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Multi-Enzyme Cascade in Packed bed Reactors for Sialyllactose Synthesis

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Introduction

Aim: Process optimisation by modelling of progress curves for 3'-sialyllactose (SL) synthesis in a continuous packed bed reactor (PBR).

- SL is a human milk oligosaccharide (HMO) and plays an important role in the growth and development of infants [1].
- Multi-enzyme cascades are often challenging. A model can provide information on the effect of parameters for process optimisation [2].
- Development of resilient biocatalysts and design of a suitable reactor are both present challenges for continuous flow technologies [3].
- Covalent immobilisation of enzymes can increase enzyme stability and lead to robust biocatalysts [4].
- The results of the kinetics and modelling of the first sub-cascade (**Figure 1**, shaded blue) are presented.

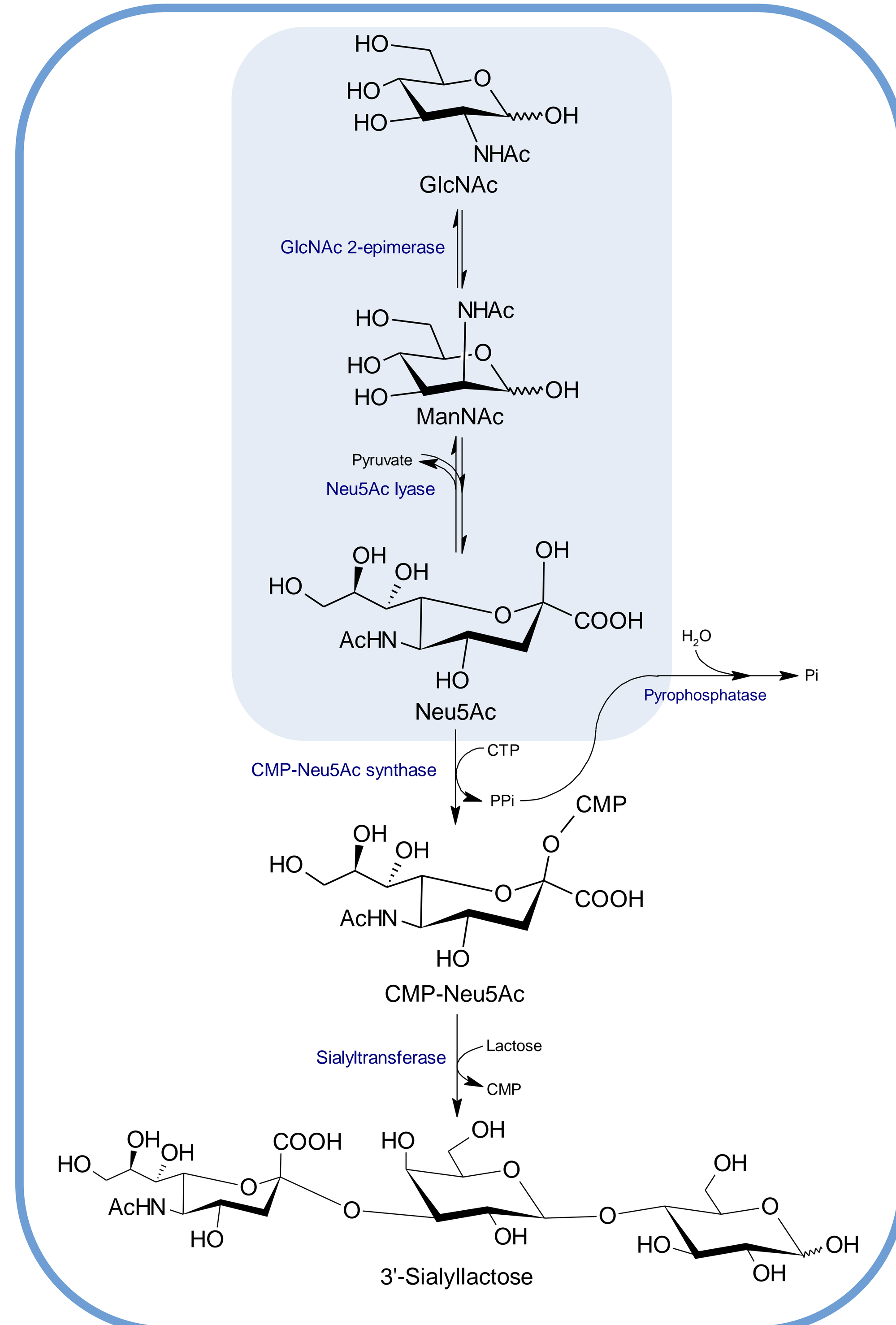

Figure 1. Multi-enzyme cascade for the conversion of GlcNAc, pyruvate, CTP and lactose to SL (GlcNAc: *N*-acetylglucosamine, ManNAc: *N*-acetylmannosamin, Neu5Ac: *N*-acetylneuraminic acid, PP$_i$: pyrophosphate, P$_i$: phosphate, CTP: cytidine triphosphate, CMP: cytidine monophosphate).

