Organic Process Research & Development

ARTICLE

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Practical Aspects of Integrated Operation of Biotransformation and SMB Separation for Fine Chemical Synthesis

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ABSTRACT: Integrated operation of biotransformation and simulated moving bed (SMB) separation is an attractive option for high-yield manufacturing of commercially relevant compounds such as rare sugars and sialic acids from equilibrium-limited isomerase- or aldolase-catalyzed reactions. Here, we present the first lab-scale implementation of such a process using the production of D-psicose, which is currently under consideration as low calorie sweetener, by D-tagatose epimerase-catalyzed epimerization from D-fructose as a model system. While a typical batchwise eprimerization of D-fructose would stop at 25%, a yield of 97% was obtained when operating the fully integrated process consisting of SMB, enzyme membrane reactor (EMR) and nanofiltration (NF) for a number of days with absolute product purities. Next to the proof of principle, important process characteristics such as startup time, stability and robustness were investigated. By pre-equilibrating the NF unit to the projected conditions, startup times could be reduced to the contributions from EMR and SMB (in this case below 5 h) which was perfectly in line with the projected range of operation time of a few days. Robustness was probed by introduction of a perturbation, specifically a 2-fold increase in process feed concentration, which did not compromise any of the set specifications. Next, long-term operation of the respective units indicated a potential process time of at least 5 days, which could be easily extended in the future by engineering a more stable enzyme variant and implementing a cleaning-in-place approach for SMB column regeneration. In summary, the principle feasibility of such process integration for fine chemical synthesis could be successfully demonstrated.

INTRODUCTION

Integration of product formation and separation constitutes an attractive solution for overcoming the yield-limitation of reactions with an unfavorable position of the thermodynamic equilibrium. Selective *in situ* recovery of the product (ISPR) from the reaction space in principle allows for complete conversion of substrate to product and hence 100% yield.[1−3] Such a process intensification strategy could potentially shift novel reaction routes from the academic arena into the realm of industrial application.[4] Prime candidates for such an approach are aldolase- and isomerase-catalyzed reactions that provide direct access to interesting molecule classes such as rare or unnatural saccharides and saccharide-like compounds. Classical chemical synthesis of these compounds requires frequently intensive protection group chemistry,[5] motivating the development of alternative and, in particular, biocatalytic routes where enzyme regioselectivity can facilitate the synthesis. However, the thermodynamic equilibrium of those direct biocatalytic reactions frequently favors the substrate,[6] and the resulting limitation in product yields clearly offsets the advantages of regioselectivity and also of sustainable reaction conditions (mild temperature and pH, aqueous solvent).

In order to increase the yield in such reactions with unfavorable equilibrium selective removal of product from the reaction space while retaining the substrate is required. Since the product and substrate of a single biotransformation often do not differ substantially with respect to their physicochemical properties, their separation requires either a highly selective auxiliary phase or a separation technology that exploits multiple equilibrium stages. The former option often cannot be realized due to the lack or the price of such a phase[1] while for the latter option a proven technology—column chromatography—is available.[7] Application of column chromatography does not permit *in situ* separation in the strict sense since a reactive chromatography approach is not applicable to the reaction types of interest, which are isomerizations and additions for isomerases and aldolases, respectively.[8] Therefore, a spatial separation of reaction and separation is required.

In principle, such a spatially separated ISPR scheme can be realized by sequential batch or continuous operation. Sequential operation of reaction and separation batches requires a number of repetitive cycles for obtaining high yields.[9] In contrast, continuous operation potentially provides directly the product in high yield and high purity (Figure 1). Additionally, simulated moving bed technology (SMB) as the technical realization of continuous chromatography, typically provides higher productivity than batch chromatography at comparable levels of solvent consumption[10] and has evolved into a standard tool for large-scale separations.[11] Furthermore, current industrial examples demonstrate the feasibility and benefit of continuous operation of biotransformations which is considered an emerging key technology in white biotechnology.[12,13] However, integration of these advanced technologies poses some unique challenges to the selection of material and process design. The direct coupling requires the use of the same solvent in all unit operations which is by no means a trivial constraint as established preparative chromatography often relies on organic solvents.[14] Hence, selection of an interoperable mobile phase/reaction medium constitutes an important optimization variable for such a process.

Special Issue: INTENANT

Received: June 16, 2011
Published: July 22, 2011

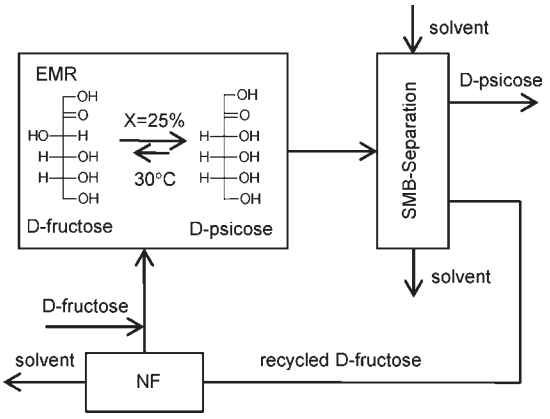

Figure 1. Locally separated *in situ* product removal scheme for the production of D-psicose from D-fructose. The reaction takes place in the enzyme membrane reactor (EMR). The mixture of D-fructose and D-psicose is separated via SMB. D-Fructose is concentrated in a nanofiltration (NF) device before re-entering the EMR. This setup enables theoretically a chemical yield of 100%.

