle of the cycle a model of CYP3A4 is depicted comprising the heme group as catalytical center. The data of the solved crystal structure are derived from Yano et al (Yano et al. 2004).

To detect the activity of different CYPs, standard substrates are commonly used, although many substrates can be converted by multiple CYPs, which enables the turnover of a wide range of substrates. This redundancy provides potential for multiple applications of CYPs in biotechnology. Basic research on CYPs has the potential to further expand their usefulness in various applications. Understanding the catalytic process is a recurring topic and is particularly interesting in relation to monooxygenases. Crystallography and structural analysis of these unique enzymes have been frequently published in order to gain better insight into the protein orientation and catalytic process. (Šrejber et al. 2018). Although the structural analysis of membrane proteins is usually challenging several structures already could be solved (Manikandan und Nagini 2018).

The development of novel pharmaceuticals and the utilization of CYPs as biocatalysts are the primary driving forces behind the production and investigation of members of the cytochrome P450 enzyme family.

1.4.4. Applications for CYPs

CYPs for drug discovery

Drugs, like other xenobiotics, undergo modification and excretion through the hepatic pathway (Zanger et al. 2008). Among the CYP enzymes responsible for drug oxidation, CYP3A4/5, CYP2C9, CYP2D6, and CYP2C19 play a major role, accounting for approximately 79% of drugs oxidation (Zanger et al. 2008) (Kumar et al. 2012). Some drugs are designed to undergo bioactivation by CYPs to form active components (John S Walsh und Gerald T Miwa 2011). Given that CYPs determine the half-life of drugs, it is important to consider that drugs can interfere with the enzymatic pathway of CYPs, leading to malfunction of natural mechanisms (Kumar et al. 2012). Prominent examples are ibuprofen, haloperidol, sildenafil and diazepam (Lynch und Price 2007). In cases where a substance has a function of increasing or decreasing CYP activity, it can result in side effects or treatment failure when a second CYP-dependent drug is metabolized through the same pathway.

Screening mechanisms that rely on CYPs for drug discovery would be advantageous, given their extensive influence in drug metabolism. Failure to account for CYP inhibition or induction can lead to the withdrawal of several drugs from the market, causing significant financial losses for pharmaceutical companies. By utilizing screening assays, potential drug-drug interactions that are implicated by CYPs can be anticipated and addressed. This issue has also been comprehensively addressed in a publication (Kumar et al. 2012). While this application does not necessarily require a high yield of protein, the challenge lies in modifying the potential drug to avoid interactions with CYPs in the human body.

CYPs as bioreactors

The monooxygenation process to produce metabolites typically requires high-temperature organic reactions. The use of catalysts that can perform monooxygenations could reduce the cost and energy resources required for many processes in the pharmaceutical industry. A comprehensive review has been published that addresses the use of CYPs as a bioreactor for the production of many different small molecules (Martinez und Rupasinghe 2013). The CYPs 2C9, 102 and 3A4 are prominent examples that are currently used in fermentation processes (Kumar 2010). The primary limitations of using CYPs as biocatalysts include low

recombinant synthesis yield and low enzyme activity, inadequate expression of CYP redox proteins, deficient cofactor recycling, and susceptibility to substrate and product inhibition and product degradation. Possible strategies to address these challenges involve optimizing the growth medium and bioreactor conditions, as well as implementing protein engineering approaches (Kumar 2010; Kumar et al. 2012).

1.4.5. Synthesis strategies for CYPs

Due to their vast range of potential applications, there is a high demand for the production of active CYPs, such as their use as enzyme reactors in research, for substrate screenings, or in the drug discovery process. The utilization of isolated microsomes derived from hepatocyte endoplasmic reticulum for CYP expression and analysis is hindered by the coexistence of various endogenous CYP variants, thereby impeding the specific analysis of individual CYP enzymes. In this context, cross-reactivity poses a particular challenge. This limitation necessitates alternative approaches to enable the focused examination of mono CYPs. Recombinant expression of CYPs has been extensively investigated (Hausjell et al. 2018). However, eukaryotic CYPs are particularly difficult to produce due to the complex co-factor requirements (the heme group) and the availability of a redox partner (CPR). Furthermore, CYPs are membrane-bound, which presents challenges for cell-based production. Nonetheless, the expression of different active CYPs in pro- and eukaryotic cells has been demonstrated multiple times (Durairaj und Li 2022). In many cases, the motivation was to gain insight into protein structure and function but also application oriented synthesis is becoming the aim of the synthesis of CYPs.

CYP expression in E. coli

E. coli is widely used for recombinant protein expression owing to its ease of handling and high growth rate. Nevertheless, synthesizing intricate eukaryotic proteins in *E. coli* presents a challenge due to the lack of inner organelles that anchor eukaryotic membrane proteins. Moreover, prokaryotic cells cannot perform most post-translational modifications. These challenges have been tackled by modifying various factors relevant to protein synthesis of CYPs. One approach involves modifying the hydrophobic N-terminus of CYPs with the sequence MALLLAVF(L), which has been attempted by several research groups (Hausjell et al. 2018). However, this method yielded a maximum of only 20 mg/L (approximately 350 nmol/L) of CYPs (CYP2D6 and CYP3A4) (Pan et al. 2011). with similar yields

obtained for other CYPs. An alternative strategy involves N-terminal deletion. However, different long truncations at the N-terminus resulted in similarly low yields (below 500 nmol/l) (Hausjell et al. 2018).

Typically, the redox partner CPR is co-expressed to enhance the yield of active CYPs. Less commonly, CYPs are expressed as fusion proteins with POR. Moreover, co-expressing the prokaryotic chaperones GrOES/EL increased the yield of active bacterial 304 P450 isoforms by supporting the folding mechanism (Ichinose und Wariishi 2013). Notably, the co-expression of an auxiliary protein usually results in lower CYP expression yields but a higher ratio of active enzyme. Since heme is essential for the catalytic function of CYPs, the heme precursor δ-ala-leuvenic acid is typically supplied to the growth medium. This has been demonstrated to be advantageous for increased expression of active CYPs (Faiq et al. 2014).

Apart from the addition of δ-ala-leuvenic acid during cell cultivation, adjustments to temperature, pH levels, translation inducer concentration, and dissolved oxygen concentrations have been made to achieve an acceptable CYP yield. By combining these optimizations, it is possible to produce around 1 µmol/L of any eukaryotic CYP in a prokaryotic cell-based expression system. The highest yield was achieved by Ichinose et al. (2013), who expressed the fungal CYPs CYP5037E1 and CYP5149A1 at a concentration of more than 2 µmol/L (Ichinose und Wariishi 2013).

1.4.6. CYP expression in yeasts

Eukaryotes, such as yeasts, have the capability of performing numerous post-translational modifications and possess specialized organelles such as the endoplasmic reticulum and the Golgi apparatus that are required for proper anchoring of eukaryotic membrane proteins such as CYPs. Consequently, the N-terminal anchor poses fewer problems in these organisms. Nonetheless, it is also possible to produce soluble CYPs in eukaryotic systems by modifying the N-terminus. Co-expression of auxiliary factors also plays a crucial role in the eukaryotic expression system. Although CPR is endogenously present in the yeast endoplasmic reticulum, co-overexpression of CPR is advantageous as it maintains the proportion of both proteins at an optimal level (Neunzig et al. 2013; Ohgiya et al. 1997; Hamann und Møller 2007; Ding et al. 2001).

There are limited studies on the production of CYPs in yeasts, with the most successful being the expression of CYP73A1 in S. cerevisiae, which achieved a maximum yield of 4 µg/mL (Schoch et al. 2003). Further optimizations for CYP production in eukaryotic systems have yet to be explored. Despite the lower protein yields, using a eukaryotic cell type for CYP expression may be advantageous for producing the enzymes in a more native state.

1.4.7. CYP expression in *Sf*21-cells and CHO-cells

In addition to yeast cells, several studies have also investigated the expression of CYPs in other eukaryotic cell lines, such as *Sf*21 and CHO cells (Lee et al. 1995) (Ding, S., et al. 1997) (Augustin et al. 2013). However, the main motivation behind these studies was not the isolation and characterization of the enzymes, but rather the effects of their overexpression in different cellular contexts. CYP3A4, which has a major impact on existing xenobiotics and is of high interest to both research and the pharmaceutical industry, was the focus of several studies (Kumar et al. 2012; Martinez und Rupasinghe 2013; Tompkins et al. 2010; Guengerich 1999; Guttman und Kerem 2022). CHO cells, in particular, are of great pharmaceutical relevance due to their similarity to human cells. Additionally, proteins produced in CHO cells have post-translational modifications that are more similar to those of native human proteins compared to proteins produced in lower eukaryotes.

1.4.8. Cell-free synthesis of CYPs

The efficient production of active CYPs through recombinant expression is a significant challenge that hinders their widespread application in different fields (Hausjell et al. 2018). Despite numerous attempts to improve protein yields in in vivo expression systems, the yields obtained are far from being profitable for industrial purposes, especially for the utilization of CYPs as bioreactors. CYPs are membrane-bound proteins that are typically difficult to express in *in vivo* expression systems. After protein synthesis, overexpressed membrane proteins are often found in inclusion bodies and are usually inactive (Rogl et al. 1998). Moreover, CYPs require co-factors, such as a heme group and an electron delivery system, to be active. These co-factors must be integrated into the overexpressing cells and must remain active even after cell-lysis and potential protein purification steps. In this case, the limitations of cell-based protein production systems have been reached. However, the utilization of a cell-free protein production platform can overcome the

challenges that hinder efficient CYP production (Thoring et al. 2016). In vitro protein production platforms are available that provide the necessary factors for gene transcription and protein translation. One such platform is a CFPS system based on CHO cell lysates, which enables gentle protein production without the need for harsh cell lysis after protein synthesis. Moreover, CHO cell lysates contain endogenous microsomes that facilitate the translocation of membrane proteins and all endoplasmic reticulum-associated post-translational modifications, such as partial N-glycosylation and disulfide bridges (Sachse et al. 2014). Compared to cell-based expression systems, the reaction chamber of CFPS is directly accessible, providing an open system that allows for straightforward addition of additional components to the reaction, such as heme or heme precursors (such as δ-aminolevulinic acid, glucose, glycine) and heme-producing enzymes, to achieve active CYPs (Kwon et al. 2013). It is possible to modify the cells utilized for lysate production, thereby allowing for the integration of CPR into the endogenous microsomes and creation of an ideal reaction environment for CYPs. Recent studies have demonstrated the transient modification of CHO cells with CPR for this purpose (Schulz et al. 2021). In a straightforward manner, the desired CYP enzyme can be synthesized in the translationally active modified CHO lysate. The utilization of genetically modified lysates, which already overexpress CPR, is now proposed for the cell-free synthesis of CYPs, potentially leading to a general improvement of the synthesis system. A possible synthesis process for these translationally active lysates is depicted in Figure 6. Subsequently, various screenings can be performed without the need for purification or processing steps, using 96 or 384-well plates. Additional general advantages and limitations of cell-free synthesis for drug development have been recently addressed (Dondapati et al. 2020). These advantages primarily encompass the site-directed incorporation of non-canonical amino acids and the facilitated targeted artificial post-translational modification of a protein. In addition to natural post-translational modifications such as glycosylation or lipidation, tags, linkers or fluorescent markers can also be incorporated at any desired positions within the protein (Zemella et al. 2018).

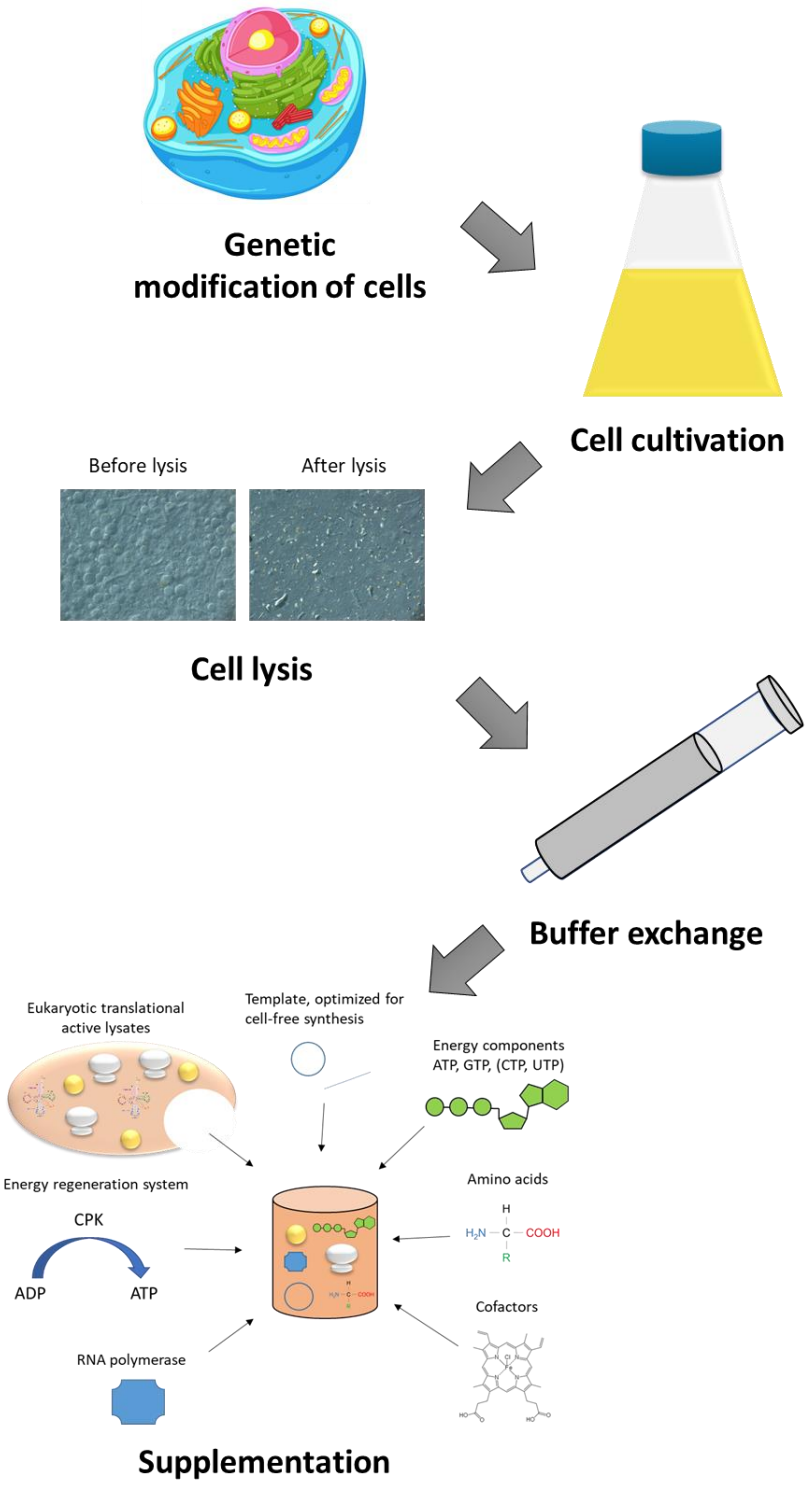


Figure 6: Schematic representation of the process for preparing a translationally active cell lysate, with various modifications that can be carried out during lysate production. The process requires specific modifications for the synthesis of different protein classes to achieve increased synthesis efficiency.

2. Aim of work

CFPS is a platform technology that enables rapid synthesis of a selected protein for various applications. The open system allows many adaptation of the synthesis parameters to a specific protein family to ensure reliable synthesis with efficient yields of functional proteins. The extent of these adaptations depends on both the protein class and the desired application methods. The aim of this study was to establish CFPS in translationally active eukaryotic cell lysates for various enzyme classes. For this purpose, model enzymes from three different classes were selected as a proof of concept. GH78 was selected for esterases, while multiple UPOs were selected for unspecific peroxidases and CYP1A2, CYP2B6, and CYP3A4 were selected for human monooxygenases. Indicators of successful synthesis of the target proteins was enzyme activity per volume of reaction mixture. In contrast to previous publications in the field of CFPS, where the target protein yield was the main indicator of successful cell-free synthesis, the focus in this study was on functionality, which can be quantified very well, especially with enzymes. Using standardized activity assays, functionality can be determined immediately after cell-free synthesis, without delay. This enables reliable comparison of different synthesis conditions, as well as modified or similar enzymes. Ultimately, this work aims to demonstrate new possibilities of applying CFPS in the context of various multifunctional enzymes.

3. Material and Methods

3.1. Material

Table 1: List of used chemicals, enzymes, cells and solutions.

Chemicals / Enzymes/ Cells /Solutions	Supplier
^{14}C-Leucine	Perkin Elmer, Inc.; Baesweiler, Germany
ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)	Merck KGaA; Darmstadt, Germany
Acetone, analytical grade	Carl Roth GmbH und CO. KG; Karlsruhe, Germany
Adenosine triphosphate (ATP)	Biotech rabbit, Hennigsdorf, Germany
Agar Agar	Roth GmbH & Co. KG, Nürnberg Germany
Agarose, low melting	Roth GmbH & Co. KG, Nürnberg Germany
Amino acids	Merck KGaA; Darmstadt, Germany
Bovine Serum Albumin (BSA)	Sigma-Aldrich GmbH; Taufkirchen, Germany

Blasticidin S -hydrochlorid	Merck KGaA; Darmstadt, Germany
Casein hydrolysate	Carl Roth GmbH und CO. KG; Karlsruhe, Germany
Caspase Inhibitor Z-VAD-FMK	Promega; Madison, USA
CHO-K1 cells (suspension cell line)	Franhofer IZI-BB, Potsdam, Germany
CHO-K1-CPR cells (suspension cell line)	AG Küpper, BTU Cottbus – Senftenberg, Germany
CHO-K1-CPR-CYP3A4 cells (suspension cell line)	AG Küpper, BTU Cottbus – Senftenberg, Germany
CYP3A4 (HL3): sc-53850 (antibody)	Santa Cruz Biotechnology, Inc., Dallas, Texas, USA
CYPOR (F-10): sc-25270 (antibody)	Santa Cruz Biotechnology, Inc., Dallas, Texas, USA
Dimethyl sulfoxide	Sigma-Aldrich GmbH; Taufkirchen, Germany
Dithiolthreitol (DTT)	Life Technologies GmbH; Carlsbad, USA
Ethylene diamine tetraacetic acid (EDTA)	Biomol GmbH; Hamburg, Germany

Efavirenz	Fisher Scientific GmbH, Schwerte, Germany
Gibco™ Human Microsomes, 50 Donors	Thermo Fisher Scientific, Waltham, Massachusetts, USA
Glutathione (GSH)	Roth GmbH & Co. KG, Nürnberg Germany
Glycine	Merck KGaA; Darmstadt, Germany
Goat anti-Mouse IgG (H+L) Secondary Antibody, HRP	Thermo Fisher Scientific, Waltham, Massachusetts, USA
Hemin (porcine), 97+%	Alfa Aesar, Haverhill, Massachusetts, USA
HotStar HiFidelity polymerase	Qiagen GmbH; Leipzig, Germany
Imidazole	Merck KGaA; Darmstadt, Germany
LB-medium (Lennox)	Roth GmbH & Co. KG, Nürnberg Germany
Magnesium acetate tetrahydrate	Merck KGaA; Darmstadt, Germany
Midazolam	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany

2-(N-morpholino)ethanesulfonic acid (MES)	Roth GmbH & Co. KG, Nürnberg Germany
Ni-NTA Magnetic beats	Qiagen GmbH; Leipzig, Germany
5-Nitro-1,3-benzodioxol ≥98%	Thermo Fisher Scientific, Waltham, Massachusetts, USA
Phenacetin	Fisher Scientific GmbH, Schwerte, Germany
Poly G	Sigma-Aldrich GmbH; Taufkirchen, Germany
Potassium acetate	Merck KGaA; Darmstadt, Germany
Sodium acetate (NaOAc)	Merck KGaA; Darmstadt, Germany
Sodium chloride (NaCl)	Merck KGaA; Darmstadt, Germany
Sodium dodecyl sulphate (SDS)	Serva Electrophoresis GmbH; Heidelberg, Germany
L-Alanyl-L-Glutamine	Sigma-Aldrich GmbH; Taufkirchen, Germany
T7 RNA polymerase	Agilent, Santa Clara, California, USA

Testosterone	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Trichloroacetic acid TCA	Roth GmbH & Co. KG, Nürnberg Germany
Trypsin/EDTA (0.05% / 0.02%)	Merck, Biochrome GmbH; Berlin, Germany
Tween-20	Sigma-Aldrich GmbH; Taufkirchen, Germany
UPO from *Agrocybe Aegerita*	AG Scheibner BTU Cottbus – Senftenberg, Germany
Zeocin selection reagent	Thermo Fisher Scientific, Inc.; Rockford, USA

Table 2: List of commercially received buffers and media

Buffer	**Supplier**
CutSmart Buffer (10x)	New England Biolabs GmbH; Frankfurt am Main, Germany
ECL-Western Blotting reagent	GE-Healthcare; Munich, Germany
Fast Digest Green Buffer (10x)	Thermo Fisher Scientific, Inc.; Rockford, USA
Hydroxyethylpiperazine ethane sulfonic acid (HEPES)	Roth GmbH & Co. KG, Nürnberg Germany

L(+)-Glutamine 200 mM 100X	VWR International GmbH, Darmstadt, Germany
LDS Sample Buffer (4 x), NuPAGE	Life Technologies GmbH; Carlsbad, USA
ProCHO 5 Protein-free CHO Medium	Lonza Group AG, Basel, Switzerland
SimplyBlue - SafeStain	Thermo Fisher Scientific, Inc.; Rockford, USA
Scintillation cocktail, quicksafe A	Zinsser Analytic GmbH; Frankfurt am Main, Germany
Serva DNA Stain Clear G	Serva Electrophoresis GmbH; Heidelberg, Germany

Table 3: Composition of buffers, solutions and media components

Buffers / Solutions	**Composition**
CD-TT-Mix (10x)	HEPES-KOH (pH 7.6, 300 mM), Mg(OAc)2 (39 mM), KOAc (2250 mM), Aminosäuren (je 1 mM), Spermidin (2,5 mM)
CD-EN-Mix (5x)	92.5 mM creatine phosphate, 8.75 mM ATP, 1.5 mM CTP, 1.5 mM GTP
IRES-EN-mix (5x)	100 mM creatine phosphate, 8.75 mM ATP, 1.5 mM CTP, 1.5 mM UTP, 1.5 mM GTP, 0.5 mM m7G(ppp)G-CAP1
LDS sample buffer (4x)	50 mM DTT, NuPAGE LDS Sample Buffer
Tris buffered saline (TBS) (10 x)	1 M Tris, 87.7 g sodium chloride

Tris buffered saline + Tween (TBST)	1x TBS, 0.1% (v/v) Tween-20
IRES-TT-mix (10x)	300 mM HEPES-KOH (pH 7.6), 2250 mM KOAc, 2.5 mM spermidine, 1 mM of the 20 standard amino acids each (Merck), 39 mM $Mg(OAc)_2$
IRES-TTG-mix (10x)	300 mM HEPES-KOH (pH 7.6), 2250 mM K(OGlu), 2.5 mM spermidine, 1 mM of the 20 standard amino acids each (Merck), 39 mM $Mg(OGlu)_2$
Refolding buffer A	50 mM Tris HCL, 0.6 M Guanidinium hydrochloride, 2 M Urea, 0.7 mM GSSG, 0.1 mM DTT, 10% Glycerol

Table 4: Kit list

Kit	**Supplier**
Cytochrome P450 Reductase Activity Assay Kit	Abcam, Cambridge, UK
ECL Plus Western Blotting Detection System	GE-Healthcare; Munich, Germany
iBlot Dry Blotting System	Thermo Fisher Scientific, Inc.; Rockford, USA
P450-Glo™ Assays	Promega, Madison, Wisconsin, USA

Plasmid-Prep Kit (Mini,Midi,Maxi)	Thermo Fisher Scientific, Inc.; Rockford, USA
Qiaquick PCR Purification kit	Qiagen GmbH; Leipzig, Germany
RTS 100 Wheat Germ CECF Kit	RiNA GmbH, Berlin Germany

Table 5: List of devices

Device	**Manufacturer**
Agarose gel imager (Gel iX Imager)	Intas Science Imaging Instruments GmbH; Göttingen, Germany
Agarose gel system (PerfectBlue Gelsystem)	Peqlab Biotechnologie GmbH; Erlangen, Germany
Amersham Typhoon RGB	GE-Healthcare; Munich, Germany
Centrifuge 5415 R	Eppendorf Wesseling-Berzdorf, Germany
Consort EV series power supplies	Merck, Biochrome GmbH; Berlin, Germany
GFL heating bath	GFL Gesellschaft für Labortechnik; Grossburgwedel, Germany

Flask Incubator 40/40R	Eppendorf; Wesseling-Berzdorf, Germany
Floor-standing clean bench Biowizard Standard	Kojair, Erlab, Cologne, Germany
IX53 Inverted Microscope	Olympus Co., Tokio, Japan
iBlot Gel Transfer System	Invitrogen; Carlsbad, USA
Ice machine Scotsman	Algeco GmbH, Kehl, Germany
Multitron Incubator Shaker	Infors Gmbh, Eisenbach, Germany
Magnetic stirrer RH basic 2	IKA-Werke GmbH & CO. KG, Freiburg, Germany
Microscope DM IL	Leica; Wetzlar, Germany
Microwave oven	Privileg, Bauknecht Hausgeräte GmbH, Stuttgart Germany
Nano drop 2000c spectrophotometer	Thermo Fisher Scientific, Inc.; Rockford, USA
pH-meter, WTW series pH720	Xylem Analytics Germany Sales GmbH & Co. KG, Weilheim, Germany
Photo scanner perfection 1200	EPSON Deutschland GmbH, Meerbusch, Germany
Platform orbital shaking device Unimax 1010	Heidolph Instruments GmbH & Co. KG; Schwabach, Germany

Precision Scale AZ214, M-POWER Series Analytical Balance 210 x 0.0001 g	Sartorius AG; Göttingen, Germany
Liquid Scintillation Counter Hidex 600SL	Hidex Deutschland Vertrieb GmbH, Mainz, Germany
SDS-electrophoresis system	Life technologies GmbH; Carlsbad, USA
SW 55 Ti Rotor Package, Swinging Bucket, Titanium, 6 x 5 mL, 55,000 rpm, 368,000 x g	Beckman Coulter GmbH; Krefeld, Germany
Thermomixer comfort	Eppendorf; Wesseling-Berzdorf, Germany
Thermomixer C-thermo top	Eppendorf; Wesseling-Berzdorf, Germany
Thermocycler (DNA Engine, PTC-200)	Bio-Rad Laboratories GmbH; Munich, Germany
TriStar LB 943 Multimode Micro Plate Reader	Berthold Technologies GmbH & Co.KG, Bad Wildbad, Germany
Unigeldryer 3545 D	UniEquip Laborgerätebau- und Vertriebs GmbH; Planegg, Germany
Ultrapure water system Arium (611 VF)	Sartorius AG; Göttingen, Germany
Vacuum filtration system (FH225V)	Hoefer, Inc.; Holliston, USA
Vibrax (VXR basic)	IKA Werke GmbH und Co. KG; Staufen, Germany

Vortex-Genie 2	Scientific Industries, Inc., New York, USA

Table 6: List of lab supplies

Lab supplies	**Manufacturer**
96-well tissue culture test plates	TPP; Trasadingen, Switzerland
Amicon Ultra-0.5 Centrifugal Filter Unit (30 kDa and 100 kDa cut-off)	Merck, Biochrome GmbH; Berlin, Germany
Blotting paper, 1.0 mm	Carl Roth GmbH und Co. KG; Karlsruhe, Germany
Cell culture TC-flask T75	Sarstedt; Nümbrecht, Deutschland
Centrifugation tubes (15 mL / 50 mL)	Sarstedt; Nümbrecht, Deutschland
Tube, Thinwall, Ultra-Clear™, 5 mL, 13 x 51 mm	Beckman Coulter GmbH; Krefeld, Germany
Filter paper MN GF-3	Machery-Nagel GmbH & Co. KG, Düren Germany
Filter tips SafeSeal Professional	Biozym Scientific GmbH; Hessisch Oldendorf, Germany
Multipipette E3/E3x	Eppendorf; Wesseling-Berzdorf, Germany
PC Erlenmeyer Shaker Flasks	VWR International GmbH, Darmstadt, Germany

Powerpette Pro Pipet Filler pipettor	VWR International GmbH, Darmstadt, Germany
Precast gels (NuPAGE®, 10% Bis-Tris Gel with MES SDS buffer)	Life Technologies GmbH; Carlsbad, USA
Serological pipettes	Sarstedt; Nümbrecht, Deutschland
Storage Phosphor Screen	GE-Healthcare; Munich, Germany
Scintillation vials	Zinsser Analytic GmbH; Frankfurt am Main, Germany
Thoma cell counting chamber (0.1 mm deep, C-Square: 0.0025 mm^2)	Marienfeld Superior; Lauda-Königshofen, Germany
Two chamber dialysis devices	Biotech rabbit, Hennigsdorf, Germany
Tubes (1.5 mL / 2 mL)	Eppendorf; Wesseling-Berzdorf, Germany

Table 7: List of used software and online tools

Software/online tool	**Supplier**
BioDoc Analyze (Agarose Gel Imager)	Biometra; Göttingenn, Germany
Clone Manager 8	Scientific & Educational Software, Sci-Ed; Cary, USA
Geneius	Eurofins Genomics Europe Shared Services GmbH, Ebersberg, Germany

Mikrowin 2000	Berthold Technologies GmbH & Co. KG; Bad Wildbad, Germany
NanoDrop 2000c Software	Thermo Fisher Scientific, Inc.; Rockford, USA
Origin	OriginLab cooperation, Northampton, USA)

3.2. Methods

3.2.1. Template generation

Templates for the synthesis of GH78 (amino acid sequence in the appendix) in cell-free systems were manufactured by Biocat GmbH (Heidelberg, Germany). The protein encoding sequence was integrated in a pUC57-1.8 k-vector backbone comprising further regulatory factors for CAP-independent protein synthesis using a CrPV-IRES (Brödel et al. 2013). Templates for protein synthesis in WG lysates were generated through Gibson assembly. For this purpose, the protein coding sequence was extracted from the pUC57 vector and cloned in a pIVEX1.3 vector. Codon optimization was performed using the "geneius" online tool (https://www.geneius.de/GENEius). Based on the original DNA sequence (XPO), sequence optimization was performed for *Spodoptera frugiperda* (*Sf*21), *Cricetulus griseus* (CHO) and *Saccharomyces cerevisiae* (SCE). Codon optimizations consider the availability of translation components in the respective organism, like tRNA and aminoacyl-tRNA synthetases. While endogenous synthetases from the lysates (*Sf*21 and CHO) are used during CFPS tRNAs originating from S. cerevisiae are supplemented to the lysates. Since the limiting factor is unknown, codon optimizations to all three organisms were performed.

