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A Cell-Free Multi-enzyme Cascade Reaction for the Synthesis of CDP-Glycerol

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CDP-glycerol is a nucleotide-diphosphate-activated version of glycerol. In nature, it is required for the biosynthesis of teichoic acid in Gram-positive bacteria, which is an appealing target epitope for the development of new vaccines. Here, a cell-free multi-enzyme cascade was developed to synthetize nucleotide-activated glycerol from the inexpensive and readily available substrates cytidine and glycerol. The cascade comprises five recombinant enzymes expressed in *Escherichia coli* that were purified by immobilized metal affinity chromatography. As part of the cascade, ATP is regenerated in situ from polyphosphate to reduce synthesis costs. The enzymatic cascade was characterized at the laboratory scale, and the products were analyzed by high-performance anion-exchange chromatography (HPAEC)-UV and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). After the successful synthesis had been confirmed, a design-of-experiments approach was used to screen for optimal operation conditions (temperature, pH value and $MgCl_2$ concentration). Overall, a substrate conversion of 89% was achieved with respect to the substrate cytidine.

Introduction

Using vaccines to prevent bacterial infections is an appealing alternative to treatment with antibiotics. Earlier bacterial vaccines often consisted of attenuated pathogens.[1] Later, carbohydrate structures on the surface of bacteria were found to be attractive vaccine targets. The first carbohydrate vaccine was commercially released in 1983 under the name of Pneumo-Vax (Merck and Co.), it consisted of capsular polysaccharides isolated from 14 *Pneumonia* serotypes.[2] As polysaccharide vaccines are unable to elicit a T-cell dependent immune response,[3] glycoconjugate vaccines were developed. They consist of an isolated polysaccharide antigen, usually obtained from pathogen culture, which is fused with a highly immunogenic carrier protein. Recent developments in the field have introduced the use of synthetically produced polysaccharides that can sometimes outperform the natural ones in terms of immunization efficacy and ease of access,[4] while still being safe to use.[5] Some Gram-negative bacteria produce poly(glycosylglycerol phosphate) capsule polymers that can be utilized as antigens for the development of new glycoconjugate vaccines, potentially against pathogens like *Actinobacillus pleuropneumoniae*, *Neisseria meningitis*, and *Bibersteinia trehalosi*.[6] Structurally similar polymers are Gram-positive wall teichoic acids. Several biological functions have been described for teichoic acids such as providing a phosphate reserve,[7] regulating autolysin sensitivity[8] and aiding the assimilation of cations.[9] In addition, wall teichoic acids are interesting vaccine targets.[10] It is possible to synthetize these polymers in vitro through enzymatic reactions, but this process requires CDP-glycerol as a substrate for the wall teichoic acid polymerizing enzymes.[11]

CDP-glycerol is not commercially available; this hinders the research into the synthesis of glycerol-phosphate-containing polymers. The chemical synthesis of CDP-glycerol has been described previously.[12] Unfortunately, this synthesis method is particularly laborious and had low yields of substrate conversion. In a later effort, the enzymatic synthesis using glycerolphosphate cytidylyltransferase (tagD) and glycerol kinase (glpK) was described.[11a,13] This enzymatic one-step reaction synthesis of CDP-glycerol starts from CTP and glycerol-3-phosphate, both of which are expensive at around €999 per 50 g and around €245 per 25 mg, respectively (Carbosynth, 2022). Here, we describe an enzymatic cascade reaction that can synthetize CDP-glycerol from the inexpensive substrates cytidine and glycerol (Figure 1), for which prices are €794 per 2 kg of cytidine (Carbosynth, 2022) and around €10 per L of glycerol depending on the provider.

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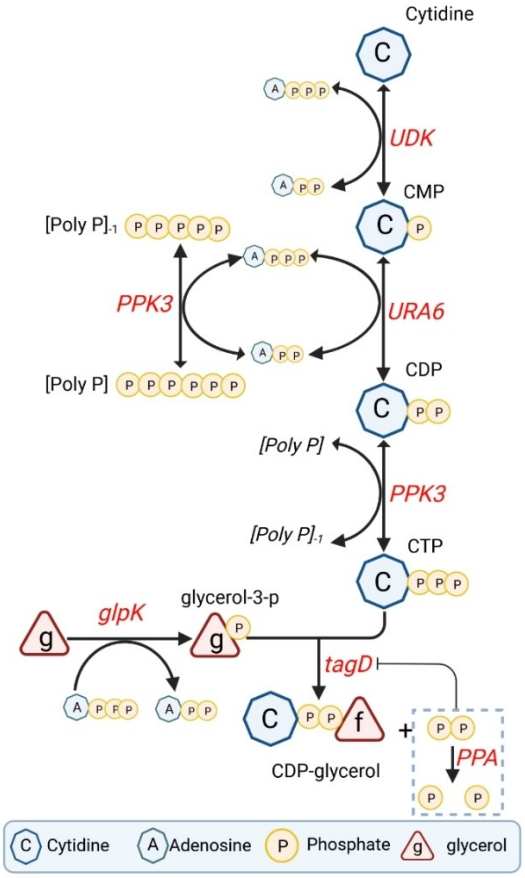

Figure 1. Multi-enzyme cascade with ATP regeneration for the synthesis of CDP-glycerol from cytidine, glycerol and polyphosphate. (Created with Biorender).