Arguably, the production of rare sugars by enzyme-catalyzed epimerization and isomerisation reactions constitutes presently one of the most promising areas of application for such a process integration strategy: Conveniently, chromatographic separation of sugars is already based on the use of ion exchangers employing enzyme compatible aqueous solution as mobile phase;[15−17] rare sugars like D-psicose, D-allose, or D-tagatose have lately attracted considerable interest as functional food component (e.g., low-calorie sweeteners and prebiotics[18−20]) or precursors for pharmaceuticals;[21,22] and the currently available set of isomerases and epimerase enzymes enables to obtain most hexose stereoisomers (including those mentioned above) from cheap starting materials such as D-glucose, D-fructose, D-galactose, or L-sorbose.[23]

In the scope of this work a lab-scale process installation was developed and implemented that realizes integrated operation of bioreactor, SMB and nanofiltration to manufacture the rare but commercially attractive sugar D-psicose[24] from D-fructose. To our knowledge this is the first report on such an integrated process. Next to demonstrating the proof of principle, other practical aspects that equally determine the applicability of a process such as stability, startup and process robustness were evaluated. D-Psicose formation from D-fructose was selected as a model biotransformation, because next to the commercial interest for D-psicose, suitable enzymes—the D-tagatose epimerases (DTEs)—from different sources[25,26] as well as a suitable chromatography material[27] were reported before.

MATERIALS AND METHODS

Chemicals. D-Psicose of high purity was produced in-house by epimerization of D-fructose using DTE and subsequent purification of D-psicose by stand-alone SMB separation (using a similar design approach as described in the Results section). D-Psicose was crystallized from the collected SMB extract by first concentration by evaporation and subsequent drowning out by addition of ethanol. Crystals were washed with ethanol and dried in the exsiccator. D-Psicose purity of >99.5% was confirmed by HPLC analysis. All other chemicals were of analytical grade and purchased from Sigma-Aldrich (Buchs, Switzerland). Ultrapure water was obtained from a GenPure Water system (TKA Thermo, Germany).

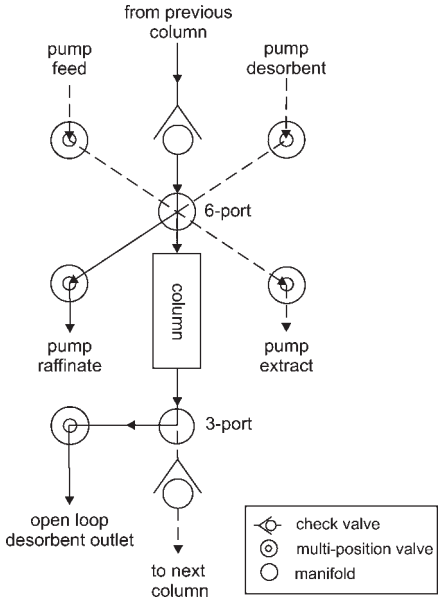

Figure 2. Setup for a single column in the 2−2−2−2 SMB displaying all possible flow paths (dotted arrows). The solid arrows indicate the flow path for a column located in zone 4 (solid arrows) when no desorbent recycling is employed (open loop).

Enzyme Preparation. DTE (E.C. 5.3.1.) from *Agrobacterium tumefaciens* was overexpressed in *Escherichia coli* BL21(DE3) from the expression plasmid pET-30a after growth in M9 medium supplied with glucose as the carbon source and induction with 0.05 mM IPTG at 18 °C. Cells were harvested by centrifugation, resuspended in aqueous buffer (containing 50 mM 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris), adjusted to pH 8.0 with HCl and 1 mM $MnCl_2$), and disrupted in a high-pressure homogenizer (Haskel Hochdrucksysteme, Wesel, Germany) at 1200 bar pressure drop over the orifice. The cell-free extract (CFE) was clarified from cell debris by centrifugation at 6000*g* for 30 min at 4 °C and stored at −80 °C. In order to guarantee complete saturation of the enzyme's active site with Mn^{2+} ions all aliquots of thawed CFE were incubated with 1 mM $MnCl_2$ at 4 °C for 180 min prior to further use. Before use in the integrated process the activity of each aliquot was determined by measuring D-psicose formation using 200 mM D-fructose as initial substrate concentration in 50 mM Tris buffer (pH 8.0) at 30 °C. The initial substrate concentration was chosen to be significantly above the previously estimated K_m of 24 mM.[26] One unit (U) of DTE was defined as the amount of enzyme producing 1 μmol min^{-1} of D-psicose at 30 °C and pH 8.0.

Operation of the Enzyme Membrane Reactor (EMR). Biocatalysis was performed in a jacketed 10-mL continuous stirred tank reactor (Julich Fine Chemicals, Julich, Germany) equipped with an AMICON regenerated cellulose membrane (nominal cutoff of 10 kDa) purchased from Millipore Cooperation (Bedford, MA, U.S.A.). Unless stated otherwise, the EMR was operated at 30 °C with a magnetic stirrer rotating at 200 rpm and using 100 mM D-fructose in 15 mM Tris pH 8.0 as substrate solution. The reactor was loaded with 500 U of DTE. Initial investigations did not indicate any observable side reactions when using CFE as judged by the mass conservation of D-fructose and D-psicose.

