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Reaction Engineering of Biocatalytic Enantioselective Reduction: A Case Study for Aliphatic Ketones

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ABSTRACT: Previously, it could be demonstrated, that the monophasic, enzymatic reduction of aliphatic 2-ketones into the corresponding (*R*)-2-alcohols is an adequate and viable method as carried out in a cascade of two enzyme−membrane reactors (Leuchs, S.; Na'amnieh, S. N.; Greiner, L. *Green Chemistry* **2013**, *15*, 167−176.). In the present work, the process metrics of the ketone reduction were calculated. A cost analysis revealed that the enzyme costs are negligible, but the cost for nicotinamide cofactor $NADP^+$ is dominating the overall cost of the chemical raw material followed by the ionic liquid (TEGO IL K5) used as solubiliser and the buffer. The overall cost of chemicals was €148/$kg_{product}$. To assess the environmental impact of the process, the *E*-factor ($kg_{waste}/kg_{product}$) 132 and the process mass intensity 133 (PMI, $kg_{substrate}/kg_{product}$) were calculated. A process model based on initial rate experiments was elaborated and used to improve the process under cost and environmental aspects. Applying several measures to enhance the cofactor utilisation, the cost base could be reduced by 65% and the *E*-factor (PMI) to 17 (18).

INTRODUCTION

Today, biocatalysis is not restricted to lab-scale use and fundamental academic research but has made its way to mainstream industrial production.[1,2] Prominent examples of large-scale biocatalytic processes yielding low-cost products are, for example, the production of acrylamide, L-aspartate, and high fructose corn syrup.[2,3] Nevertheless, the unique stereoselectivity (enantio- as well as diastereoselectivity) and regioselectivity of most enzymes makes biocatalysis most suitable for the pharmaceutical and fine chemical industries.[4,5] While hydrolases dominate process biocatalysis, several oxidoreductases have also made it to the production level,[3,5,6] mainly because reductive cofactor regeneration can be routinely performed.[7−9] Most of the biocatalytically produced substances possess at least one chiral centre in which the stereo centre originates from the biogenic starting material or alternatively is created during the biocatalytic conversion.[5]

In view of limited patent lifetimes, especially in pharmaceutical development, time to market is an important issue. Typically, at the beginning subkilogram quantities are sufficient to allow for primary assessment of the product. However, in order to make the most of the patent lifetime, soon multikilogram amounts of the respective product are required.[4]

Catalysts in general, and enzymes in particular, respond to changes in reaction conditions sensitively. At a minimum, a reaction comprises the enzymes, oxidising and reducing agents, and their respective products. This alone imposes a multivariate nonlinear optimisation problem for productivity and stability. From a practical perspective, other substances, e.g. cofactors in all forms, buffer, solubilisers, and other additives, as well as temperature and pH will further add to the overall complexity. In order to assess the best conditions for production, hundreds of experiments can easily be necessary to cover the whole range of all parameters, which rapidly exceeds experimental capacity and time. In such cases, process modeling can help to reduce the experimental effort and to quickly assess process alternatives or improvement strategies. In doing so, it is important to carefully define the aim of the process development. For example, this can be based on space-time-yield (STY), enzyme productivity, a decrease in environmental impact (in order to make the calculation quick and easy the *E*-factor defined by Sheldon[10,11] can be used for a primary environmental assessment), conversion, reduced cost for raw material, reduction of side-product formation, or ease of downstream processing. According to the literature, we listed key values to be obtained for an economically viable industrial process. However, these values are guidelines that depend on the desired product (Table 1). Defining maximum conversion as the modelling goal will always lead to higher residence times and catalyst concentrations. This will normally not lead to a

Table 1. Key values to be obtained for an economically viable (bio-)catalytic industrial process

	bulk	fine-chemical	pharma
product amount (t per year)[10]	10^4−10^6	10^2−10^4	10−10^3
typical cost[12] /(€/kg)	1(−5)	>15	>100
E-factor[10] ($kg_{waste}/kg_{product}$)	<1−5	5−50	25−100
STY[5] /g L^{-1} h^{-1}		>0.1	>0.001
$c_{Product}$[5]/g L^{-1}		>1	>0.1
catalyst productivity/ ($kg_{product}/kg_{enzyme}$)[12]	5000−20000	670−1700	100−250