3.2.2. Lysate preparation

Lysate preparation of *Sf*21 and CHO lysates was performed as described earlier (Brödel et al. 2014; Stech et al. 2012). CHO cells were grown exponentially in suspension cultures in well-controlled fermenters at 37 °C using the ProCHO 5 serum-free medium (Lonza). *Sf*21 cells were grown in fermenters at 27 °C in an

animal component-free insect cell medium. Cells were harvested at a density of approximately 1-4x10^6 cells/mL. Collected cells were centrifuged at 200 x g for 10 min and washed once with a buffer consisting of 40 mM HEPES-KOH (pH 7.5), 100 mM NaOAc (CHO)/100 mM KOAc (*Sf*21), and 4 mM DTT. The cell pellet was resuspended to achieve a density of approximately 1-5 x 10^8 cells/mL. Cell disruption was accomplished by syringing the harvested cell pellet through a 20-gauge needle, followed by centrifugation at 10.000xg for 10 min to remove the nuclei and cell debris. The supernatant was applied to a size-exclusion chromatography column (Sephadex G-25, GE Healthcare) and the elution fractions (each 1 ml) with an RNA content above an absorbance of above 100 at 260 nm were pooled. Residual mRNA was digested by micrococcal nuclease (S7) treatment. In this respect, 10 U/mL S7 nuclease (Roche) and 1 mM $CaCl_2$ (final concentration) were added to the eluate and the reaction mixture was incubated for 2 min (CHO)/ 10 min (*Sf*21) at room temperature. The reaction was inactivated by the addition of 6.7 mM EGTA (f.c.). Finally, translationally active lysates were immediately shock-frozen in liquid nitrogen and stored at -80 °C in order to preserve maximum activity.

3.2.3. Cell-free protein synthesis

Production of proteins in translationally active lysates derived from cultured insect cells (*Sf*21) and Chinese hamster ovary (CHO) cells was performed as previously described (Thoring und Kubick 2018; Stech et al. 2012) in batch based and continuous exchange cell-free (CECF) systems. A standard batch reaction mixture was composed of 40 % (v/v) processed cell lysate. Further standard supplements comprise HEPES-KOH (pH 7.6, f.c. 30 mM; BioMol GmbH, Hamburg, Germany), KOAc (f.c. 135 mM, Merck, Darmstadt, Germany), $Mg(OAc)_2$ (f.c. 3.9 mM, Merck) and amino acids (complete 100 µM, Merck), spermidine (f.c. 0.25 mM, Sigma-Aldrich, St. Louis, USA). Creatine phosphate (20 mM; Roche, Grenzach, Germany), ATP (f.c. 1.75 mM, biotechrabbit GmbH), CTP (f.c. 0.3 mM, biotechrabbit GmbH), GTP (f.c. 0.3 mM, biotechrabbit GmbH), UTP (f.c. 0.3 mM, biotechrabbit GmbH) and 0.1 mM (f.c.) m^7G(ppp)G (Prof. Edward Darzynkiewicz, Warsaw University, Poland) were added as energy resources to the reaction. PolyG (biomers.net GmbH , Ulm, Germany) primer was supplemented to the reaction at a final concentration of 4.5 µM in the batch mode. Plasmid DNA (f.c. 60 ng/µl) and 1 U/µl T7 RNA polymerase (f.c.) (Agilent technologies, Waldbronn, Germany) were added to start the reaction. For the conformation of successful CFPS, ^{14}C-labeled leucine (f.c. 30

µM, specific radioactivity 46.15 dpm/pmol; Perkin Elmer, Baesweiler, Germany) was added to the reaction. In the course of the work KOAc and $Mg(OAc)_2$ were replaced by KGlu (Alfa Aesar GmbH & Co KG, Karlsruhe, Germany) and $Mg(Glu)_2$ (Alfa Aesar GmbH & Co KG) in a f.c. of 135 mM and 3.9 mM respectively, which has been observed to increase the enzymatic activity of GH78 in batch reactions. Batch reactions were incubated for 3 h and CECF reactions were incubated for 24 h at 600 rpm unless otherwise noted. A standard CECF reaction consisted of a reaction mix (50 µl) and a feeding mix (1000 µl). Reaction mix was composed like a batch reaction without $m^7G(ppp)G$ cap analogue. Sodium azide was supplemented to the reaction to prevent microbial growth during cell-free synthesis. The feeding mix contained HEPES-KOH, salts, amino acids and nucleotides in the same concentration as in the reaction mixture and 0.02 % of sodium azide. CECF synthesis was performed in a commercially available two chamber dialysis device (biotechrabbit GmbH) for 24 h and 600 rpm in a thermomixer (Eppendorf). Concentrations of 11 µM ^{14}C leucine (specific radioactivity 9.9 dpm/pmol) supplemented to the CECF reaction was to enable further qualitative and quantitative analysis of cell-free synthesized proteins.

Cell-free synthesis based on WG lysates were performed according to the RTS 100 Wheat Germ CECF Kit Manual (biotechrabbit GmbH). Reaction mixtures were incubated at 21 °C for 24 h at 900 rpm.

3.2.4. Protein yield quantification by TCA precipitation

To validate successful protein synthesis, radioactive labeling of the proteins with ^{14}C-leucine was performed which allows quantitative analysis through scintillation counting as described earlier (Stech et al. 2012). Triplicates of 3 µL aliquots were taken from the cell-free translation reaction mixture, the microsomal fraction and the supernatant, which were previously separated by centrifugation for 10 min at 16,000 x g. 3 ml 10 % TCA was added to the samples and heated at 80 °C for 15 min in a water bath. The samples were subsequently cooled down on ice for at least 30 min. Precipitated protein solution was sucked on a filter using a vacuum filtration system. Proteins retained on the surface of the filter paper were washed twice with 5 % TCA and twice with acetone. The filter papers were transferred to scintillation tubes, covered in 3 ml scintillation cocktail and incubated on an orbital shaker for 30 min. Quantification of radiolabeled proteins was performed by liquid scintillation

counting using a LS6500 Multi-Purpose scintillation counter. The received values from scintillation counting are measured in counts per minutes (cpm) which are subsequently converted to disintegrations per minute (dpm). Protein yields were calculated using the measured counts, the molecular weight of the synthesized protein, the specific radioactivity A_{spec} (Equation 1) and the total number of leucines were present in the target protein (Equation 2).

$$A_{spec} = \frac{c_{14C-Leu} \cdot A_{14C-Leu\ Stock}}{c_{total\ Leu}} \qquad \text{(Equation 1)}$$

$$concentration\left[\frac{\mu g}{ml}\right] = \frac{measured\ counts\left[\frac{dpm}{ml}\right] \cdot molecular\ weight\left[\frac{\mu g}{pmol}\right]}{A_{spec}\left[\frac{dpm}{pmol}\right] \cdot \#_{leucines\ in\ the\ protein}} \qquad \text{(Equation 2)}$$

3.2.5. Acetone precipitation and SDS-PAGE for qualitative protein determination

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography were used for the analysis of protein homogeneity and molecular weight in vitro translation products. Cell-free reactions were subjected to centrifugation at 16,000 x g for 10 minutes to fractionate the components. Aliquots of 5 µL from the translation mixture, supernatant, and microsomal fraction were diluted in 45 µL of water and precipitated with 150 µL of acetone at 4 °C for at least 15 minutes. The precipitated proteins were pelleted by centrifugation at 16,100 x g for 10 minutes at 4 °C. The protein pellets were dried for 1 hour at 45 °C and re-suspended in 40 µL of LDS sample buffer. The samples were loaded onto 10% SDS-PAGE precast gels and electrophoresed at 185 V for 35-45 minutes. The gels were stained for 1 hour using SimplyBlue - SafeStain and destained overnight in water. Subsequently, the gels were dried (Unigeldryer) for 70 minutes at 70 °C. The dried gels were placed on a phosphor screen for a minimum of 24 h, and radiolabeled proteins were visualized using a phosphor-imager system.

3.2.6. GH78 activity measurement

The α-L-rhamnosidase activity of cell-free synthesized GH78 was assayed photometrically using *p*-nitrophenyl α-L-rhamnopyranoside (*p*-NPRP; Sigma-Aldrich) as substrate (Gallego Custodio et al. 1996; Romero et al. 1985). Each well of a 96-well microtiter plate contained 2.5 mM *p*-NPRP in 50 mM sodium acetate buffer (pH 5.0) in an overall reaction volume of 100 µL. Dependent on the enzyme

synthesis yield, different volumes of the supernatant fraction were added to the substrate to start the reaction. The supernatant fraction contains the active cell-free synthesized GH78 enzymes. The reaction proceeded for 2 h at 37 °C (unless otherwise noted) and was stopped by adding 40 µL sodium carbonate solution (1 M) to each well (resulting in a pH around 11). Absorbance at 405 nm was determined using a microplate reader (Mithras² LB 943, Berthold). The amount of synthesized *p*-nitrophenol was calculated by using a calibration curve.

Enzymatic activity over time was determined using the above-described assay (with 100 µL *p*-NPRP) with stepwise stopping of the reaction after different time points. For kinetic measurements, different concentrations of *p*-nitrophenyl α-L-rhamnopyranoside (0.1-2.5 mM) were applied. After adding the enzyme, mixtures were incubated at 37 °C and the reactions were stopped after 30 to 120 minutes by adding 40 µL of 1 M sodium carbonate solution. Kinetic data (K_m, k_{cat}) were calculated by non-linear regression using the "Origin" software (OriginLab).

3.2.7. GH78 FLAG-Tag purification

Purification of GH78 was performed by FLAG-Tag protein capture using Anti-FLAG M2 Magnetic Beads (Sigma-Aldrich). 20 µL magnetic bead suspension (50%) was loaded onto a small column and washed twice with 200 µL TBS. A cell-free translation mixture containing the synthesized enzyme was centrifuged at 4 °C and 16.000 × g for 10 min. 500 µL of the supernatant were added to the agarose and incubated over night at 4 °C on a rotator. The binding supernatant (BSN) was removed and the agarose was washed five times with 500 µL TBS (W1-5). Subsequently, the agarose was incubated with 50 µL elution buffer (3 x FLAG-peptide, 100 ng/µL in TBS) two times (E1-2). Each fraction was collected individually and analyzed by autoradiography and scintillation counting. GH78 rhamnosidase activity was determined as described above.

3.2.8. GH78 FLAG-Tag immobilization and activity determination

The immobilization of GH78 was performed similar to the purification. 20 µL magnetic bead suspension (50 %) was loaded onto a small column and washed twice with 200 µL TBS. A cell-free translation mixture containing the synthesized enzyme was centrifuged at 4 °C and 16.000 × g for 10 min. 200 µL of the supernatant were added to the agarose and incubated over night at 4 °C on a rotator. The binding

supernatant (BSN) was removed and the agarose was washed three times with 500 µL TBS (W1-3). The TBS was depleted and the beads were incubated repeatedly with 100 µL TBS buffer containing 2.5 mM *p*-NPRP. 40 µL of a 1 M $NaCO_3$ solution was added to the obtained 100 µL fractions, which were then analyzed by measuring the absorbance at 405 nm with a microplate reader.

3.2.9. NBD assay

The assay was performed in a 96-well plate. The enzyme (UPO) was added to the wells along with the reaction buffer (Calcium phosphate, f.c. 50 mM, pH 7.0) and substrate 5-nitro-1,3-benzodioxol (NBD) (f.c. 0.5 mM). To initiate the reaction, hydrogen peroxide in a final concentration 0.003 % was applied. The absorbance at 425 nm was measured using a photometer after 5 minutes. The amount of substrate turnover was determined from the measured values using the specific absorption of the reaction product 4-nitrocatechol (ε_{425} = 9.700 M^{-1} cm^{-1}).

3.2.10. ABTS assay

The assay was conducted in a 96-well plate. The enzyme (UPO) was added to the wells along with the reaction buffer (disodiumhydrogenphosphate, f.c. 50 mM, Sodiumcitrate f.c. 25 mM, pH 5) and substrate 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (f.c. 0,3 mM). To initiate the reaction, hydrogen peroxide in a final concentration 0.003 % was applied. The absorbance at 420 nm was measured using a photometer after 5 minutes. The amount of substrate turnover was determined from the measured values using the specific absorption of the reaction product $ABTS^{\cdot+}$ (ε_{420}=36 mM^{-1} cm^{-1}).

3.2.11. Western blot

Western blotting and subsequent antibody detection were used for the identification of endogenous and de novo synthesized CYP3A4 and CPR in the translation mixture of the cell-free synthesis reaction. SDS-PAGE was performed like described above. Proteins were blotted on a PVDF membrane with an iBlot device. The membrane was washed three times with TBS and subsequently blocked with 2 % Bovine Serum Albumin (BSA) over night at 4 °C. After three washing steps with TBS/T, the membrane was incubated with the primary antibody at a concentration of 0.4 µg/mL in 2 % BSA for three hours at room temperature. The blot was washed three times with TBS/T and incubated with a secondary Horse Radish Peroxidase (HRP) linked

antibody at a final concentration of 0.5 µg/mL in 2 % BSA at room temperature for one hour. Three final washing steps in TBS/T were performed. Chemiluminescent signals were detected after incubation with ECL detection reagent. The primary antibody used for the detection of CPR was “CYPOR (F-10): sc-25270” (Santa Cruz Biotechnology), the primary antibody used for the detection of CYP3A4 was “CYP3A4 (HL3): sc-53850” (Santa Cruz Biotechnology, Dallas, Texas, USA).

3.2.12. Fluorescence Microscopy

Confocal laser scanning microscopy was used to analyze protein translocation. In preparation, the microsomal fraction was separated from the rest of the translation mixture as described above. 5 µL of the MF were diluted in 15 µL PBS and transferred on chambered Coverslips (ibidi GmbH), The samples were analyzed by confocal laser scanning microscopy using a LSM 510 Meta (Zeiss). Therefore, samples were excited with an argon laser at 488 nm, and the emission signals were recorded with a bandpass filter in the wavelength range from 505 nm to 550 nm. Photobleaching was performed using an argon laser at 488 nm with 100 % laser intensity. After photobleaching pictures were taken each minute for 14 minutes.

3.2.13. CPR activity assay

CPR activity was determined by “Cytochrome P450 Reductase Activity Assay Kit” (Abcam, Cambridge, UK) using the manufacturers protocol. The activity was determined directly in the translationally active lysate of wild-type (wt) CHO cells and CHO-CPR cells. Additionally, the microsomal fraction was isolated as described previously. The activity was quantified using a calibration curve that was generated with supplements supplied by the kit.

3.2.14. CYP activity assays

For CYP activity measurement, ”P450-Glo™ Assays” (Promega) were used. CYP1A2 activity was detected by Luciferase-ME turnover (V8772), CYP2B6 was detected by Luciferin-2B6 turnover (V8321) and CYP3A4 was detected by Luciferin-IPA turnover (V9001). The CYP reaction was performed according to the Promega “P450-Glo™ Assays” protocol except the CYP reaction time was prolonged to 1 h unless otherwise noted. The CYP reaction temperature was set at 37 °C. The NADPH Regeneration System (V9510) was used for the supply of NADPH during the assay. Three control approaches were performed, one with buffer control, one designed as

no template control and one control, using human liver cell microsomes (Gibco™ Human Microsomes, 50 Donors) (Thermo Fisher Scientific) as positive control. Human microsomes were tested at a final concentration of 0.4 mg/mL. If not otherwise noted 5 µL of the microsomal fraction of the cell-free reaction were applied as samples to the activity assay. For response condition adjustments, CYP activities were usually expressed as percentage of the highest CYP activity during the assay. For CYP activity quantification, a standard curve was prepared using beetle luciferin (Promega) according to the protocol.

3.2.15. Indirect substrate screening

Luciferase-based assays were also used as a preliminary screening procedure for the turnover of various potential CYP substrates. The substrates testosterone (Sigma-Aldrich Chemie GmbH), midazolam (Sigma-Aldrich Chemie GmbH), efavirenz (Fisher Scientific GmbH), and phenacetin (Fisher Scientific GmbH) were solved at a concentration of 3 mM in 100 % methanol. As a control for a sterol that is not known to be turned over by CYPs, cholesterol was prepared in the same way. Cell-free CYP synthesis and isolation of the microsomal fraction was performed as described above. The luciferase based CYP activity assay was performed using 5 µL of the microsomal fraction of the cell-free synthesis of CYP1A2, CYP2B6 and CYP3A4. A final concentration of 200 µM of the analyzed substrate was added to the CYP reaction in parallel to the specific luciferase substrate. A vehicle control with methanol was performed to exclude an influence of the solvent. Changes in the turnover of the luciferase product indicate an interaction of the test substrate with the tested CYP. Changes in luminescence signal were expressed in percentage with reference to the result from the batch without addition of a substrate.

3.2.16. Statistical analysis

The collected data, unless otherwise indicated, were obtained in triplicates. The standard deviation is represented using error bars. Excel Data Analysis tools were used for statistical analysis, especially to test for statistical significance between two independent samples. After F-test for variance, a variance corresponding t-test was performed for records that were normally distributed.

4. Results

4.1. Cell-free protein synthesis of GH78

4.1.1. Template design

CFPS in CHO and *Sf*21-based systems is a well-established process, with ample literature available on the cultivation of cells, template design, and successful translation of target proteins. However, the focus of this results section is on the specifics of synthesizing complex enzymes using eukaryotic-based cell-free systems. Since such systems are typically used only for specific applications like screenings, the first enzyme that was chosen for production was a biologically stable enzyme. A DNA template for GH78, a glycosidase hydrolase from *Xylaria polymorpha*, was constructed for translation-active lysates. The template included regulatory sequences such as IRES and untranslated N0 and C0 regions containing transcription regulatory sequences. The encoded region was codon-optimized to *Sf*21, with additional constructs using other codon optimizations as the work progressed. After the initial use of the templates for syntheses, the target protein was checked for successful synthesis, using radioactive ^{14}C-leucine added to the synthesis reaction during cell-free protein synthesis. The resulting proteins were detected via autoradiography using SDS-PAGE. Figure 7A shows the initial synthesis of GH78 (*Sf*21 codon optimized) in a CHO cell-based and an *Sf*21 cell-based translational active lysate. Figure 7B presents an autoradiogram of different GH78 constructs, selected based on a template screening by Stephanie Hahn during her related master's thesis ("Charakterisierung und Labeling zellfrei synthetisierter Enzyme aus den Pilzen *Xylaria polymorpha* und *Agrocybe aegerita*", S. Hahn, 2021, unpublished). The autoradiogram provides a qualitative indication of protein production success, with all templates producing a protein with a size of approximately 100 kDa, and a weak band at approximately 55 kDa in most constructs. A no-template-control (NTC) yielded no relevant background signal.

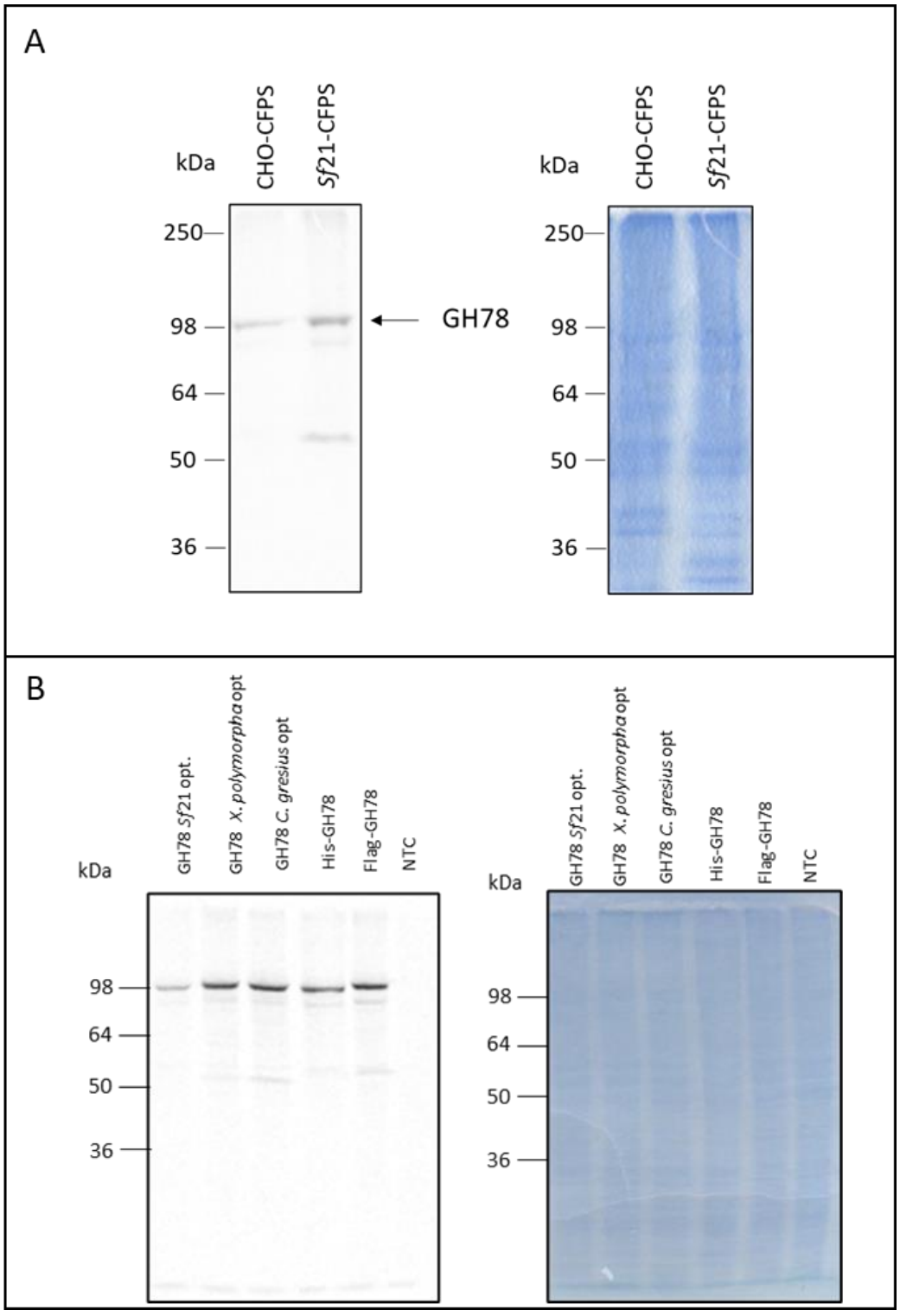


Figure 7: A) Qualitative analysis of GH78 synthesis in an in a CHO cell based and in a Sf21 cell-based B) Qualitative analysis of GH78 construct variants synthesized in a CHO lysate-based cell-free reaction. An autoradiography based on a 10% SDS-PAGE (right) visualizes the size of the ^{14}C-labeled proteins in the cell-free reaction. The respective utilized DNA template is labeled above the images. The autoradiograph (B) was performed by S. Hahn and is already published (Knauer et al. 2022) .

Based on the band intensity, it can already be assumed that the target protein quantity in the synthesis of the initial *Sf*21 codon optimized construct is lower than in the other constructs. Enzyme activity quantification is required to determine which of the generated templates results in the production of the highest volume activity of GH78.

GH78 is an enzyme whose functionality can be demonstrated relatively easily by means of a substrate conversion assay. GH78 is an α-L-rhamnosidase that catalyzes hydrolysis of ester bonds in rhamnose-containing glycosides (at the α-C atom). This allows the use of an artificial substrate for the quantification of the activity of the enzyme. When p-NPRP is hydrolyzed by GH78, rhamnose as well as para-nitrophenol are formed. Para-nitrophenol is a dye and can be detected using an optical spectroscope. This allows the amount of converted substrate to be detected and thus the relative activity of the synthesized enzyme to be determined.

In an initial experiment, the volumetric activity of GH78 synthesized with different templates (template sequences in the appendix) in parallel was determined. Prior to sampling for the assay, a portion of the sample was centrifuged in a reaction vessel at 16,000 x g for 10 minutes. This forms a pellet with the endogenous microsomes of the lysates in which, among other things, membrane-bound proteins are found. Since GH78 is a soluble protein that is not bound to the membrane and can be found in the supernatant fraction. 5 µL of a supernatant fraction of each sample was used for the activity assay. The amount of substrate reacted was calculated relative to the total amount of substrate in the assay and the different template for GH78 synthesis were compared (Figure 8).

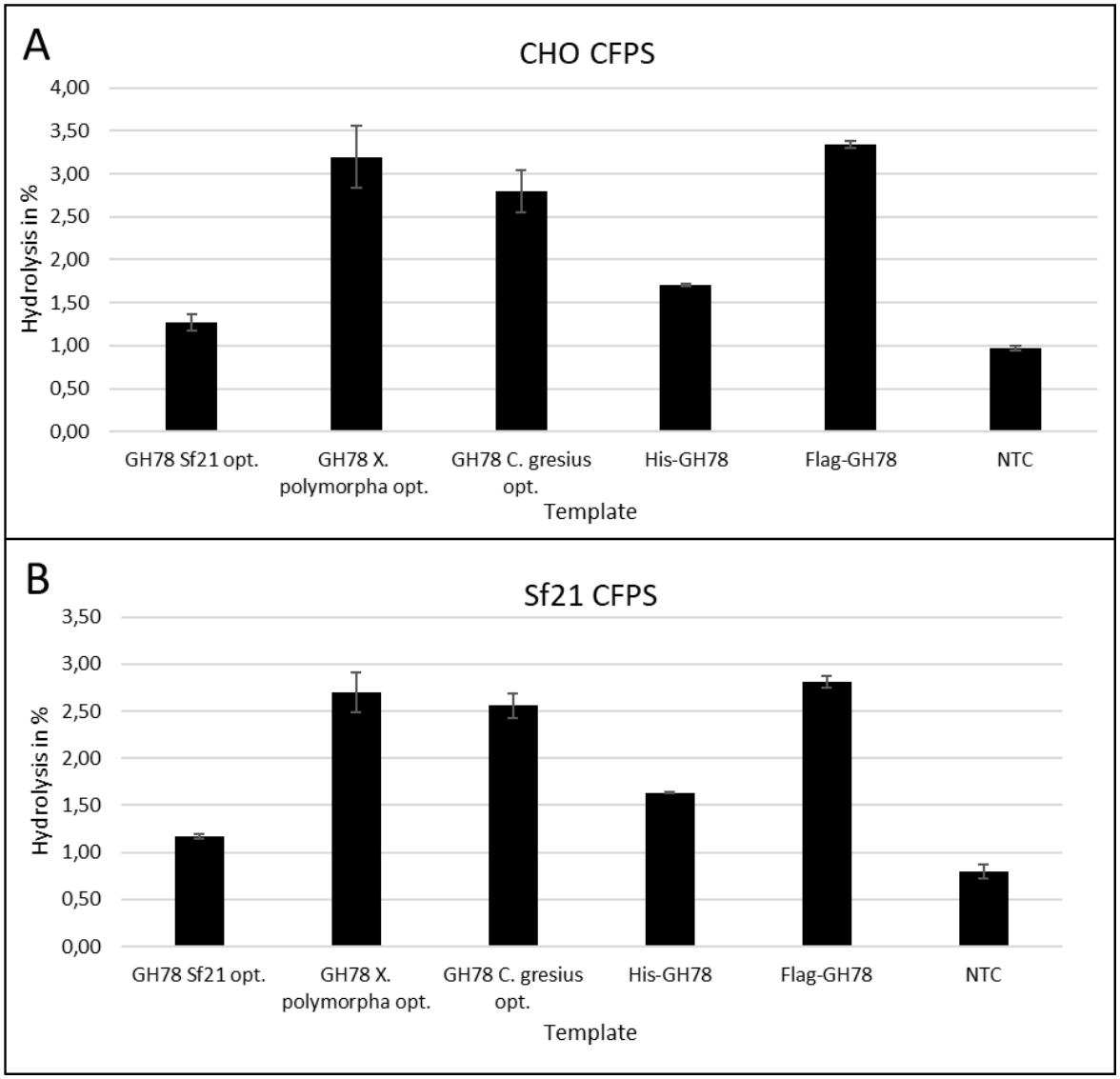


Figure 8: Enhancing synthesis efficiency by modifying the template for cell-free protein synthesis in a A) CHO and a B) Sf21 lysate-based cell-free protein synthesis system. p-NPRP assay of cell-free synthesized GH78 using the supernatant fraction compared to an NTC is depicted. Columns represent the percentage of hydrolyzed substrate after an incubation time of 2 h at 37 °C. Standard deviation was calculated from triplicates. The data are published (Knauer et al. 2022).