SMB Columns. DOWEX 50 WX4-400 was purchased in H^+-form from Sigma Aldrich (Buchs, Switzerland) and converted to

dx.doi.org/10.1021/op200160e |*Org. Process Res. Dev.* 2012, 16, 323–330

the Ca^{2+}-form by incubation with a 300 mM Ca_2Cl solution for 1 h. The pretreated material was packed into PEEK columns (150 mm × 7 mm I.D.) obtained from Omnilab (Mettmenstetten, Switzerland). The external bed porosities of all columns were measured using dextran (average molecular weight = 100−200 kDa) as a nonadsorbing tracer. An average porosity of 0.41 ± 0.01 was determined. Adsorption isotherms were determined by analysis of finite injection HPLC experiments using different sample concentrations[28] of D-psicose and D-fructose (0.05−1.4 M) and water as mobile phase.

SMB Setup. The implementation of a laboratory-scale SMB-plant based on the Amersham ÄKTAbasic-10 system (GE Healthcare, Uppsala, Sweden) was described in detail previously.[28] The SMB was operated in open-loop arrangement with eight columns in a 2−2−2−2 configuration (Figure 2). At the inlet of each column a six-port manifold was placed which connected the columns to each other and to multiposition valves that directed the SMB inlet and outlet flows, namely feed, eluent, extract and raffinate. Check valves (CV-3000, Upchurch Scientific, Oak Harbor, U.S.A.) were installed between columns to ensure correct flow direction. Back pressure relief valves (1000 psi, Upchurch Scientific, Oak Harbor, U.S.A.) were implemented downstream of the feed and eluent pump in order to accommodate for local pressure buildup during valve switching. An additional multiposition valve that was connected to a three-port manifold installed at the outlet of each column was dedicated to direct the waste outlet stream from zone IV in order to realize the open-loop arrangement. The SMB installation including eluent reservoirs was thermostatted at 25 °C by housing the system in a refrigerated incubator. The SMB unit was controlled with a modified version of the UNICORN software (GE Healthcare, Uppsala, Sweden).[29,30] SMB design was performed on the basis of the triangle theory.[31]

Operation of the Nanofiltration (NF). Concentration of sugars was carried out using a custom designed cross-flow NF device (MMS AG, Urdorf, Switzerland) with an integrated gear pump (Scherzinger, Furtwangen, Germany). The NF membrane NF-90 (FilmTec Cooperation, Minneapolis, U.S.A.), a polyamide membrane with a nominal cutoff of 90 Da,[32] was positioned in the jacketed annular membrane compartment, providing a surface area of 28 cm^2. The NF feed was supplied by a MERCK L-6200A HPLC pump (VWR, Nyon, Switzerland). Constant flux through the membrane was achieved by using a LIQUI-FLOW flow controller (Bronkhorst, Reinach, Switzerland) which was installed at the outlet of the retentate stream. The NF unit and the flow controller were controlled by a Labview program (National Instruments, Austin, U.S.A.) that was provided by the NF manufacturer. For regeneration the membrane was washed for 2−3 h with pure water.

Quantification of Concentrations. Samples were analyzed on an Agilent 1200 series HPLC system equipped with a RI-101 detector using a self-packed DOWEX50 WX4-400(Ca^{2+}) column (150 mm × 7 mm I.D.) or an AMINEX Fast Carbohydrate (Pb^{2+}) column with a Carbo-P guard column (100 mm × 7.8 mm I.D.) (Biorad, Reinach, Switzerland). The former column was employed with pure water as mobile phase at 85 °C and a flow rate of 2 mL min^{-1} and was used exclusively for D-fructose quantification. D-Psicose quantification was conducted with the latter column using the same mobile phase at 25 °C and 0.5 mL min^{-1}. Next, a DIONEX LC DX-300 system equipped with a guard column CarboPac PA1 column (50 mm × 4 mm I.D.) and a CarboPac PA1 (250 mm × 4 mm I.D.) was used for confirming purities when high accuracy was required. Samples were eluted employing an isocratic concentration of 25 mM aqueous NaOH at a flow rate of 2.5 mL min^{-1}. They were detected via triple pulsed amperometry using an ED detector with a gold electrode (all Dionex, Sunnyvale, U.S.A.).

Table 1. Summary of performance parameters of each unit in integrated process

operational unit	performance parameters	
EMR	equilibrium constant [−]	
	0.33	
SMB	Henry constants [−]	
	D-fructose	D-psicose
	0.99	2.55
	HETP at 1 mL min^{-1} [cm]	
	D-fructose	D-psicose
	0.30	0.43
NF	hexose rejection [%]	
	>98	

Performance Parameters. In order to evaluate the performance of the integrated system and its respective subunits different parameters were applied. Conversion X in the reactor was defined as the ratio of concentrations of product (here, D-psicose) $c_{Psi,EMR,out}$ leaving the reactor and substrate (D-fructose) $c_{Fru,EMR,in}$ entering the reactor.

$$X = \frac{c_{Psi,EMR,out}}{c_{Fru,EMR,in}}[-]$$

The purity PU of the SMB raffinate stream was defined as ratio of the concentration of the less retained component (D-fructose) to the total concentration of the binary mixture (D-fructose and D-psicose) in the raffinate. The extract purity PU_{Ex} was defined with respect to the more retained component, D-psicose.