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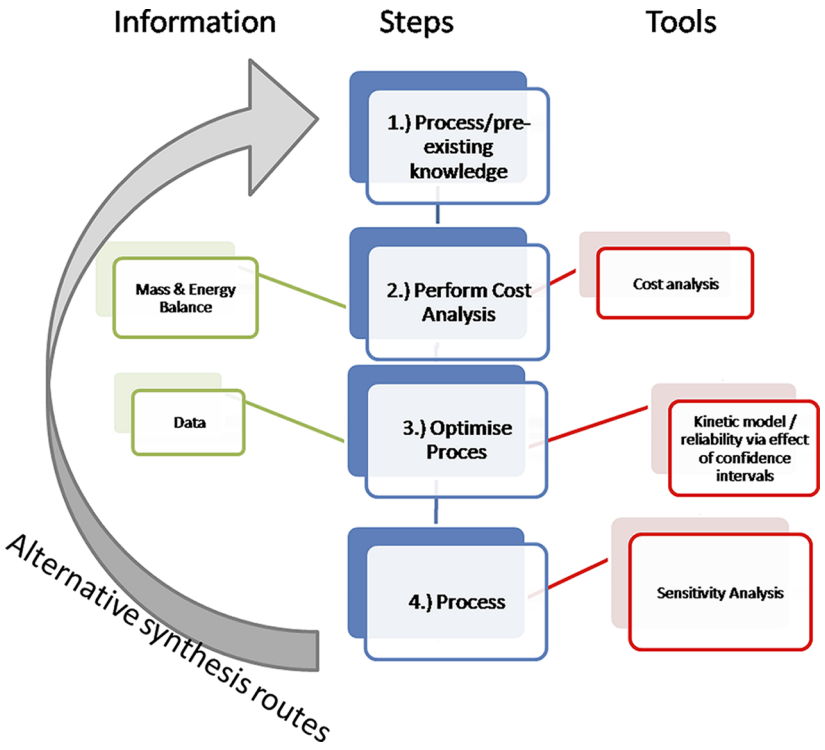

Figure 1. Proposed work flow for optimisation.

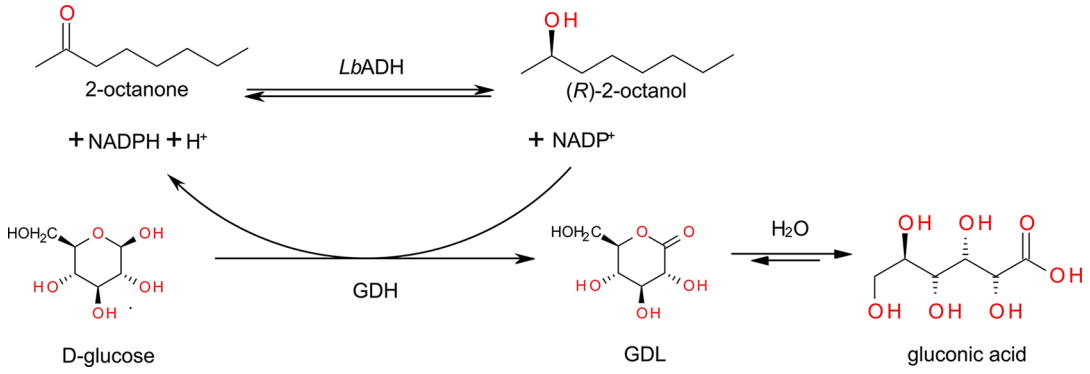

Figure 2. Reaction scheme of the synthesis of aliphatic, enantiopure alcohols.

more economic process. Thus, in this case, a conversion range should be used. Most probably, all aspects end up leading to lower overall production costs, which may be the result of reduced costs of the raw materials or a reduction in the waste produced and thus lower costs for wastewater treatment and waste disposal (see also ref 2). For this reason a scheme to improve and develop a process based on pre-existing knowledge was derived and has been applied throughout the present paper (Figure 1).