The design of the cascade reaction was largely based on enzyme cascades for the synthesis of sugar nucleotides established previously in our group by Mahour et al.[14]

After establishing the enzyme cascade reaction, optimization of the process variables is necessary to achieve an industrially relevant process. In the field of biocatalysis, cascade reactions are often screened using parameters with random variations. In other cases, computational models have been established to predict best inputs for optimizing biocatalytic processes.[15] The latter approach is well suited to optimize biocatalyst and substrate loads.[16] However, with more complex cascade reaction set ups and additional factors like pH value and temperature to be taken into account for optimization, the selection of kinetics and proper parametrization becomes very challenging.[17] On the other hand, a design-of-experiments (DoE)[18] approach is quite promising for successfully improving biocatalytic processes, especially when the scale-up for industrial production is targeted. The approach has not yet been used for enzyme cascade reactions synthetizing CDP-glycerol or similar molecules, but there is a variety of examples from different enzyme chemistries, in which DoE was used to improve biocatalytic reactions. For instance, an enzymatic process for the production of (−)-Ambrox was improved by screening for temperature, pH value and substrate concentrations. Here, the titers improved from around 20 to $>30\ g\,L^{-1}$.[19] In another recent example, DoE screening facilitated the selection of a suitable enzyme and reaction conditions for the reductive amination of cyclohexanone with cyclopropylamine.[20]

In the present work, the optimal pH value, temperature and co-factor concentration to increase the product titer were determined using a fractional factorial DoE set up. The substrate conversion yield was increased from 10% in the first successful cascade implementation to around 89% with the setup identified after DoE screening. The addition of polyphosphate kinase (PPA), an enzyme that degrades the side product diphosphate into monophosphates, did not have a positive effect on yields and the pH value had the most significant effect on product formation.

Results and Discussion

In this section, results from the various steps of the process are shown. First, biocatalyst production, particularly the expression of the recombinant enzymes and purification, is addressed. Second, proof of concept experiments performed to confirm the synthesis of CDP-glycerol from the starting substrates CTP/cytidine and glycerol, are shown. Third, the results of a DoE approach, which was used to identify factors that are relevant to increase the substrate conversion yield, are presented.

Enzyme expression and purification

Each recombinant enzyme was expressed in *Escherichia coli* and purified by immobilized metal affinity chromatography (IMAC; Figure S1 in the Supporting Information). Enzyme stocks are stored in 50% glycerol. However, to control glycerol concentration, a second stock of each enzyme was produced and stored without glycerol.

In the stock with glycerol, glycerol-3-phosphate cytidylyltransferase (tagD) was purified to a final concentration of $28\ g\,L^{-1}$ after buffer exchange and concentration. A total amount of protein of 28 mg was obtained from 200 mL bacterial culture. Without glycerol, the total amount of enzyme was 28.6 mg. As a comparison, Park et al. reported a total amount of 46.3 mg enzyme from a 1 L bacterial culture. The pET expression system was also used in their study but anion exchange chromatography was used for purification instead of affinity chromatography.[21]

Glycerol kinase (GlpK) was purified to a final concentration of $1.6\ g\,L^{-1}$. The total amount of protein was 6.4 mg from a 200 mL bacterial culture. Without glycerol, the total amount of enzyme was 12.1 mg. Previous purifications of this enzyme were reported by Kastumi et al. and Koga et al., who obtained a total amount of 5 mg and 9.2 mg recovered protein from a 1 L culture, respectively.[22] Thus, the amount of protein obtained is well in the range within the previously reported purifications for this enzyme.

Polyphosphate kinase 3 (PPK3) was purified to a final concentration of 0.24 g L^{-1} and the total amount of purified protein was 0.96 mg from a 200 mL bacterial culture. Without glycerol, the total amount of enzyme was 14 mg from a 200 mL culture. This enzyme precipitated during the elution of the chromatographic step. The precipitation of PPK3 is a recurring characteristic that has been reported in literature previously.[14d,23] Most likely, this is due to it being a membrane-associated protein.[24]

With glycerol, UMP-CMP kinase 3 (URA6) was purified to a final concentration of 1.32 g L^{-1}. The total protein obtained was 2.64 mg from a 200 mL bacterial culture. Without glycerol, the total amount of enzyme was 31.5 mg. In the work of Mahour et al., a total amount of 42.84 mg was obtained from a 500 mL bacterial culture.[14a]

Uridine kinase (UDK) was purified to a concentration of 0.91 g L^{-1}. the total amount of protein obtained was 3.64 mg from a 200 mL culture. Without glycerol, the total amount of enzyme was 11.2 mg from a 200 mL culture. To our knowledge, there are no studies reporting the recombinant overexpression and purification of this enzyme. One study by Valentin-Hansen reported a total target protein recovery of 0.6 mg from a 10 L bacterial culture, although no overexpression was done.[27] As shown in Figure S1, the enzyme stock solution contained impurities. However, the expected enzymatic activity was observed (data not shown) and it could be used for the enzyme cascade reactions.

PPA could be purified to a concentration of 12.34 g L^{-1} and a total protein amount of 61.7 mg was obtained from a 200 mL cell culture. Without glycerol, the total amount of enzyme was 46 mg from a 200 mL culture. One study reported a total amount of 2.05 mg recovered from a 500 mL cell culture and another reported 53 mg from a 500 mL cell culture.[14a,d] The amount of enzyme recovered in this work is within the range of the previously reported amounts for this enzyme. A summary of each total purified enzyme, and how they compare to previous literature, is shown in Table 1.

Table 1. Summary of purified enzyme stocks. The highest amount of total purified target protein from a bacterial culture is compared to previously reported purifications.