$$PU_{Ex} = 100\frac{c_{Psi,Ex}}{c_{Psi,Ex} + c_{Fru,Ex}}$$

$$PU_{Raff} = 100\frac{c_{Fru,Raff}}{c_{Fru,Raff} + c_{Psi,Raff}}[\%]$$

The ability of the membrane to retain the applied monosaccharides was expressed by the rejection REJ that denoted the distribution of concentrations between the retentate (c_{Ret}) and the permeate side (c_P). Since the task assigned to the nanofiltration was concentration of the substrate, the rejection was expressed in terms of D-fructose concentration.

$$REJ = 1 - \frac{c_{Fru,P}}{c_{Fru,Ret}}[-]$$

The total throughput TP of the integrated system was calculated from the extract port by:

$$TP = Q_{Ex}c_{Psi,Ex}\ [mg\ h^{-1}]$$

The yield Y was calculated from the ratio of throughput and fed substrate-mass flow:

$$Y = 100\frac{Q_{Ex}c_{Psi,Ex}}{Q_S c_{Fru,S}}[\%]$$

Process Simulations. SMB operation was simulated using a SMB node model combined with a transport chromatography model[14] implemented in Matlab (Mathworks, Natick, U.S.A.). Simulations of coupled EMR and SMB operation were performed

Table 2. Flow rates used in integrated operation throughout this work and steady-state concentrations of D-fructose and D-psicose measured in the proof-of-concept-run

stream	flow rate [mL min^{-1}]	concentrations	D-fructose [g L^{-1}]	D-psicose [g L^{-1}]
Q_S	0.08	c_S	18	0
$Q_{EMR,in}$	0.26	$c_{EMR,in}$	18	0
$Q_{EMR,out}$	0.26	$c_{EMR,out}$	13.5	4.5
$Q_{SMB,in}$	0.25	$c_{SMB,in}$	13.5	4.5
Q_{Raff}	0.44	c_{Raff}	7.67	0
Q_{Ex}	0.47	c_{Ex}	0	2.37
$Q_{D,in}$	1.10	$c_{D,in}$	0	0
$Q_{D,out}$	0.44	$c_{D,out}$	0	0
Q_P	0.25	c_P	0.25	0
Q_{Ret}	0.18	c_{Ret}	18.3	0

applying a CSTR model for the EMR[33] and the SMB node model combined with a transport dispersive model.[27] These combined models were implemented in gProms (PSE, London, UK). The applied process parameters are summarized in Tables 1 and 2.

RESULTS AND DISCUSSION

Process Description. Integrated operation of SMB, bioreactor, and NF holds great potential for the production of fine chemicals from thermodynamically limited reactions. In order to demonstrate the principle feasibility of this novel process concept, an integrated setup was realized with lab-scale equipment. Specifically, the process setup consisted of three major unit operations: (i) a 10-mL EMR; (ii) a lab-scale SMB based on an Amersham ÄKTAbasic system; and (iii) a custom-made cross-flow NF (Figure 3). In detail, the devised process concept works as follows. In fully integrated mode the substrate feed entering the system Q_S is combined with the recycled retentate stream from the NF unit Q_{Ret} and fed to the enzyme reactor $Q_{EMR,in}$. The substrate is partially converted into product in the EMR (depending on the adjusted residence time, supplied enzyme amount, and enzyme kinetics), and the obtained reaction mixture of product and substrate is directed to the SMB as stream $Q_{SMB,in}$.

The SMB unit then allows separation of binary mixtures into two outlet streams at opposing locations relative to the inlet flow. The more retained component is obtained as stream Q_{Ex} at the extract port located upstream (with respect to the fluid flow) of the inlet port. The less retained component is collected as stream Q_{Raff} at the raffinate port downstream of the inlet. Fresh eluent as stream $Q_{D,in}$ is supplied to zone I assigned to column regeneration. For the model process no solvent recycling was implemented (open-loop configuration). Therefore, a fraction of eluent left the system from zone IV of the SMB as stream $Q_{D,out}$. For a detailed description of the SMB process and the tasks assigned to the different zones please refer to ref 34.

The substrate stream (assigned to the raffinate stream in Figure 3) is directed to a NF unit in order to counterbalance the dilution introduced in the SMB separation. Depending on the rejection of the D-fructose and D-psicose by the applied membrane, the substrate mass flow is divided into a concentrated retentate stream Q_{Ret} and a diluted permeate stream Q_P. Concentration factors can be adjusted by controlling the retentate flow Q_{Ret} in order to ensure that the substrate concentration in the retentate c_{Ret} meets the design criteria. In this case it was specified to equal the feed concentration c_S.

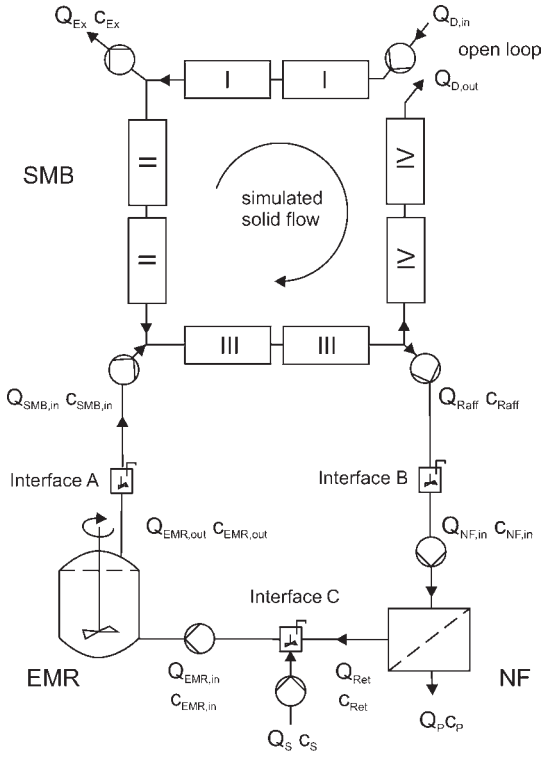

Figure 3. Flow scheme of the fully integrated process employing EMR, open-loop SMB, and substrate recycling over the NF.