The assay revealed that the choice of template has a significant impact on the volume activity of GH78. The FLAG-tagged construct results in the highest absolute enzyme activity per volume of cell-free reaction mixture. This is almost three times higher than the initial construct used without TAG. Codon-optimized templates derived from *X. polymorpha* and *C. gresius* also resulted in relatively high volumetric activity of GH78. However, the standard codon optimization for eukaryotic systems

using *Sf*21 codon optimization led to one-third of the volumetric activity of GH78 observed with the FLAG-GH78 construct. In the *Sf*21 CFPS, the volume activity ratios obtained with different templates are quite similar to those observed in the CHO CFPS. The absolute volume activity is slightly lower with each template.

These initial results led to the utilization of the FLAG-GH78 template for the subsequent experiments. The template optimization will be revisited in chapter 4.1.3 when employing the CECF modification.

4.1.2. Reaction adjustments in batch synthesis

After initial synthesis, establishment of an activity assay and selection of a suitable template, various parameters were adapted in the cell-free reaction. For this purpose, concentrations of individual supplements were changed in the cell-free reaction in several preliminary experiments and the effect on the volume activity of the supernatant fraction was determined by activity assays carried out in parallel (master`s thesis, S. Hahn, unpublished). Furthermore, reaction parameters such as reaction time and reaction temperature were varied. In the *Sf*21 and CHO-based cell-free synthesis of GH78, the change in temperature and the use of potassium and magnesium glutamate led to an increase in volume activity.

Adjustment of protein synthesis temperature

GH78 was synthesized in a cell-free batch reaction at different temperatures. Yields in the translation supernatant as well as in the supernatant fraction and volume activity in the supernatant fraction were compared. Temperature matching was performed for the CHO based batch system (Figure 9) as well as the *Sf*21 based batch

system (Figure 10). For both systems, temperatures were selected in 3 K steps below the respective standard temperatures.

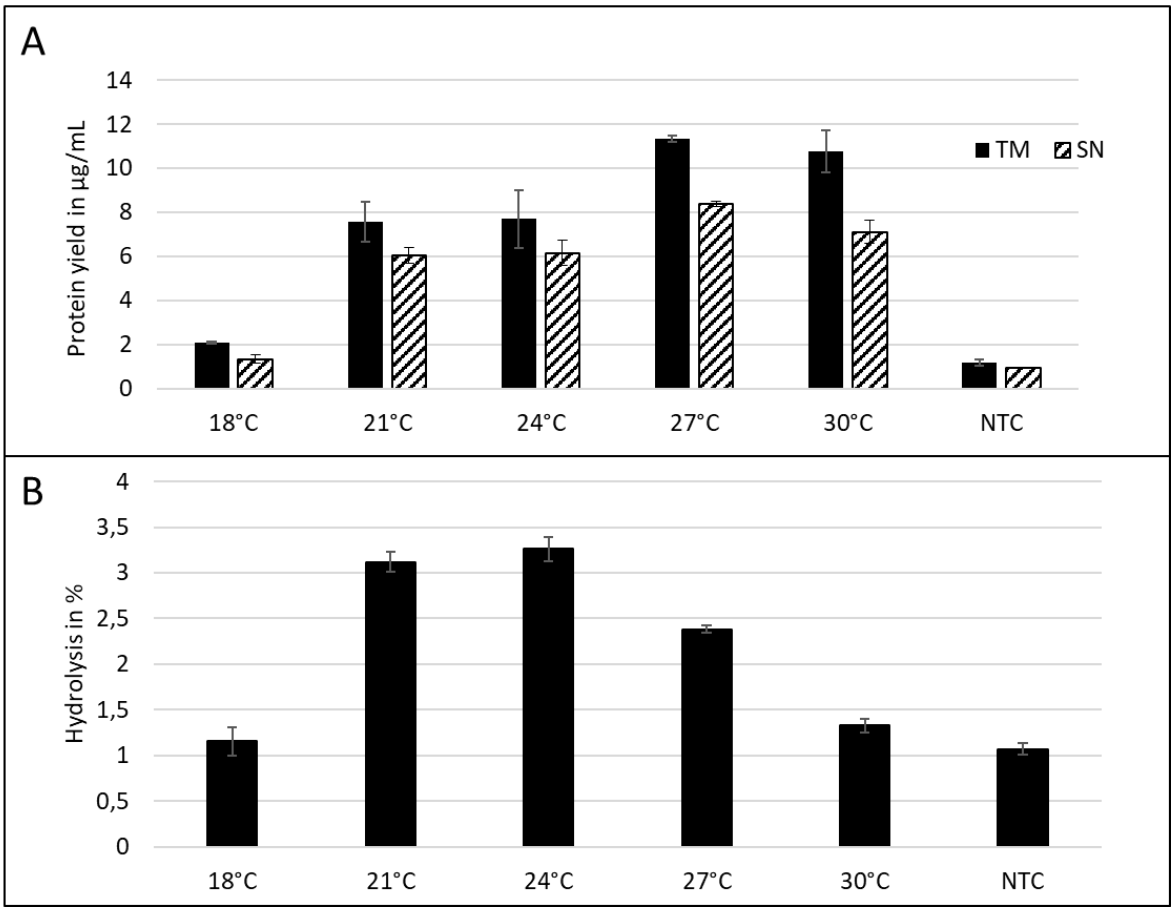


Figure 9: Comparison of GH78 yield and activity in CHO-lysate batch protein synthesis systems at different synthesis temperatures. a) Total protein yield determined by scintillation counting of GH78 produced under varying temperatures in the translation mixture (TM) and in the supernatant fraction (SN) after centrifugation at 16,000 × g. Standard deviation was calculated from triplicates. The NTC was performed at 24 °C. b) p-NPRP assay of cell-free synthesized GH78 using 3 µL of the supernatant fraction. Bars represent the percentage of hydrolyzed substrate after an incubation time of 2 h. The standard deviation was calculated from triplicates. The data are published (Knauer et al. 2022).

In the CHO-based cell-free system, a reduction in temperature results in a reduction in target protein yield in the translational mix. In the supernatant, the yield is highest at 27 °C at 8 µg/mL. Volume activity peaks at 24 °C and decreases as temperature increases and decreases.

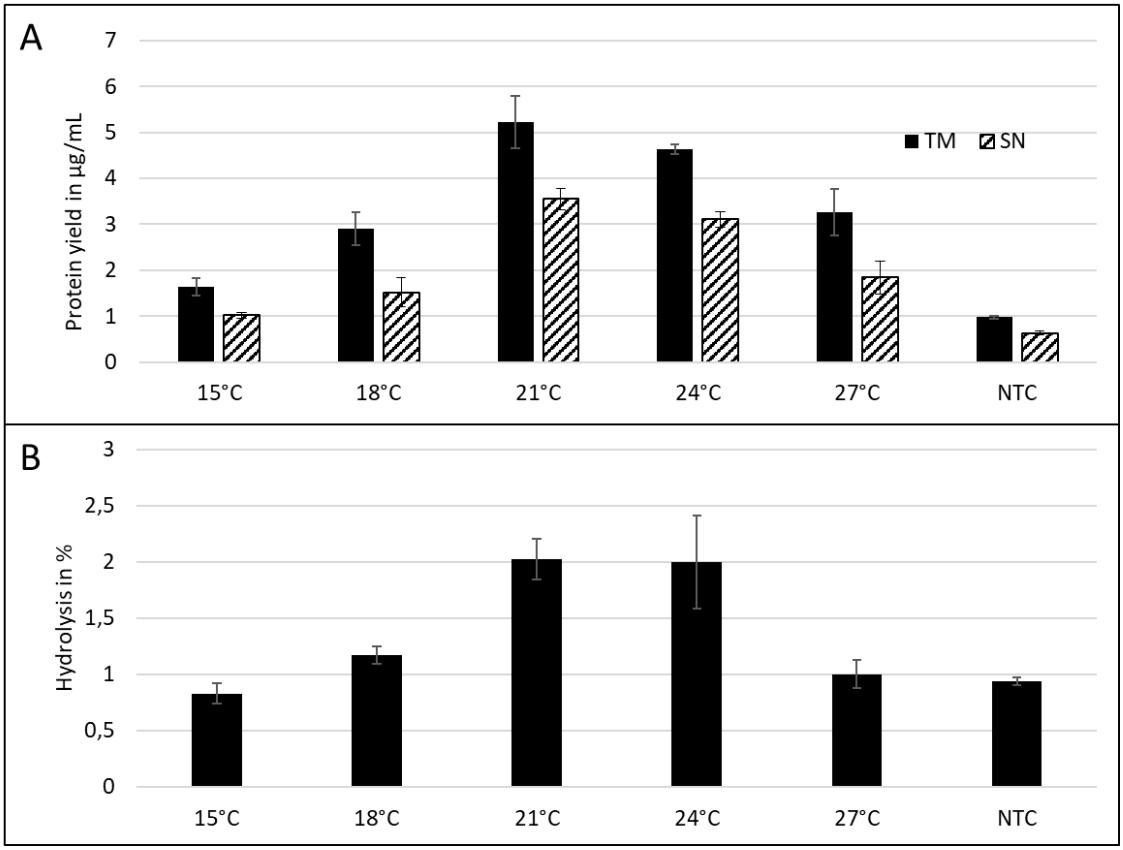


Figure 10: Comparison of GH78 yield and activity in Sf21-lysate batch protein synthesis systems at different synthesis temperatures. a) Total protein yield determined by scintillation counting of GH78 produced under varying temperatures in the translation mixture (TM) and in the supernatant fraction (SN) after centrifugation at 16,000 × g. Standard deviation was calculated from triplicates. The NTC was performed at 24 °C. b) p-NPRP assay of cell-free synthesized GH78 using 3 µL of the supernatant fraction. Bars represent the percentage of hydrolyzed substrate after an incubation time of 2 h. The standard deviation was calculated from triplicates. The data are published (Knauer et al. 2022).

For the *Sf*21-based system, the highest yield and volume activity can be measured between 21 °C and 24 °C. At higher and lower temperatures, there is a decrease in volume activity and target protein yield.

Exchange of the anions in CFPS

The exchange of potassium and magnesium acetate with potassium and magnesium glutamate had a strong effect on the *Sf*21 based batch synthesis of GH78 in preliminary experiments. Comparison of yields and volume activity when the anions were exchanged shows a doubling of volume activity in the *Sf*21 based system (Figure 11). In the CHO based system, the activity increases only slightly by exchanging the

anions. However, the yields of the target protein decrease significantly when the anions are exchanged in the CHO based cell-free synthesis in the supernatant fraction, whereas an increase in yield was measured for *Sf*21.

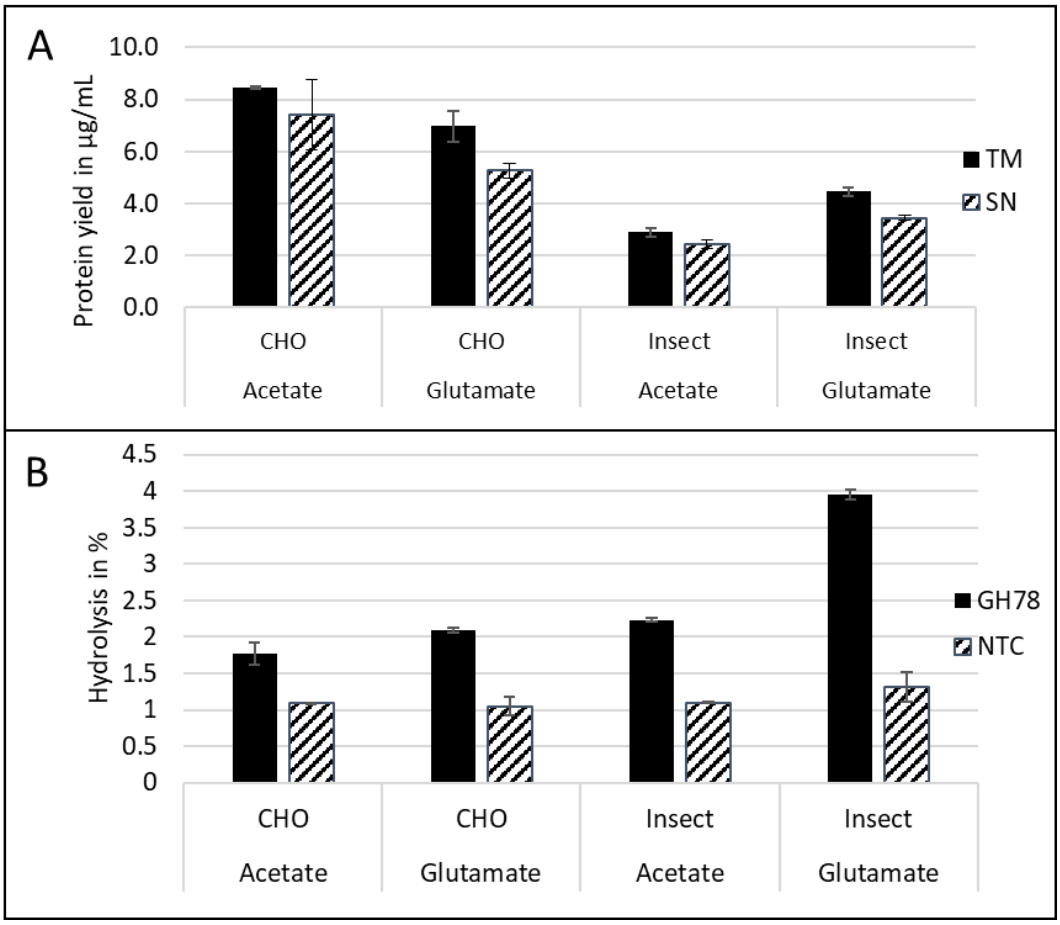


Figure 11: Comparison of GH78 yield and activity in CHO and Sf21 () cell-free protein synthesis systems using two different counter anions (acetate and glutamate). a) Total protein yield was determined by scintillation counting.in the translation mixture (TM) and in the supernatant fraction (SN) after centrifugation at 16,000 × g. b) p-NPRP assay of cell-free synthesized FLAG-GH78 using 5 µL of the supernatant fraction compared to an NTC. The bars represent the percentage of hydrolyzed substrate after an incubation time of 2 h at 37 °C. The data are published (Knauer et al. 2022).

4.1.3. Syntheses of GH78 in a continuous exchange cell-free protein synthesis format

Cell-free synthesis in batch format is usually limited to about 3 h reaction time. This is mainly due to by-products such as pyrophosphate, which are formed during protein synthesis. By utilizing a dialysis device, it is possible to extend the CFPS. For this purpose, certain devices are used in which the reaction chamber in which the

protein synthesis reaction takes place is connected to a feeding chamber via a dialysis membrane. The by-products diffuse across the membrane into the feeding chamber and are thus diluted in the reaction chamber. Thus, protein synthesis can be prolonged to up to 48 h. This also usually increases the target protein concentration in the translation mixes. Whether the CECF process also leads to an increased amount of functional protein varies from protein to protein and can only be determined in functionality assays.

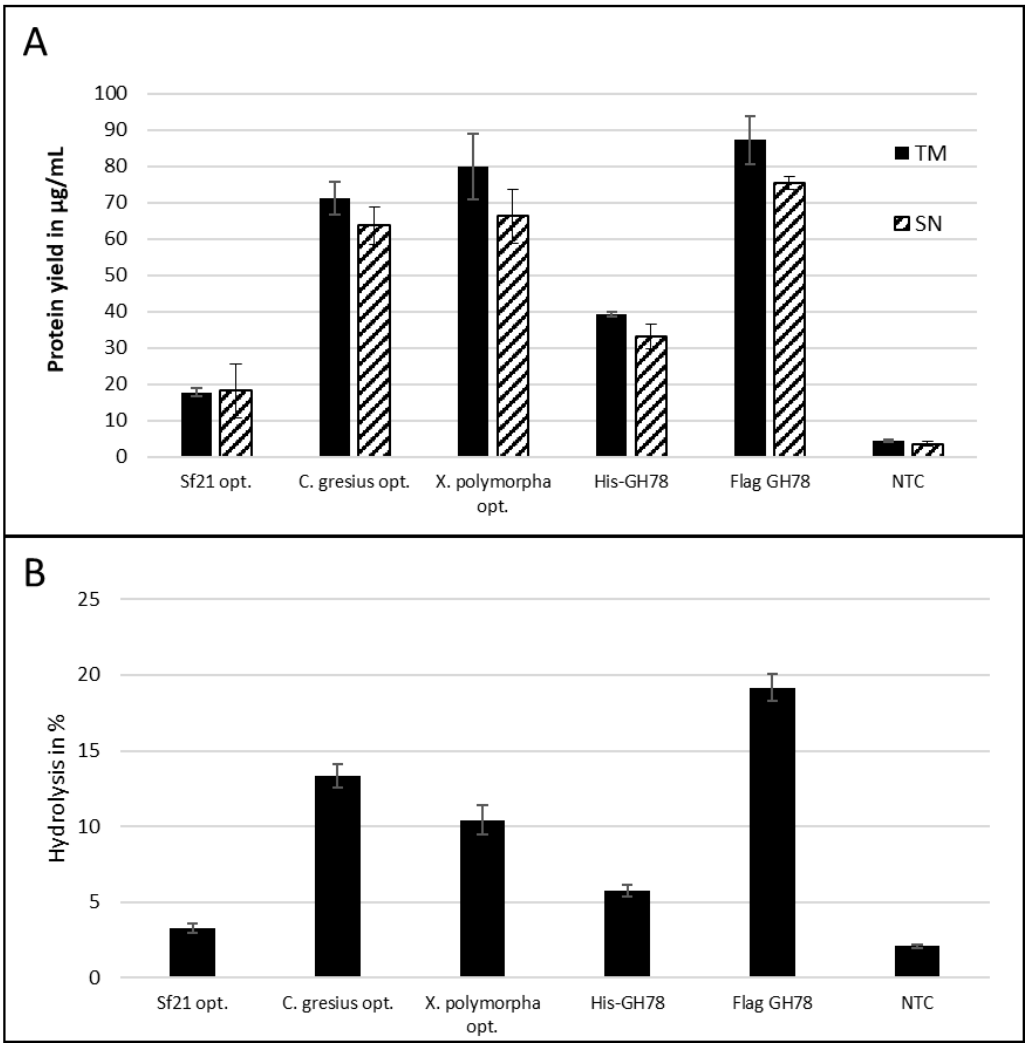


Figure 12: Enhancing synthesis efficiency by modifying the template for cell-free protein synthesis in a CHO cell-free protein synthesis system. GH78 yield determination by scintillation counting in the translation mixture (TM) and in the supernatant fraction (SN) after centrifugation at 16,000 × g in a CECF formatted system. The standard deviation was calculated from triplicates. p-NPRP assay of cell-free synthesized GH78 using the supernatant fraction compared to an NTC is depicted (B). Columns represent the percentage of hydrolyzed substrate after an incubation time of 2 h at 37 °C. Standard deviation was calculated from triplicates. The data are published (Knauer et al. 2022).

GH78 was synthesized in a CECF format using CHO lysates as well as *Sf*21 lysates and the yields and activities were determined. For the *Sf*21 system, the CECF synthesis showed only minor improvements in activity and yield compared to the batch format (data not shown). However, the volume activity of the enzyme in the CHO system is significantly higher than in the classical batch system. Again, the five different templates were compared (Figure 12). the FLAG GH78 achieved the highest volume activity as well as protein yield in comparison to the other generated templates. Compared to the synthesis in the batch system, the protein yields and volume activity increased by 900%. Consequently, there is no loss of relative enzyme activity by changing the synthesis format.

To complement the choice of a suitable template, another template was created that both has the FLAG TAG and was optimized for *C. gresius* codon. This codon-optimized FLAG-GH78 template did not result in increased protein yield or volume activity compared to the originally used FLAG-GH78 template. (Figure 13)

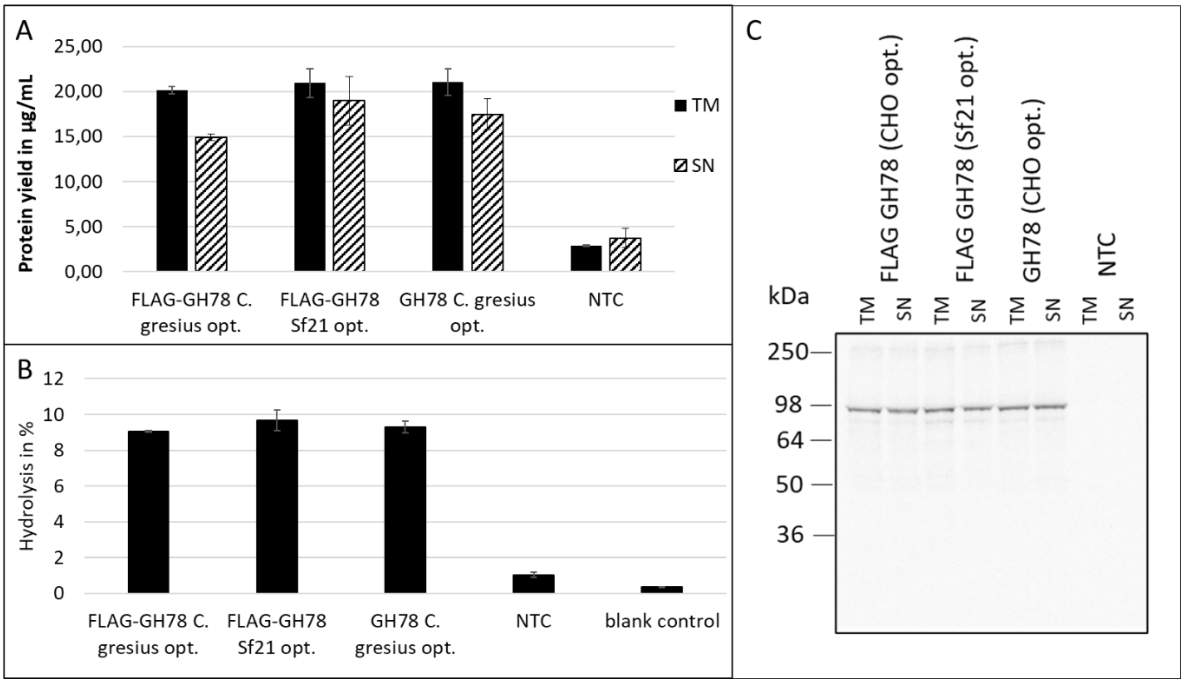


Figure 13: Template adaption of GH78. The templates FLAG-GH78 (C. gresius optimized), FLAG-GH78 (Sf21 optimized) and GH78 (CHO optimized) were compared in a CHO lysate based cell-free protein synthesis (CECF). A) Total protein yield determined by scintillation counting of GH78 produced with the three different templates in the translation mixture (TM) and in the supernatant fraction (SN) after centrifugation at 16,000 × g. A no template control (NTC) was performed. Standard deviation was calculated from triplicates. B) p-NPRP assay of cell-free synthesized GH78 using 3 µL of the supernatant fraction. Bars represent the percentage of hydrolyzed substrate after an incubation time of 2 h. The standard deviation was calculated from triplicates. C) Qualitative analysis of GH78 construct variants synthesized in a CHO lysate-based cell-free reaction. An autoradiography based on a 10% SDS-PAGE visualizes the size of the ^{14}C-labeled proteins in the cell-free reaction.

Adjustment of the synthesis temperature in CECF

Analogous to the batch format, the synthesis temperature was also adjusted for the CECF format (Figure 14). GH78 was synthesized at temperatures between 15 °C and 33 °C in CECF format.

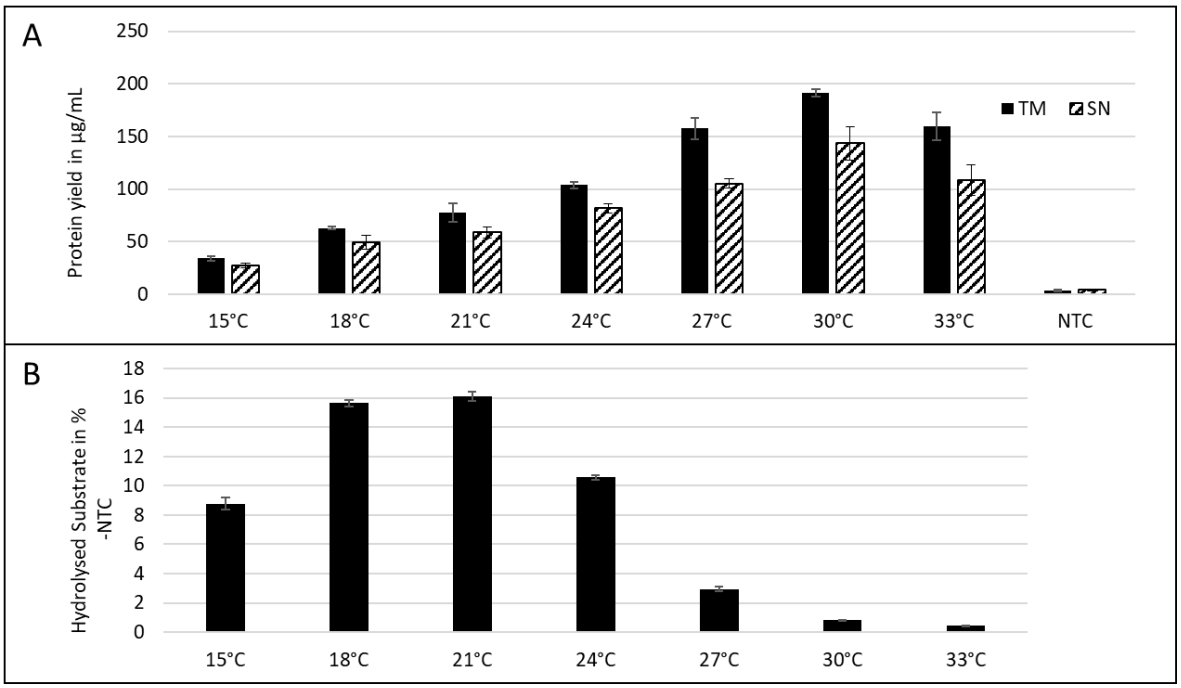


Figure 14: Comparison of GH78 yield and activity in CHO- CECF-formatted protein synthesis systems at different synthesis temperatures. A) Total protein yield determined by scintillation counting of GH78 produced under varying temperatures in the translation mixture (TM) and in the supernatant fraction (SN) after centrifugation at 16,000 × g. Standard deviation was calculated from triplicates. The NTC was performed at 24 °C. B) p-NPRP assay of cell-free synthesized GH78 using 3 µL of the supernatant fraction. Bars represent the percentage of hydrolyzed substrate after an incubation time of 2 h. The standard deviation was calculated from triplicates. The data are published (Knauer et al. 2022).

Similar to the batch format, the optimum of volume activity tends to be at lower temperatures between 18 °C- and 21 °C. However, the highest target protein yield can be achieved at the default temperature of 30 °C. The relative amount of soluble target protein decreases with increasing temperature.