Enzyme	Total target protein in this work [mg]	Bacterial culture volume [mL]	Total target protein previously reported [mg]	Bacterial culture volume [mL]
tagD	28	200	46.3,[21] 10–15[25]	1000
glpK	12.1	200	5, 9.2[22a,26]	1000
PPK3	14	200	57.85[14a]	500
URA6	31.5	200	42.84[14a]	500
UDK	11.2	200	0.6[27]	10000
PPA	61.7	200	53[14d]	500

Synthesis of CDP-glycerol from CTP and glycerol

The synthesis of CDP-glycerol was first tested in a cascade reaction using only the enzymes tagD and glpK as biocatalyst and CTP and glycerol as substrates. This cascade reaction was performed as a preliminary test to identify the presence of CDP-glycerol as a product. The main species identified after 18 h (Figure 2, blue) of incubation were ADP and CDP-glycerol. It was observed that ATP was fully used and transformed to ADP and AMP. Similarly, CTP was fully transformed into CMP, CDP and CDP-glycerol.

Identification of CDP-glycerol

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was used to confirm the presence of CDP-glycerol and it could be identified as a particular peak during HPAEC-UV analysis. The results from this analysis are summarized in Figures 3 and S2.

CDP-glycerol was identified by its mass at 476.05 *m/z*. As the sample used for this analysis was taken from the acascade reactionmixture, other prominent intermediates such as AMP, ADP and CDP could also be identified. The precursor ion corresponding to CDP-glycerol [*M*−H](−) was analyzed by MS/MS to confirm its identity (Figure S2). In addition, two unidentified compounds were observed (Figure S3). The first compound eluting at around 6 min is most likely an unexpected side product from the cascade reaction.

Although ATP is not known to be a substrate for tagD,[28] the mass balance suggests that an adenosine containing compound different than AMP, ADP or ATP was formed. Through MS analysis, the unidentified compound was identified at 500.07 *m/z* and it is likely to be ADP-glycerol (Figure 3). The second compound eluting shortly before 10 min is hypothesized to be a species of adenosine tetraphosphate, as the enzyme polyphosphate kinase has been reported to synthetize

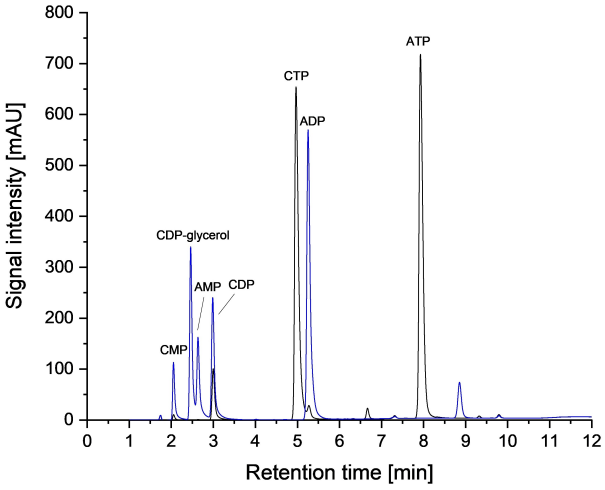

Figure 2. HPAEC-UV chromatogram of enzyme cascade reaction with tagD and glpK as catalysts. In black: time zero, in blue: 18 h of incubation.

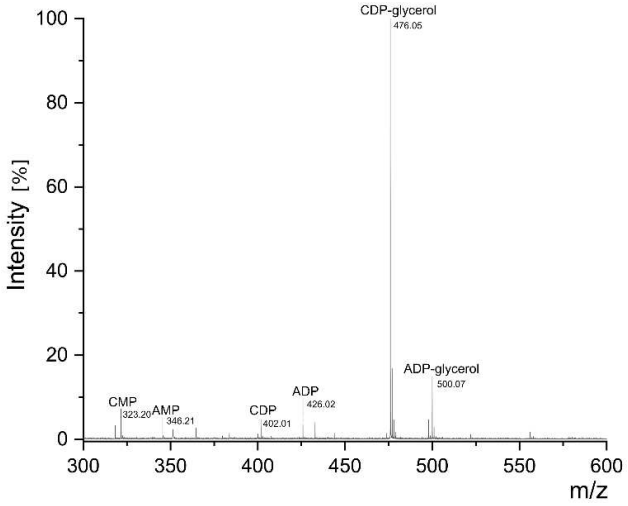

Figure 3. MALDI-TOF-MS of the cascade reaction using only the enzymes tagD and glpK; 1 μL 9-aminoacridine (10 mg L^{-1}) was used as a matrix; [*M*−H](−) ions were detected in reflectron negative-ion mode.

tetraphosphates.[29] The formation of both side products was not considered for the optimization of the cascade reaction.

Synthesis of CDP-glycerol from cytidine and glycerol

Dilution of enzyme stocks in 50% glycerol is a common practice to avoid damage to the enzyme during freezing. As glycerol is the starting substrate for the cascade, reactions were tested with and without storing the enzymes in glycerol. The cascade reaction using only a controlled amount of glycerol resulted in CDP-glycerol production (Figure 4A). Accordingly, this confirms for the first time that CDP-glycerol can be produced from glycerol and cytidine by the designed cascade with active in-situ ATP regeneration.