Small stirred vessels were implemented at the interfaces before and after each unit in order to assess the concentration profiles. Therefore, the input flows were adjusted to slightly higher rates (0.05−0.10 mL min^{-1}) than those of the outlet flows. This demanded the use of additional feed pumps for each unit operation (Figure 3).

Proof of Concept. Identification of suitable operating points for the integrated process required a detailed characterization of each unit operation. Adsorption isotherms and plate numbers (as an indicator for column efficiency) were determined by analysis of HPLC elution profiles. For both components, D-fructose and D-psicose, linear isotherms were determined for the projected concentration ranges. D-Fructose was much less retained than D-psicose (Table 1) which is in line with the previously reported adsorption behavior for this system.[27] Application of the triangle theory[31] allowed identification of a safe SMB operating point with flow rate ratios $m_I = 2.50$, $m_{II} = 1.12$, $m_{III} = 1.85$, and $m_{IV} = 0.56$ and a switch time of 10 min. The significant safety margin with respect to the theoretical optimum at the vertex of the triangle can be explained by the low efficiency of the applied columns. Detailed SMB simulation accounting for the observed slow mass transfer predicted complete separation for the selected operating point. This was also confirmed in a run of the SMB alone using a feed of 75 mM D-fructose and 25 mM D-psicose, which yielded absolute purities at both SMB outlets.

Preliminary investigations using DTE in the enzyme membrane reactor indicated that the enzyme was partially inactivated in the projected operation period of a number of days. In order to ensure constant conversion a large amount of CFE containing 500 U of DTE, was supplied to the EMR. At this loading, loss of

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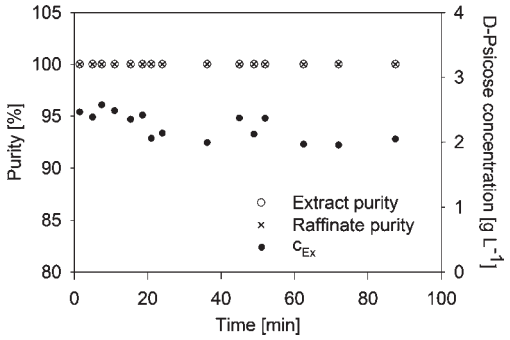

Figure 4. Proof-of-concept run in fully integrated mode. The product concentrations (c_{Ex}) and raffinate and extract purities were determined experimentally over 87 h. t = 0 represents the start of full process integration.

75% of the enzyme activity would lead only to a reduction in the degree of conversion from 98 to 95%. Effectively, the mixture of D-fructose and D-psicose that left the reactor was virtually at equilibrium for 10 days. Consequently, EMR operation could be adequately characterized by the equilibrium constant of the epimerization (Table 1).

Initial NF experiments had demonstrated that the applied membrane retained hexoses almost completely (Table 1) and that no significant fouling occurred when operated under conditions similar to those projected for integrated operation. On the basis of the rejection, the concentration factor and the corresponding permeate and retentate flow rates were determined (Table 2) in order to concentrate D-fructose to the process feed level.

On the basis of the obtained descriptions and the operating points that had been validated in stand-alone operations, appropriate flow rates were determined for a fully integrated operation (Table 2). The obtained elution order in HPLC experiments assigned the raffinate stream to the less retained D-fructose and the extract to the more retained D-psicose. Hence, the recycle was implemented at the raffinate port (Figure 3). The concentration of substrate D-fructose in the feed was set to 100 mM (18 g L^{-1})—a relatively low concentration for sugar production but representative of fine chemical synthesis[35] and fully sufficient for this proof of principle.

Next, the fully integrated process was implemented and operated at steady state (as judged by the overall mass balance of inlet and outlet flows) for 87 h. D-Psicose could be produced in very high purity (>99.5%, which was our analytical limit) (Figure 4). Due to the incomplete rejection (>98%) in the NF unit, small amounts of D-fructose were lost in the permeate, leading to a D-psicose yield based on D-fructose of 97%. The adjusted flow rates of the NF yielded, as projected, a concentration of D-fructose to the feed level, proving the feasibility of the recycling strategy. The throughput for the system was 67 mg h^{-1} of D-psicose. However, please note that the throughput was not optimized and could be easily increased by a larger substrate feed and optimized design in the future.

Interestingly, the pH values measured at the interfaces A, B, and C and the extract fractions differed moderately, with the pH lowered in the raffinate to 7.0 and increased in the extract to 8.5. This indicated that Tris (pK_a of 8.3), the compound that we used for buffering the system at pH 8.0, was to some extent retained in its noncharged form by in the ion-exchange stationary phase, leading to an acidification of the raffinate side (SMB zones III and IV). This would have escaped our attention as Tris is poorly detectable in the used matrix with RI or UV−vis detectors. To maintain a pH value above 7.0 in the EMR, the Tris-buffered process feed flow (Q_S) was adjusted to pH 9, resulting in a pH of 7.4 in the EMR feed. In this way, integrated operation could successfully be conducted for three days without notable shifts in pH in the respective sections. However, in order to obtain D-psicose in high purity with respect to the buffer agent, we are currently screening for a suitable buffer system that is not retained by the stationary phase.

