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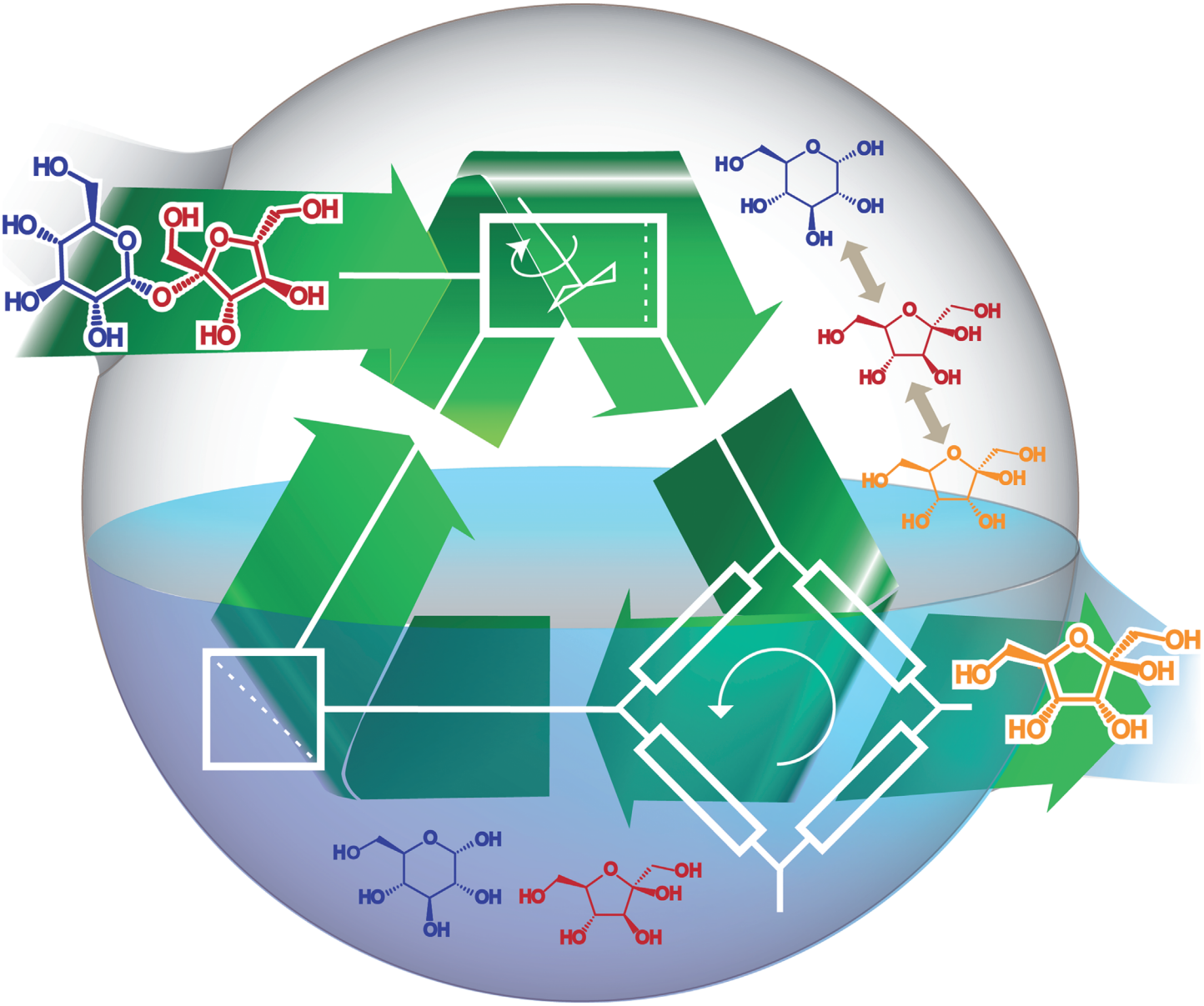
A Separation-Integrated Cascade Reaction to Overcome Thermodynamic Limitations in Rare-Sugar Synthesis**

*Nina Wagner, Andreas Bosshart, Jurek Failmezger, Matthias Bechtold, and Sven Panke**