In this work we focused on the enantioselective reduction of aliphatic ketones into their corresponding alcohols using oxidoreductases, since there is no chemical alternative available that yields similar enantiomeric excess (ee).[13−16] Long-chain enantiopure aliphatic alcohols are an interesting raw material in the pharmaceutical industry. They have also been used for the production of liquid crystals with interesting optical properties.[17]

EXPERIMENTAL SECTION

All continuous and initial rate experiments as well as stability tests were carried out as described by Leuchs et al.[1] All chemicals were purchased from Carl-Roth, Karlsruhe, Germany. C_8-alcohols and -ketones were from Sigma-Aldrich, Steinheim, Germany. Enzymes were from X-Zyme GmbH/Johnson Matthey Catalysts, Düsseldorf, Germany. The parameter estimation, process modelling, and process improvements were performed using MATLAB2009a from Mathworks.

RESULTS AND DISCUSSION

Process Pre-existing Knowledge. Alcohol dehydrogenase from *Lactobacillus brevis* (*Lb*ADH, EC 1.1.1.2) exhibits outstanding enantioselectivity, robustness, and flexibility in a wide variety of reaction conditions.[18] The substrate scope of this enzyme is broad, and its ability to convert long-chain aliphatic ketones into the desired (*R*)-2-alcohols has been proven in many batch syntheses,[19,20] as well as in monophasic,[1,19] and biphasic[20−22] continuous experiments. Water-

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solubility restrictions in single-phase approaches can be overcome by applying an ionic liquid (IL) as a solubiliser.[1,19,23] Kinetics[21,23] and stability tests[1,23] reveal the applicability of *Lb*ADH in many processes. Among these possibilities, the monophasic continuous synthesis with an ionic liquid as solubiliser[1] was chosen for further assessment and development.

*Lb*ADH requires NADPH as a cofactor or redox equivalent.[18,24] For *in situ* cofactor regeneration, an enzyme-coupled approach with glucose dehydrogenase from *Bacillus* spp. (GDH, EC 1.1.1.47) was chosen (Figure 2). To enhance the solubility of the aliphatic compounds (2-ketones and 2-alcohols), a solubiliser was used, in this case the ionic liquid (IL) TEGO IL K5.[1,19] A cascade of two enzyme−membrane reactors (EMRs) was employed for the synthesis of enantiopure (*R*)-2-alcohols (2-octanol, 2-nonanol, 2-decanol) (Figure 3). In the outlets of reactors 1 and 2, flow cells were

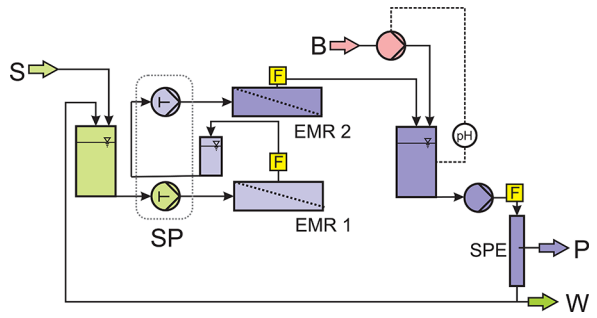

Figure 3. Flow-scheme of the process to be improved according to Leuchs et al.;[1] S = substrate solution, SP = syringe pump, P = product, B = NaOH solution, W = waste, EMR = enzyme−membrane reactor, f = flow cell, SPE = solid-phase extraction.

integrated for automatic online GC measurements. Due to the stoichiometric formation of gluconic acid and the resulting decrease in pH, the pH had to be adjusted to recycle the aqueous stream. This was achieved by dosing NaOH solution with the aid of a pH-stat immediately after reactor 2. The product and remaining substrate were removed by passing the solution through a stainless steel column filled with solid-phase extraction (SPE) material, HR-P (Macherey-Nagel). The product was eluted from the column by washing with *n*-heptane which was selected because of its lower toxicity compared to that of *n*-hexane.[25] Ninety percent of the aqueous stream was recycled after recharging with 2-octanone, glucose (*via* fresh solution), and $NADP^+$. Previous work led to the choice of the reaction conditions, such as the best buffer, pH, type and concentration of the salt, and type and concentration of the solubiliser.[1] All these conditions elicited a very stable and promising process with more than 1000 h of continuous operation and very low deactivation. Thus, this process was chosen for further development with 2-octanone as the model substrate representing the class of poorly water-soluble aliphatic ketones. The continuous synthesis of (*R*)-2-octanol was taken as the base case; its conditions are detailed in Figure 2[1] and Table 2. All alternatives were compared to the results obtained for these conditions.