4.1.4. The utilization of commercially available wheat germ lysates

In addition to the in-house-produced translationally active lysates, commercially available wheat germ (WG) lysates were used as a comparison for the production of GH78. However, a different DNA vector had to be used for this synthesis system, which limits the comparability of the results. Nevertheless, this system is suitable as a benchmark for the performance of eukaryotic cell-free systems (Table 8).

4.1.5. Determination of kinetic parameters

After optimization of the reaction parameters, the kinetic parameters of the cell-free synthesized GH78 were determined. For this purpose, several reactions were carried out under the same conditions with different substrate concentrations and the initial reaction rate was determined. The data were plotted on a Michaelis-Menton diagram (Figure 15). From the data, the Michaelis constant as well as the maximum rate can be determined by non-linear regression (Equation 3).

$$v_0 = \frac{v_{max} \cdot [S]}{K_m + [S]} \quad \text{(Equation 3)}$$

Enzymes produced in CHO lysates, *Sf*21 lysates and wheat germ lysates were compared. The wheat germ CFPS system is a commercial CFPS system which was used here as a comparison to the in-house animal cell-free systems.

To exclude any influence of the lysate on the turnover reaction, the supernatant fraction of the different syntheses was used in the same volume in the assay. The amount of enzyme present was determined by TCA precipitation and included in the calculation of the turnover number k_{cat} (Equation 4).

$$k_{cat} = \frac{v_{max}}{[E]} \quad \text{(Equation 4)}$$

Part of the data (CHO based CFPS) was recorded by Ms. Stephanie Hahn and is also included in her master's thesis. The complete data have also already been published in a journal (Knauer et al. 2022). The calculated kinetic benchmarks are summarized in Table 8.

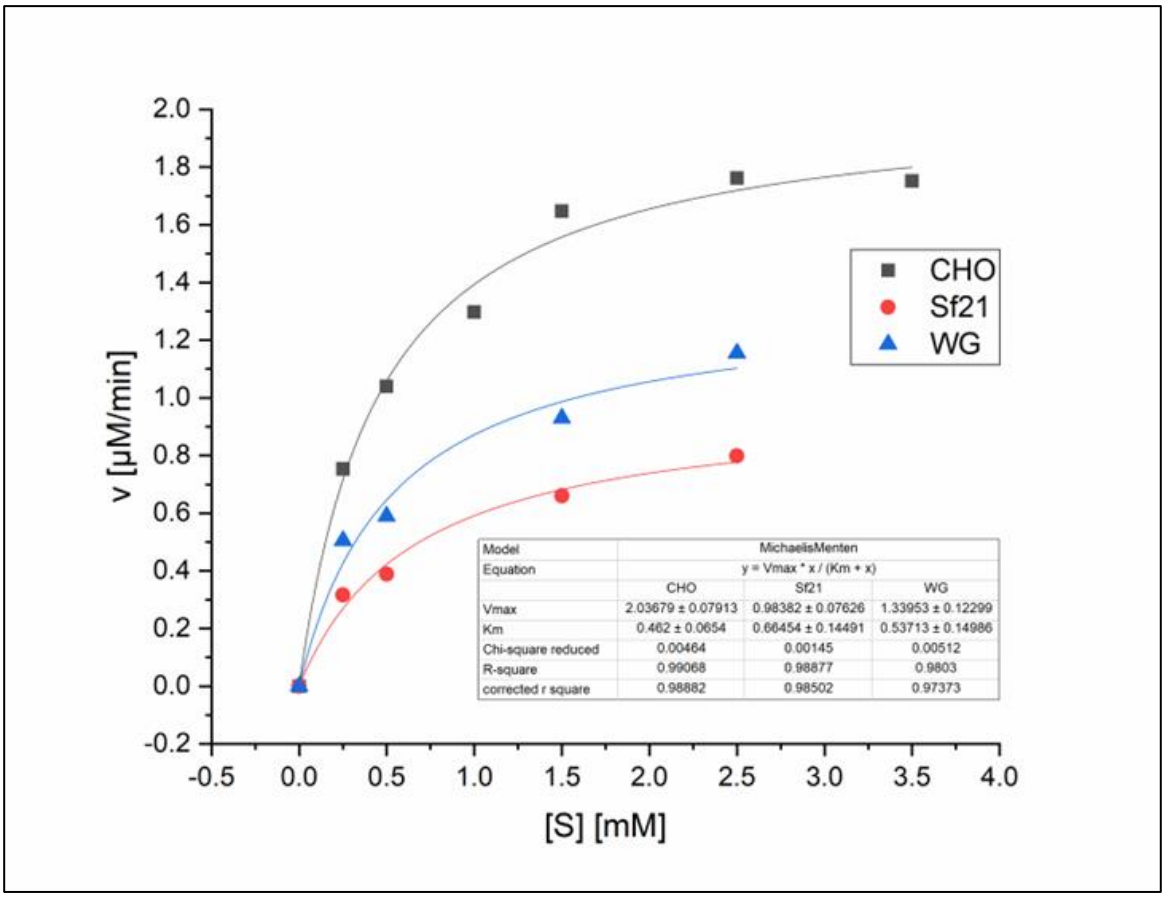


Figure 15: Michaelis Menton-Plots for GH78 produced in CHO, wheat germ and Sf21 CFPS. The plots served to estimate the kinetic constants for GH78 produced in the different eukaryotic lysate based CFPS. Kinetic constants were determined with "Origin 2019" via non-linear regression. Final enzyme concentrations in the assay were 3.7 nM (wheat germ), 1.7 nM (Sf21) and 6,6 nM (CHO). Target protein yield and start reaction velocity v for each substrate concentration was determined in triplicates. Data or CHO CFPS were collected by S. Hahn. Adapted from (Knauer et al. 2022)

The volume activity in the CHO cell-free system is higher than the synthesis in *Sf*21 lysates as well as the synthesis in wheat germ lysates. However, since the enzymes are present at different concentrations in the supernatant fractions, it is useful to compare the concentration-independent k_{cat} and K_m parameters. The GH78 prepared in *Sf*21 has both higher exchange number and catalytic efficiency than the GH78 enzymes prepared in CHO or wheat germ lysate-based cell-free syntheses (Table 8). However, the values of the catalytic efficiency (k_{cat}/K_m) are of the same order of magnitude and differ only slightly.

Table 8: Kinetik benchmarks of GH78 produced in different translational active lysates. Data of CHO CFPS were collected by Stephanie Hahn The data are published (Knauer et al. 2022).

Cell-free system	**Template**	**V_{max} [µM/min]**	**c [nM]**	**K_m [mM]**	**k_{cat} [s^{-1}]**	**k_{cat}/K_m [$mM^{-1} s^{-1}$]**
WG CECF	GH78-His pIVEX1.3	1.3 ± 0.1	3.7 ± 0.2	0.5 ± 0.1	6.0 ± 0.8	12 ± 4
***Sf*21 CECF**	GH78 pUC57-1.8k	0.98 ± 0.08	1.7 ± 0.1	0.7 ± 0.1	9.9 ± 0.9	14 ± 3
CHO CECF	FLAG-GH78 pUC57-1.8k	2.03 ± 0.08	6.6 ± 0.4	0.46 ± 0.07	5.1 ± 0.5	11 ± 3

Despite the higher catalytic efficiency of GH78 synthesized in *Sf*21 CFPS, the CHO CFPS system has a higher resource efficiency due to the higher synthesis efficiency. Therefore, the CHO CFPS system was further used for the following experiments.

4.1.6. Immobilization

Many applications for enzymes require that the enzyme used is immobilized on a surface. The use of affinity tags is one way to ensure this. With the help of the FLAG tag, which is bound as a fusion peptide to the recombinantly produced enzyme, GH78 was immobilized. For this purpose, magnetic beats were used, which were provided with anti-FLAG antibodies and are commercially available.

After cell-free synthesis in CECF format, the supernatant fraction was incubated overnight on the magnetic beads. The binding supernatant (BSN) was removed after incubation. Successful immobilization can be determined using TCA yield determination (Figure 16 A). The difference between target protein yield in the BSN and in the SN fraction suggests the amount of immobilized target protein. After subsequent washing steps, the immobilized enzyme can be identified on the magnetic beats using the NPRP activity assay. The activity assay can be repeated as

often as desired by repeatedly adding the substrate to the beads. In this case, the beads were incubated with the substrate ten times for 30 minutes. Supernatant was removed each time and the amount of substrate converted was determined (Figure 16 B). The substrate conversion initially increases slightly to 7% by the 3rd replicate, then slowly decreases to 5.5% by the 5th replicate, and then remains approximately constant. The cell-free synthesized enzyme is thus easy to immobilize and can be used several times without losing much activity.

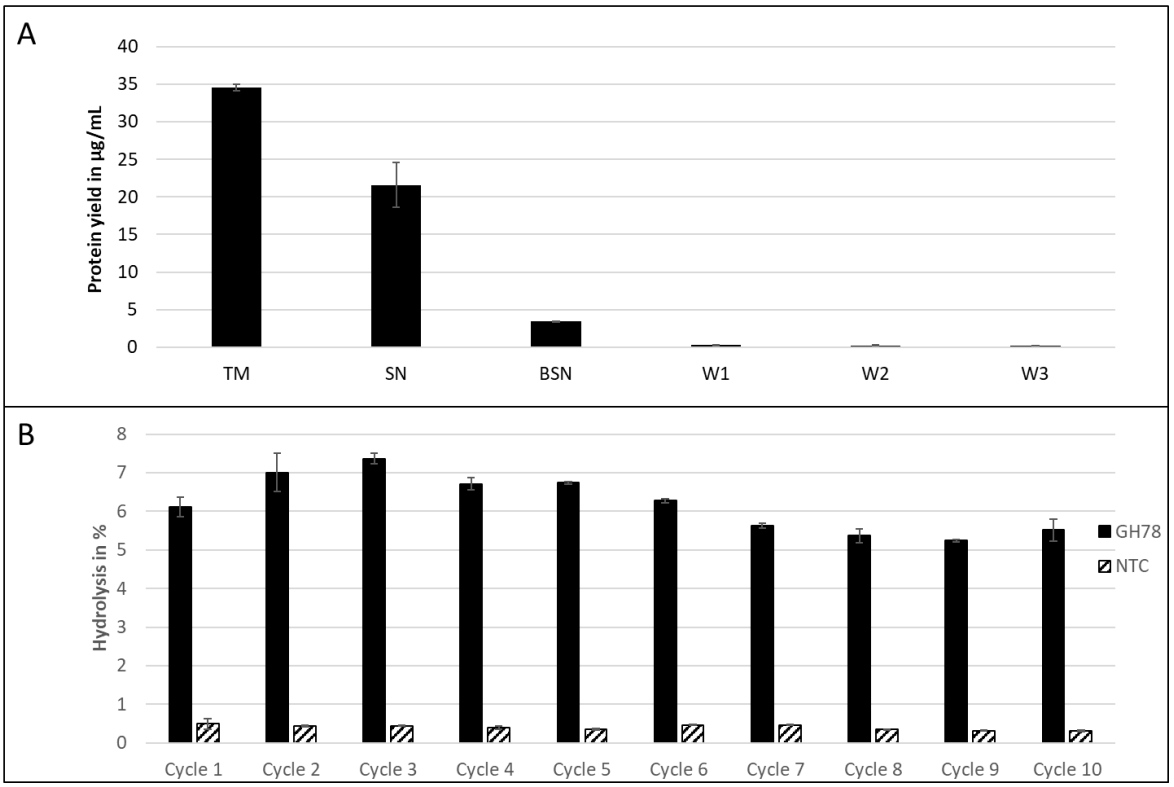
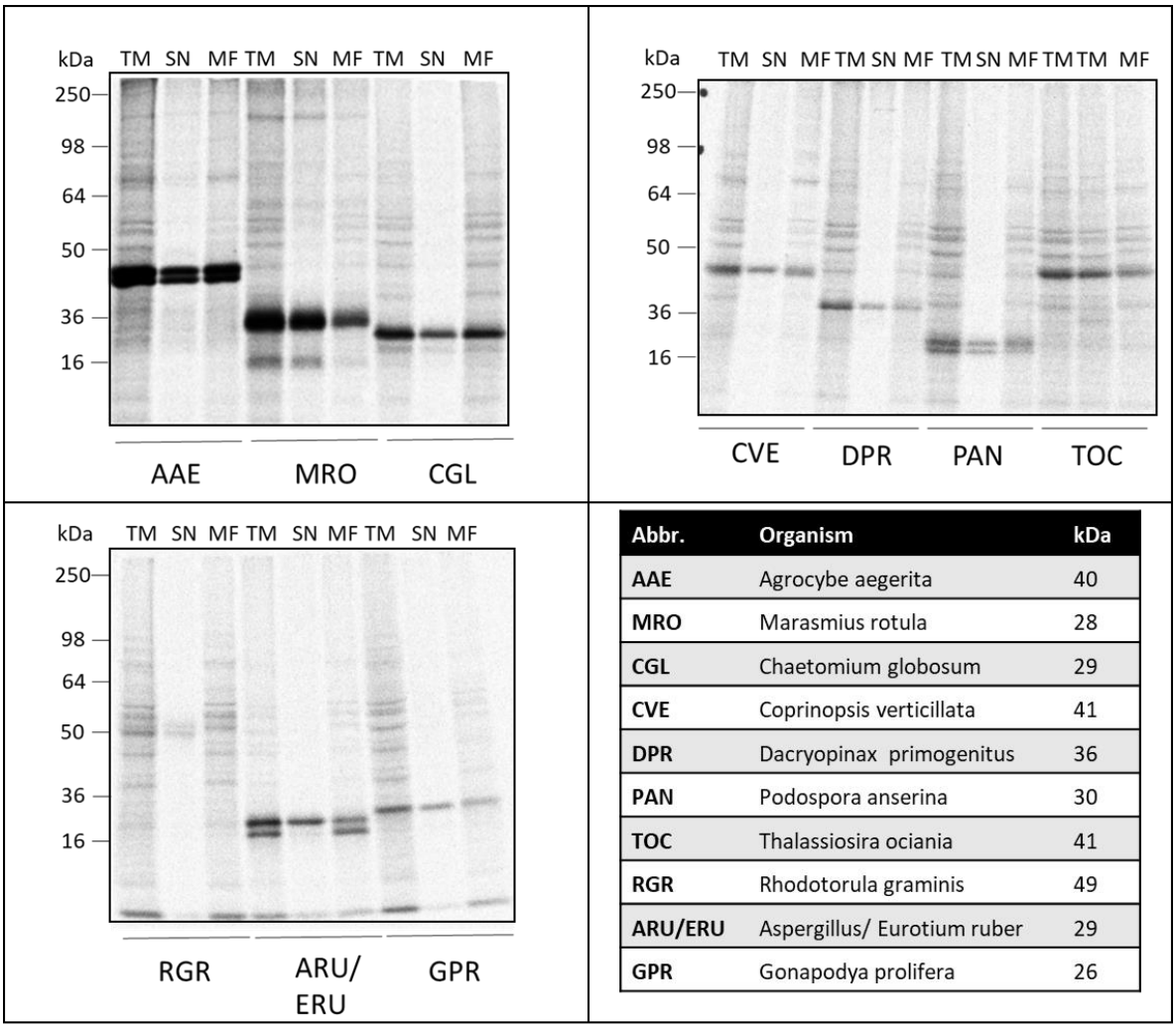


Figure 16: Immobilization/binding of FLAG-GH78 onto magnetic Anti-FLAG M2 beads. A) Scintillation results depicting the yield of GH78 in each fraction during immobilization and washing. Fractions include TM (translation mixture), SN (supernatant after 10 min centrifugation at 16,000 x g), BSN (binding supernatant), and W1-3 (washing fractions 1-3). Standard deviation was calculated from triplicates. B) Hydrolysis of immobilized GH78 using p-NPRP at room temperature. The reaction buffer was ten times exchanged and incubated for 15 min with magnetic Anti-FLAG M2 beads at a time. Values from the no-template control were subtracted from those received from immobilized GH78.

4.2. Cell free synthesis of UPOs

After successful cell-free synthesis and subsequent characterization of a fungal enzyme, the knowledge gained will also be used for other enzymes. Monooxygenases are of particular interest here. These include non-specific peroxygenases (UPOs) from various fungi as well as cytochromeP450, in particular from human hepatocytes. Templates of 10 different UPOs were designed, and three different activation assays were established.

Initial syntheses of 10 different UPOs were performed in the CHO lysate-based cell free system. Proteins produced in these processes were radiolabeled using ^{14}C-leucine. This allowed detection of the target proteins on an autoradiogram after SDS-PAGE. All UPOs could be detected in the expected size in the autoradiographs. In addition to CFPS, the CHO system allows translocation of proteins with signal sequence into system-specific microsomes. By fractionating the sample after the synthesis reaction, the microsomal fraction can be separated from the supernatant fraction (soluble components). In the autoradiographs, it can be seen for each UPO that part of the target protein is localized in the microsomal fraction (Figure 17).



Abbr.	Organism	kDa
AAE	Agrocybe aegerita	40
MRO	Marasmius rotula	28
CGL	Chaetomium globosum	29
CVE	Coprinopsis verticillata	41
DPR	Dacryopinax primogenitus	36
PAN	Podospora anserina	30
TOC	Thalassiosira ociania	41
RGR	Rhodotorula graminis	49
ARU/ERU	Aspergillus/ Eurotium ruber	29
GPR	Gonapodya prolifera	26

Figure 17: Qualitative analysis of cell-free UPO synthesis via SDS-PAGE based autoradiography. Ten different potential UPO sequences were used to generate templates for the cell-free synthesis of UPOs in translational active CHO lysates. After cell-free synthesis 5 µL of each fraction (Translation mixture, supernatant and microsomal fraction) of each reaction synthesis were applied to an SDS-PAGE. The synthesized proteins are visualized through radioactive labeling during the synthesis following autoradiography of the SDS-PAGEs.

The various UPOs were synthesized analogously to GH78 in both batch and CECF formats. AAE UPO is the most established member of the UPOs regarding recombinant synthesis. The synthesis of AAE UPO also resulted in the highest target protein yields in eucaryotic lysate-based cell-free protein synthesis. A yield comparison of CHO and *Sf*21 CECF system is shown exemplarily for all UPOs (Figure 18).

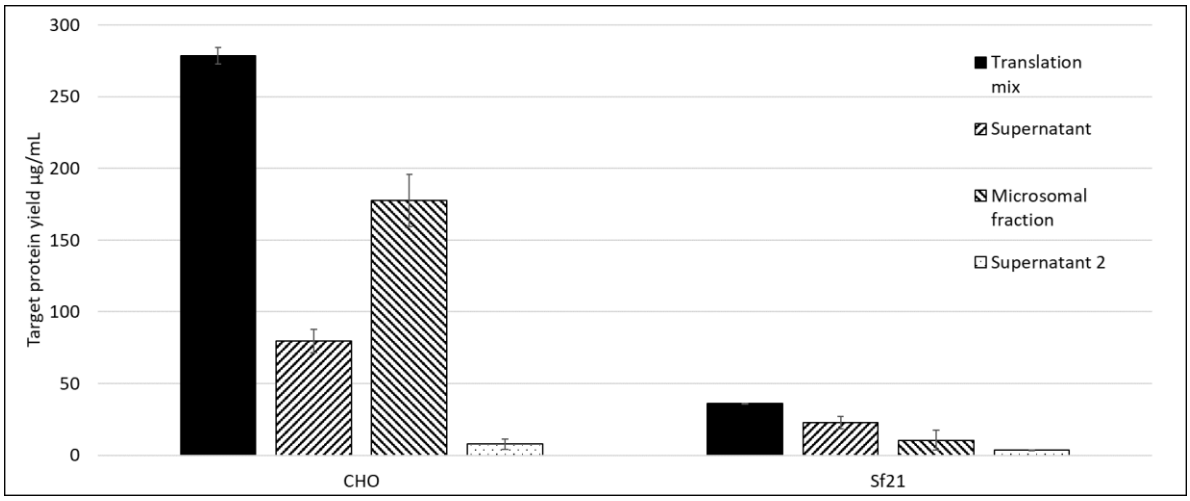


Figure 18: Protein yield of the AAE UPO protein after synthesis in a CHO and Sf21 continuous exchange cell-free synthesis. AAE UPO yield determination by scintillation counting in the translation mixture, in the microsomal fraction and in the supernatant fraction after centrifugation at 16,000 × g. After lysis of the endogenous microsomes for 20 min with Refolding buffer A, a second centrifugation step at 16,000 x g was performed to receive and quantify the target protein yield of the soluble content of the microsomes (supernatant 2). The standard deviation was calculated from triplicates.

In addition to target protein yield in translation mixture and supernatant also the yield in microsomal fraction and supernatant 2 were analyzed. The letter to fractions inherits produced enzyme that was translocated into the endogenous microsomes. The protein yields in the translation mix reached nearly 300 µM in the CHO-based system. However, a large proportion of these produced proteins were found to be insoluble in the microsomal fraction. On the other hand, a total of only 40 µM of target protein was produced in the *Sf*21 system, with approximately 25 µM present in the supernatant and about 15 µM in the microsomal fraction. In both synthesis systems, the yield in the supernatant 2 fraction was close to zero.

Activity assays for the UPO from *Agrocybe aegerita (AAE UPO)* were established (Figure 19) using naturally produced enzyme which was provided by cooperation partners (AG Scheibner, BTU CS). An NBD and an ABTS assay were chosen for detection of UPO activity. For both assays standard curves were generated to determine turnover rates of the substrates.

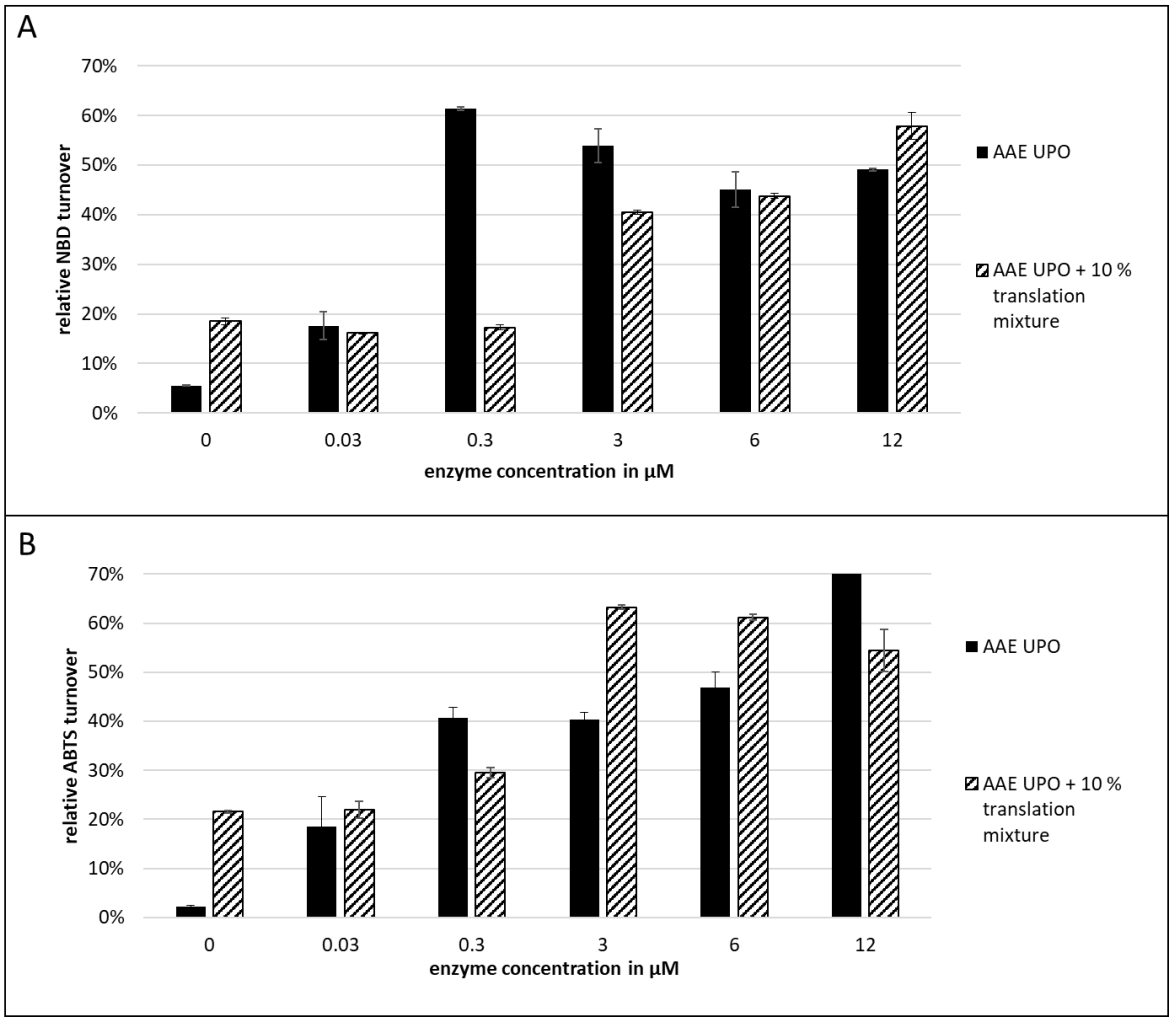


Figure 19: Effect of cell-free synthesis components on activity assays for UPO activity detection. The natural produced and purified Enzyme AAE UPO was used to determine the detection range in an NDB (A) and an ABTS (B) assay. Additionally, the detection range of an AAE UPO samples that were spiked into a translation mixture of a standard cell-free protein synthesis reaction was determined.

By applying different concentrations of the enzyme to the assay the detection range and especially the detection limit can be estimated. Additionally, the effect of the translation mixture on the detection limit of the activity assay was tested by spiking different concentrations of the naturally produced AAE UPO to a translation mixture of a standard CHO lysate-based cell-free reaction without template (NTC). While the detection limit for the wild-type enzyme in both assays is below 30 nM the detection limit for the spiked samples is between 30 nM and 300 nM in the ABTS assay. In the NBD assay the detection limit for spiked AAE UPO is even higher between 300 nM and 3 µM. The production of these concentrations of enzyme by cell-free protein synthesis only for performing an activity assay is very resource intensive. Multiple

attempts of buffer exchange and purification methods were not successful to detect active cell-free produced peroxygenases.

4.3. Cell-free synthesis of membrane-associated enzymes from the cytochrome P450 family.

4.3.1. Generation of a modified CHO-CPR Lysate

Modified CHO-K1 cell lines were generated and provided by cooperation partners (AG Küpper, BTU-CS). These cell lines are a CPR overexpressing (CHO-CPR),and a CPR and CYP3A4 overexpressing (CHO-CPR-CYP3A4) CHO-K1 cell line. Translationally active cell lysates were prepared from these modified cell lines. Therefore, modified CHO-K1 cell-lines were cultivated similar to wild-type CHO-K1 cells described earlier (Thoring et al. 2016). Zeocin (f.c. 300 µg/mL) and Blasticidin (f.c. 3 µg/mL) were used as selection antibiotics. A doubling rate of about 48 h of CHO-CPR and CHO-CPR-CYP3A4 compared to the wild-type cell line with a doubling rate of about 24 h slowed down the process but did not prevent the achievement of sufficiently high cell densities. The different cell-lines could all be harvested in the exponential growth phase. Typical growth conditions in the fermenter are shown exemplarily for CHO-CPR cells (Figure 20). After buffer exchange and supplementation of the raw lysate translational active lysates of the modified cell-lines were generated similar to the process of wild-type cell-lysate generation. With total target protein yields of around 40 µg/mL at optimal conditions, the protein translation in the modified lysates is in the same range as classical CHO based cell free protein synthesis. After validation of translational activity, the lysates from CHO-CPR cells and CHO-CPR-CYP3A4 cells were additionally analyzed for their CPR and CYP activity.

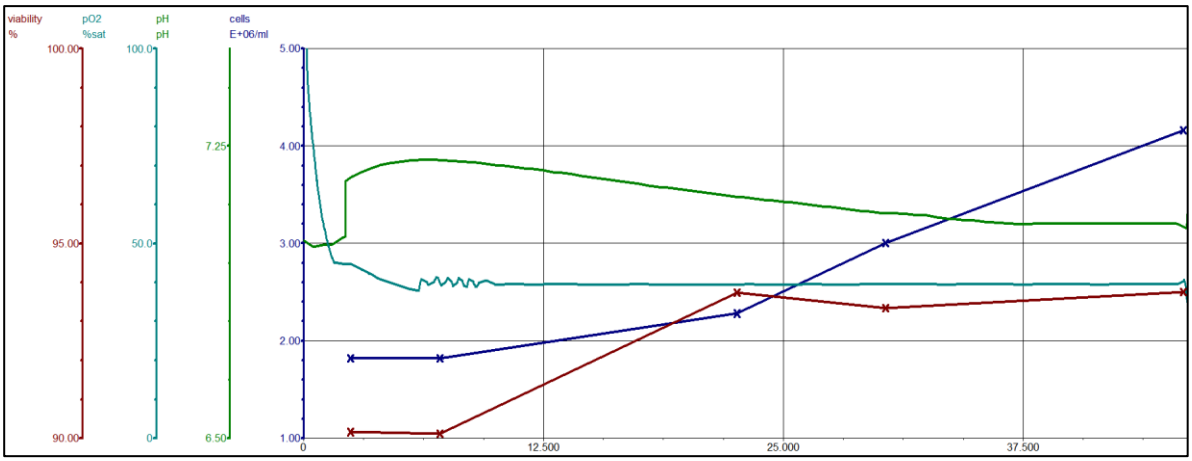


Figure 20: Growth of CHO-CPR cells in a 5 L fermenter. The different colored lines show the growth density (blue), the viability (red) the oxygen level (turquoise) and the pH (green) over the fermentation time of 48 h.