No CDP-glycerol was produced within 18 h in the cascade reaction set up with enzymes that contained 50% glycerol (Figure 4B). Our hypothesis is that the high amount of glycerol introduced with the enzymes favors the use of ATP by glpK. This results in an excess of glycerol-3-phosphate, preventing other ATP dependent reactions to take place. This is supported by literature, where the K_m for ATP in glpK has been reported to be as low as 0.0078 mM for the wild type enzyme,[30] while the lowest K_m for ATP in UDK has been reported to be 0.062 mM.[31] Or in other words, consumption of ATP by the enzyme glpK is favored. The initial concentration of glycerol seems to be a critical factor for the cascade reaction to produce CDP-glycerol, and the enzyme cascade reaction cannot be properly controlled when enzymes are stored in glycerol. Fortunately, alternative additives to glycerol exist (dimethyl sulfoxide, mannitol), should they be needed for long-term storage and also immobilization of enzymes is a promising alternative strategy.[32]

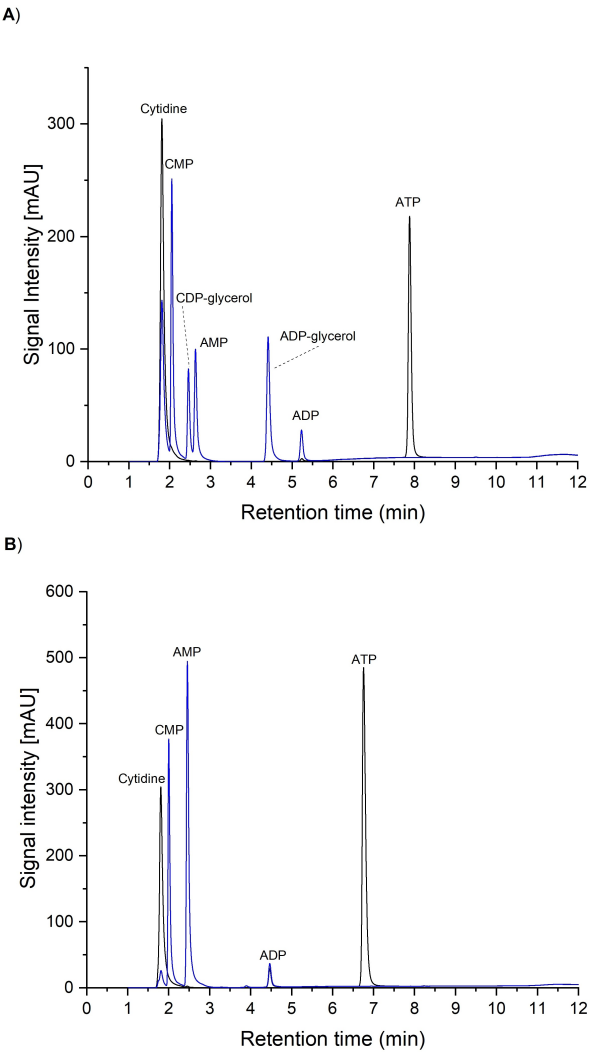

Figure 4. Enzyme cascade reaction with tagD, glpK, UDK, URA6, PPK3 and PPA as catalysts. Enzymes stored A) without glycerol. In black: time zero, in blue: 18 h of incubation and B) in 50% glycerol.

Effect of PPA on the cascade reaction

Two independent cascade reactions were conducted, one with and one without PPA. The purpose was to observe if degrading the side product diphosphate into monophosphates would result in a cascade reaction yielding higher amounts of CDP-glycerol. The concentration of the resulting components over time, measured by HPAEC-UV, is shown in Figure 5.

The final concentration of CDP-glycerol was close to 40 mM in the cascade reaction without the enzyme PPA which indicates a substrate conversion yield of around 70%. Conversely, the assay that included PPA in the reaction mix achieved only around 30 mM of CDP-glycerol after 24 h of cascade reaction, which corresponds to a substrate conversion yield of around 50%.

It is not known why the product yield was higher in the absence of PPA. The enzyme PPA is widely used in cascades for the synthesis of nucleotide derivatives to remove inhibiting diphosphate from the cascade reaction. However, as shown in