Abstract: *Enzyme cascades combining epimerization and isomerization steps offer an attractive route for the generic production of rare sugars starting from accessible bulk sugars but suffer from the unfavorable position of the thermodynamic equilibrium, thus reducing the yield and requiring complex work-up procedures to separate pure product from the reaction mixture. Presented herein is the integration of a multienzyme cascade reaction with continuous chromatography, realized as simulated moving bed chromatography, to overcome the intrinsic yield limitation. Efficient production of* D*-psicose from sucrose in a three-step cascade reaction using invertase,* D*-xylose isomerase, and* D*-tagatose epimerase, via the intermediates* D*-glucose and* D*-fructose, is described. This set-up allowed the production of pure psicose (99.9 %) with very high yields (89 %) and high enzyme efficiency (300 g of* D*-psicose per g of enzyme).*

One-pot cascade reactions are considered an attractive alternative to more conventional step-by-step synthetic schemes. They frequently rely on enzymes as myriads of these selective biocatalysts evolved to operate under broadly similar reaction conditions. This combination enables compact one-pot reaction schemes which avoid laborious protection/deprotection steps and intermediate isolation.[1] Among many other benefits,[2] running concomitantly multiple reactions in one vessel allows combination of critical steps, having unfavorable position of the reaction equilibrium, with energetically and highly favorable steps at the end of the cascade to achieve high yield, and indeed this has been exploited in a number of elegant synthetic cascade strategies.[3]

However, the scope of cascade reactions could be substantially broadened if the thermodynamic requirement on the last reaction could be removed. A case in point is the production of rare sugars: here, the existence of multiple chemically similar hydroxy groups requires demanding protection-group chemistry and the use of selective enzymes appears particularly attractive.[4] In fact, cascade reactions with a maximum of only three consecutive enzymatic steps already allow the synthesis of 13 of the 20 rare standard hexoses from either D-glucose, D-fructose, D-galactose (from natural sources), or L-sorbose (from Reichstein's vitamin C process[5]).[6] While this could be highly attractive for increasing the flexibility and compactness of rare sugar synthesis, these cascades consist of isomerases and epimerases with the corresponding unfavorable equilibrium positions,[7] thus severely reducing the yield and thus preventing such cascades from implementation.

A promising possibility to alleviate this problem in a potentially generic way is to compensate for the absence of the energetically favorable final reaction by online integration of the cascade reaction and product removal in a continuous process (Figure 1 a). However, the complexity of the cascade reaction imposes a number of challenging boundary conditions, in particular on the selected separation technique: 1) the technique needs to be sufficiently selective to separate the product from substrate and intermediate(s); 2) the technique needs to be highly efficient to produce the target compound in industrially relevant amounts and productivities; and 3) the continuous operation of reaction and separation requires both to broadly operate under the same reaction conditions.

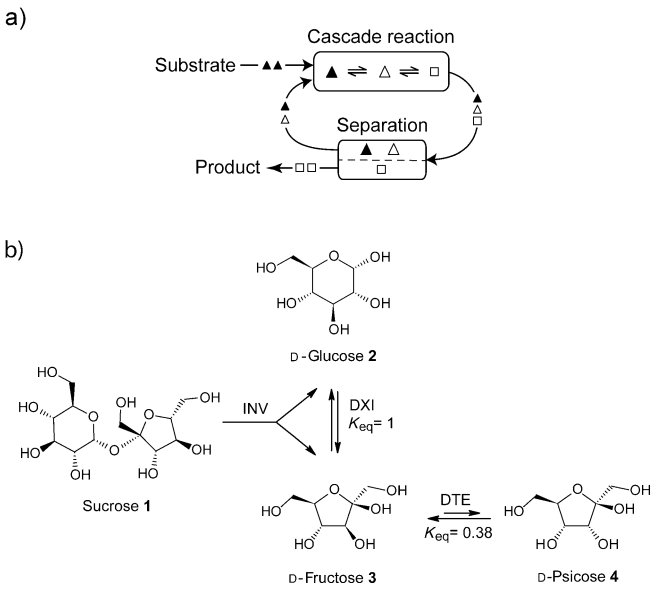


Figure 1. a) Integrated operation of enzyme cascade reaction and product removal. b) Enzyme cascade reaction for the manufacturing of the rare sugar D-psicose (**4**) from sucrose (**1**) with the yield limited by the position of the equilibria of the isomerization and the epimerization reactions. Enzymes that participate in the reaction: invertase (INV), D-xylose isomerase (DXI), D-tagatose epimerase (DTE). Equilibrium constants of the iso- and epimerase reactions at 50 °C are indicated.