4.3.2. CPR activity validation of the generated CHO-CPR-lysates

Since CPR as CYP co-enzyme is mandatory for any CYP activity, CPR has to be active in the cell lysate derived from wild-type CHO cells. By engineering CHO K1 cells a more than threefold increased CPR activity could be detected (Figure 21 A).The processed lysates form CHO-K1 CPR cells were centrifuged to separate the endogenous microsomes from the soluble componence of the lysate at 16.000 x g for 10 minutes. The activity of CPR can be detected in particular in the microsomal fraction with about 90 % (Figure 21 B). Low CPR activity could also be measured in the supernatant fraction.

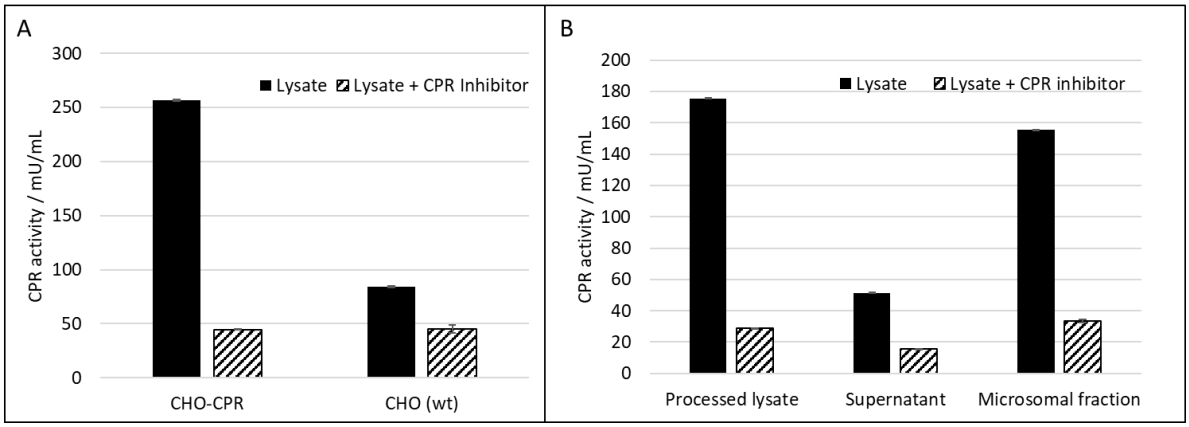


Figure 21: Validation of the CPR activity in the generated lysates by Cytochrome P450 reductase activity assay kit (colorimetric) (ab204704). The assay was performed according to the manufactures protocol. Diphenyleneiodonium chloride was used as inhibitor control. A) CHO-CPR cells were lysed and processed according to protocols for the generation of translationally active lysates. Two lysates were generated: CHO-K1 S CPR and CHO-K1 wt cell lysates. B) CHO-K1 S CPR lysate was fractionated by centrifugation at 16,000 x g for 10 minutes. CPR activity of the supernatant, the Microsomal (Pellet) fraction and the processed lysate (lysate before centrifugation) were compared. Standard deviations were calculated from triplicate analysis (n = 3)

To characterize the CPR activity of the lysates on CYPs further, cell-free synthesized CYP3A4 was produced in the translationally active lysates. CYP3A4 serves as model protein for the cell-free synthesis of CYPs.

4.3.3. Cell-free synthesis of CYP 3A4 in CHO-CPR lysates

CYP3A4 was produced in a batch-based cell-free synthesis in the CHO-CPR lysate, CHO-CPR-CYP3A4 lysates and wild-type CHO lysates. As a negative control a cell-free reaction without the addition of any DNA template (NTC) was performed. The presence of CPR and CYP3A4 in the translation mixture from each batch reaction was visualized via antibody detection on a western blot (Figure 22 A+B). In the anti-CPR western blot, well-defined bands are detectable at approximately 90 kDa. However, these are less prominent in the wild-type CHO lysate than in the modified lysates (Figure 22 A). A well-defined band at 60 kDa in the anti CYP3A4 western blot can be detected in any sample where CYP3A4 has been synthesized in a cell-free manner (Figure 22 B). In addition, a much weaker band at 60 kDa can be detected in the NTC of CHO-CPR-CYP3A4 lysates. Autoradiography was used to reveal the successful cell-free synthesis of CYP3A4 in the samples containing the template

(Figure 22 C). Similar to the anti-CYP3A4 western-blot well-defined band at the level of about 60 kDa are detected in the samples containing the DNA template. However, no bands in any NTC are observed here.

A CYP3A4 specific luminescent assay by Promega (Luminescent Assays and Screening Systems for Measuring CYP Activity) was performed for enzyme activity determination (Figure 22 D). The highest CYP3A4 activity was measured in the CHO-CPR Lysate after cell-free CYP3A4 synthesis followed by the CHO-CPR-CYP3A4 synthesis under the same condition. The CYP3A4 activity in the wild-type CHO lysate was threefold lower than in the CHO-CPR lysate. In the NTCs of the CHO-CPR and the wild-type CHO lysate, practically no CYP activity was detected. In the NTC of the CHO-CPR-CYP lysate low CYP3A4 activity could be detected similar to the wild-type CHO lysate after CYP3A4 synthesis.

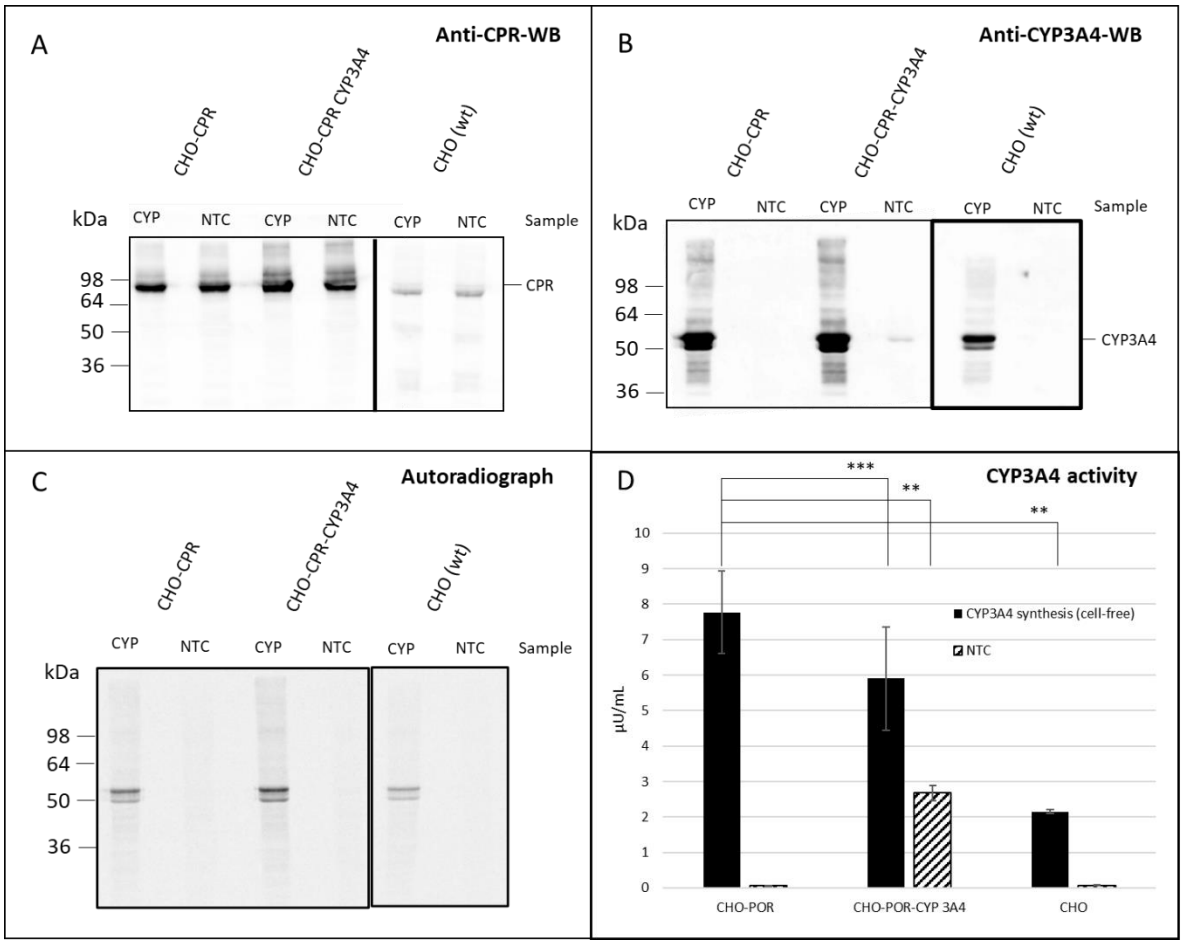


Figure 22: Characterization of translationally active CHO lysates derived from genetically modified CHO cells after cell-free synthesis of CYP3A4. CYP3A4 (57 kDa) and CPR (82 kDa) were identified in the translation mixture through SDS-PAGE (10 %) and subsequent western blotting with anti CYP3A4 antibodies (A) and CPR antibodies (B) followed by a secondary HRP linked antibody. Cell-free synthesis of CYP3A4 was compared with no-template-controls (NTC) in each sample. An autoradiograph (C) allows the detection of radioactive labeled cell-free synthesized proteins in the cell-free reaction. The activity of CYP3A4 in the different lysates was determined by an IPA-Luc assay (D) after CYP3A4 cell-free synthesis and in a no-template control (NTC). Standard deviations were calculated from triplicate analysis (n = 3)

4.3.4. Adaption of reaction conditions

Heme is a cofactor of CYPs and is therefore mandatory for its function. Adaption of the amount of supplemented heme to the cell free reaction is therefore necessary. Heme was supplemented in different concentrations to different batches of the cell-free reaction. The CYP activity in the microsomal fraction was determined by the Luciferase based CYP3A4 activity assay. A heme concentration of 5 µM resulted in

the highest CYP3A4 activity, which was more than twofold higher compared to the control without supplementation (Figure 23).

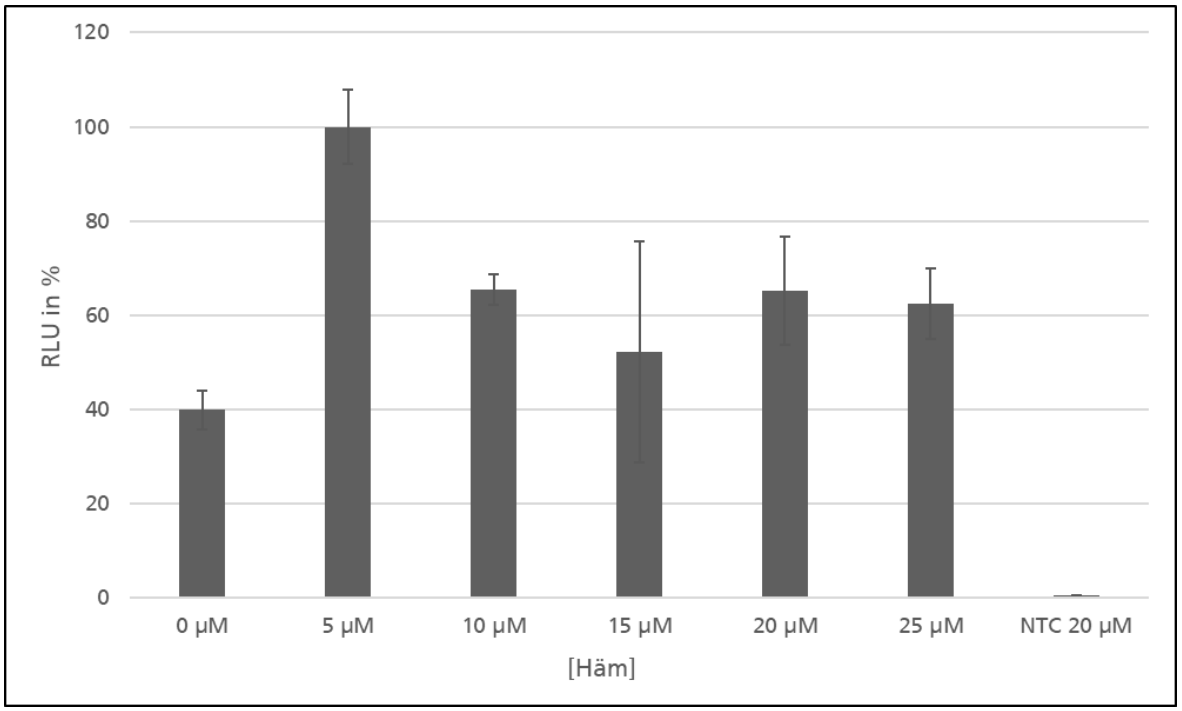


Figure 23: Influence of heme concentration on CYP3A4 activity during cell-free protein synthesis. The synthesis reaction was performed in a batch mode for 3 h. The activity was determined using an IPA luciferase activity assay. Standard deviations were calculated from triplicate analysis (n = 3)

The supplementation of higher concentrations of heme results in equally reduced activity at a two-thirds level compared to the 5 µM heme supplemented sample. The concentration of 5 µM heme was used in all subsequent reactions.

4.3.5. Localization of cell-free produced CYPs

CYPs are membrane associated proteins, but in contrast to most trans-membrane proteins they are only N-terminal anchored in the membrane and have a partially lipophilic surface that is oriented to a membrane. The translocation process is therefore different to other membrane proteins that have been produced already successfully by CFPS. Localization and the influence of signal sequences are therefore an important issue for the cell free synthesis of CYPs. The localization of the cell-free produced CYPs was analyzed using confocal laser scanning microscopy. For this purpose, templates for CYP3A4-eYFP fusion proteins were generated. Additionally, a template containing a melittin signal sequence (Mel-CYP3A4-eYFP)

was generated. Both templates were used for the CFPS in the modified CHO-lysates. The microsomal fraction was analyzed using confocal microscopy (Figure 24). Both templates led to a co-localization of the target protein with the microsomes.

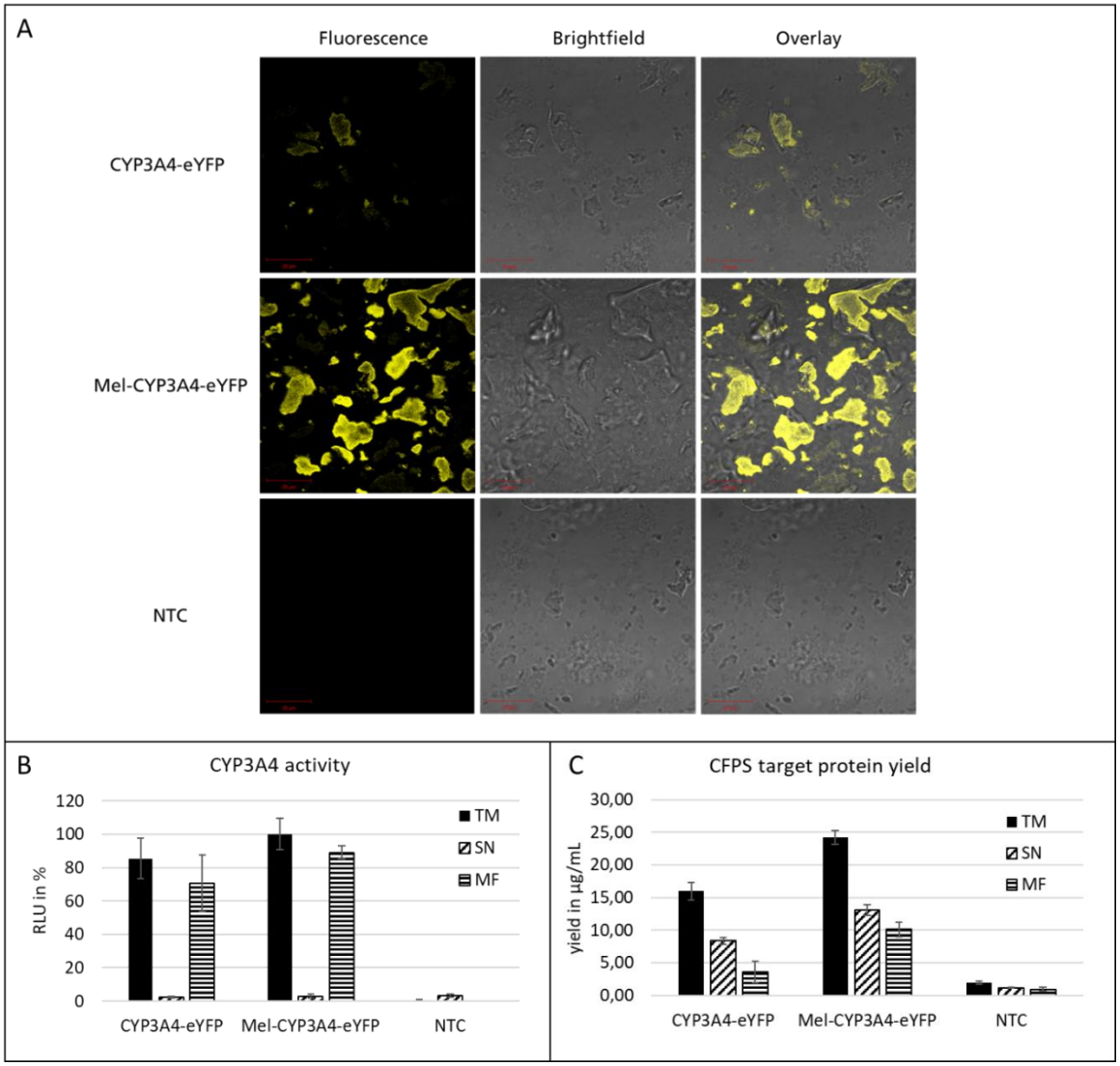


Figure 24: A) Confocal microscopy images of cell-free synthesized CYP3A4-eYFP and Mel-CYP3A4-eYFP. The proteins were synthesized in batch mode for 3 h. The microsomal fraction of the cell-free reaction was analyzed. The fluorescent image, a brightfield image and an overlay of both images are shown. NTC = no template control; translation reaction without DNA template. B) Determination of CYP3A4-eYFP and Mel-CYP3A4-eYFP yield and enzyme activity after cell-free synthesis in the translation mixture (TM), the microsomal fraction (MF) and the supernatant fraction (SN). Enzyme activity was determined by an IPA-luciferase assay (Promega). C) Additionally, the yield of cell-free produced proteins was determined via radioactive labeling followed by TCA precipitation and scintillation counting. Standard deviations were calculated from triplicate analysis.

Apparently, the addition of a melittin signal sequence, led to a clearly visible increased co-localization of the produced CYPs with the microsomes. In the analysis of CYP3A4 activity, such a difference is not discernible to this extent. The addition of the melittin signal sequence results in a 30 % increase in activity (Figure 24 B). The analysis of protein yield from CFPS correlates with the increase in translocation induced by the melittin signal sequence. The yield in the microsomal fraction is nearly four times higher with the melittin signal sequence (Figure 24 C).

The co-localization was further analyzed by comparing the microsomal fraction of the CYP-sample with the microsomal fraction of an NTC to which the supernatant fraction of the CFPS sample was added. Fluorescence microscopy reveals a similar image as in the of both samples (Figure 25 A). The CYPs of the supernatant fraction are not active despite being co-localized with the microsomes of the NTC batch (Figure 25 C) and despite having a higher target protein yield than the CYPs in the microsomal fraction (Figure 25 B).

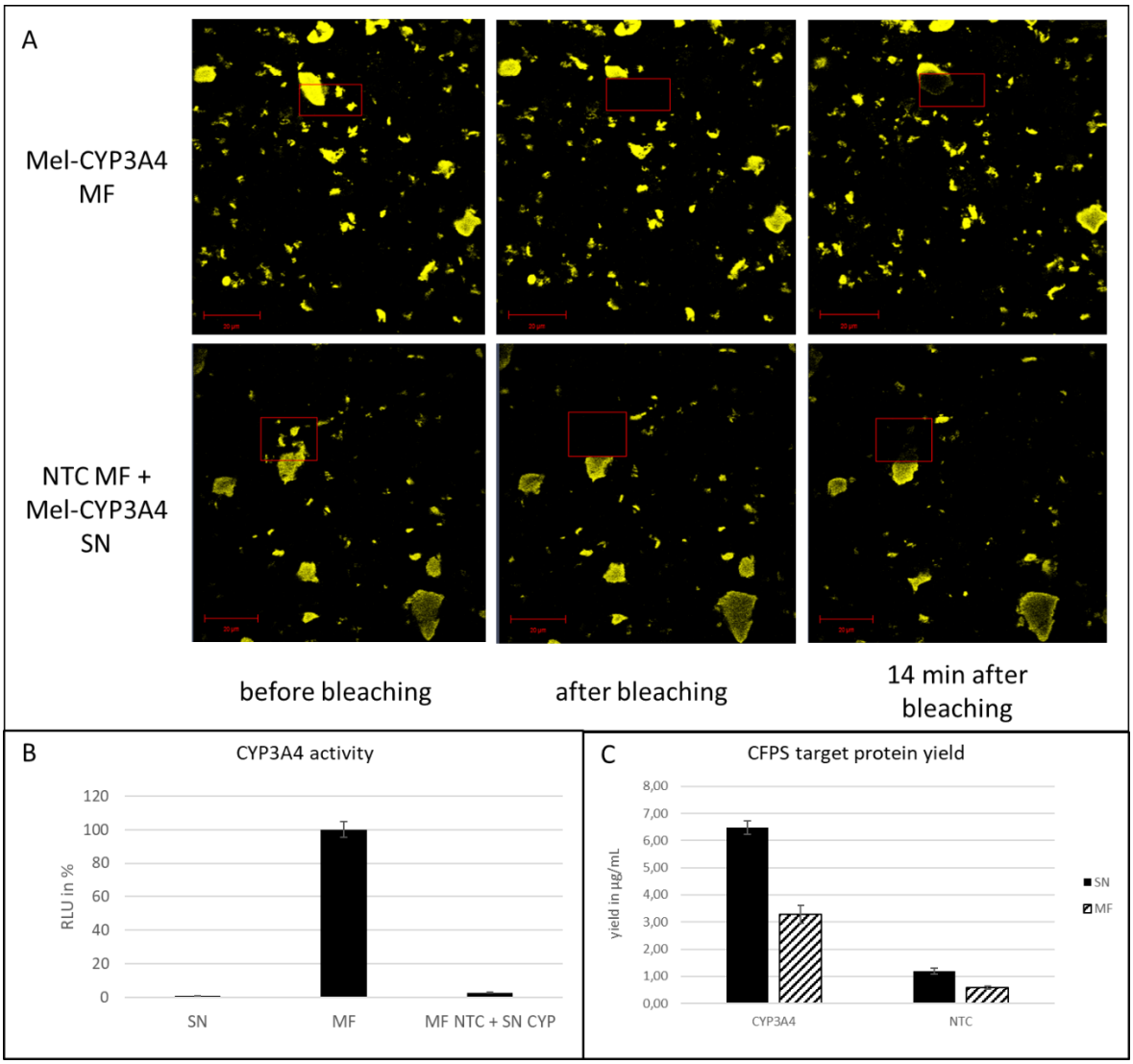


Figure 25: A) Confocal microscopy images of cell-free synthesized Mel-CYP3A4-eYFP. The proteins were synthesized in batch mode for 3 h. The microsomal fraction was analyzed and compared to the microsomal fraction of an NTC to which the supernatant fraction of the CYP batch was added. Photo bleaching of the region in the red rectangle was photo bleached. The images before after and 14 min after photo bleaching are depicted. B) Relative activity of CYP3A4 in the supernatant fraction (SN) the microsomal fraction (MF) and in the Microsomal fraction of an NTC to which the supernatant fraction of the CYP batch was added. Standard deviations were calculated from triplicate analysis (n = 3). C) Yield determination of cell-free produced proteins via radioactive labeling followed by TCA precipitation and scintillation counting. Standard deviations were calculated from triplicate analysis (n = 3).

Photo bleaching was performed in the area of the red rectangle. It resulted in a loss of fluorescence activity in the microsomes with no significant recovery. At the time

of photo bleaching the diffusion rate of the cell-free produced CYPs is therefore low and there is no further attachment or detachment of CYPs from the microsomes.

4.3.6. Synthesis of different CYPs and turnover of pharmaceutical relevant CYP substrates

The application of CFPS enables the quick synthesis and analyses of different proteins via template exchange. Besides CYP3A4, CYP1A2 and CYP2B6 were synthesized in the modified cell-free system using the same adapted reaction conditions. Yield determinations by scintillation counting and activity assays were performed using the corresponding luciferase-based assay (Figure 26 A). All three CYPs were active in the microsomal fraction with almost zero background. Cell-free produced CYP2B6 had the highest activity (15 µU/mL) followed by CYP3A4 (4 µU/mL) and CYP1A2 (2 µU/mL)

An indirect activity assay using the luciferase-based assay will identify potential CYP substrates and inhibitors in a screening procedure. For this purpose, various known pharmaceutical relevant CYP substrates (testosterone, midazolam, efavirenz and phenacetin) were used as a proof of principle. The CYP substrates were added to the CHO lysate originating microsomes after cell-free synthesis during the CYP luciferase-based activity assay. The substrates were added in a final concentration of 200 µM. A sample without additional substrate and with cholesterol as non-interacting control substance were prepared as a reference. Due to competitive turnover, adding of CYP substrates should lead to a decrease of luciferase signal due to competitive substrate turnover in batches with interacting substrates (Figure 26 B).

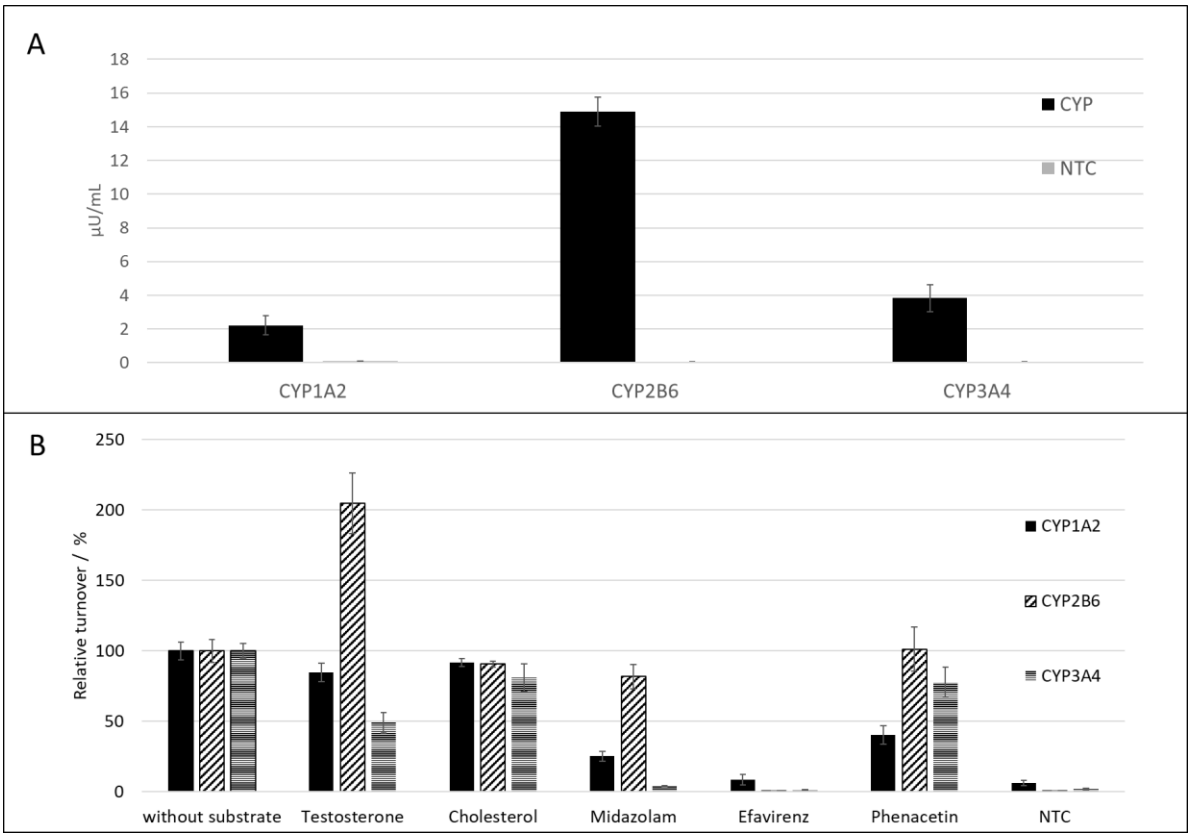


Figure 26: A) Activity determination of different CYPs in the microsomal fraction (MF) after 16,000 x g centrifugation. Standard deviations were calculated from triplicate analysis (n = 3) B) Screening of different pharmaceutical relevant CYP substrates in relation to a sample without additional substrate. different CYP substrates were added during the CYP activity assay in a concentration of 200 µM. Cholesterol as a steroid that is not known to be a CYP substrate was used as control substrate. A reduction of Luc signal implies the competitive turnover of the CYP substrate or an Inhibition of the CYP activity during the assay. Standard deviations were calculated from triplicate analysis (n = 3)

In the completive assay CYP1A2, CYP2B6 and CYP3A4 were analyzed on their activity changes after adding different CYP substrates. CYP1A2 assay luciferase activity was reduced by all tested substrates while midazolam and efavirenz had the highest impact. Cyp2B6 assay luciferase activity reduction was only observed after addition of efavirenz. The supplementation of testosterone led to a 100 % increase of luciferase activity. CYP3A4 assay luciferase activity was significantly reduced by testosterone, midazolam and efavirenz. The reference substrate cholesterol did not lead to either an increase or a decrease in the conversion of the luciferin substrate across all tested CYPs.