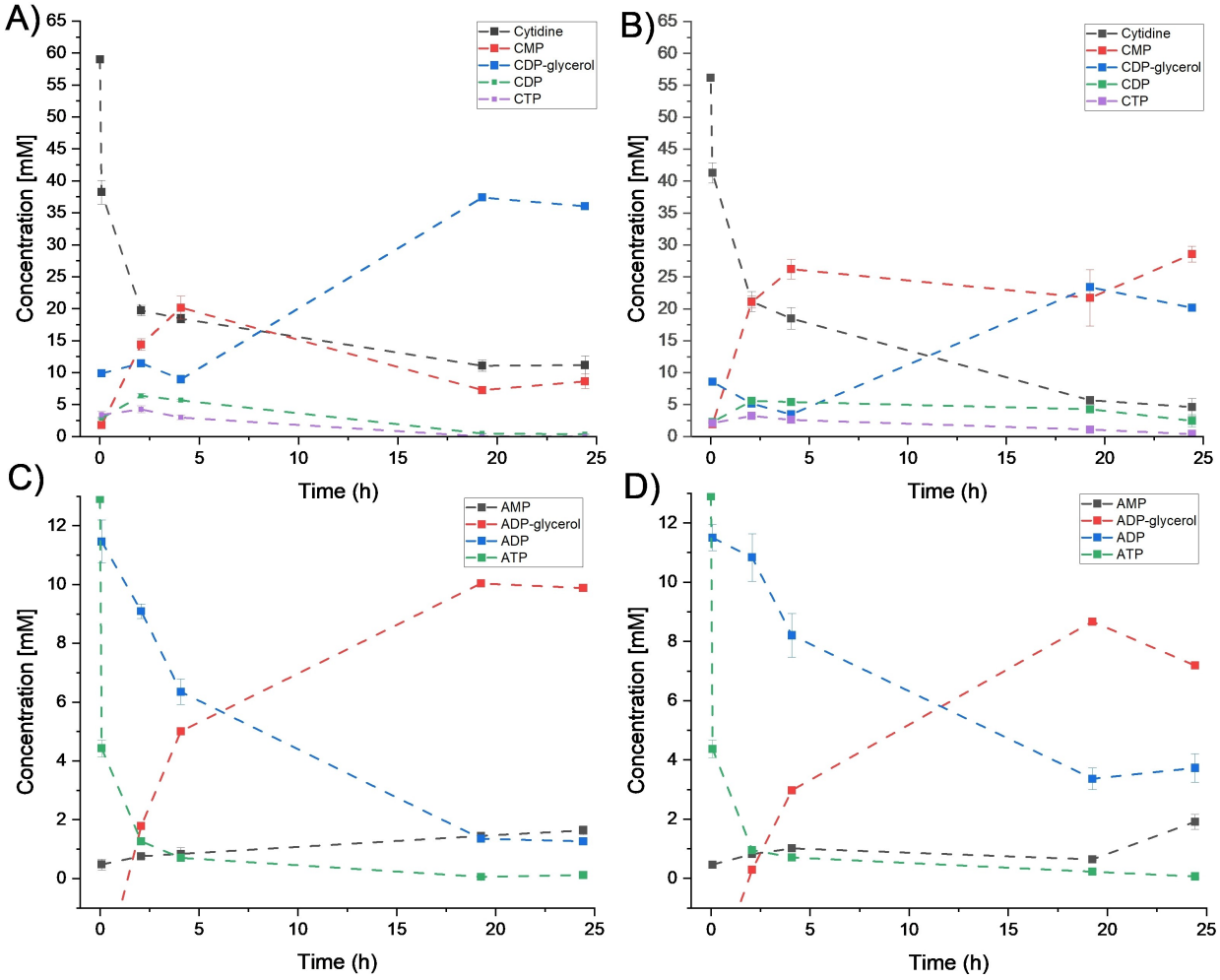

Figure 5. Enzyme cascade reaction for the synthesis of CDP-glycerol without PPA: A) cytidine- and C) adenosine-containing components. Cascade reaction for the synthesis of CDP-glycerol with PPA: B) cytidine- and D) adenosine-containing components. Cascade reactions were performed in biological triplicates; error bars show standard deviation.

Figure 5, no clear benefit could be observed from adding PPA to the cascade reaction. Park and collaborators reported that PPi had an inhibitory effect on tagD which can be due to a rapid equilibrium random order mechanism, and reported an apparent inhibition constant of 0.51 mM.[21] This constant, however, was calculated by varying glycerol-3-phosphate concentrations in a range outside of the substrate concentrations used for this study. Since adding PPA did not increase the product titer, it was decided to optimize the reaction cascade without adding PPA.

DoE set up

DoE approaches allow to reduce the total number of experiments needed to screen a certain set of factors and statistically analyze the data. For an in depth view on the methodology, we recommend the well-known text by Box, Hunter and Hunter,[33] and the DoE specific text by Kuehl.[18] Here, a central composite orthogonal fractional-factorial design with the three factors temperature, co-factor ($MgCl_2$) concentration and pH value was selected for optimization. The following ranges were used for the experimental set up: temperature from 16 °C to 49 °C, pH value from 6.7 to 8.7 and $MgCl_2$ concentration from 0 to 116 mM. Experiments for each combination were performed in triplicates except for the center point, which was performed nine times. The chosen response was the average peak area of CDP-glycerol measured by HPAEC-UV (in mAU*min). Initial concentrations of each component used in the experiments are provided in Table S2 and the results of the 51 independent cascade reaction runs performed are shown in Table S3.

DoE model fit and results

Using MODDE® (see the Experimental Section), we fitted the obtained 51 responses to a quadratic polynomial model and evaluated the model fit. In Figure 6A, a sensitivity analysis showing the effects of the three different factors over the response is depicted. The bars in green represent the change in the response as each factor is varied within its range; a positive value indicates an increase of the measured output response

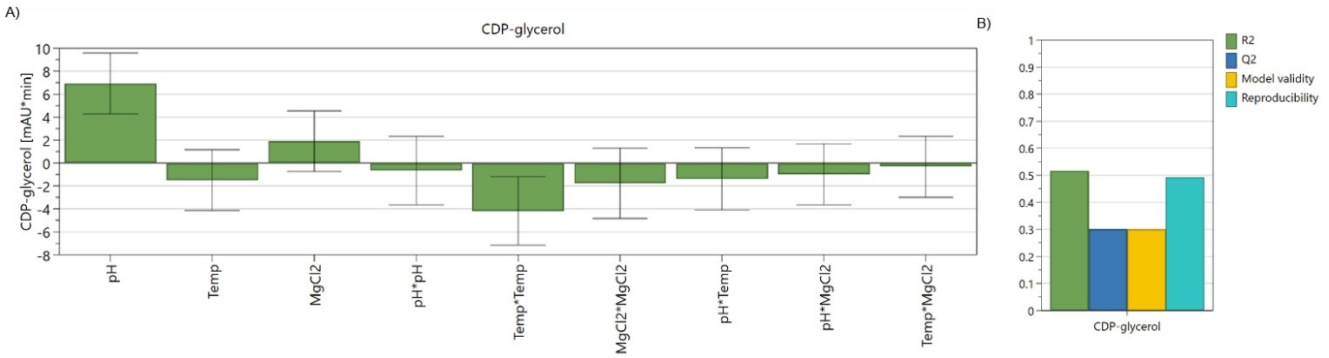

Figure 6. A) Effect graph for each factor. Green bars represent the magnitude of a positive or negative influence on the response, while the error bars represent the significance of the response. Single-factor terms are included as well as interaction and quadratic terms for each factor. B) Summary of fit. *R2*: goodness of fit; *Q2*: prediction precision; model validity: a test for diverse model problems; reproducibility: variation within replicates.