While these are formidable prerequisites, continuous countercurrent chromatography, technically realized as simulated moving bed (SMB) chromatography,[8] often fulfills these criteria.[9] In fact, single enzymatic or chemical reactions have been integrated with SMB chromatography before.[10]

Herein, we present the practical integration of a cascade reaction with SMB chromatography to enable the highly efficient production of the rare sugar D-psicose (**4**) from sucrose (**1**), and it involves three biocatalytic steps, the last two of which employ thermodynamically unfavorable isomerase- and epimerase-catalyzed reactions. The sugar **4**, like many of the 20 rare sugars in the standard set of hexoses,[4] has a high potential as either low-calorie sweetener or active pharmaceutical ingredient.[4] The manufacture of **4** was recently demonstrated from D-fructose (**3**) using the enzyme D-tagatose epimerase (DTE).[10b] We took advantage of the availability of sugar-converting enzymes from either natural

[*] N. Wagner, A. Bosshart, J. Failmezger, Dr. M. Bechtold, Prof. S. Panke
Department of Biosystems Science and Engineering, ETH Zurich
Mattenstrasse 26, 4058 Basel (Switzerland)
E-mail: sven.panke@bsse.ethz.ch

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sources (invertase; INV) or our own enzyme engineering efforts (xylose isomerase (DXI) and DTE[11]) to set up a continuous three-step cascade consisting of the hydrolysis of **1** into D-glucose (**2**) and **3**, the isomerization of **2** to **3**, and the epimerization of **3** to **4** (Figure 1b). This was integrated with ligand-exchange chromatography[12] using a Ca^{2+}-substituted stationary phase for the separation of the sugar mixture using water as the liquid phase.

To realize an integrated set up, we addressed first the cascade reaction. We selected enzymes from thermophilic organisms, as they usually exhibit high thermal and operational stability and thus can be employed at elevated temperatures, which are required in sugar processing to reduce microbial contamination and the viscosity of typically highly concentrated feed streams.[13] High temperatures also increase catalytic activity and can shift reaction equilibria towards a more favorable position. We recruited INV (TnINV)[14] and DXI (TnDXI)[15] from the extreme thermophilic bacterium *Thermotoga neapolitana*, and completed the set with a previously thermostabilized DTE from *Pseudomonas cichorii* (PcDTE Var8).[11]

We next compared the specific activities of the three catalysts. The isomerization step was substantially slower ($k_{cat} = 1.56\ s^{-1}$) for TnDXI Mut1F1, a three-site mutant of the wild-type enzyme[16] (see Table S3 in the Supporting Information), than the other two steps in the cascade ($k_{cat} = 118\ s^{-1}$ (TnINV) and $k_{cat} = 36\ s^{-1}$ (PcDTE Var8), all at 50 °C). We therefore increased its specific activity for **2** by a factor of four by iterative saturation mutagenesis,[17] thus resulting in the variant TnDXI-2 (see Chapter S3).

Subsequently, we optimized the joint operation of all three enzymes. Activity profiles at different pH values were recorded (see Figure S6) and a working pH of 7.0 was assigned for cascade operation, as a trade-off of the optimum operating pH values of the individual biocatalysts. To determine the optimal temperature for the cascade, we examined the influence of temperature on thermal activation, unfolding, and irreversible deactivation of each biocatalyst in an isothermal enzyme-membrane reactor (EMR) setup as described before.[18] Briefly, an EMR was continuously supplied with fresh starting material, and conversion at different temperatures was determined by HPLC from periodically collected samples of the outlet stream.