Startup Procedure. Next to the principle aspect of achieving close to maximum yield, the applicability of such an integrated process is further defined by the time required for the system to reach steady state or at least to obtain the product in high purity at relevant concentrations. Startup of such a system with all units initially devoid of substrate and product and integrated from the start will take significantly longer than the sum of the individual equilibration times of the involved units. This argument can easily be made by assuming that concentration steps propagate as ideal fronts through the system, depending on the residence times of each unit. In an integrated setup the process feed becomes initially diluted when combined with, at that time, substrate-free solvent from the NF. This front of reduced concentration will propagate through the system and eventually reach the retentate recycle. In this way, a new and less diluted concentration front is generated at the interface of process feed and recycle that again propagates through the system. This stepwise increase in retentate concentrations will continue until the feed concentration is reached.

Obviously, more efficient strategies would be preferred for startup. Initial experiments clearly indicated that the startup of the substrate-free NF constituted a limiting factor with very long equilibration times (>20 h with a feed of 100 mM D-fructose and a concentration factor of 5). The considerable startup period of the concentration device is due to the high dead volume (approximately 40 mL) and the inherently slow equilibration of cross-flow filtration with a partial retentate recycle. Even though the concentration factor applied in the integrated operation was only about half that value (VCF = 2.4), NF startup times in this order of magnitude are clearly not feasible when the entire processing time is only on the order of a number of days. Therefore, we chose to prefill the retentate side of the NF unit with a D-fructose solution adjusted to the concentration projected for steady-state operation of the integrated process (in this case, 100 mM D-fructose). The rationale behind this approach was that the retentate recycle could be instantly established when connecting the thus pre-equilibrated NF to the sequence of EMR and SMB after startup. Obviously, this can only be sustained if the set concentration factor yields the projected retentate concentration from the SMB raffinate in stationary operation which seems perfectly feasible with careful design.

Having identified a short-cut startup for the NF unit, different strategies for EMR-SMB startup were evaluated. Sequential startup of EMR and SMB was tested by connecting the SMB to the EMR after uniform concentration profiles at the EMR outlet were obtained for a prolonged period of time (Figure 5). Roughly both units produced close to steady-state concentrations after 120 min of operation. After constant extract and raffinate concentrations were obtained (after 600 min), the recycle loop with the pre-equilibrated NF unit was implemented. Integration was established by connecting the retentate flow Q_{Ret} to the interface vessel C and simultaneously reducing the feed stream Q_S from 0.26 to 0.08 mL min^{-1}. As expected, the implementation of the pre-equilibrated NF did not perturb the stationary state of the EMR−SMB sequence as judged

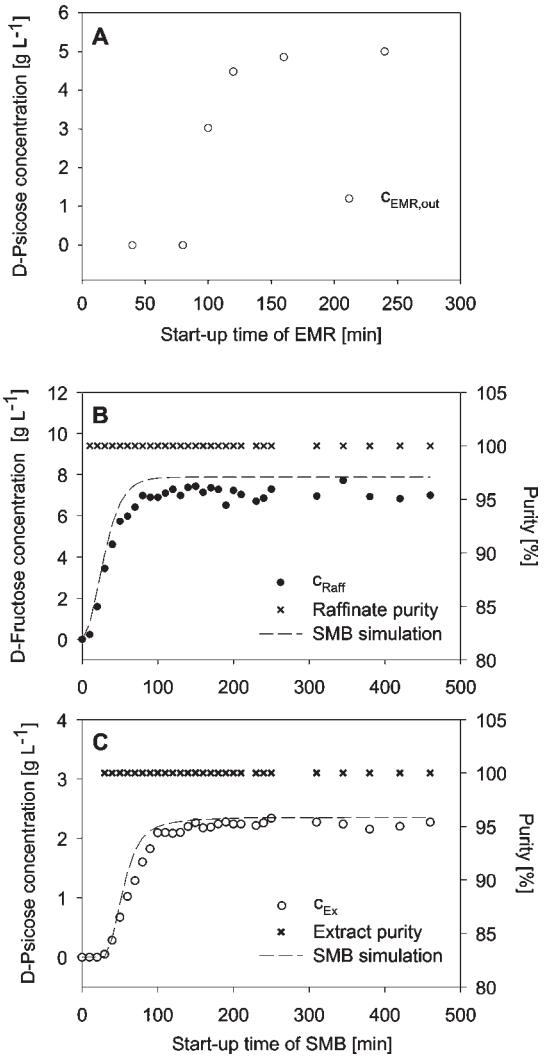

Figure 5. Concentration profiles and purities obtained after sequential startup of EMR and SMB and coupling after 300 min. Profiles for (A) D-psicose concentration in the outlet stream of the EMR $Q_{EMR,out}$, (B) D-fructose concentration and purity of the SMB raffinate stream Q_{Raff}, (C) D-psicose concentration and purity of the SMB extract stream Q_{Ex}. Dashed lines represent the model prediction using a detailed SMB model.