and a negative value a decrease. The error bars represent the significance of the effect for a confidence interval (±95%) of a particular factor; it is considered non-significant when the range of the error includes the zero value. Although we have reason to believe that all chosen factors influence the output of the reaction (i.e., for each enzyme there must be a temperature optimum), the overall sensitivity analysis shows that only the pH value has a significant positive effect on the output in the selected design space. In addition, no significant interactions between factors were found.

A summary of key results is shown in Figure 6B. With $R2 = 0.51$ and $Q2 = 0.30$, a model validity of 0.299 and a reproducibility of 0.49 (one outlier; Table S2), the model can be accepted.

DoE optimization results

In a next step, the built-in optimizer of MODDE®, which uses a response surface methodology, was used for identifying an approximation to the combination of factors that yielded the highest response for CDP-glycerol synthesis. These values were a pH of 8.76, a temperature of 29.2 °C and a $MgCl_2$ concentration of 58.52 mM. A peak area value of 28 mAU min was predicted by the model under this conditions. (An approximation of the concentration was not possible as a CDP-glycerol reference standard was not available). The optimization result is illustrated as a response contour plot in Figure 7.

Optimized cascade reaction

To validate the optimization, a cascade reaction experiment was performed using the optimal pH value, temperature and $MgCl_2$ concentration predicted by the DoE model. The conditions for the cascade reaction were the same as before in terms of enzyme/substrate concentrations; the resulting time courses are shown in Figure 8. The amount of CDP-glycerol reached 31.2 mM (peak area of 22.5 mAU min) after 24 h, which corresponded to a substrate conversion yield of 89%. The concentrations of all intermediate compounds (cytidine, CMP, CDP, CTP, AMP, ADP and ATP) were below 2.5 mM. ATP was fully depleted, meaning the cascade reaction would not continue even when left for a longer period. Although the prediction of a peak area of 28 mAU min was not reached, a significant improvement was achieved, as the substrate conversion yield was increased from 10%, in the earlier trials, to 89% after screening with DoE. This clearly demonstrates the usefulness of DoE approaches in improving cell-free enzyme cascade reactions. Furthermore, due to the high product concentration obtained, relative to other intermediate compounds, the use of this approach resulted in optimized reaction

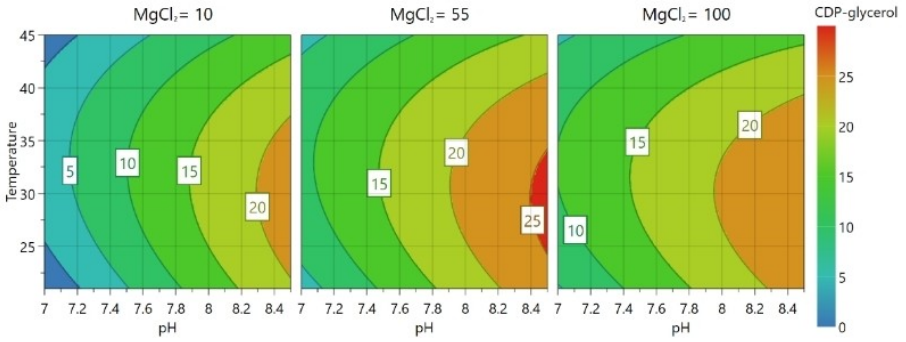

Figure 7. Response 4D contour plot, graphic visualization of the predicted CDP-glycerol yield.

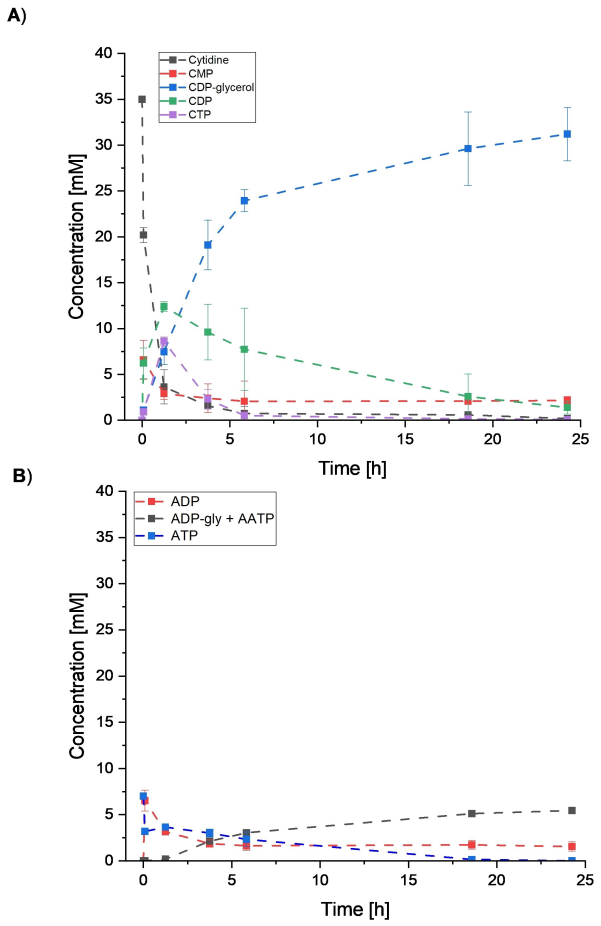

Figure 8. Optimized cascade reaction for the synthesis of CDP-glycerol. A) Cytidine- and B) adenosine-based components. Cascade reactions were performed in biological triplicate; error bars: standard deviation.

conditions that will facilitate the establishment of downstream processing steps for product purification in the future.