The resulting curves were fitted to biocatalyst inactivation models using a previously described procedure[19] (see Figure S9), thus resulting in inactivation rate constants of each biocatalyst species at 50 °C (TnINV: $3 \times 10^{-7}\ s^{-1}$, TnDXI-2: $1.4 \times 10^{-4}\ s^{-1}$, PcDTE Var8: $2.9 \times 10^{-6}\ s^{-1}$; see the Supporting Information). This study allowed us to calculate the amount of enzyme that needed to be injected periodically into the EMR to compensate for inactivated enzyme and thus to keep the conversion of substrate into intermediates and product constant[18] over a period of at least two days with a feed concentration of 171 $g\,L^{-1}$ (500 mM) for **1** (Figure 2). An excellent agreement between the experimental and simulated concentrations could be observed. As the hydrolysis of **1** is irreversible under these reaction conditions, we reasoned that the separation during integrated operation can be reduced from a quaternary to a ternary problem by providing sufficient TnINV in the EMR to run the first reaction essentially to completion.

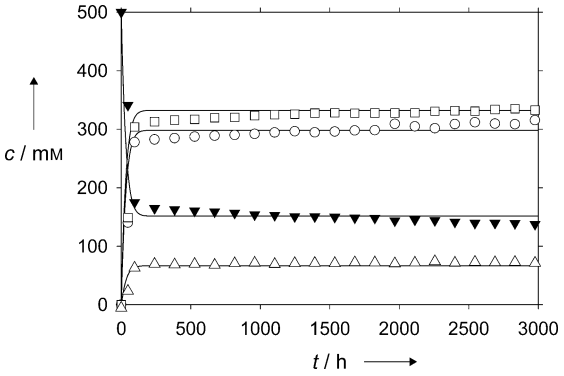

Figure 2. Constant production of **4** over 3000 min from **1** by a three-enzyme cascade in an EMR at 50 °C, with concentrations of **1** (filled triangles), **2** (empty squares), **3** (empty circles), and **4** (empty triangles) as indicated. The solid lines indicate simulated concentrations of the respective compounds taking enzyme inactivation into consideration.

Next, we investigated the multicomponent separation of **2**, **3**, and **4** by SMB using a lab-scale plant employing eight DOWEX 50 WX4-400 (Ca^{2+}) chromatographic columns. A four-zone SMB, a standard technique for the continuous separation of sugars,[20] was used, with each zone characterized by a designated flow-rate ratio m, representing the ratio of net liquid phase flow to simulated stationary phase flow, and a periodic shifting time, t_{sw}, of the outlet ports. This reflects the simulated countercurrent movement of the stationary phase. The sugar feed stream is provided between the two central zones II and III (Figure 3).

The compound that is retained most strongly is transported to the extract port upstream between zone I and II, whereas the compound that is retained more weakly is carried in direction of the fluid flow movement (against the simulated

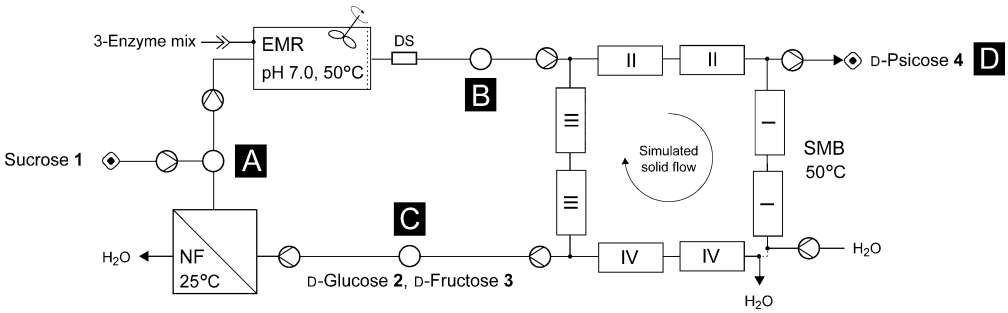

Figure 3. Direct-coupling of a multienzyme-cascade reaction, SMB separation, and NF recycling for the continuous production of **4**. The letters indicate interfaces at which sampling took place.

