Development of a cell-free enzymatic cascade for the synthesis of GDP-fucose - modeling and optimization.

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In nature, glycans can be found in their free form or conjugated to macromolecules like proteins, where they fulfil essential biological functions. They can be synthetized or modified to show particular characteristics. For example, specific antigen glycosylation patterns can result in a better immune response of vaccines and supplementing baby milk formulas with synthetic human milk oligosaccharides can support early development of infants. As new functions and potential applications for glycans are unravelled, there is a growing demand to develop economical synthesis routes that can facilitate tailoring conjugated glycans and enable the synthesis of complex structures.

The nucleotide sugar GDP-fucose is the substrate for the biosynthesis of fucosylated glycans. However, GDP fucose is scarcely available with prices exceeding 120 €/mg. This represents a bottleneck for the commercial production of specialty glycans. To address this challenge, we have established a scalable, cell-free enzymatic cascade reaction to synthetize GDP-fucose from the inexpensive substrates polyphosphate, GMP and fucose. Cell free synthesis has the potential to achieve higher substrate conversion yields and titres than fermentation-based platforms. The initial design of the enzymatic cascade was based on previous work of our research group [1]. A mathematical model comprising enzyme kinetics was established and used to simulate two optimization problems: (a) to maximize the final product titre while keeping a constant enzyme load, and (b) to minimize biocatalyst load without reducing the substrate conversion yield. Experimental data was used for parameter estimation with the software COPASI as a platform for all simulation and optimization steps. After using this approach, both model predictions were experimentally validated, in (a) the substrate conversion yield (GMP to GDP-fucose) was increased from 51.2% to 90.3%, reaching a product titre of 19.86 mM. Additionally, in (b) the biocatalyst load was decreased by 36.2 % while keeping a substrate conversion yield of 51% [2].

Overall, this work demonstrates the potential of rationally designed modelling and optimization approaches to improve cell-free synthesis methods. Results obtained pave the way for the commercial use of the technology. Through the spin-off company eversyn® (www.eversyn.de) low-cost nucleotide sugars and specialty glycans for applications in (bio-)pharma and nutrition will be available in the near future.

[1] Mahour, R., et al., *ChemCatChem* **2021**, *13(8): 1981-1989*.

[2] Huber, N. & Alcala-Orozco, E.A., et al., *Metabolic Engineering*, **2024**, *8*1, 10-25.