Only three parameters were chosen for the optimization: temperature, pH value and $MgCl_2$ concentration. These parameters were chosen because they are known to have an influence on enzyme activity and it is often challenging to establish a kinetic model that predicts the cascade reaction behavior when pH value or temperature change. This is especially the case when multiple enzymes are being used for reactions taking place at the same time. On the other hand, that is not the case for parameters like the starting substrate and enzyme concentrations, where kinetics are better understood. We believe that a further optimization of cascade reactions will be possible in the future by using a combination of both, DoE approaches and kinetic modeling, to optimize a broader set of parameters at the same time.

Conclusions

Glycans for manufacturing conjugate vaccines against bacterial infections can be readily produced by biocatalytic synthesis. However, the accessibility and costs of substrates such as CDP-glycerol is a major hurdle for production on the gram scale and beyond. Here, we have developed a cascade of five recombinant enzymes for the cell-free, one-pot synthesis of CDP-glycerol. This paves the way for the production of a so-far commercially unavailable compound from inexpensive and readily available substrates. Through the screening of cascade reaction conditions in a DoE approach, the yield of the cascade was increased from 10 to 89% with respect to cytidine. The final product titer after a batch time of 24 h was 31.2 mM CDP-glycerol. Using the presented cascade, CDP-glycerol can be produced as a precursor for the enzymatic synthesis of teichoic acids and, thus, facilitate research directed towards novel glycoconjugate vaccines.

Experimental Section

Bacterial strains and vectors: An *E. coli* BL21(DE3) strain (New England Biolabs GmbH) was used as a host for recombinant enzyme expression using a recommended high efficiency transformation protocol.[34] Gene sequences of all enzymes used were codon optimized for expression in *E. coli*, modified to feature a 6x His-tag at the N-terminus and inserted in a pET28a(+) vector by BioCat GmbH.

Pathway design: The enzymes were selected based on their suitability to be used at the same reaction conditions, sequences were retrieved from the online resource Uniprot. The enzymes and experimental conditions used are shown in Table 2. They comprise the ATP dependent UDK, which transfers phosphate from ATP to cytidine to form CMP; URA6, which transfers phosphate from ATP to CMP to form CDP; glpK, which forms glycerol-3-phosphate from glycerol and ATP. CDP is converted to CTP under the consumption of polyphosphate by PPK3. The latter also catalyzes the regeneration of ATP from ADP and polyphosphate. The in situ regeneration of nucleotide triphosphate from polyphosphate by polyphosphate kinases was established by our group earlier for sugar nucleotide

Table 2. Summary of enzymes used for the CDP-glycerol cascade.

Gene	Enzyme	EC no.	Organism	Reaction
tagD	glycerol-3-phosphate cytidylyltransferase	2.7.7.39	*Bacillus subtilis*	Gly3P+CTP →CDP − glycerol+PPi
UDK	uridine kinase	2.7.1.48	*Escherichia coli*	cytidine+ATP →CMP+ADP
URA6	UMP-CMP kinase 3	2.7.4.14	*Arabidopsis thaliana*	CMP+ATP →CDP+ADP
PPK3	polyphosphate kinase 3	2.7.4.1	*Ruegeria pomeyori*	NDP+PolyPn →NTP+PolyPn − 1
glpK	glycerol kinase	2.7.1.30	*Thermococcus kodakarensis*	glycerol+ATP →Gly3P+ADP
PPA	inorganic pyrophosphatase	3.6.1.1	*Pasteurella multocida*	PPi → 2Pi

synthesis.[14] CDP-glycerol is synthesized from CTP and glycerol-3-phosphate by tagD. A co-product of the last step is diphosphate, which can have an inhibitory effect on tagD. Therefore, PPA can be added to the cascade reaction to degrade diphosphate into monophosphate and, in theory, drive the cascade reaction towards the product side (Figure 1).[21]

***E. coli* culture**: A pre-culture was prepared with 25 mL Terrific Broth medium (yeast extract, 24 g L^{-1}; tryptone, 12 g L^{-1}; glycerol, 4 g L^{-1}; KH_2PO_4, 2.3 g L^{-1}; K_2HPO_4, 16.4 g L^{-1}) supplemented with 50 μg mL^{-1} kanamycin in 100 mL shake flasks. The medium was inoculated with one isolated colony from an agar plate and incubated at 37 °C with rotational shaking at 150 rpm for 18 h. The main culture consisted of 200 mL TB media supplemented with 50 μg mL^{-1} kanamycin in 1 L shake flasks. The media was inoculated to an OD_{600} of 0.1 and incubated at 37 °C and 150 rpm until an OD_{600} of 0.8-1 was reached. Then, protein expression was induced by addition of 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) followed by incubation at 16 °C for 8–16 h. The wet biomass was harvested by centrifugation at 7000 *g* for 10 min; the resulting pellet was recovered and stored at −20 °C for further processing.