5. Discussion

5.1. Cell-free production of enzymes

Enzymes represent indispensable tools in various domains of biotechnological research and industrial applications for catalyzing and modulating a diverse array of chemical reactions (McKelvey und Murphy 2018). This applies particularly for the animal feed and food industry, as well as the pharmaceutical sector and research endeavors (Adrio und Demain 2014). Owing to their high substrate and reaction specificity, as well as their chemical stability, enzymes are facile to manipulate and enable the execution of numerous reactions at ambient temperatures, which would require energy-intensive and high-temperature approaches in organic chemistry procedures, and within considerably shorter time frames (Hevilla et al. 2021). The execution of reactions under such conditions is generally vital for organisms, as a lot of biological processes are supported and regulated by enzymes. Consequently, alterations in conditions affecting the native enzymes of an organism are of particular significance in pharmacological research (Meghwanshi et al. 2020).

The production and isolation of enzymes is highly valuable for both research and practical applications. To isolate an enzyme, an organism that naturally produces the desired enzyme can be utilized, and the enzyme can be extracted in a pure form via cell lysis and subsequent biochemical separation techniques (Nghi et al. 2012; Ho und Yin Sze 2018; Bannerman und Nicolet 1976). If the enzyme is already secreted by the organism, the cell lysis step can be skipped. This method generally yields an isolated enzyme that closely resembles its native form, as it undergoes all the steps of synthesis and processing akin to a naturally occurring enzyme. However, this method does have certain limitations in terms of enzyme modifiability. Although genetic modifications can be performed in the organism's genome, they can lead to a loss of cell viability or division capability, rendering isolation unfeasible (Zhang et al. 2019). Furthermore, the quantity of enzyme production in an unmodified organism may be insufficient compared to other methods of enzyme production. As a result, the most prevalent method of producing enzymes and proteins, in general, is through overexpression in another species. Popular hosts for protein overexpression are *E. coli*, *S. cerevisiae* or *Sf*21 cells (Greene 2004).

However, even the standard method of overexpression of enzymes in cultured cells also has problems and limitations. Firstly, overexpression of foreign genes can lead

to the accumulation of toxic byproducts or metabolic intermediates that may damage or kill the host cells (Zemella et al. 2015; Rosano und Ceccarelli 2014; Zuppone et al. 2019). This toxicity can be exacerbated by the metabolic burden imposed on the cells by overexpression of foreign genes, leading to reduced growth and productivity. In addition, many enzymes require specific post-translational modifications such as glycosylation, phosphorylation, or proteolytic processing, which may not occur in the heterologous expression system, leading to misfolding, loss of activity or even insolubility (Mital et al. 2021). The overexpression of enzymes in cultured cells may not be easily scalable to industrial production levels due to the high costs associated with maintaining large cell cultures and the difficulties of scaling up cell-based systems (Sivashanmugam et al. 2009).

CFPS as an alternative enzyme synthesis method might overcome these limitations in future (Thoring und Kubick 2018). To date, significant progress has been made in the cell-free production of certain enzymes. For instance, the NO synthetase was successfully synthesized outside of cellular systems (Tian et al. 2022). Additionally, phosphatases and kinases were produced in a cell-free manner (Goshima et al. 2008; Thoring et al. 2017). Moreover, luciferases have been successfully manufactured in a cell-free environment (Brödel et al. 2014). The feasibility of CFPS for enzyme production shall be further assessed through the analysis of various model enzymes in eukaryotic protein production systems.

CFPS is a promising but currently underutilized tool in the field of enzymology, despite its well-established potential as evidenced in previous publications (Rolf et al. 2019; Tinafar et al. 2019). One particular advantage of CFPS over traditional cell-based methods is its potential for streamlining enzyme discovery, both for identifying natural enzymes via high-throughput experiments and for generating novel, artificially evolved or engineered enzymes (Tinafar et al. 2019). Additionally, CFPS allows for efficient characterization of proteins across multiple dimensions, as demonstrated in this thesis with an enzyme of significance for basic research, ecology, and industry. To aid enzymologists seeking to employ CFPS in their investigations, this may be a practical guide for facilitating straightforward and specific approaches.

CFPS has only been sporadically employed in enzymology thus far. However, it offers a significant potential for screening applications that are required for various

optimization processes in the production of enzymes for economic use. A good example of this is enzyme engineering through rational design, which requires screening of different gene templates to improve enzyme performance for the desired application (Fisher et al. 2014). Such improvements may include increasing the specific activity of an enzyme. Nevertheless, there are also other ways to enhance absolute enzyme activity in a cell- free reaction assay, for instance, by increasing the translation rate and, thus, producing a higher enzyme yield. These modifications are usually easier to implement and may potentially lead to improvements in enzyme activity for multiple enzymes in one synthesis reaction. One way to achieve this is by modifying the N-terminus of the enzyme, which directly affects translation initiation (Hershey et al. 2012).

5.2. Cell-free synthesis of GH78

Based on the qualitative detection methods of autoradiography and the NPRP activity assay, it can be assumed that the desired target protein was successfully synthesized in the cell-free system. The presence of a secondary band at 55 kDa in the autoradiogram is a known degradation product caused by proteases for this enzyme (Nghi et al. 2012). Due to initially low enzyme yields, several optimizations were performed, with the alteration of the template sequence proving to be particularly effective. Similar to the trans-acting factors referred to as ITAFs (King et al. 2010; Godet et al. 2019), it is highly probable that the "cis-acting factors" or sequence context may also have an impact on the initiation of IRES-mediated translation. Hence, a modified template sequence was used to potentially enhance the yield of the target protein and consequently, the concentration of the active enzyme produced. One effective adaptation of the template sequence involves the usage of alternative codons (Mauro und Chappell 2014). This does not alter the protein sequence but results in a different mRNA sequence and fold, thereby influencing translation. The *Sf*21 optimized coded sequence is usually employed for CFPS, but it leads to reduced synthesis efficiency in comparison to *C. gresius* and *X. polymorpha* codon optimization. Along with the codon optimizations, the addition of N-terminal tags improved GH78's synthesis in cell-free systems heavily. Similar to previous studies (Booth et al. 2018) (Aslantas und Surmeli 2019) these modifications affected the enzymes activity and stability, indicating the crucial role of the mRNA sequence context immediately after CrpV-IRES. Due to the simultaneous increase in both target protein yield and absolute enzyme activity

resulting from the modifications, the increase in activity is mainly attributable to enhanced translation efficiency.

There is evidence from several studies suggesting that the mRNA structure has a significant influence on the effectiveness of translation initiation mediated by the Internal Ribosome Entry Site (IRES). The dynamic structure of mRNA forms loops that can both facilitate and impede elongation factor-dependent steps in the IRES initiation process (Ruehle et al. 2015; Dreher 2010; Simon und Miller 2013). The lack of mRNA structure analysis hinders the ability to establish whether it is indeed the cause of the significant variation in protein synthesis observed in this study. To achieve sustainable improvements in IRES-based initiation mechanisms in protein synthesis, a more structured approach incorporating analysis of specific mRNA secondary structures would be necessary. Conducting such a study using CFPS would be feasible and first approaches have already been made with bacterial CFPS (Li et al. 2022). As demonstrated by this study, even modest screenings can lead to substantial improvements in synthesis conditions. Interestingly, the optimization of templating appears to be independent of lysate type, at least in *Sf*21 and CHO lysates, which can be attributed to the similarity of translation processes across these cell-free systems.

Cell-free protein synthesis typically enables optimization and adaptation to the specific enzyme of interest, particularly for *de novo* synthesis of a particular protein type in a cell-free system. Among the factors that have the greatest impact on the target enzyme activity, the reaction temperature is significant (Hodgman und Jewett 2013) , as it affects not only the protein yield but is also a critical factor for protein folding (Guo et al. 2012). In the case of GH78 synthesis, temperature screening showed that the default temperature of 27 °C, which is considered optimal for *Sf*21 lysate-based CFPS (Stech et al. 2014) in fact resulted in the highest protein yield. For the CHO cell-free system, a temperature of 30 °C resulted in maximal protein yield, which is considered the default temperature for these lysates. However, this does not necessarily mean that all of the synthesized protein is soluble, correctly folded, and active. Lower synthesis rates have led to a more consistent protein folding process, promoting correct folding and less aggregation, and hence higher relative activity (Nedialkova und Leidel 2015). It is also worth noting that GH78 folding is optimal at lower synthesis temperatures, as the protein is of fungal origin and therefore needs to be active and correctly folded in this moderate temperature range (A'Bear et al.

2014). In contrast to eukaryotic CFPS, synthesis at low temperatures is challenging to achieve for recombinant in vivo synthesis in yeast (usually 30 °C) or E. coli (usually 30 or 37 °C) (Hodgman und Jewett 2013; Levine et al. 2019). Given the increasing relevance of fungal enzymes for research and applications, this information will undoubtedly be useful for future approaches addressing similar enzymes.

Moreover, the addition of additives or replacement of components in cell-free reaction mixtures can impact enzyme activity, in conjunction with the optimization of synthesis temperature. For example, previous research has demonstrated that substitution of acetate with glutamate can enhance luciferase activity in a yeast lysate-based CFPS system (Hodgman und Jewett 2013). This finding is applicable to the production of GH78 in an *Sf*21 lysate-based CFPS system as well. Given that glutamate is a predominant anion in cells, it is possible that it could also stabilize protein synthesis, particularly during protein folding processes in cell-free systems, thereby improving relative enzyme activity (Jewett und Swartz 2004; Record et al. 1998). For other fungal enzymes, the introduction of cofactors may play an even more significant role and remains a bottleneck in the successful synthesis of active fungal oxidoreductases such as peroxygenases UPOs (Martin Hofrichter et al. 2015).

Kinetic analysis of GH78 proteins synthesized in *Sf*21, CHO, and wheat germ CFPS systems revealed only minor differences. Michaelis-Menten constants for NPRP hydrolysis were similar for enzyme proteins produced in all three systems, whereas a higher k_{cat} was observed for GH78 produced in the *Sf*21 cell lysate-based CECF system, indicating a higher ratio of active enzyme synthesis. However, a lower absolute amount of active GH78 was produced in *Sf*21-based CFPS compared to CHO and wheat germ CFPS, likely due to slower enzyme production promoting the generation of a higher percentage of correctly folded active enzyme (O'Brien et al. 2014). Wild-type GH78 produced with *X. polymorpha* exhibited superior kinetic data (Knauer et al. 2022) but required several days for production (Nghi et al. 2012). It should be noted that cell-free produced GH78 may exhibit different post-translational modifications, such as the lack of complete glycosylation compared to the wild-type protein, which could affect its behavior in the NPRP assay. Although glycosylation is not critical for enzyme solubility or activity, it can alter its conformation (Chang et al. 2017; Shental-Bechor und Levy 2008). Furthermore, the specific activity of GH78 for different substrates may be altered due to a lack of glycosylation. Surprisingly, the absence of sugars on the enzyme's surface can have

both stimulating and inhibiting effects on the specific activities of enzymes, as previously described for proteases (Goettig 2016) and other fungal hydrolases such as esterases (Bonzom et al. 2019).

The high yield synthesis of GH78 using the CECF system enabled immobilization and purification of the enzyme, as well as screening, kinetic and structural analyses. His-Tag binding, a straightforward immobilization method (Datta et al. 2013)(Puri et al. 2010), was employed for both purification and immobilization of the enzyme to monitor its activity in the immobilized state. This provides a basis for future applications such as protein arrays, enzyme purification and the development of enzyme reactors. Enzyme immobilization is increasingly important for industrial and medical applications, especially if performed directly after protein synthesis. The immobilized GH78 showed reusability after repeated substrate incubation in a small reactor, indicating the potential for enhanced stability of immobilized enzymes. Increased stability of immobilized enzymes has been previously reported in the literature (Fernandez-Lopez et al. 2017). The manner of immobilization influences the stability and activity of the immobilized enzyme, with differences observed between rigid and non-rigid immobilization methods (Mateo et al. 2007). In the case of immobilization using FLAG affinity tags, non-rigid immobilization is employed. It is possible that alternative methods utilizing shorter linkers may be better suited for this purpose. The observed decrease in GH78 activity after the first cycle of substrate incubation may be attributed to partial denaturation of the enzyme, resulting in reduced catalytic activity. Optimization of reaction conditions of the activity assay reaction like an increased pH can be explored to enhance the stability of the immobilized enzyme. Furthermore, the numerous buffer exchanges and centrifugation steps involved in the process can weaken the interactions between enzymes and beads, potentially leading to a loss of activity. In addition, purified GH78 at higher concentrations can be utilized for structural studies, such as X-ray crystallography. Rhamnosidases are of great significance to the chemical and medical industries (reviewed in (Yadav et al. 2010)) and their application potential may be further broadened through enzyme engineering and site-directed mutagenesis. Possibilities include enhancing specific catalytic efficiencies and extending the enzymatic functionalities. For instance, site-directed mutagenesis of a similar enzyme has led to an increased yield of reverse hydrolysis (Xu et al. 2016).

Alpha-L-rhamnosidase from Alternaria sp. L1 hydrolysis activity was enhanced by altering critical amino acids at the active site of the enzyme.

CFPS allows for the facile synthesis of mutated templates and rapid enzymatic characterization of the resulting mutant enzymes, providing an advantage over other synthesis modes. Site-directed mutagenesis targeted at different domains of the enzyme may also shed light on which domain is responsible for which catalytic reaction. However, the lack of structural data currently renders a rational design mutagenesis approach for GH78 from *Xylaria polymorpha* unfeasible.

The results of this study demonstrate the suitability of using eukaryotic cell-lysates based CFPS for the synthesis of fungal proteins and suggest that it can improve the performance of analytical approaches. The use of GH78 from X. polymorpha allowed for the screening of reaction conditions, providing a wide range of experimental opportunities for gaining deeper insights into the nature of GH78 proteins and improving their industrial application. The gained knowledge can also be applied to other fungal enzymes that are more difficult to synthesize, providing a foundation for the successful synthesis of active fungal oxidoreductases, such as the industrially relevant UPOs. Immobilization methods, as demonstrated with GH78, may also be applicable to the reaction conditions of other fungal proteins. In the near future, high-throughput screenings of fungal enzyme mutants may be well-suited for cell-free systems, including targeted enzyme engineering approaches.

5.3. Cell free synthesis of UPOs

UPOs are enzymes found in fungi that catalyze monooxygenations of carbon atoms in aliphatic compounds. Such enzymatic function is relatively rare in nature and is also relevant to the synthetic industry especially for C-H-oxyfunctionalization (Monterrey et al. 2023). An extension of the usability of this enzyme class can replace many reaction steps, especially in the field of organic chemistry. CFPS can lead to an expansion of the pool of usable UPOs, for example, by screening potential UPO sequences and the de novo recombinant synthesis of the respective enzymes.

However, it is necessary to develop a synthesis platform that allows for the uncomplicated synthesis and analysis of the synthesized enzymes. Despite its good performance in synthesizing GH78, the CHO lysate-based CFPS system is currently unsuitable. Although several known UPOs can be easily produced using the CHO-

based cell-free system with above-average yields, as demonstrated using radioactive labeling, SDS-PAGE autoradiography, and TCA precipitation, the analysis of the enzymes is very challenging.

As a well-researched enzyme, AAE UPO was used as a model enzyme to test the feasibility of analytical methods (Molina-Espeja et al. 2015). Due to the strong influence of the components of the synthesis reaction on the colorimetric activity assays, the synthesized model enzyme AAE UPO cannot be reliably analyzed directly after synthesis. Such hurdles significantly hinder reliable screening procedures, as further purification or buffer exchange steps are required, which cannot be parallelized well. However, even these steps are not straightforward to perform for the model enzyme. For example, adding an N- or C-terminal tag is unfavorable, as both ends of the protein are near the enzyme's active center and are therefore inaccessible (Rotilio et al. 2021). Another bottleneck for successful UPO production is the integration of the heme group. Heme is added as an additive in the cell-free synthesis, but whether this co-factor is successfully incorporated into each enzyme is uncertain. Meaningful optimizations of the synthesis reaction can only be performed if the enzyme's activity is quantifiable.

For these reasons, it is useful to consider alternative synthesis methods. For example, *Sf*21 and HEK-based synthesis systems allow for the translocation of the synthesized proteins into endogenous microsomes, which resemble the endoplasmic reticulum. By using a signal sequence preceding the protein, translocation of the synthesized proteins can be initiated. Similar methods have enabled the production of antibody fragments that depend on the oxidative redox potential in microsomes (Haueis et al. 2022). Such redox potentials may also be conducive to heme incorporation in UPOs.

Recently, it has been achieved to produce small amounts of active UPOs from *Agrocybe aegerita*, *Marasmius rotula* and *Podospora anserina* using a modified translationally active *Sf*21 lysate (Walter et al. 2022). In this process, glutathione was employed to adjust the redox potential, while CHAPS was utilized as a stabilization component during fractionation. As mentioned earlier and as anticipated, heme is also a critical component in the production of active UPOs. By performing buffer exchanges after the protein synthesis, the negative effect on the activity assays was bypassed.

5.4. Cell-free synthesis of CYPs

For many years, recombinant expression of membrane proteins has posed a challenge (Kang und Tullman-Ercek 2018; Pandey et al. 2016). More than half of all pharmacologically relevant proteins are membrane-bound (Hauser et al. 2017). Consequently, there is great interest in being able to produce a wide range of membrane proteins in vitro quickly, inexpensively and with minimal effort. Recent progress in cell-free protein synthesis has enabled successful synthesis of various toxic and membrane-bound proteins for research and development purposes (Sachse et al. 2014; Ramm et al. 2022; Dondapati et al. 2020; Khambhati et al. 2019; Fenz et al. 2014). However, there have been only a few studies on cytochromes P450 (CYPs), one of the most pharmaceutically relevant groups of membrane proteins. The cytochrome P450 family is one of the largest known protein families, found in all living organisms, and catalyzes various reactions such as the assimilation of carbon sources, synthesis of hormones and secondary metabolites, or the degradation of xenobiotics (Šrejber et al. 2018). Recombinant expression of heme-containing, membrane-bound oxidoreductases such as cytochromes P450 has been attempted numerous times but with limited success, mainly due to the lack of cofactors and a suitable membrane environment (Hausjell et al. 2018). Even prokaryotic-based cell-free protein synthesis has not yet yielded significant breakthroughs for the cost-efficient synthesis of cytochromes P450 (Kwon et al. 2013). However, using vesicle-containing eukaryotic cell extracts for cell-free protein synthesis overcomes most restrictions and allows for the development of convenient cytochrome P450 substrate screening systems. The availability of endogenous microsomes from the endoplasmic reticulum in which cytochrome P450 reductase is located, and in which cytochromes P450 are translocated after their cell-free synthesis, is of outstanding advantage in this context.

5.5. CPR in translationally active CHO lysates

The activity of CYPs is dependent on the electron transfer of CPR, and therefore, it is crucial to examine the localization and activity of CPR in translationally active lysates (Neunzig et al. 2013; Riddick et al. 2013). Although CPR activity was observed in wild-type CHO cells, elevated levels of CPR activity were found in CHO lysates that overexpressed human CPR. Given the structural similarity between human and CHO CPR (Ohgiya et al. 1997), western blotting confirmed the presence of CPR in both modified CHO and in the wild-type CHO lysates. Endogenous CPR can facilitate the electron transfer required for CYP-catalyzed reactions, and basal CPR activity was detected in lysates derived from wild-type CHO cells.

The presence of CPR in CHO cells is not unexpected, as it serves as an electron donor for a variety of enzymatic reactions. These reactions encompass beside CYP-catalyzed processes predominantly observed in hepatocytes, as well as squalene monooxygenase, cytochrome b5, methylsterol monooxygenase, stearoyl-CoA desaturase, and sterol-C5-desaturase activities (Porter 2012). The production of CPR-enriched CHO-CPR lysates via the use of CHO cells specifically designed for CYP synthesis resulted in a threefold increase in CPR activity due to CPR overexpression. CPR activity is primarily localized in the microsomal fraction, and the microsomes in CHO lysates are derived from the endoplasmic reticulum (ER) of cells where both CPR and most CYPs are naturally located (Tydén et al. 2014).

Hence, it can be concluded that the natural translocation of CPR into the microsomes enables proper localization and folding of CPR. This makes the generated CHO-CPR lysates suitable for the production of various CYPs (Schulz et al. 2021). To characterize the generated lysates, CYP3A4 was used as a model CYP due to its high frequency of analysis and its responsibility for the degradation of most xenobiotics in the human body (Klein und Zanger 2013). Cell-free synthesis of CYP3A4 in the modified lysates resulted in a fourfold increase in total CYP activity per volume of cell-free reaction compared to synthesis in conventional lysates, demonstrating a significant improvement in CYP synthesis efficiency in a cell-free system. The generated CHO-CPR lysates constitute a fundamental platform for the cell-free synthesis of a diverse range of enzymes, as CPR serves as a co-factor for numerous Oxidoreductases.

5.6. CYP synthesis and reaction condition adaption

The primary advantage of CFPS is the development of high-throughput screening systems for biomolecules (Contreras-Llano und Tan 2018). This platform technology enables the parallelized synthesis of different CYPs and variants without the need for time-consuming cloning and fermentation (Khambhati et al. 2019). The use of CHO cell-based CFPS is a promising technology for various medical applications, as this cell line is established for the synthesis of complex membrane-bound proteins in eukaryotic expression systems. In this study, we demonstrate for the first time a hybrid model that uses both cell-based and CFPS methods together. The lack of CYP background activity in the CHO cells is an additional advantage for CYP applications. To prevent unnecessary resource consumption during CFPS, CPR, which is required for all CYPs, can be included in the cell-free system by modifying the CHO cells used for lysate production (Stech et al. 2017). When comparing the cell-free production of the model protein CYP3A4 to the cell-based approach using the same cell lysis procedure, the cell-free method demonstrated higher CYP activity per unit volume of lysate. Luciferase-based assays are ideal for quantifying CYP activity ratios in different approaches, while the use of mass spectrometry enables specific determination of substrate turnover (Kim et al. 2015; Bell et al. 2008). The Western blot analysis reveals that the quantity of CYP synthesized via cell-free approach is significantly greater than that of the parallel cell-based approach using CHO-CPR-CYP3A4 cells. The difference in the synthesis level seems to be even more pronounced than the difference in activity. This could be attributed to incomplete membrane integration or aggregation of the cell-free synthesized CYPs. Hence, there is immense potential for even higher CYP activities and optimization of reaction parameters to achieve optimal CYP synthesis conditions. Endoplasmic reticular CYPs, which are integral membrane proteins, exhibit targeting consistent with the ER targeting signal hypothesis (Walter und Johnson 1994). These microsomal CYPs are anchored to the ER membrane by a single transmembrane domain near the N-terminus, which often contains an ER targeting domain and stop transfer signal. The Pro-rich regions located around amino acid residues 35-40 in various microsomal CYPs play a crucial role in facilitating proper folding of the cytosolic exposed catalytic domain of the protein (Avadhani et al. 2011). During translation, CYPs are synthesized on membrane-free ribosomes in the cytosol, and upon emergence of the hydrophobic N-terminus from the ribosome, translation is terminates through the

binding of the signal recognition particle (Sakaguchi et al. 1984). A better comprehension of the translocation mechanisms in a eukaryotic CFPS system is essential for a more efficient synthesis of membrane proteins. Of particular significance are the translocon interactions and the translation process during co-translational translocation that are critical for the appropriate localization and activity of CYPs.

In addition to CPR, heme represents a crucial cofactor for CYPs, which is essential for their proper function (Correia et al. 2011). Thus, ensuring adequate availability of heme during cell-free synthesis is of great importance. However, heme is a toxic molecule with hydrophobic and reactive properties that can lead to a reduction in protein activity (Jeney et al. 2002; Ponka 1999), negatively affecting the amount of active CYPs. Furthermore, a certain basal concentration of heme is already present in the cell-free system, as basal CYP activities can be detected even without the addition of exogenous heme (Ponka 1999). The optimal temperature for cell-free synthesis of CYPs is a critical parameter, particularly for enzyme stability. Recent studies have suggested that lower temperatures, around 24 °C, promote the highest yield of active enzyme by allowing for more reliable protein folding mechanisms, albeit at the expense of slower synthesis rates. To further enhance folding efficiency at conventional synthesis temperatures, the utilization of chaperones for CYPs has been proposed, although none have been identified to date (Prozorovskiĭ et al. 2001). The correlation between enzyme activity and temperature may also be influenced by translocation dynamics, which can be studied using CYP-eYFP fusion proteins. In particular, the co-localization of fluorescently labeled CYPs and microsomes can be detected using confocal microscopy. It is anticipated that a majority of the enzymes initially localize to the microsomal surface with the N-terminal anchor, guided by the signal recognition particle, and become integrated on the outer side of the microsomes. Since eYFP is located at the C-terminus of CYP3A4, the fluorescence signal should co-localize with the microsomes. It should be noted that even posttranslational addition of CYPs to microsomes can result in co-localization, due to the inherent hydrophobicity of the membrane-adjacent regions of CYP3A4 (Avadhani et al. 2011). The strong hydrophobicity of proteins, leading to an increased affinity for microsomes, can be primarily attributed to the high proportion of hydrophobic amino acids present in their structure (Baylon et al. 2013). The utilization of the melittin signal sequence on the CYP template leads to a significantly

higher translocation efficiency. This has been demonstrated in other membrane proteins and secretory proteins produced through CFPS as well (Stech et al. 2012; Thoring et al. 2017). However, the enzyme activity has only increased by a small factor. Therefore, it appears that translocation is not the sole factor influencing the activity of cell-free synthesized CYP3A4. Aggregations due to the high concentration of enzymes in the microsomes could potentially be a factor negatively affecting the enzyme activity (Guigas und Weiss 2016). Passive incorporation of the enzyme into the membrane can also be a cause for low activity despite a significantly increased yield. This hypothesis was confirmed through a comparison of the microsomal fraction with a NTC sample where CYPs were added from the SN fraction. The non-translocated CYPs exhibited non-specific binding to the membrane. As expected, these non-translocated CYPs are also not enzymatically active due to their missing connection to CPR (Riddick et al. 2013). Fluorescence bleaching experiments demonstrate a similar effect in microsomes containing functionally incorporated CYP proteins as well as in those with subsequently added CYP protein. This lack of strong fluorescence signal recovery at the bleached site suggests that the proteins are relatively static within the microsomes, with no additional proteins capable of binding to them (Cai et al. 2022).