Process Evaluation. *Economic Assessment.* In order to identify possible bottlenecks in the base process,[1] a cost analysis was carried out, taking into account the costs of all the chemicals (substrates, buffer, enzymes, and cofactor) (Figure 4). The substances which dominated cost were the cofactor

Table 2. Base case conditions for the continuous synthesis of (*R*)-2-octanol

substance	concentration
ADA-buffer[a]	150 mmol L^{-1}
$MgCl_2$	20 mmol L^{-1}
TEGO IL K5	100 g L^{-1}
2-octanone	60 mmol L^{-1}
glucose	200 mmol L^{-1}
*Lb*ADH	66.7 mg L^{-1}
GDH	280 mg L^{-1}
$NADP^+$	0.1 mmol L^{-1}
other conditions	values
pH_{inlet}	7.5
$\dot{V}$	4 mL h^{-1}
τ	3.75 h
T	25 °C

[a]ADA = *N*-(2-acetamido)iminodiacetic acid.

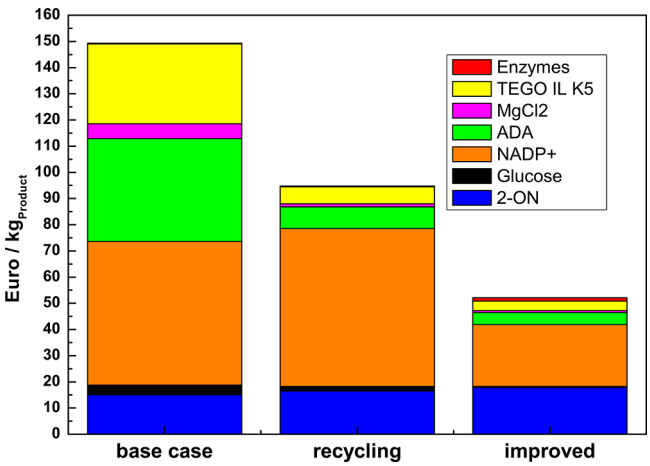

Figure 4. Cost distribution for the base case as presented in Leuchs et al.;[1] base case: without recycling of the aqueous phase; recycling: with recycling of the aqueous phase; recycling: with recycling of the aqueous phase; improved: improved synthesis with reduced cofactor concentration.

$NADP^+$, accounting for 36.7% of the entire expenditure, the ionic liquid TEGO IL K5 (20.4%), and the ADA-buffer[26] (26.3%). The overall cost was €149/$kg_{product}$. In contrast to many other biocatalytic processes, the cost analysis revealed that the enzyme costs were relatively small. Their contribution to the overall cost was less than 1% . Depending on the case, for a fine chemical, enzyme, or catalyst the costs can be up to 5% of the selling costs.[12] Thus, the enzyme productivity is not subject to possible improvements.

Environmental Assessment. In general, a process should be judged not only by its costs but also by its impact on the environment. An easy method to get a first idea about a process's environmental impact is the *E*-factor ($kg_{waste}/kg_{product}$) introduced by Sheldon.[10,11,27] In contrast to a full assessment of the environmental impact of a process, the *E*-factor only considers the mass of waste. The American Chemical Society Green Chemistry Institute Pharmaceutical Roundtable favors another measure, the process mass intensity (PMI).[28] To allow for a comparison with other processes, we calculated both the *E*-factor and PMI of the respective experiments. The *E*-factor for the base case was 132 (including water), and the PMI, 133. Amongst others,[10,11] benchmarks for the *E*-factor were given.