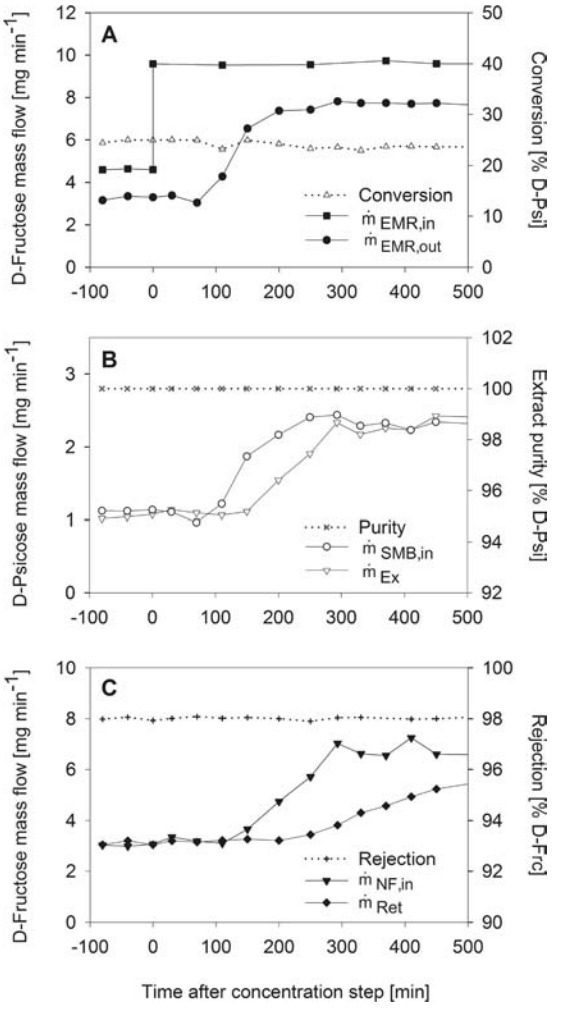

Figure 6. Response of the coupled EMR−SMB−NF system to a concentration step in the process feed introduced at $t = 0$. For better comparison, the state at the respective outlets is characterized by the mass flow in the respective units (A) EMR, (B) SMB, and (C) NF.

by the invariant purities and concentrations (data not shown) at the SMB outlets.

The evolution of EMR and SMB outlet concentration profiles is characterized by an initial lag phase with little of either of the involved species in the outlet, then a steep increase in concentrations followed by a slow approximation of the eventual steady-state concentration (Figure 5). Arguably, the transition between steep increase and slow approximation constitutes the appropriate time point for coupling the EMR to the SMB and collection of D-psicose at relevant concentrations of the SMB extract. For both units this transition occurred after approximately 120 min, yielding a minimal startup time of roughly 240 min. Please note that SMB purities were not compromised during the whole startup procedure.

Next, starting the process with initially coupled EMR and SMB constitutes another promising option that in principle enables fast equilibration as the evolving concentration profile from the reactor would support SMB startup. Simulation of coupled EMR−SMB operation supported this argument, suggesting an equilibration time of approximately 300 min, at which time point the deviation from the steady-state mass balance was predicted to be smaller than 2%. The extract concentration should reach a usable level (arbitrarily set at 90% of the steady-state concentration) after approximately 200 min.

In summary, there are options for efficient startup of the integrated process that allow a reduction of the startup period to less than the sum of contributions of SMB and EMR. For the investigated system, the startup could be performed in 200−300 min, depending on the strategy, which is definitely acceptable for the projected operation time. In general, similar startup strategies should be applicable to large-scale operation.

Robustness. For industrial application it is important that the process can withstand at least to some extent fluctuations in the operating variables. Such fluctuation can originate from deteriorating materials such as the NF membrane or the stationary phase, resulting in reduced rejection in NF operation or changes in the adsorption behavior of the SMB. Next, enzyme inactivation at some point results in decreasing conversion in the reactor, leading to gradually changing SMB feed concentrations. In order to derive a general impression about how fluctuations propagate

dx.doi.org/10.1021/op200160e |Org. Process Res. Dev. 2012, 16, 323–330

through the system and how they affect the performance of the integrated process, the feed concentration profile was subjected to a step gradient. On the basis of the derived equilibration times we assumed that perturbations would migrate slowly through the systems, and hence the effect of the recycle could only be accessed by excessively long experimentation times. Thus, for the sake of simplicity EMR, SMB, and NF were operated in sequence without recycle, and the response to a 2-fold increase in feed concentration was recorded for almost 9 h. Due to the excess of supplied biocatalyst the conversion in the reactor remained unaffected by the concentration step, and consequently the concentrations at the reactor outlet gradually increased to twice the initial value over approximately 200 min (Figure 6). Please note that the difference in the startup equilibration time is due to the presence of interface C in this experiment (Figure 3). The SMB unit reached a steady state with the new concentration regime after approximately 300 min without changes in the purity of extract or raffinate. Since the applied chromatographic system relies on linear isotherms (meaning there is no competition between D-fructose and D-psicose at the stationary-phase binding sites and no capacity limitation), both species move independently of each other and of their respective concentrations.[7] Consequently, the obtained constant extract and raffinate purities upon a change in feed concentration are fully supported by linear chromatography theory. The gradually increasing concentrations in the raffinate resulted in a slow increase in retentate concentrations. Due to the slow dynamics of the cross-flow filtration, the concentration unit did not reach steady state to the new concentration regime within the time frame of the experiment, confirming our initial assumption. However, the obtained results can be transferred to predict the response of the fully integrated process upon a similar shift in the process feed. In fully integrated operation the shift in the process feed will initially be diluted by the retentate recycle, resulting in a moderate increase in D-fructose concentration in the EMR feed (130 mM calculated from the flow rates given in Table 2 and assuming 100 mM D-fructose in the retentate feed). The EMR feed concentration will eventually converge to the process feed concentration, according to the mechanism described for the startup with a fully integrated system. This final state represents exactly the state of the EMR and SMB that was probed in the experiment; hence, uncompromised purities can be expected also for the integrated case. Considering that roughly after 5 h the first traces of the concentration step were observed in the retentate, the time to approach the new steady state will be presumably in the range of days, demonstrating the inertia of such integrated systems.