solid flow; Figure 3) to the raffinate port between zones III and IV. Zones I and IV are used to regenerate solid and mobile phases. The desorbent stream leaving the SMB through zone 4 is here discarded as a waste stream (open loop SMB). Although SMB chromatography is typically used for the separation of binary mixtures, it can be easily adapted for the processing of multicomponent mixtures if the product elutes either as the first or the last compound.[10a] In our case, **4** eluted after **3** and **2**. We carried out SMB experiments using a 1 M (180 $g L^{-1}$) feed solution containing the three hexoses at those concentrations which were predicted to leave the EMR during integrated operation (464 mM **2**, 409 mM **3**, 127 mM **4**). Fractions were collected from the extract, raffinate, and waste stream. We started with an operating point (No. 1 in Table 1) previously identified suitable for the separation of **3** and **4**.[21] As expected, operating point No. 1 showed high purity of **4** in the extract and high recovery of **3** (RC_{Frc}) in the raffinate stream, but only moderate recovery of **2** ($RC_{Glc} = 87\%$). The balance was found in the waste stream, thus indicating that the mobile phase was not fully regenerated. As predicted by the triangle theory for SMB-design,[22] RC_{Glc} could be easily increased up to 99.5% by stepwise decreasing the mass flow ratio in zone 4 (No. 2 to 4 in Table 1).

Table 1: SMB pre-experiments at 50°C for an increased sugar recovery in the recycling stream.

No.	SMB operating parameters					SMB performance		
	t_{sw} [min]	m_I [–]	m_{II} [–]	m_{III} [–]	m_{IV} [–]	PU_{Ex} [%]	RC_{Frc} [%]	RC_{Glc} [%]
1	5.1	1.406	0.704	1.132	0.500	99.9	99.9	87
2	5.1	1.406	0.704	1.132	0.475	99.9	99.9	94
3	5.1	1.406	0.704	1.132	0.450	99.9	99.9	97
4	5.1	1.406	0.704	1.132	0.300	99.9	99.9	99.5

Having separately identified the optimal conditions for the operation of the cascade reactions and the SMB, we next addressed integration of the reaction and separation (Figure 3; for a more detailed scheme see Figure S13). In the fully integrated mode, fresh **1** is mixed with the recycling stream enriched in **2** and **3** and then enters the EMR, where it is partially (ca. 11%) converted into **4**. The EMR-outlet stream enters the SMB unit for separation into product stream (pure **4**, leaving between zone I and II) and a mixed recycling stream containing the intermediate sugars (leaving between zone III and IV). To compensate for dilution during SMB operation, the recycling stream is concentrated by a nanofiltration (NF) device. The implementation of the optimum SMB operating point exhibiting full RC_{Frc} and RC_{Glc} (No. 4, Table 1) led to a strongly diluted raffinate stream, thus requiring unfeasibly high NF concentration factors for our lab-scale system. Thus, we adopted operating point No. 3 (Table 1) which showed decreased recovery of **2** ($RC_{Glc} = 97\%$), but allowed operation of the NF with a feasible concentration factor of 1.8 to obtain again a total sugar concentration (**2** and **3**) of 180 $g L^{-1}$ in the recycling stream. The EMR was operated at 50°C and received initially

