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Modelling of an Immobilised Enzyme Cascade in Packed Bed Reactors for Continuous *N*-Acetylneuraminic Acid Synthesis

Kristin Hölting [1,2], Miriam Aßmann [1], Paul Bubenheim [2], Andreas Liese [2], Jürgen Kuballa [1]

[1] GALAB Laboratories GmbH, Am Schleusengraben 7, 21029 Hamburg
[2] Institute of Technical Biocatalysis, Hamburg University of Technology, Denickestr. 15, 21073 Hamburg
Corresponding author: Kristin.Hoelting@galab.de

Introduction

Aim: Process optimisation by modelling for *N*-acetylneuraminic acid synthesis in a continuous packed bed reactor (PBR).

- Neu5Ac is the most abundant sialic acid and monomer of sialylated human milk oligosaccharides such as sialyllactose [1].
- It plays an important role in the regulation of biological recognition, cellular immunity, and disease being approved as a food additive [1].
- The two-step synthesis of Neu5Ac (**Fig. 1**) represents a subsection of a multi-enzyme cascade to produce sialyllactose.

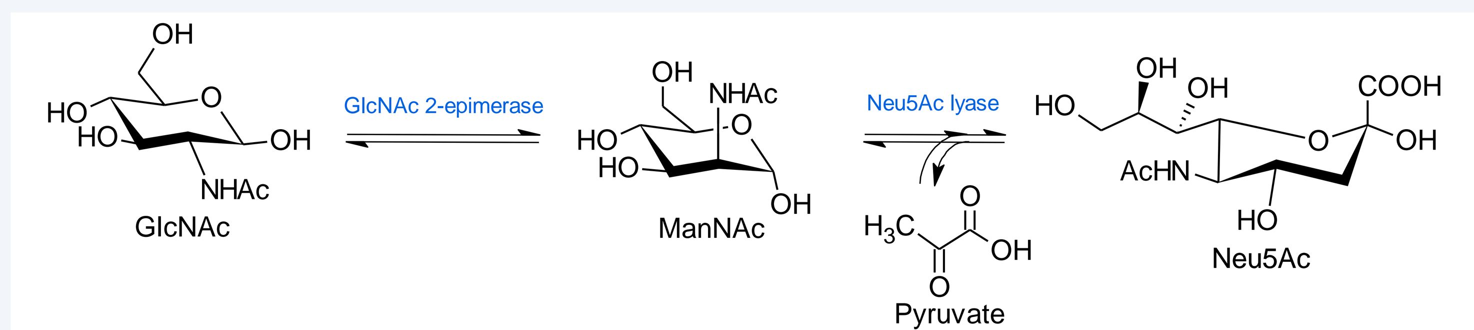

Fig. 1: Conversion of GlcNAc and pyruvate to Neu5Ac with GlcNAc 2-epimerase and Neu5Ac lyase (GlcNAc: *N*-acetylglucosamine, ManNAc: *N*-acetylmannosamin, Neu5Ac: *N*-acetylneuraminic acid).

- Enzyme cascades in industrial processes are often challenging due to the enzyme stability and the difficulties in enzyme recovery and reuse
- Immobilisation of enzymes can increase enzyme stability, influence specificity and selectivity and possibly reduce inhibition [2].
- Covalent enzyme immobilisation offers the advantage of stable bonds that prevent leaching of the enzyme [3].

Experimental Results

Kinetic characterisation:

- Kinetic parameters K_m and v_{max} were determined by non-linear fitting of Michaelis-Menten kinetics to the measured activities (**Table 2**).
- The stepwise optimisation of the kinetic constants to fit the kinetic model was carried out in Phyton.
- The least squares method was used as a criterion.

Stability of immobilised enzymes in a PBR :

- An exponential fitting curve was applied to the measured points to calculate the half-life (**Fig. 5**).
- An increased activity could be analysed for epimerase over the test period. It is not possible to calculate a half-life, but it can be given as **>28 days**.
- A half-life of **57 days** was determined for lyase.

Table 2: Kinetic parameters for immobilised epimerase and lyase in a PBR.