Process Stability. The economic feasibility of such an integrated process heavily depends on the time the process can be run without down-time. All involved unit operations are in principle prone to different sources of performance reduction. Enzymes, particularly in cell-free extracts, are potentially subject to numerous irreversible inactivation mechanisms such as proteolysis, oxidation, aggregation, loss of cofactor, or shear stress induced by vigorous stirring.[36] Therefore, the pool of active enzyme becomes gradually depleted which can be balanced by supplying sufficient initial amounts of enzyme to the reactor. Nevertheless, all enzyme reactions will show decreasing performance over a certain time scale, depending on the stability of the enzyme. High concentrations of compounds and buffering agents potentially cause swelling of the stationary phase, leading to altered velocities of the compounds and increased back pressures.[37] Next, aging effects can occur as irreversible adsorption of contaminants or chemical modifications, both of which reduce the number of available adsorption sites, resulting in changes in the adsorption behavior.[14] Membrane fouling due to irreversible adsorption of matter typically increases the pressure drop over the membrane, resulting in lower rejections and higher energy consumption.[38] In order to estimate the stability of the overall process, the respective stand-alone units were investigated with the application of conditions similar to the integrated process. The EMR supplied with 500 U of enzyme provided constant conversion for a period of 10 days. However, the back pressure of the EMR ultrafiltration membrane steadily increased which might be due to degraded proteins causing membrane fouling. Due to pressure limitations of the membrane and the reactor, the reaction was stopped at a pressure drop of 25 bar over the membrane after 10 days. During the course of the whole study the same NF-90 membrane was used, and no irreversible changes in membrane performance were observed. The observed pressure increase during operation was rather moderate, indicating a potential for operation of at least 10 days. Furthermore, please note that the reactor was heavily overloaded with enzyme (suggesting potential for much smaller protein loadings), and we have not yet applied molecular biology methods to increase enzyme stability. Adsorption behavior of the SMB columns was frequently assessed by determination of Henry constants over a period of several weeks that included a number of multiple-day SMB runs. The results indicate that retention decreased for both D-fructose and D-psicose under prolonged SMB conditions. However the columns could be regenerated by flushing them with a solution of 0.1 mM $CaCl_2$ for 24 h. After the regeneration step, the Henry constants were back to their original level. Using the current material we conservatively estimate potential process time to approximately 5 days due to the limited stability of the enzyme and the associated pressure drop increase over the membrane. However, most of the bottlenecks can be addressed with available technologies such as enzyme immobilization[39] or enzyme engineering[40,41] for biocatalyst stabilization or SMB cleaning in place approaches for regenerating the columns during SMB operation.[30]

CONCLUDING REMARKS

We have demonstrated for the first time the experimental implementation and successful operation of a fully integrated process consisting of a bioreactor, SMB separation, and concentration by nanofiltration. The yield obtained in integrated operation (97%) constitutes a drastic improvement compared to reactor standalone operation (max. 25%). Furthermore, the product D-psicose is obtained in high purity (>99.5% with respect to D-fructose) facilitating further workup. The principle feasibility of this process with respect to critical properties such as stability, robustness, and startup time was further demonstrated. In summary, we provide here a proof of concept for this integration strategy. Obviously the applied conditions were not optimized. Preliminary data on the performance of DTE suggests that the process can be operated at much higher feed concentrations, up to the molar range, and an economic process can be derived by engineering a more stable and active DTE variant. This view is supported by literature examples on operation of comparable isomerase reactions at high substrate and product concentrations[42] and successes in enzyme engineering for stabilization.[40,41] Next, in order to exploit the full potential of this strategy, model-based process design based on the optimization of operational parameters such as reactor residence time or SMB flow rates is required which holds enormous potential for productivity improvements.[43]

The obtained results can in principle be translated to all SMB−EMR−NF process schemes such as the integration of SMB enantioseparation and enzymatic racemisation for high-yield production of a single enantiomer from a racemate (the unifying topic of this special issue). Albeit, it has to be noted that SMB performance of chromatographic systems with more complex isotherms (e.g., Bi-Langmuir typically observed for chiral separations) depends largely on the applied process feed concentration.[31] Therefore, such systems would be more prone to noncompliance with specifications upon process disturbances, in particular upon concentration changes.

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ACKNOWLEDGMENT

We acknowledge support from the EU-project "INTENANT" and the Swiss National Science Foundation. We thank Prof. Marco Mazzotti and his group for providing the Matlab program used for detailed SMB simulations and Rene Pellaux for helpful discussions on the buffer separation.

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