Materials & Methods

Immobilisation:

- Aminomethacrylate carriers (**Table 1, Figure 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	Amino C6 methacrylate (Resindion S.R.L)	200-500	40-60
Lyase	Amino C2 methacrylate (Purolite™)	300-710	60-120

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

Kinetic characterisation of immobilised enzymes in a PBR:

- A binary pump was used for kinetic characterisation (**Figure 3**). This allowed rapid activity analysis at different substrate concentrations by mixing different substrates.

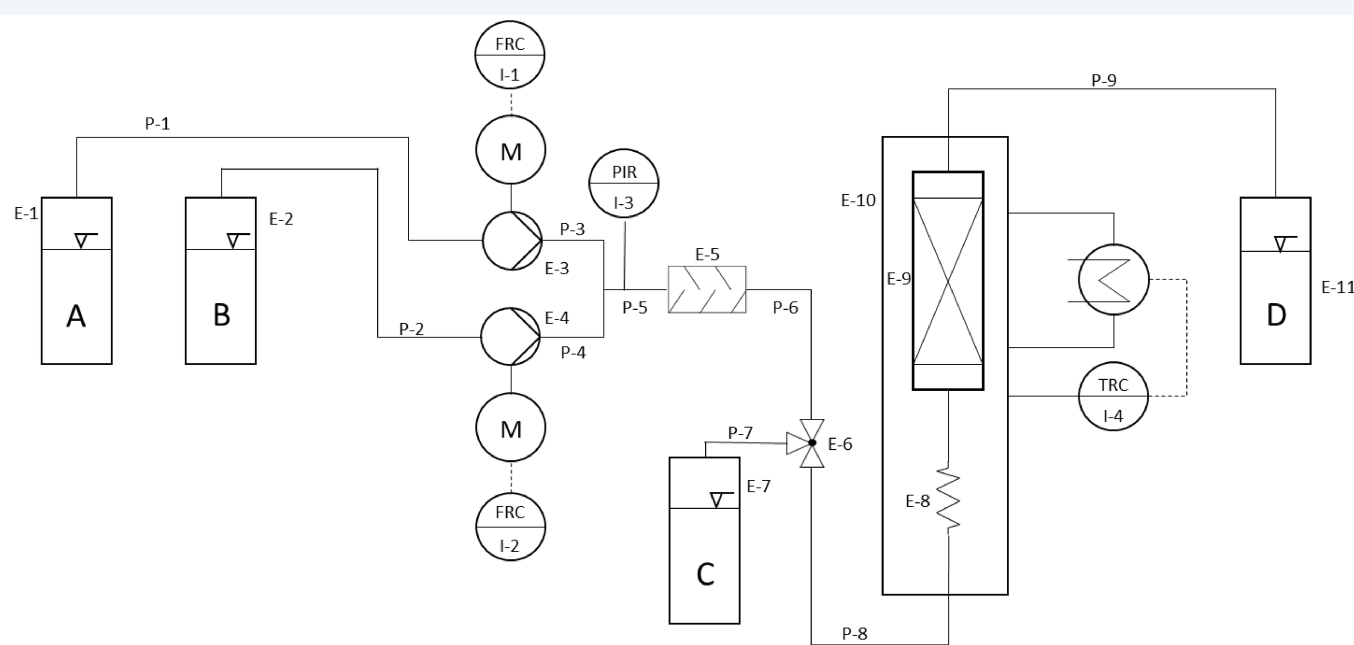

Figure 3: Experimental setup used to characterise immobilised enzymes in a PBR (E-1 & E-2: bottles with substrate; E-3 & E-4: Waters Acquity UPLC pump; E-5: mixing chamber; E-6: sample valve; E-7: sample vessel; E-8: capillary loop for substrate tempering; E-9: PBR (**Figure 4**); E-10: oven; E-11: bottle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

Figure 4. UHPLC column used as PBR (ID: 0.3 cm, length: 3 cm).

Coupled enzyme cascade:

- A column (25 cm length (z), 2.1 cm ID (D)) was filled with gently mixed 38 g immobilised epimerase (EC 5.1.3.8) and 38 g immobilised lyase (EC 4.1.3.3).

Residence time determination:

- The residence time was determined by mass flux analysis using 40 mM pyruvate solution. Absorption was measured at 320 nm.
- Signals were converted to residence time density function to calculate the mean value of the distribution.

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 Python using the least squares method as a criterion.