Enzyme	Parameter	Value	Unit
Epimerase	$v_{max,forward}$	130 ± 6	U/g
	$K_{m,forward}$	217 ± 26	mM
	$v_{max,backward}$	955 ± 55	U/g
	$K_{m,backward}$	588 ± 64	mM
Lyase	$v_{max,forward}$	354 ± 39	U/g
	$K_{m,ManNAc,forward}$	328 ± 102	mM
	$K_{m,pyruvate,forward}$	308 ± 21	mM
	$v_{max,backward}$	1396 ± 138	U/g
	$K_{m,backward}$	624 ± 101	mM

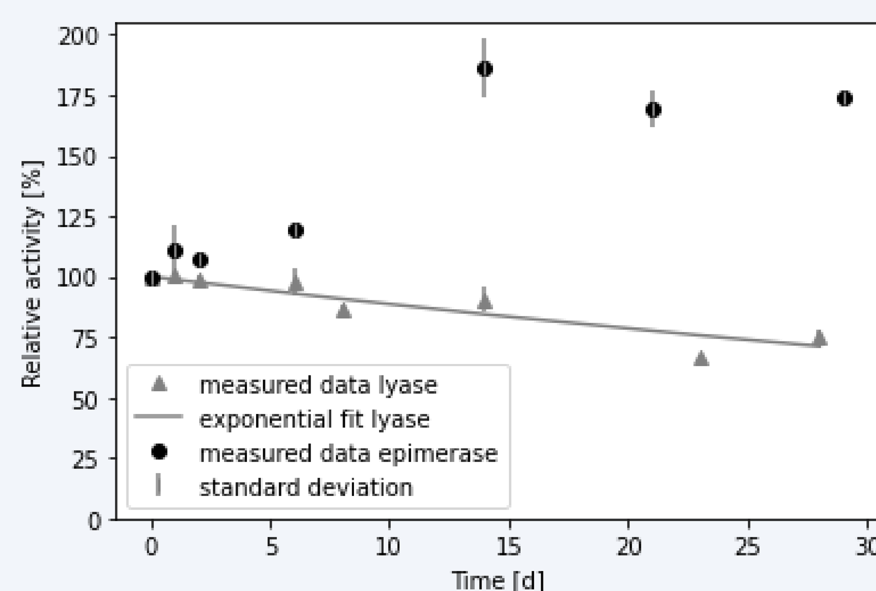

Fig. 5: Stability of immobilised epimerase and lyase in a PBR (Experimental conditions: $\dot{V}$ = 1,5 ml/min, 200 mM Tris, pH 8.0, 20 mM $MgCl_2$, T= 30 °C; Conditons activity assay epimerase: 100 mM Tris, pH 8.0, 1 mM $MgCl_2$, 1 mM ATP, 100 mM GlcNAc, $\dot{V}$ = 1.5 ml/min, T= 30 °C, 25 mg immobilisate, quantifiation of ManNAc by ManDH-Assay; lyase: 100 mM Tris, pH 8.0, 100 mM ManNAc, 250 mM pyruvate, $\dot{V}$ = 1.5 ml/min, T= 30 °C, 205 mg immobilisate, quantification of Neu5Ac by HPLC).

Materials & Methods

Immobilisation:

- Aminomethacrylate carriers (**Table 1, Fig. 2**) pre-activated with glutaraldehyde were used for covalent enzyme immobilisation

Table 1: Carriers for immobilisation.

Enzyme	Carrier	Particle size/ µm	Pore diameter/ nm
Epimerase	Hexamethylamino methacrylate	200-500	40-60
Lyase	Dimethylamino methacrylate	300-710	60-120

Fig. 2: Dimethylamino methacrylate carrier (pre-activated with glutaraldehyde).

Packed bed reactor:

- An UHPLC column with a diameter of 0.3 cm and a length of 3 cm was used as packed bed reactor (PBR) (**Fig. 3**). The reactor was filled with immobilised enzyme.

Fig. 3: UHPLC column used as PBR (ID: 0.3 cm, length: 3 cm).

Kinetic characterisation of immobilised enzymes in a PBR:

- A binary pump was used for kinetic characterisation (**Fig. 4**). This allowed rapid activity analysis at different substrate concentrations by mixing different substrates.
- Experimental conditions: 200 mM Tris buffer, 20 mM $MgCl_2$, pH 8.0, T= 30 °C, $\dot{V}$ = 1.5 ml/min, various substrate concentrations.