Immobilized metal affinity chromatography (IMAC): The wet biomass was suspended in lysis/binding buffer (50 mM Tris-HCl pH 8, 300 mM NaCl and 20 mM imidazole) using 10 mL buffer per gram of wet biomass. Cells were lysed using high-pressure homogenization (Maximator GmbH, Germany) at 1,000 bar for 5 min. Cell debris was removed by centrifugation at 12000 *g* and 4 °C for 20 min. The supernatant was recovered for following purification steps.

Affinity chromatography was performed using an ÄKTA start instrument with 5 mL His-trap HP columns (Cytiva, Sweden) Binding buffers composed of 50 mM Tris-HCl at pH 8, 300 mM NaCl and 20 mM imidazole and elution buffers composed of 50 mM Tris-HCl pH 8, 300 mM NaCl and 300 mM imidazole were used. Column equilibration was carried out using the binding buffer followed by elution of all nonbinding proteins by a wash step with a 20/80 % mixture of the elution buffer and binding buffer. His-tagged enzymes were eluted with 100 % isocratic elution buffer. Elution samples were collected in 4 mL fractions. The fractions containing the eluted protein(s) were pooled. The pooled samples were then concentrated, and imidazole was removed through a buffer exchange step using Amicon® Ultra 4–10 kDa centrifugal filters (Merck Millipore) with a centrifugal force of 6000 g for 30 min per step. The storage buffer consisted of 50 mM Tris·HCl pH 8 and 300 mM NaCl. The final protein solutions were stored directly at 4 or -20 °C in 50 % volume glycerol. Enzyme stocks were analyzed for purity using SDS-PAGE with 12 % precast gels (BIORAD Laboratories, Inc.) and Coomassie staining; the concentration was determined by bicinchoninic acid (BCA) assays with the Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific).

Enzyme cascade reactions: The one-pot enzymatic cascade reactions were carried out in 1.5 mL Eppendorf micro tubes at 37 °C and 500 rpm rotational shaking using a total reaction volume of 200 μL, unless stated otherwise. To start a cascade reaction, a mix with all enzymes was prepared and added to another mix containing the reaction substrates, buffers and cofactors. After mixing the solution shortly, vials were incubated in a Thermoblock (Eppendorf AG). Sampling was done by removing the appropriate volume from the cascade reaction to achieve a concentration of less than 100 μM in 1 mL of Milli-Q water. The cascade reaction was stopped by heating to 90 °C for 5 min and stored at −20 °C. The reaction buffer consisted of Tris·HCl with $MgCl_2$, ATP, polyphosphate, CTP/cytidine and glycerol. Glycerol content was approximately 10 % *v/v* when enzyme stock solutions with glycerol were used. Enzymes were used in a concentration ranging from 0.01–0.5 g L^{-1}. A summary of all concentrations and conditions used for cascade reactions in this work is shown in Table S2.

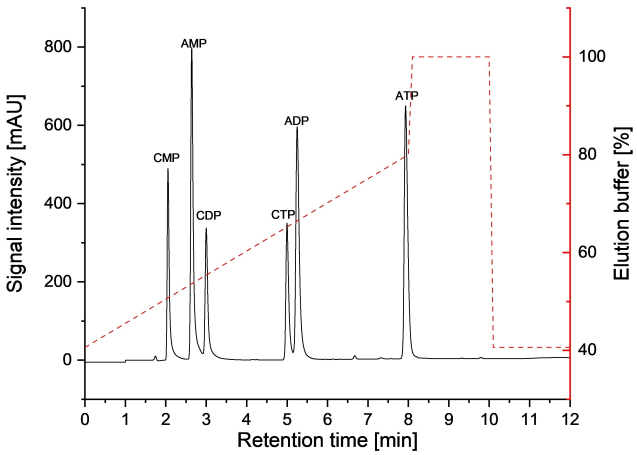

Figure 9. HPAEC of reference standards for CMP, CDP, CTP, AMP, ADP, and ATP at a concentration of 100 μM. Detection by a UV-Vis detector at a wavelength of 260 nm. For the elution, 1 M NaOAc in 1 mM NaOH buffer was applied (red).

DoE: For the DoE approach, the software package MODDE® version 12.1 (Sartorius) was used.

HPAEC-UV: An IC5000 anion-exchange chromatography system (ThermoFisher Scientific Inc.) was employed to quantify reactant concentrations.

The stationary phase consisted of a Dionex CarboPac TM PA200 column (3 mm×250 mm) with the corresponding guard column (3 mm×50 mm). The sample injection volume was 25 μL and the elution and separation of reactants was achieved through an optimized eluent gradient (Table S1) using Milli-Q water and a 1 M NaOAc in 1 mM NaOH solution as elution buffer. The different species present in the cascade reaction were identified by comparing their elution time with an external reference standard (Figure 9). The reference standard was prepared in concentrations varying from 2 to 100 μM to enable quantitative analysis. The concentrations of CDP-glycerol and ADP-glycerol were calculated from the mass balance analysis of the other cytidine-/adenosine-containing components.

MALDI-TOF/TOF MS: The analysis was carried out on an ultrafleXtreme MALDI-TOF/TOF MS (Bruker Daltonics). Briefly, 1 μL 9-aminoacridine (9-AA, 10 mg mL^{-1} in acetone) was spotted on a MTP AnchorChip 800/384 TF MALDI target (Bruker Daltonics). Subsequently 1 μL sample was applied onto the dried matrix layer. Pure ethanol was added to the dried samples for recrystallization. Measurements were carried out in reflectron negative ion mode and LIFT negative ion mode.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: biocatalysis · CDP-glycerol · design of experiments · multi-enzyme cascades

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