5.7. Application of the cell-free CYP production platform

The aim of cell-free CYP synthesis is to develop a screening system that enables the parallel analysis of various CYPs (Khambhati et al. 2019). As a proof of concept, human CYP1A2 and CYP2B6 were synthesized, demonstrating the system's capability to expand to CYPs from other gene families. These CYPs, which play a significant role in CYP metabolism with 10% (CYP1A2), 5% (CYP2B6), and 20% (CYP3A4) contribution, are highly regarded in both research and industry (Šrejber et al. 2018). Similar to CYP3A4, no significant activity was detected for the other CYPs in the negative control (NTC), indicating the suitability of the CHO cell-free system for mono-CYP microsome generation without interference from endogenous CYPs in cell-line based production. Especially these mono-CYP microsomes can contribute to advancements in the research of these enzymes, such as in structural elucidation or interaction studies, and also expedite the drug approval process by providing more accurate identification of CYP-drug interactions (Lynch und Price 2007). The turnover of pharmaceutically relevant CYP substrates by cell-free produced CYPs was indirectly measured through competitive turnover in luciferase

substrate-based CYP assays. This approach provides a straightforward screening method for identifying CYP substrates and inhibitors, which is of great significance for pharmaceutical applications (McDonnell und Dang 2013). Interactions of the tested CYPs with their substrates can lead to a decrease in luciferase assay activity, as observed in this study for all three CYPs with several known substrates. Among these substrates, the steroid hormone testosterone is likely the most extensively studied, particularly with respect to its metabolism by CYP3A4 (Usmani und Tang 2004; Dai et al. 2001). Cholesterol, as a sterol control substance exhibiting high structural similarity to testosterone, was utilized in the assay. Notably, a distinct difference between both substrates was observed for CYP3A4. Furthermore, interactions of CYP3A4 with midazolam and efavirenz (Hariparsad et al. 2004; Xiao et al. 2019) were confirmed during the assay. An allosteric activation of the Luciferase enzyme can be assumed in the case of an increase in Luc activity compared to the control batch, as observed for CYP2B6 with the substrate testosterone. This finding was surprising as the method had previously demonstrated efficacy with the known CYP2B6 substrate efavirenz, which was included in the assay (Sukasem et al. 2012). The present study reveals an atypical kinetics characteristic of substrate activation by testosterone, which has been observed previously for CYP2B6 (Ekins et al. 1998). Notably, this atypical kinetics was found to be present only in certain variants of CYP2B6 (Ariyoshi et al. 2001). The previously postulated mechanism of auto-activation of testosterone oxidation through altered enzyme conformation by Ekins et al. (1998) cannot explain the observed activation of luciferase substrate oxidation in this study. Instead, it is more likely that an allosteric conformational change is responsible for the observed activation. The presence of multiple substrate binding sites that lead to positive cooperation between dissimilar substrates may be a plausible model in this case. Such a "two binding pocket" hypothesis has been previously described for CYP3A4, but with negative cooperativity regarding testosterone (Roberts und Atkins 2007). The proposed "two binding pocket" model would provide an explanation for the observed 50% reduction in activity of CYP3A4 with testosterone in the competitive assay, in contrast to the 100% reduction observed for Midazolam and Efavirenz. Additionally, the broad substrate spectrum of CYP1A2 has been confirmed in previous studies (Zhou et al. 2009), with interaction observed for all applied substrates except testosterone and cholesterol. Nonetheless, further analytical steps,

particularly mass spectrometry, are required following the initial screening of various substrates.

The CHO cell-free protein production platform shows great promise as a new technology for synthesizing CYPs and could potentially facilitate future CYP analysis approaches. This platform may be particularly useful for screening purposes, including analyses of mutations, isoforms, and genetic variants, as well as substrate and inhibitor screenings. The potential combination of this system with phase 2 enzymes may also lead to novel applications, such as the development of an artificial in vitro liver, in which all enzymes can be tailored to meet the specific needs of the user.

5.8. Conclusion

The production of enzymes through CFPS is possible in small quantities and can simplify certain applications. The quality of the enzymes produced through CFPS is comparable to those synthesized through cell-based methods. Specific applications such as immobilizations or substrate binding assays can be carried out in parallel. The endogenous microsomes, a unique feature of eukaryotic CFPS systems, allow for the production of both soluble and membrane-bound enzymes. However, the synthesis costs of cell-free production remain economically feasible only for applications requiring small amounts of enzyme. Additionally, adapting the system to various enzyme classes can be time-consuming and expensive. In this study, the adaptation of the CFPS system to two enzyme classes was completed, while adaptation for one enzyme class was in progress. In this context, the adaptation of synthesis temperature to lower temperatures, template design, and genetic modification of cells for the production of translationally active lysates have led to significant improvements in the absolute enzyme activity per component utilized. In conclusion, the successful production of active mono-CYP microsomes using eukaryotic CFPS has been achieved, showcasing great potential for applications in both industry and research.

Bibliography

A'Bear, A. Donald; Jones, T. Hefin; Kandeler, Ellen; Boddy, Lynne (2014): Interactive effects of temperature and soil moisture on fungal-mediated wood decomposition and extracellular enzyme activity. In: *Soil Biology and Biochemistry* 70, S. 151–158. DOI: 10.1016/j.soilbio.2013.12.017.

Adrio, Jose L.; Demain, Arnold L. (2014): Microbial enzymes: tools for biotechnological processes. In: *Biomolecules* 4 (1), S. 117–139. DOI: 10.3390/biom4010117.

Ana Conesa, Peter J. Punt, Cees A.M.J.J. van den Hondel (2002): Fungal peroxidases: molecular aspects and applications. In: *Journal of biotechnology* 93, S. 143–158.

Arnau, José; Yaver, Debbie; Hjort, Carsten M. (2019): Strategies and Challenges for the Development of Industrial Enzymes Using Fungal Cell Factories. In: *Grand Challenges in Fungal Biotechnology*, S. 179–210. DOI: 10.1007/978-3-030-29541-7_7.

Aslantas, Yaprak; Surmeli, Nur Basak (2019): Effects of N-Terminal and C-Terminal Polyhistidine Tag on the Stability and Function of the Thermophilic P450 CYP119. In: *Bioinorganic chemistry and applications* 2019, S. 8080697. DOI: 10.1155/2019/8080697.

Augustin, Ewa; Borowa-Mazgaj, Barbara; Kikulska, Agnieszka; Kordalewska, Milena; Pawłowska, Monika (2013): CYP3A4 overexpression enhances the cytotoxicity of the antitumor triazoloacridinone derivative C-1305 in CHO cells. In: *Acta pharmacologica Sinica* 34 (1), S. 146–156. DOI: 10.1038/aps.2012.132.

Avadhani, Narayan G.; Sangar, Michelle C.; Bansal, Seema; Bajpai, Prachi (2011): Bimodal targeting of cytochrome P450s to endoplasmic reticulum and mitochondria: the concept of chimeric signals. In: *The FEBS journal* 278 (22), S. 4218–4229. DOI: 10.1111/j.1742-4658.2011.08356.x.

Bannerman, E. N.; Nicolet, J. (1976): Isolation and characterization of an enzyme with esterase activity from Micropolyspora faeni. In: *Applied and environmental microbiology* 32 (1), S. 138–144. DOI: 10.1128/aem.32.1.138-144.1976.

Baylon, Javier L.; Lenov, Ivan L.; Sligar, Stephen G.; Tajkhorshid, Emad (2013): Characterizing the membrane-bound state of cytochrome P450 3A4: structure, depth of insertion, and orientation. In: *Journal of the American Chemical Society* 135 (23), S. 8542–8551. DOI: 10.1021/ja4003525.

Bell, Leslie; Bickford, Shari; Nguyen, Phong Hung; Wang, Jianling; He, Timothy; Zhang, Bailin et al. (2008): Evaluation of fluorescence- and mass spectrometry-based CYP inhibition assays for use in drug discovery. In: *Journal of biomolecular screening* 13 (5), S. 343–353. DOI: 10.1177/1087057108317480.

Bonzom, Cyrielle; Hüttner, Silvia; Mirgorodskaya, Ekaterina; Chong, Sun-Li; Uthoff, Stefan; Steinbüchel, Alexander et al. (2019): Glycosylation influences activity, stability and immobilization of the feruloyl esterase 1a from Myceliophthora thermophila. In: *AMB Express* 9 (1), S. 126. DOI: 10.1186/s13568-019-0852-z.

Booth, William T.; Schlachter, Caleb R.; Pote, Swanandi; Ussin, Nikita; Mank, Nicholas J.; Klapper, Vincent et al. (2018): Impact of an N-terminal Polyhistidine Tag on Protein Thermal Stability. In: *ACS omega* 3 (1), S. 760–768. DOI: 10.1021/acsomega.7b01598.

Brödel, Andreas K.; Sonnabend, Andrei; Kubick, Stefan (2014): Cell-free protein expression based on extracts from CHO cells. In: *Biotechnology and bioengineering* 111 (1), S. 25–36. DOI: 10.1002/bit.25013.

Brödel, Andreas K.; Sonnabend, Andrei; Roberts, Lisa O.; Stech, Marlitt; Wüstenhagen, Doreen A.; Kubick, Stefan (2013): IRES-mediated translation of membrane proteins and glycoproteins in eukaryotic cell-free systems. In: *PloS one* 8 (12), e82234. DOI: 10.1371/journal.pone.0082234.

Bundy, Bradley C.; Swartz, James R. (2010): Site-specific incorporation of p-propargyloxyphenylalanine in a cell-free environment for direct protein-protein click conjugation. In: *Bioconjugate chemistry* 21 (2), S. 255–263. DOI: 10.1021/bc9002844.

Cai, Ning; Lai, Alvin Chi-Keung; Liao, Kin; Corridon, Peter R.; Graves, David J.; Chan, Vincent (2022): Recent Advances in Fluorescence Recovery after Photobleaching for Decoupling Transport and Kinetics of Biomacromolecules in Cellular Physiology. In: *Polymers* 14 (9). DOI: 10.3390/polym14091913.

Carlson, Erik D.; Gan, Rui; Hodgman, C. Eric; Jewett, Michael C. (2012): Cell-free protein synthesis: applications come of age. In: *Biotechnology advances* 30 (5), S. 1185–1194. DOI: 10.1016/j.biotechadv.2011.09.016.

Chang, Xiaoyu; Xu, Bo; Bai, Yingguo; Luo, Huiying; Ma, Rui; Shi, Pengjun; Yao, Bin (2017): Role of N-linked glycosylation in the enzymatic properties of a thermophilic GH 10 xylanase from Aspergillus fumigatus expressed in Pichia pastoris. In: *PloS one* 12 (2), e0171111. DOI: 10.1371/journal.pone.0171111.

Chiba, Camila Hiromi; Knirsch, Marcos Camargo; Azzoni, Adriano Rodrigues; Moreira, Antonio R.; Stephano, Marco Antonio (2021): Cell-free protein synthesis: advances on production process for biopharmaceuticals and immunobiological products. In: *BioTechniques* 70 (2), S. 126–133. DOI: 10.2144/btn-2020-0155.

Datta, Sumitra; Christena, L. Rene; Rajaram, Yamuna Rani Sriramulu (2013): Enzyme immobilization: an overview on techniques and support materials. In: *3 Biotech* 3 (1), S. 1–9. DOI: 10.1007/s13205-012-0071-7.

Davies, M. V.; Kaufman, R. J. (1992): The sequence context of the initiation codon in the encephalomyocarditis virus leader modulates efficiency of internal translation initiation. In: *Journal of virology* 66 (4), S. 1924–1932. DOI: 10.1128/jvi.66.4.1924-1932.1992.

Ding, S.; Yao, D.; Deeni, Y. Y.; Burchell, B.; Wolf, C. R.; Friedberg, T. (2001): Human NADPH-P450 oxidoreductase modulates the level of cytochrome P450 CYP2D6 holoprotein via haem oxygenase-dependent and -independent pathways. In: *The Biochemical journal* 356 (Pt 2), S. 613–619. DOI: 10.1042/0264-6021:3560613.

Ding, S., et al. (1997): High Levels of Recombinant CYP3A4 Expression in Chinese Hamster Ovary Cells Are Modulated by Coexpressed Human P450 Reductase and Hemin Supplementation. In: *Archives of Biochemistry and Biophysics* (348), S. 403–410.

Dondapati, Srujan Kumar; Stech, Marlitt; Zemella, Anne; Kubick, Stefan (2020): Cell-Free Protein Synthesis: A Promising Option for Future Drug Development. In: *BioDrugs : clinical immunotherapeutics, biopharmaceuticals and gene therapy*. DOI: 10.1007/s40259-020-00417-y.

Dong, Yanpeng; Yan, Jiang; Du, Huiqian; Chen, Miao; Ma, Ting; Feng, Lu (2012): Engineering of LadA for enhanced hexadecane oxidation using random- and site-directed mutagenesis. In: *Applied microbiology and biotechnology* 94 (4), S. 1019–1029. DOI: 10.1007/s00253-012-4035-y.

Dreher, Theo W. (2010): Viral tRNAs and tRNA-like structures. In: *Wiley interdisciplinary reviews. RNA* 1 (3), S. 402–414. DOI: 10.1002/wrna.42.

Durairaj, Pradeepraj; Li, Shengying (2022): Functional expression and regulation of eukaryotic cytochrome P450 enzymes in surrogate microbial cell factories. In: *Engineering Microbiology* 2 (1), S. 100011. DOI: 10.1016/j.engmic.2022.100011.

Eggenreich, Britta; Willim, Melissa; Wurm, David Johannes; Herwig, Christoph; Spadiut, Oliver (2016): Production strategies for active heme-containing peroxidases from E. coli inclusion bodies - a review. In: *Biotechnology reports (Amsterdam, Netherlands)* 10, S. 75–83. DOI: 10.1016/j.btre.2016.03.005.

Esteves, Francisco; Rueff, José; Kranendonk, Michel (2021): The Central Role of Cytochrome P450 in Xenobiotic Metabolism-A Brief Review on a Fascinating Enzyme Family. In: *Journal of xenobiotics* 11 (3), S. 94–114. DOI: 10.3390/jox11030007.

Faiq, Muneeb A.; Ali, Mashook; Dada, Tanuj; Dada, Rima; Saluja, Daman (2014): A novel methodology for enhanced and consistent heterologous expression of unmodified human cytochrome P450 1B1 (CYP1B1). In: *PloS one* 9 (10), e110473. DOI: 10.1371/journal.pone.0110473.

Fernandez-Lopez, Laura; Pedrero, Sara G.; Lopez-Carrobles, Nerea; Gorines, Beatriz C.; Virgen-Ortíz, Jose J.; Fernandez-Lafuente, Roberto (2017): Effect of protein load on stability of immobilized enzymes. In: *Enzyme and microbial technology* 98, S. 18–25. DOI: 10.1016/j.enzmictec.2016.12.002.

Finnigan, James D.; Young, Carl; Cook, Darren J.; Charnock, Simon J.; Black, Gary W. (2020): Cytochromes P450 (P450s): A review of the class system with a focus on prokaryotic P450s. In: *Advances in protein chemistry and structural biology* 122, S. 289–320. DOI: 10.1016/bs.apcsb.2020.06.005.

Fisher, Amanda K.; Freedman, Benjamin G.; Bevan, David R.; Senger, Ryan S. (2014): A review of metabolic and enzymatic engineering strategies for designing and optimizing performance of microbial cell factories. In: *Computational and structural biotechnology journal* 11 (18), S. 91–99. DOI: 10.1016/j.csbj.2014.08.010.

Gallego Custodio, M. V.; Otamendi, F. P.; Vidal, D. R. (1996): Production and characterization of anAspergillus terteus α-l-rhamnosidase of oenological interest. In: *Zeitschrift für Lebensmittel-Untersuchung und Forschung* (203), S. 522–527.

Godet, Anne-Claire; David, Florian; Hantelys, Fransky; Tatin, Florence; Lacazette, Eric; Garmy-Susini, Barbara; Prats, Anne-Catherine (2019): IRES Trans-Acting Factors, Key Actors of the Stress Response. In: *International journal of molecular sciences* 20 (4). DOI: 10.3390/ijms20040924.

Goettig, Peter (2016): Effects of Glycosylation on the Enzymatic Activity and Mechanisms of Proteases. In: *International journal of molecular sciences* 17 (12). DOI: 10.3390/ijms17121969.

Goshima, Naoki; Kawamura, Yoshifumi; Fukumoto, Akiko; Miura, Aya; Honma, Reiko; Satoh, Ryohei et al. (2008): Human protein factory for converting the transcriptome into an in vitro-expressed proteome. In: *Nature methods* 5 (12), S. 1011–1017. DOI: 10.1038/nmeth.1273.

Greene, James J. (2004): Host cell compatibility in protein expression. In: *Methods in molecular biology (Clifton, N.J.)* 267, S. 3–14. DOI: 10.1385/1-59259-774-2:003.

Gregorio, Nicole E.; Levine, Max Z.; Oza, Javin P. (2019): A User's Guide to Cell-Free Protein Synthesis. In: *Methods and protocols* 2 (1). DOI: 10.3390/mps2010024.

Guengerich, F. P. (1999): Cytochrome P-450 3A4: regulation and role in drug metabolism. In: *Annual review of pharmacology and toxicology* 39, S. 1–17. DOI: 10.1146/annurev.pharmtox.39.1.1.

Guigas, Gernot; Weiss, Matthias (2016): Effects of protein crowding on membrane systems. In: *Biochimica et biophysica acta* 1858 (10), S. 2441–2450. DOI: 10.1016/j.bbamem.2015.12.021.

Guo, Minghao; Xu, Yangfan; Gruebele, Martin (2012): Temperature dependence of protein folding kinetics in living cells. In: *Proceedings of the National Academy of Sciences of the United States of America* 109 (44), S. 17863–17867. DOI: 10.1073/pnas.1201797109.

Guttman, Yelena; Kerem, Zohar (2022): Dietary Inhibitors of CYP3A4 Are Revealed Using Virtual Screening by Using a New Deep-Learning Classifier. In: *Journal of agricultural and food chemistry* 70 (8), S. 2752–2761. DOI: 10.1021/acs.jafc.2c00237.

Hamann, Thomas; Møller, Birger Lindberg (2007): Improved cloning and expression of cytochrome P450s and cytochrome P450 reductase in yeast. In: *Protein expression and purification* 56 (1), S. 121–127. DOI: 10.1016/j.pep.2007.06.007.

Hatakka, Annele (1994): Lignin-modifying enzymes from selected white-rot fungi: production and role from in lignin degradation. FEMS Microbiology Reviews, 13(2-3), 125-135. DOI: 10.1111/J.1574-6976.1994.TB00039.X.

Haueis, Lisa; Stech, Marlitt; Kubick, Stefan (2022): A Cell-free Expression Pipeline for the Generation and Functional Characterization of Nanobodies. In: *Frontiers in bioengineering and biotechnology* 10, S. 896763. DOI: 10.3389/fbioe.2022.896763.

Hausjell, Johanna; Halbwirth, Heidi; Spadiut, Oliver (2018): Recombinant production of eukaryotic cytochrome P450s in microbial cell factories. In: *Bioscience reports* 38 (2). DOI: 10.1042/BSR20171290.

He, Mingyue; Stoevesandt, Oda; Taussig, Michael J. (2008): In situ synthesis of protein arrays. In: *Current opinion in biotechnology* 19 (1), S. 4–9. DOI: 10.1016/j.copbio.2007.11.009.

Hershey, John W. B.; Sonenberg, Nahum; Mathews, Michael B. (2012): Principles of translational control: an overview. In: *Cold Spring Harbor perspectives in biology* 4 (12). DOI: 10.1101/cshperspect.a011528.

Hevilla, Víctor; Sonseca, Agueda; Echeverría, Coro; Muñoz-Bonilla, Alexandra; Fernández-García, Marta (2021): Enzymatic Synthesis of Polyesters and Their Bioapplications: Recent Advances and Perspectives. In: *Macromolecular bioscience* 21 (10), e2100156. DOI: 10.1002/mabi.202100156.

Hinnebusch, Alan G.; Lorsch, Jon R. (2012): The mechanism of eukaryotic translation initiation: new insights and challenges. In: *Cold Spring Harbor perspectives in biology* 4 (10). DOI: 10.1101/cshperspect.a011544.

Ho, Jason Chen Kok; Yin Sze, Lim (2018): Isolation, identification and characterization of enzyme-producing lactic acid bacteria from traditional fermented foods. In: *Bioscience Horizons: The International Journal of Student Research* 11. DOI: 10.1093/biohorizons/hzy004.

Hodgman, C. Eric; Jewett, Michael C. (2013): Optimized extract preparation methods and reaction conditions for improved yeast cell-free protein synthesis. In: *Biotechnology and bioengineering* 110 (10), S. 2643–2654. DOI: 10.1002/bit.24942.

Hofrichter, Martin (2002): Review: lignin conversion by manganese peroxidase (MnP). In: *Enzyme and microbial technology* 30 (4), S. 454–466. DOI: 10.1016/S0141-0229(01)00528-2.

Hofrichter, Martin; Kellner, Harald; Herzog, Robert; Karich, Alexander; Kiebist, Jan; Scheibner, Katrin; Ullrich, René (2022): Peroxide-Mediated Oxygenation of Organic Compounds by Fungal Peroxygenases. In: *Antioxidants (Basel, Switzerland)* 11 (1). DOI: 10.3390/antiox11010163.

Hofrichter, Martin; Kellner, Harald; Herzog, Robert; Karich, Alexander; Liers, Christiane; Scheibner, Katrin et al. (2020): Fungal Peroxygenases: A Phylogenetically Old Superfamily of Heme Enzymes with Promiscuity for Oxygen Transfer Reactions. In: Helena Nevalainen (Hg.): Grand challenges in fungal biotechnology. Cham, Switzerland: Springer (Grand Challenges in Biology and Biotechnology), S. 369–403.

Hofrichter, Martin; Steinbüchel, Alexander (Hg.) (2001): Lignin, humic substances and coal. Weinheim: Wiley-VCH (Biopolymers, biology, chemistry, biotechnology, applications / ed. by A. Steinbüchel ; Vol. 1).

Hofrichter, Martin; Ullrich, René (2014): Oxidations catalyzed by fungal peroxygenases. In: *Current opinion in chemical biology* 19, S. 116–125. DOI: 10.1016/j.cbpa.2014.01.015.

Hrycay, Eugene G.; Bandiera, Stelvio M. (2015): Monooxygenase, peroxidase and peroxygenase properties and reaction mechanisms of cytochrome P450 enzymes. In: *Advances in experimental medicine and biology* 851, S. 1–61. DOI: 10.1007/978-3-319-16009-2_1.

Ichinose, Hirofumi; Wariishi, Hiroyuki (2013): High-level heterologous expression of fungal cytochrome P450s in Escherichia coli. In: *Biochemical and biophysical research communications* 438 (2), S. 289–294. DOI: 10.1016/j.bbrc.2013.07.057.

Jang, Yun-Woo; Lee, In-Kyoung; Kim, Young-Sook; Seok, Soon-Ja; Yu, Seung Hun; Yun, Bong-Sik (2009): Chemical Constituents of the Fruiting Body of Xylaria polymorpha. In: *Mycobiology* 37 (3), S. 207–210. DOI: 10.4489/MYCO.2009.37.3.207.

Jewett, Michael C.; Swartz, James R. (2004): Mimicking the Escherichia coli cytoplasmic environment activates long-lived and efficient cell-free protein synthesis. In: *Biotechnology and bioengineering* 86 (1), S. 19–26. DOI: 10.1002/bit.20026.

John S Walsh; Gerald T Miwa (2011): Bioactivation of Drugs: Risk and Drug Design. In: *Annual Review of Pharmacology* 51 (1), S. 145–167. DOI: 10.1146/annurev-pharmtox-010510-100514.

Karich, Alexander; Ullrich, René; Scheibner, Katrin; Hofrichter, Martin (2017): Fungal Unspecific Peroxygenases Oxidize the Majority of Organic EPA Priority Pollutants. In: *Frontiers in microbiology* 8, S. 1463. DOI: 10.3389/fmicb.2017.01463.

Katsimpouras, Constantinos; Stephanopoulos, Gregory (2021): Enzymes in biotechnology: Critical platform technologies for bioprocess development. In: *Current opinion in biotechnology* 69, S. 91–102. DOI: 10.1016/j.copbio.2020.12.003.

Kim, Young-Hoon; Bae, Young-Ji; Kim, Hyung Soo; Cha, Hey-Jin; Yun, Jae-Suk; Shin, Ji-Soon et al. (2015): Measurement of Human Cytochrome P450 Enzyme Induction Based on Mesalazine and Mosapride Citrate Treatments Using a Luminescent Assay. In: *Biomolecules & therapeutics* 23 (5), S. 486–492. DOI: 10.4062/biomolther.2015.041.

King, Helen A.; Cobbold, Laura C.; Willis, Anne E. (2010): The role of IRES trans-acting factors in regulating translation initiation. In: *Biochemical Society transactions* 38 (6), S. 1581–1586. DOI: 10.1042/BST0381581.

Kinne, Matthias; Poraj-Kobielska, Marzena; Ralph, Sally A.; Ullrich, René; Hofrichter, Martin; Hammel, Kenneth E. (2009): Oxidative cleavage of diverse ethers by an extracellular fungal peroxygenase. In: *The Journal of biological chemistry* 284 (43), S. 29343–29349. DOI: 10.1074/jbc.M109.040857.

Kirill A. Martemyanov; Alexander S. Spirin; Anatoly T. Gudkov (1997): Direct expression of PCR products in a cellfree transcription/translation system: synthesis of antibacterial peptide cecropin. In: *FEBS letters* 414, 268 270.

Klein, Kathrin; Zanger, Ulrich M. (2013): Pharmacogenomics of Cytochrome P450 3A4: Recent Progress Toward the "Missing Heritability" Problem. In: *Frontiers in genetics* 4, S. 12. DOI: 10.3389/fgene.2013.00012.

Knauer, Jan Felix; Liers, Christiane; Hahn, Stephanie; Wuestenhagen, Doreen A.; Zemella, Anne; Kellner, Harald et al. (2022): Cell-free production of the bifunctional glycoside hydrolase GH78 from Xylaria polymorpha. In: *Enzyme and microbial technology* 161, S. 110110. DOI: 10.1016/j.enzmictec.2022.110110.

Kobayashi, Kaoru; Urashima, Kikuko; Shimada, Noriaki; Chiba, Kan (2002): Substrate specificity for rat cytochrome P450 (CYP) isoforms: screening with cDNA-expressed systems of the rat. In: *Biochemical pharmacology* 63 (5), S. 889–896. DOI: 10.1016/s0006-2952(01)00843-7.

Komar, Anton A.; Hatzoglou, Maria (2011): Cellular IRES-mediated translation: the war of ITAFs in pathophysiological states. In: *Cell cycle (Georgetown, Tex.)* 10 (2), S. 229–240. DOI: 10.4161/cc.10.2.14472.

Komisarz, Karolina; Majka, Tomasz M.; Pielichowski, Krzysztof (2022): Chemical and Physical Modification of Lignin for Green Polymeric Composite Materials. In: *Materials (Basel, Switzerland)* 16 (1). DOI: 10.3390/ma16010016.

Kumar, Sanjay; Sharma, Rajiv; Roychowdhury, Abhijit (2012): Modulation of Cytochrome-P450 Inhibition (CYP) in Drug Discovery: A Medicinal Chemistry Perspective. In: *Current Medicinal Chemistry* (19), S. 3605–3621.

Kumar, Santosh (2010): Engineering cytochrome P450 biocatalysts for biotechnology, medicine and bioremediation. In: *Expert opinion on drug metabolism & toxicology* 6 (2), S. 115–131. DOI: 10.1517/17425250903431040.

Kwon, Yong-Chan; Oh, In-Seok; Lee, Nahum; Lee, Kyung-Ho; Yoon, Yeo Joon; Lee, Eun Yeol et al. (2013): Integrating cell-free biosyntheses of heme prosthetic group and apoenzyme for the synthesis of functional P450 monooxygenase. In: *Biotechnology and bioengineering* 110 (4), S. 1193–1200. DOI: 10.1002/bit.24785.

Lee, C. A.; Kadwell, S. H.; Kost, T. A.; Serabjitsingh, C. J. (1995): CYP3A4 Expressed by Insect Cells Infected with a Recombinant Baculovirus Containing Both CYP3A4 and Human NADPH-Cytochrome P450 Reductase Is Catalytically Similar to Human Liver Microsomal CYP3A4. In: *Archives of Biochemistry and Biophysics* (319), S. 157–167.

Levine, Max Z.; Gregorio, Nicole E.; Jewett, Michael C.; Watts, Katharine R.; Oza, Javin P. (2019): Escherichia coli-Based Cell-Free Protein Synthesis: Protocols for a robust, flexible, and accessible platform technology. In: *Journal of visualized experiments : JoVE* (144). DOI: 10.3791/58882.

Li, Jiaojiao; Tang, Mengtong; Qi, Hao (2022): Codon-Reduced Protein Synthesis With Manipulating tRNA Components in Cell-Free System. In: *Frontiers in bioengineering and biotechnology* 10, S. 891808. DOI: 10.3389/fbioe.2022.891808.

Liers, Christiane; Arnstadt, Tobias; Ullrich, René; Hofrichter, Martin (2011): Patterns of lignin degradation and oxidative enzyme secretion by different wood- and litter-colonizing basidiomycetes and ascomycetes grown on beech-wood. In: *FEMS microbiology ecology* 78 (1), S. 91–102. DOI: 10.1111/j.1574-6941.2011.01144.x.