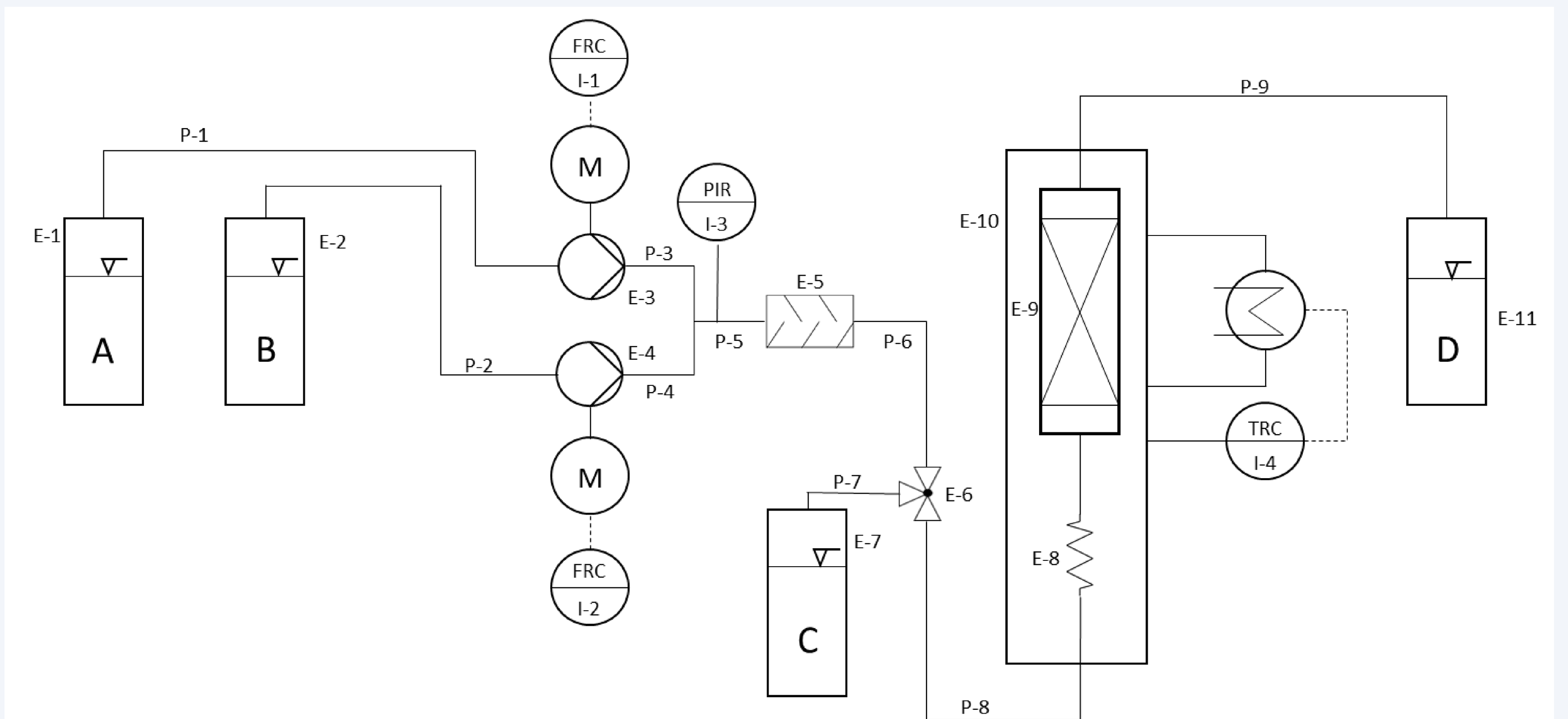

Fig. 4: Experimental setup used to characterise immobilised enzymes in PBR (E-1 & E-2: bottles with substrate; E-3 & E-4: Waters Acquity UPLC pump; E-5: mixing chamber; E-6: sample valve; E-7: sample vessel; E-8: capillary loop for substrate tempering; E-9: PBR; E-10: oven; E-11: bottle with product; P-1 bis P-9: capillaries).

Stability of immobilised enzymes in a PBR:

- Stability tests were carried out by continuously pumping a buffer solution through the PBR. The buffer was recycled in the process.
- Experimental conditions: 200 mM Tris buffer, 20 mM $MgCl_2$, pH 8.0, T= 30 °C, $\dot{V}$ = 1.5 ml/min.

Conclusion & Outlook

- Kinetic parameters were determined for epimerase and lyase.
- Substrate excess inhibition was not observed.
- To model the cascade, the inhibition constants has to be determined.
- Residence times must be determined to model catalysis in the PBR.
- The high stability of the covalently immobilised enzymes demonstrates their suitability for use in PBR.
- Enzyme stability will be part of the model for packed bed lifetime estimation.

References: [1] X. Zhang et al., 'Microbial production of sialic acid and sialylated human milk oligosaccharides: Advances and perspectives', Biotechnology Advances, vol. 37, no. 5, pp. 787–800, 2019; [2] R. A. Sheldon and S. van Pelt, 'Enzyme immobilisation in biocatalysis: Why, what and how', Chemical Society Reviews, vol. 42, no. 15, pp. 6223–6235, 2013; [3] P. De Santis, et al., 'The rise of continuous flow biocatalysis – fundamentals, very recent developments and future perspectives', Reaction Chemistry & Engineering, vol. 5, no. 12, pp. 2155–2184, 2020.

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