Kinetic model:

- The concentration (c_i) was calculated over the length of the PBR (z) using the Runge-Kutta method in Python according to equation 1.

$$\frac{\partial c_i}{\partial z} = \frac{r_i}{u_z} = \frac{v_i \cdot m_{immo} \cdot \bar{t}}{V_{PBR}} \qquad (1)$$

- The concentration dependent activities (v_i) were calculated using Michaelis-Menten equations.
- The residence time ($\bar{t}$) of 48 min was determined by mass flux analysis.
- The volume of the PBR (V_{PBR}) of 64 mL was calculated using the determined residence time (equation 2).

$$V_{PBR} = \frac{\pi}{4} \cdot D^2 \cdot z \cdot \frac{\bar{t}_{theory}}{\bar{t}_{measured}} \qquad (2)$$

Model validation:

- The change in concentration (c_i, Equation 1) over the PBR length (z, Equation 1) was calculated by the model.
- The model fit was compared to the measured Neu5Ac concentration at the end of the PBR.
- The model can predict the Neu5Ac concentration with only 6% deviation (**Figure 5**).

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

Enzyme	Parameter	Value*	Unit
Epimerase	$v_{max,forward}$	234 ± 3	U/g
	$K_{m,forward}$	266 ± 10	mM
	$v_{max,reverse}$	797 ± 46	U/g
	$K_{m,reverse}$	294 ± 45	mM
	$K_{I,pyruvate}$	940 ± 107	mM
Lyase	$v_{max,forward}$	294 ± 22	U/g
	$K_{m,ManNAc,forward}$	249 ± 64	mM
	$K_{m,pyruvate,forward}$	229 ± 11	mM
	$v_{max,reverse}$	151 ± 6	U/g
	$K_{m,reverse}$	205 ± 19	mM
	$K_{I,GlcNAc}$	418 ± 40	mM

* Experimental conditions: $\dot{V}$= 1.5 ml·min^{-1}, T= 30 °C, 200 mM Tris, pH 8.0, 20 mM MgCl$_2$, varying substrate concentrations.

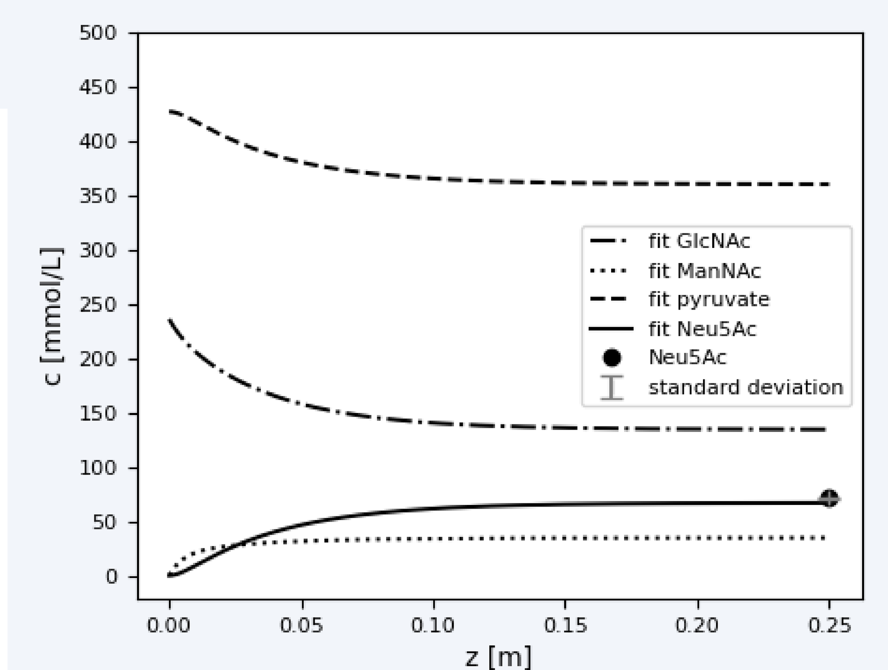

Figure 5. Concentration over the length of the PBR for model validation of the coupled reaction with epimerase and lyase (reaction conditions: PBR with 250 mm length and 21 mm ID, 38 g immobilised epimerase, 38 g immobilised lyase, $\dot{V}$= 1.5 ml·min^{-1}, T= 30 °C, 200 mM Tris, pH 8.0, 1 mM MgCl$_2$, 236 mM GlcNAc, 427 mM pyruvate, 1 mM ATP, Neu5Ac was quantified by HPLC).

Conclusion & Outlook

- Kinetics and residence times were determined for epimerase and lyase.
- The kinetic model can predict Neu5Ac concentration produced in a PBR with 6% deviation and can be applied for process otimisation.
- The progress curve modelling approach has been proven to be applicable.
- Further kinetic studies for CMP-Neu5Ac synthase, pyrophosphatase and sialyltransferase will be carried out to extend the model for process optimisation of the whole multi-enzyme cascade.

References: [1]: Bruggencate et al., Functional Role and Mechanisms of Sialyllactose and Other Sialylated Milk Oligosaccharides. Nutrition Reviews 2014, 72, 377–389.
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