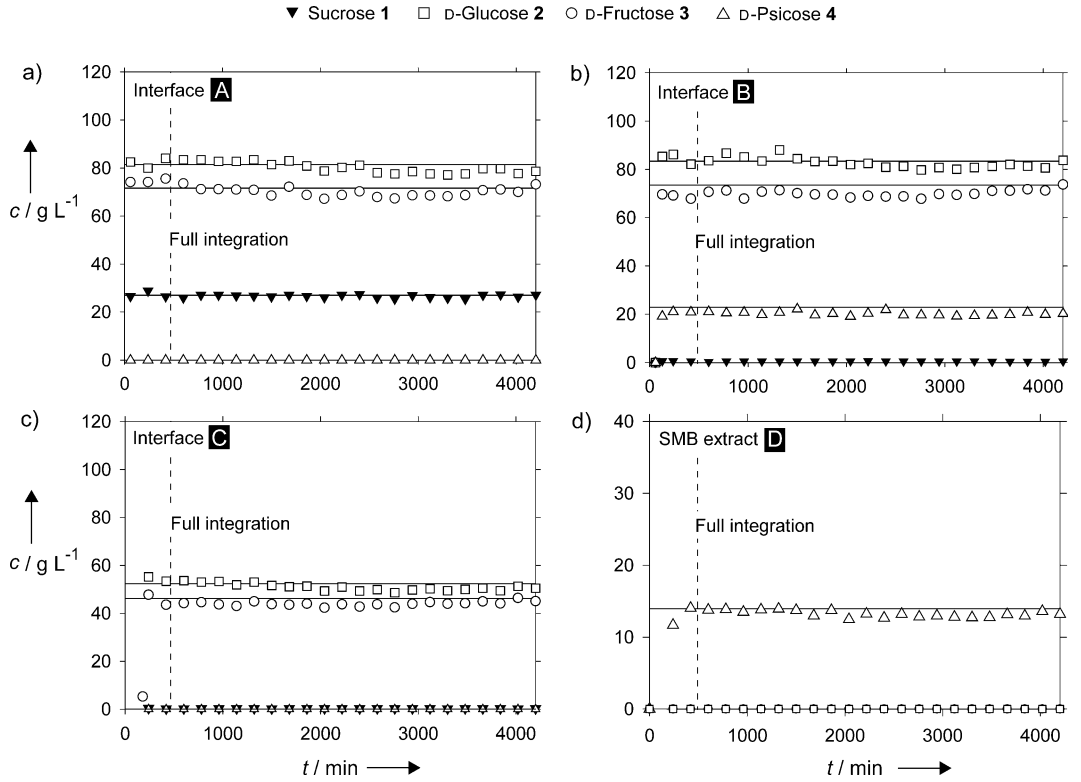

Figure 4. Concentration profiles of the starting material **1**, intermediates **2** and **3**, and the product **4** during operation of the integrated process. Samples were taken at different sampling interfaces (compare Figure 3): a) Interface A (located between NF and EMR), b) Interface B (located between EMR and SMB), c) Interface C (located between SMB and NF) and d) at the extract port D. The time point of full integration is indicated by a dashed line in each plot.

1.5 mg mL^{-1} TnINV, 8.9 mg mL^{-1} TnDXI-2, and 0.95 mg mL^{-1} PcDTE Var8. During the operation, 1.50 μg TnINV, 0.37 mg TnDXI-2, and 4.12 μg PcDTE Var8 were injected every 30 minutes to compensate for losses resulting from biocatalyst inactivation. To shorten the settling time, a feed solution containing **1**, **2**, and **3** at the projected steady-state target concentrations was used during the start-up (see Chapter S10). Finally, for monitoring the process during the integrated operation, three stirred sampling reservoirs (A, B, and C in Figure 3) were installed in the interfaces of the unit operations.

Stable operation of the integrated process was accomplished for more than 4000 minutes (Figure 4), with 3800 minutes of it in a fully integrated manner. The process immediately delivered 12.5 g L^{-1} of **4** in very high purity ($PU_{Ex} \geq 99.9\,\%$). Taking into account the incomplete rejection of the NF membrane for **2** and **3**, the theoretical process yield was 92 % based on **1**, and we found an experimental yield of 89 % by comparing the molar mass flows of **1** entering the process and **4** leaving the system through the extract port (see Chapter S10). This deviation resulted from the small amount of **2** leaving the system in the SMB waste stream, which could be easily circumvented on lager scale by further reducing the setpoint for m_{IV} and employing a NF unit which can cope with the higher dilution of the raffinate flow. The experimental yield compares well with a process yield of approximately 14 %, which could be maximally obtained by running the same cascade reaction in batch mode. A total of 146 mg enzyme was provided over the investigated run time of 4200 minutes, thus translating into a total turnover of 0.3 kg of **4** per gram of total enzyme and a remarkably high EMR space time yield of 1.6 kg L^{-1} day^{-1}. We produced preparative amounts of pure **4** (14 g day^{-1}) in the course of this work and it could be isolated from the aqueous phase in high yields (≥ 85 %) according to a previously described protocol.[21]

In summary, we demonstrate an efficient and, given the achieved yield and productivity, eminently suitable strategy for the synthesis of the rare sugar **4** by employing epimerases and isomerases. Given the diversity of aqueous-media-compatible stationary phases and the wide range of hexose-interconverting iso- and epimerases,[4] we argue that this integrated synthetic strategy enables general access to a variety of rare hexoses.

Keywords: biocatalysis · carbohydrates · enzymes · isomerases · synthetic methods

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