Liu, David V.; Zawada, James F.; Swartz, James R. (2005): Streamlining Escherichia coli S30 extract preparation for economical cell-free protein synthesis. In: *Biotechnology progress* 21 (2), S. 460–465. DOI: 10.1021/bp049789y.

Lynch, Tom; Price, Amy (2007): The effect of cytochrome P450 metabolism on drug response, interactions, and adverse effects. In: *American family physician* 76 (3), S. 391–396.

Manikandan, Palrasu; Nagini, Siddavaram (2018): Cytochrome P450 Structure, Function and Clinical Significance: A Review. In: *Current drug targets* 19 (1), S. 38–54. DOI: 10.2174/1389450118666170125144557.

Marques, Rita; Lacerda, Rafaela; Romão, Luísa (2022): Internal Ribosome Entry Site (IRES)-Mediated Translation and Its Potential for Novel mRNA-Based Therapy Development. In: *Biomedicines* 10 (8). DOI: 10.3390/biomedicines10081865.

Martin Hofrichter; Harald Kellner; Marek J Pecyna; René Ullrich (2015): Fungal Unspecific Peroxygenases: Heme-Thiolate Proteins That Combine Peroxidase and Cytochrome P450 Properties. In: *Advances in experimental medicine and biology* 851, S. 341–368. DOI: 10.1007/978-3-319-16009-2_13.

Martinez, Carlos A.; Rupasinghe, Sanjeewa G. (2013): Cytochrome P450 Bioreactors in the Pharmaceutical Industry: Challenges and Opportunities. In: *Current Topics in Medicinal Chemistry* (13), S. 1470–1490.

Mateo, Cesar; Palomo, Jose M.; Fernandez-Lorente, Gloria; Guisan, Jose M.; Fernandez-Lafuente, Roberto (2007): Improvement of enzyme activity, stability and selectivity via immobilization techniques. In: *Enzyme and microbial technology* 40 (6), S. 1451–1463. DOI: 10.1016/j.enzmictec.2007.01.018.

Mauro, Vincent P.; Chappell, Stephen A. (2014): A critical analysis of codon optimization in human therapeutics. In: *Trends in molecular medicine* 20 (11), S. 604–613. DOI: 10.1016/j.molmed.2014.09.003.

McDonnell, Anne M.; Dang, Cathyyen H. (2013): Basic review of the cytochrome p450 system. In: *Journal of the advanced practitioner in oncology* 4 (4), S. 263–268. DOI: 10.6004/jadpro.2013.4.4.7.

McKelvey, Shauna M.; Murphy, Richard A. (2018): Biotechnological Use of Fungal Enzymes. In: Kevin Kavanagh (Hg.): Fungi. Biology and applications, Bd. 4. Third edition. Hoboken, NJ: John Wiley & Sons Inc, S. 201–225.

Meghwanshi, Gautam Kumar; Kaur, Navpreet; Verma, Swati; Dabi, Narendra Kumar; Vashishtha, Abhishek; Charan, P. D. et al. (2020): Enzymes for pharmaceutical and therapeutic applications. In: *Biotechnology and applied biochemistry* 67 (4), S. 586–601. DOI: 10.1002/bab.1919.

Mital, Suraj; Christie, Graham; Dikicioglu, Duygu (2021): Recombinant expression of insoluble enzymes in Escherichia coli: a systematic review of experimental design and its manufacturing implications. In: *Microbial cell factories* 20 (1), S. 208. DOI: 10.1186/s12934-021-01698-w.

Mohamad, Nur Royhaila; Marzuki, Nur Haziqah Che; Buang, Nor Aziah; Huyop, Fahrul; Wahab, Roswanira Abdul (2015): An overview of technologies for immobilization of enzymes and surface analysis techniques for immobilized enzymes. In: *Biotechnology, biotechnological equipment* 29 (2), S. 205–220. DOI: 10.1080/13102818.2015.1008192.

Molina-Espeja, Patricia; Ma, Su; Mate, Diana M.; Ludwig, Roland; Alcalde, Miguel (2015): Tandem-yeast expression system for engineering and producing unspecific peroxygenase. In: *Enzyme and microbial technology* 73-74, S. 29–33. DOI: 10.1016/j.enzmictec.2015.03.004.

Monterrey, Dianelis T.; Menés-Rubio, Andrea; Keser, Merve; Gonzalez-Perez, David; Alcalde, Miguel (2023): Unspecific peroxygenases: The pot of gold at the end of the oxyfunctionalization rainbow? In: *Current Opinion in Green and Sustainable Chemistry* 41, S. 100786. DOI: 10.1016/j.cogsc.2023.100786.

Mureev, Sergei; Kovtun, Oleksiy; Nguyen, Uyen T. T.; Alexandrov, Kirill (2009): Species-independent translational leaders facilitate cell-free expression. In: *Nature biotechnology* 27 (8), S. 747–752. DOI: 10.1038/nbt.1556.

Nebert, Daniel W.; Wikvall, Kjell; Miller, Walter L. (2013): Human cytochromes P450 in health and disease. In: *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* 368 (1612), S. 20120431. DOI: 10.1098/rstb.2012.0431.

Nedialkova, Danny D.; Leidel, Sebastian A. (2015): Optimization of Codon Translation Rates via tRNA Modifications Maintains Proteome Integrity. In: *Cell* 161 (7), S. 1606–1618. DOI: 10.1016/j.cell.2015.05.022.

Neunzig, Ina; Widjaja, Maria; Peters, Frank T.; Maurer, Hans H.; Hehn, Alain; Bourgaud, Frédéric; Bureik, Matthias (2013): Coexpression of CPR from various origins enhances biotransformation activity of human CYPs in S. pombe. In: *Applied biochemistry and biotechnology* 170 (7), S. 1751–1766. DOI: 10.1007/s12010-013-0303-2.

Nghi, Do Huu; Bittner, Britta; Kellner, Harald; Jehmlich, Nico; Ullrich, René; Pecyna, Marek J. et al. (2012): The wood rot ascomycete Xylaria polymorpha produces a novel GH78 glycoside hydrolase that exhibits α-L-rhamnosidase and feruloyl esterase activities and releases hydroxycinnamic acids from lignocelluloses. In: *Applied and environmental microbiology* 78 (14), S. 4893–4901. DOI: 10.1128/AEM.07588-11.

Norouzian, D.; Hosseinzadeh, A.; Inanlou, D. N.; Moazami, N. (1999): Various techniques used to immobilize naringinase produced by Penicillium decombens PTCC 5248. In: *World Journal of Microbiology and Biotechnology* 15 (4), S. 501–502. DOI: 10.1023/A:1008980018481.

O'Brien, Edward P.; Ciryam, Prajwal; Vendruscolo, Michele; Dobson, Christopher M. (2014): Understanding the influence of codon translation rates on cotranslational protein folding. In: *Accounts of chemical research* 47 (5), S. 1536–1544. DOI: 10.1021/ar5000117.

Ogu, Chris C.; Maxa, Jan L. (2000): Drug interactions due to cytochrome P450. In: *Proceedings (Baylor University. Medical Center)* 13 (4), S. 421–423.

Ohgiya, S.; Goda, T.; Hoshino, T.; Kamataki, T.; Ishizaki, K. (1997): Establishment of a novel host, high-red yeast that stably expresses hamster NADPH-cytochrome P450 oxidoreductase: usefulness for examination of the function of mammalian cytochrome P450. In: *Archives of Biochemistry and Biophysics* 343 (2), S. 215–224. DOI: 10.1006/abbi.1997.0148.

Omura, Tsuneo (2006): Mitochondrial P450s. In: *Chemico-biological interactions* 163 (1-2), S. 86–93. DOI: 10.1016/j.cbi.2006.06.008.

Pan, Yan; Abd-Rashid, Badrul Amini; Ismail, Zakiah; Ismail, Rusli; Mak, Joon Wah; Ong, Chin Eng (2011): Heterologous expression of human cytochromes P450 2D6 and CYP3A4 in Escherichia coli and their functional characterization. In: *The protein journal* 30 (8), S. 581–591. DOI: 10.1007/s10930-011-9365-6.

Parameswaran, Binod; Varjani, Sunita; Raveendran, Sindhu (2019): Green Bio-processes. Singapore: Springer Singapore.

Patterson, Andrew D.; Gonzalez, Frank J.; Idle, Jeffrey R. (2010): XENOBIOTIC METABOLISM – A VIEW THROUGH THE METABOLOMETER. In: *Chemical research in toxicology* 23 (5), S. 851–860. DOI: 10.1021/tx100020p.

Poraj-Kobielska, Marzena; Kinne, Matthias; Ullrich, René; Scheibner, Katrin; Hofrichter, Martin (2012): A spectrophotometric assay for the detection of fungal peroxygenases. In: *Analytical biochemistry* 421 (1), S. 327–329. DOI: 10.1016/j.ab.2011.10.009.

Porter, Todd D. (2012): New insights into the role of cytochrome P450 reductase (POR) in microsomal redox biology. In: *Acta Pharmaceutica Sinica B* 2 (2), S. 102–106. DOI: 10.1016/j.apsb.2012.02.002.

Puri, Munish; Kaur, Aneet; Singh, R. S.; Schwarz, Wolfgang H.; Kaur, Amrit (2010): One-step purification and immobilization of His-tagged rhamnosidase for naringin hydrolysis. In: *Process Biochemistry* 45 (4), S. 451–456. DOI: 10.1016/j.procbio.2009.11.001.

Record, M. T.; Courtenay, E. S.; Cayley, S.; Guttman, H. J. (1998): Biophysical compensation mechanisms buffering E. coli protein-nucleic acid interactions against changing environments. In: *Trends in biochemical sciences* 23 (5), S. 190–194. DOI: 10.1016/s0968-0004(98)01207-9.

Riddick, David S.; Ding, Xinxin; Wolf, C. Roland; Porter, Todd D.; Pandey, Amit V.; Zhang, Qing-Yu et al. (2013): NADPH-cytochrome P450 oxidoreductase: roles in physiology, pharmacology, and toxicology. In: *Drug metabolism and disposition: the biological fate of chemicals* 41 (1), S. 12–23. DOI: 10.1124/dmd.112.048991.

Roberts, B. E.; Paterson, B. M. (1973): Efficient translation of tobacco mosaic virus RNA and rabbit globin 9S RNA in a cell-free system from commercial wheat germ. In: *Proceedings of the National Academy of Sciences of the United States of America* 70 (8), S. 2330–2334. DOI: 10.1073/pnas.70.8.2330.

Robinson, Peter K. (2015): Enzymes: principles and biotechnological applications. In: *Essays in biochemistry* 59, S. 1–41. DOI: 10.1042/bse0590001.

Rogl, H.; Kosemund, K.; Kühlbrandt, W.; Collinson, I. (1998): Refolding of Escherichia coli produced membrane protein inclusion bodies immobilised by nickel chelating chromatography. In: *FEBS letters* 432 (1-2), S. 21–26. DOI: 10.1016/S0014-5793(98)00825-4.

Rolf, Jascha; Rosenthal, Katrin; Lütz, Stephan (2019): Application of Cell-Free Protein Synthesis for Faster Biocatalyst Development. In: *Catalysts* 9 (2), S. 190. DOI: 10.3390/catal9020190.

Romero, C.; Manjón, A.; Bastida, J.; Iborra, J. L. (1985): A method for assaying the rhamnosidase activity of naringinase. In: *Analytical biochemistry* 149 (2), S. 566–571. DOI: 10.1016/0003-2697(85)90614-1.

Rosano, Germán L.; Ceccarelli, Eduardo A. (2014): Recombinant protein expression in Escherichia coli: advances and challenges. In: *Frontiers in microbiology* 5, S. 172. DOI: 10.3389/fmicb.2014.00172.

Rotilio, Laura; Swoboda, Alexander; Ebner, Katharina; Rinnofner, Claudia; Glieder, Anton; Kroutil, Wolfgang; Mattevi, Andrea (2021): Structural and biochemical studies enlighten the unspecific peroxygenase from Hypoxylon sp. EC38 as an efficient oxidative biocatalyst. In: *ACS catalysis* 11 (18), S. 11511–11525. DOI: 10.1021/acscatal.1c03065.

Ruehle, Marisa D.; Zhang, Haibo; Sheridan, Ryan M.; Mitra, Somdeb; Chen, Yuanwei; Gonzalez, Ruben L. et al. (2015): A dynamic RNA loop in an IRES affects multiple steps of elongation factor-mediated translation initiation. In: *eLife* 4. DOI: 10.7554/eLife.08146.

Sachse, Rita; Dondapati, Srujan K.; Fenz, Susanne F.; Schmidt, Thomas; Kubick, Stefan (2014): Membrane protein synthesis in cell-free systems: from bio-mimetic systems to bio-membranes. In: *FEBS letters* 588 (17), S. 2774–2781. DOI: 10.1016/j.febslet.2014.06.007.

Sakaguchi, M.; Mihara, K.; Sato, R. (1984): Signal recognition particle is required for co-translational insertion of cytochrome P-450 into microsomal membranes. In: *Proceedings of the National Academy of Sciences of the United States of America* 81 (11), S. 3361–3364. DOI: 10.1073/pnas.81.11.3361.

Schmid, Andreas; Hollmann, Frank; Park, Jin Byung; Bühler, Bruno (2002): The use of enzymes in the chemical industry in Europe. In: *Current opinion in biotechnology* 13 (4), S. 359–366. DOI: 10.1016/S0958-1669(02)00336-1.

Schoch, Guillaume A.; Attias, Roger; Belghazi, Maya; Dansette, Patrick M.; Werck-Reichhart, Danièle (2003): Engineering of a water-soluble plant cytochrome P450, CYP73A1, and NMR-based orientation of natural and alternate substrates in the active site. In: *Plant physiology* 133 (3), S. 1198–1208. DOI: 10.1104/pp.103.020305.

Schulz, Christian; Jung, Friedrich; Küpper, Jan-Heiner (2021): Inhibition of phase-1 biotransformation and cytostatic effects of diphenyleneiodonium on hepatoblastoma cell line HepG2 and a CYP3A4-overexpressing HepG2 cell clone. In: *Clinical hemorheology and microcirculation* 79 (1), S. 231–243. DOI: 10.3233/CH-219117.

Shental-Bechor, Dalit; Levy, Yaakov (2008): Effect of glycosylation on protein folding: a close look at thermodynamic stabilization. In: *Proceedings of the National Academy of Sciences of the United States of America* 105 (24), S. 8256–8261. DOI: 10.1073/pnas.0801340105.

Silvana Andreescu; Bogdan Bucur, and Jean-Louis Marty (2006): Affinity Immobilization of Tagged Enzymes. In: *Methods in Biotechnology: Immobilization of Enzymes and Cells, Second Edition*, S. 97–106.

Simon, Anne E.; Miller, W. Allen (2013): 3' cap-independent translation enhancers of plant viruses. In: *Annual review of microbiology* 67, S. 21–42. DOI: 10.1146/annurev-micro-092412-155609.

Sivashanmugam, Arun; Murray, Victoria; Cui, Chunxian; Zhang, Yonghong; Wang, Jianjun; Li, Qianqian (2009): Practical protocols for production of very high yields of recombinant proteins using Escherichia coli. In: *Protein science : a publication of the Protein Society* 18 (5), S. 936–948. DOI: 10.1002/pro.102.

Sorokin, Ivan I.; Vassilenko, Konstantin S.; Terenin, Ilya M.; Kalinina, Natalia O.; Agol, Vadim I.; Dmitriev, Sergey E. (2021): Non-Canonical Translation Initiation Mechanisms Employed by Eukaryotic Viral mRNAs. In: *Biochemistry. Biokhimiia* 86 (9), S. 1060–1094. DOI: 10.1134/S0006297921090042.

Šrejber, Martin; Navrátilová, Veronika; Paloncýová, Markéta; Bazgier, Václav; Berka, Karel; Anzenbacher, Pavel; Otyepka, Michal (2018): Membrane-attached mammalian cytochromes P450: An overview of the membrane's effects on structure, drug binding, and interactions with redox partners. In: *Journal of inorganic biochemistry* 183, S. 117–136. DOI: 10.1016/j.jinorgbio.2018.03.002.

Stech, Marlitt; Merk, Helmut; Schenk, Jörg A.; Stöcklein, Walter F. M.; Wüstenhagen, Doreen A.; Micheel, Burkhard et al. (2012): Production of functional antibody fragments in a vesicle-based eukaryotic cell-free translation system. In: *Journal of biotechnology* 164 (2), S. 220–231. DOI: 10.1016/j.jbiotec.2012.08.020.

Stech, Marlitt; Quast, Robert B.; Sachse, Rita; Schulze, Corina; Wüstenhagen, Doreen A.; Kubick, Stefan (2014): A continuous-exchange cell-free protein synthesis system based on extracts from cultured insect cells. In: *PloS one* 9 (5), e96635. DOI: 10.1371/journal.pone.0096635.

Thoring, Lena; Dondapati, Srujan K.; Stech, Marlitt; Wüstenhagen, Doreen A.; Kubick, Stefan (2017): High-yield production of "difficult-to-express" proteins in a continuous exchange cell-free system based on CHO cell lysates. In: *Scientific reports* 7 (1), S. 11710. DOI: 10.1038/s41598-017-12188-8.

Thoring, Lena; Kubick, Stefan (2018): Versatile Cell-Free Protein Synthesis Systems Based on Chinese Hamster Ovary Cells. In: *Methods in molecular biology (Clifton, N.J.)* 1850, S. 289–308. DOI: 10.1007/978-1-4939-8730-6_19.

Thoring, Lena; Wüstenhagen, Doreen A.; Borowiak, Maria; Stech, Marlitt; Sonnabend, Andrei; Kubick, Stefan (2016): Cell-Free Systems Based on CHO Cell Lysates: Optimization Strategies, Synthesis of "Difficult-to-Express" Proteins and Future Perspectives. In: *PloS one* 11 (9), e0163670. DOI: 10.1371/journal.pone.0163670.

Thormann, Markus N. (2006): Diversity and function of fungi in peatlands. A carbon cycling perspective. In: *Can. J. Soil. Sci.* 86 (Special Issue), S. 281–293. DOI: 10.4141/S05-082.

Tinafar, Aidan; Jaenes, Katariina; Pardee, Keith (2019): Synthetic Biology Goes Cell-Free. In: *BMC biology* 17 (1), S. 64. DOI: 10.1186/s12915-019-0685-x.

Tompkins, Leslie; Lynch, Caitlin; Haidar, Sam; Polli, James; Wang, Hongbing (2010): Effects of commonly used excipients on the expression of CYP3A4 in colon and liver cells. In: *Pharmaceutical research* 27 (8), S. 1703–1712. DOI: 10.1007/s11095-010-0170-2.

Ullrich, René; Nüske, Jörg; Scheibner, Katrin; Spantzel, Jörg; Hofrichter, Martin (2004): Novel haloperoxidase from the agaric basidiomycete Agrocybe aegerita oxidizes aryl alcohols and aldehydes. In: *Applied and environmental microbiology* 70 (8), S. 4575–4581. DOI: 10.1128/AEM.70.8.4575-4581.2004.

Walter, P.; Johnson, A. E. (1994): Signal sequence recognition and protein targeting to the endoplasmic reticulum membrane. In: *Annual review of cell biology* 10, S. 87–119. DOI: 10.1146/annurev.cb.10.110194.000511.

Walter, Ruben Magnus; Zemella, Anne; Schramm, Marina; Kiebist, Jan; Kubick, Stefan (2022): Vesicle-based cell-free synthesis of short and long unspecific peroxygenases. In: *Frontiers in bioengineering and biotechnology* 10, S. 964396. DOI: 10.3389/fbioe.2022.964396.

Wang, H.; Napoli, K. L.; Strobel, H. W. (2000): Cytochrome P450 3A9 catalyzes the metabolism of progesterone and other steroid hormones. In: *Molecular and cellular biochemistry* 213 (1-2), S. 127–135. DOI: 10.1023/a:1007124417566.

Wang, M.; Roberts, D. L.; Paschke, R.; Shea, T. M.; Masters, B. S.; Kim, J. J. (1997): Three-dimensional structure of NADPH-cytochrome P450 reductase: prototype for FMN- and FAD-containing enzymes. In: *Proceedings of the National Academy of Sciences of the United States of America* 94 (16), S. 8411–8416. DOI: 10.1073/pnas.94.16.8411.

Wang, Yonghua; Lan, Dongming; Durrani, Rabia; Hollmann, Frank (2017): Peroxygenases en route to becoming dream catalysts. What are the opportunities and challenges? In: *Current opinion in chemical biology* 37, S. 1–9. DOI: 10.1016/j.cbpa.2016.10.007.

Werck-Reichhart, D.; Feyereisen, R. (2000): Cytochromes P450: a success story. In: *Genome biology* 1 (6), REVIEWS3003. DOI: 10.1186/gb-2000-1-6-reviews3003.

Xu, Li; Liu, Xiaohong; Yin, Zhenhao; Liu, Qian; Lu, Lili; Xiao, Min (2016): Site-directed mutagenesis of α-L-rhamnosidase from Alternaria sp. L1 to enhance synthesis yield of reverse hydrolysis based on rational design. In: *Applied microbiology and biotechnology* 100 (24), S. 10385–10394. DOI: 10.1007/s00253-016-7676-4.

Yadav, Vinita; Yadav, Pramod K.; Yadav, Sarita; Yadav, K.D.S. (2010): α-l-Rhamnosidase: A review. In: *Process Biochemistry* 45 (8), S. 1226–1235. DOI: 10.1016/j.procbio.2010.05.025.

Yano, Jason K.; Wester, Michael R.; Schoch, Guillaume A.; Griffin, Keith J.; Stout, C. David; Johnson, Eric F. (2004): The structure of human microsomal cytochrome P450 3A4 determined by X-ray crystallography to 2.05-A resolution. In: *The Journal of biological chemistry* 279 (37), S. 38091–38094. DOI: 10.1074/jbc.C400293200.

Yu, Hongshan; Gong, Jinmei; Zhang, Chunzhi; Jin, Fengxie (2002): Purification and characterization of ginsenoside-alpha-L-rhamnosidase. In: *Chemical & pharmaceutical bulletin* 50 (2), S. 175–178. DOI: 10.1248/cpb.50.175.

Zanger, Ulrich M.; Klein, Kathrin (2013): Pharmacogenetics of cytochrome P450 2B6 (CYP2B6): advances on polymorphisms, mechanisms, and clinical relevance. In: *Frontiers in genetics* 4, S. 24. DOI: 10.3389/fgene.2013.00024.

Zanger, Ulrich M.; Turpeinen, Miia; Klein, Kathrin; Schwab, Matthias (2008): Functional pharmacogenetics/genomics of human cytochromes P450 involved in drug biotransformation. In: *Analytical and bioanalytical chemistry* 392 (6), S. 1093–1108. DOI: 10.1007/s00216-008-2291-6.

Zemella, Anne; Thoring, Lena; Hoffmeister, Christian; Kubick, Stefan (2015): Cell-Free Protein Synthesis: Pros and Cons of Prokaryotic and Eukaryotic Systems. In: *Chembiochem : a European journal of chemical biology* 16 (17), S. 2420–2431. DOI: 10.1002/cbic.201500340.

Zemella, Anne; Thoring, Lena; Hoffmeister, Christian; Šamalíková, Mária; Ehren, Patricia; Wüstenhagen, Doreen A.; Kubick, Stefan (2018): Cell-free protein synthesis as a novel tool for directed glycoengineering of active erythropoietin. In: *Scientific reports* 8 (1), S. 8514. DOI: 10.1038/s41598-018-26936-x.

Zhang, Yi; Geary, Timothy; Simpson, Benjamin K. (2019): Genetically modified food enzymes: a review. In: *Current Opinion in Food Science* 25, S. 14–18. DOI: 10.1016/j.cofs.2019.01.002.

Zhao, Mingzhe; Ma, Jingsong; Li, Mo; Zhang, Yingtian; Jiang, Bixuan; Zhao, Xianglong et al. (2021): Cytochrome P450 Enzymes and Drug Metabolism in Humans. In: *International journal of molecular sciences* 22 (23). DOI: 10.3390/ijms222312808.

Zuppone, Stefania; Fabbrini, Maria Serena; Vago, Riccardo (2019): Hosts for Hostile Protein Production: The Challenge of Recombinant Immunotoxin Expression. In: *Biomedicines* 7 (2). DOI: 10.3390/biomedicines7020038.

DeepL and ChatGPT were used for phrasing assistance.

List of publications

Knauer, et al. (2022): Cell-free production of the bifunctional glycoside hydrolase GH78 from Xylaria polymorpha. In: Enzyme and microbial technology 161, S. 110110. DOI: 10.1016/j.enzmictec.2022.110110.

Knauer, et al.: Synthesis of mono Cytochrome P450 in a modified CHO-CPR cell-free protein production platform. (submitted)

Appendix

Frequently used template sequences

NC-GH78-Co (*Sf*21 opt.)

ATGATATCTCGAGCGGCCGCTAGCTAATACGACTCACTATAGGGAGACCACAACGGTTTC
CCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATAAACAAAAGCAAAAATGTGATC
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GCCCTCTCTGCGGTTTTTCAGATTAGGTAGTCGAAAAACCTAAGAAATTTACCTGCTGCC
GTCAGCATCTTCCAGGTGTCCTTCGAGCACCACAGAACCGCTCTCGGAATCGGCGAGGCC
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TCCATCGAGGTGTCCAGACAGGGCAAAGCCGACGTGTTCAACGTGACCTCCTCCGACTCT
GTGCTGGTGGACTGGCCTACAGTGGCTCTGTCCTCTGCTGAGTCTGCCCTCGTCAGAGTG
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CGGACTGCTGGCCGATGATGATTGGAGTGGCGCCCAAGTGATCGCCGCCAACAAGACCA
CAGAGCAGAACGTGCCACACCAGCCTATCCTGCTGCGGAAGGACTTCTCTCTGGATGGGG
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GCGACAGAGTGGGAGATGCTGTGCTGGCTCCTGGCTGGCAGTCCTACAATCACAGACTG
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ACTGAGCGCTCCTGATGGACCTCCTATCCAGCGAGTGGAAGAGGTCCAGCTGCAAGAAGT
GATCACCACACCATCCGGCGCCACCGTGCTGGATTTTGGCCAGAATCTCGTCGGCTGGCT
GCAGCTGAATGTGACAGGACCTGCTGGCACCGCCATCAAGATGGTGCATGTGGAAGTGC
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CAGAGACTGCAGAACATCGTGGAAGCCAACGACTATAGAGTCGGCACCGGCTTCGCTGG
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GCTGATGCAGACCTCCGTGCCTTCCTGGCTGTACCAGGTGGTGGAAAACGGCACCACAAC
CTGGGAGAGATGGGACTCTCTGCTGCCTGACGGCTCTCTGAACGCCAACATGATGACCAG
CTTCAACCACTACGCCTTCGGCTCCGTGGTCAACTGGATGGTCCGAACCATTGGAGGACT
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GACATCTGCTAAGACCACCTACCTGTCTCCATACGGCAGAGTGTCTGCCGAGTGGACCGT
GAACGAGGGCACCTTCAACCTGAAGCTGATCGTGCCTCCTAACTCCAGAGCCGAAGTTGC
TCTGCCTGGCGACAATGGCAGAGTGATGAACGTCGGATCTGGCACCCACACCTTCAAGCT
GTCTGGCGTGGCCGGAAAAGAGTAATAACTAACTAACCAAGATCTGTACCCCTTGGGGCC
TCTAAACGGGTCTTGAGGGGTTTTTTGGATCCGAATTCACCGGTGATATCAT

NC-GH78-Co (*C. gresius* opt.)

ATGGCTGTTTCGATTTTCCAGGTATCTTTTGAACATCATCGCACCGCTCTTGGCATTGGCG
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CGCGTCAAAGCATATGGAGCCGATTCAGTCGATACCGATTGGAGCGAAGCCTTTCCGGTG
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CGGACCCAAGATGCCAACCGCCGTCTGGGGTGACGTGGTGGTTGCAAATGCTTGGAACG
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ACCGGACTGGCCTTGAGCCTCTATTTTGGTCTGTTCCCCGACGGCCAGGACGGCCCTGCC
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CATCTTTCAATCACTACGCCTTCGGAAGTGTGGTGAACTGGATGGTGAGGACCATCGGTG
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GCCTGACATCTGCTAAGACTACCTACCTGAGTCCATACGGCAGAGTGTCCGCTGAGTGGA
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GTGGCCCTGCCAGGCGACAACGGCAGAGTGATGAACGTGGGATCCGGAACGCATACTTT
TAAGCTGTCCGGCGTGGCCGGCAAGGAGTAA

NC-GH78-Co (*X. polimorpha*)

ATGGCAGTATCAATCTTCCAAGTCTCATTTGAGCATCATCGGACCGCCCTGGGCATCGGC
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NC-Flag-Spacer-GH78-Co (*Sf*21 opt.)

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NCM-AaeUPO-Co

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NC-CYP1A2-C0

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