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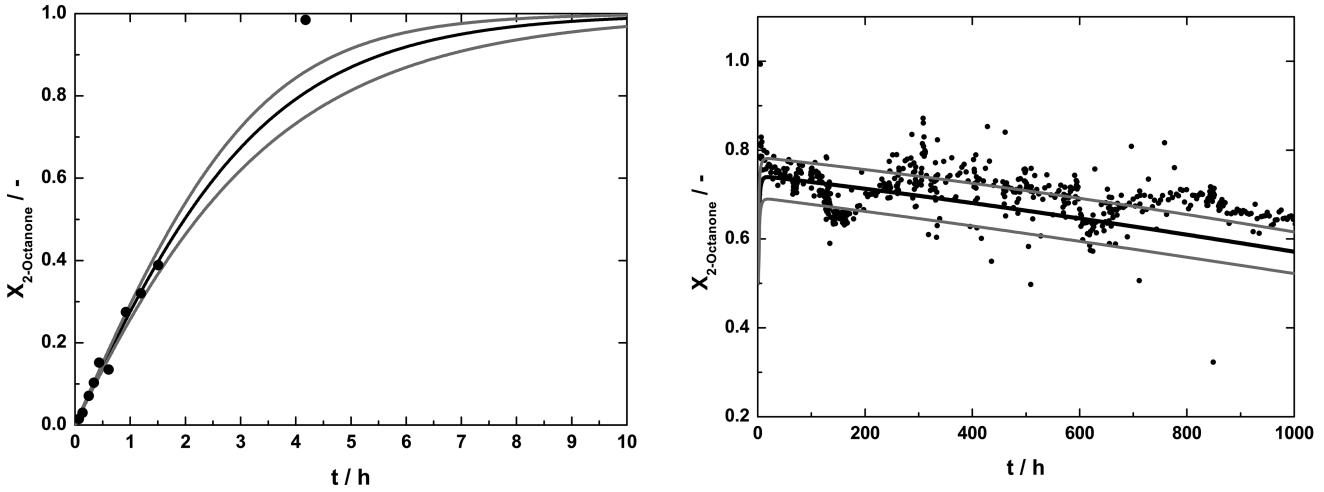

Figure 5. Effect of confidence intervals on the conversion (grey lines) in comparison to the predicted conversion using the estimated parameters (black lines) and measured conversion (black dots) in batch (left) and continuous synthesis (right, see also ref 1). $\dot{V}$ = 4 mL h^{-1}; $c_{2\text{-octanone}}$ = 60 mmol L^{-1}; c_{LbADH} = 50 mg L^{-1}; c_{GDH} = 250 mg L^{-1} (batch); c_{GDH} = 250 mg L^{-1} (conti) ; t_{total} = 10 h (batch); t_{total} = 1000 h (conti); c_{NADP^+} = 0.05 mmol L^{-1} (batch); c_{NADP^+} = 0.1 (conti).

For a bulk chemical it should not exceed 5; for fine chemicals a value between 5 and 50 is reasonable; for a pharmaceutical, values up to 100 are standard. Thus, even for a fine chemical, an *E*-factor of 132 and thus a PMI of 133 are too high and should be decreased. Sheldon[11] suggested not to include water into the calculation of the *E*-factor because exceptionally high *E*-factors may be obtained with biocatalytic processes. Whether water can be left out or should be included certainly depends on the process considered and on the available separation techniques. As we only assessed and compared improvements in a single process scheme, the inclusion or exclusion of water did not change the results qualitatively. However, as the solubiliser is considered toxic to aquatic organisms, water was included into the *E*-factors given throughout.

Bottleneck Analysis. In order to reduce the cost, the *E*-factor, and the consumption and release of the IL, which is possibly harmful to the environment,[29] recycling of 90% of the aqueous product stream was carried out. The effect of this recycling step on the amount of waste produced and chemicals consumed to produce 1 kg of product was enormous. The *E*-factor could be reduced from 132 to 27, while the PMI was reduced from 133 to 28. More benefits of this step are demonstrated in Figure 4.

The overall cost was reduced by 36.5% to €95/$kg_{product}$. Due to the recycling, the contribution of the ADA buffer was lowered to 8.8% and that of the IL to 7.0%. Although the absolute contribution of the cofactor was not changed, its contribution to the overall cost was increased to 63.5% due to the fact that the IL and the buffer were recycled but not the cofactor. Less costly than NADP(H) is NAD(H),[30] and the preference of *Lb*ADH for NADP(H) is regarded as its major drawback. So far, attempts to change the cofactor preference to NAD(H) have not been successful, and the resulting enzyme variants are not commercially available.[31,32] Instead of relying on time-consuming molecular biology to change the cofactor preference of the enzyme, we decided to use chemical engineering tools to ameliorate the productivity of the cofactor. In that way, we also wanted to demonstrate that an enzyme's preference for NADP(H) instead of NAD(H) is not a general knockout criterion. Thus, the cofactor-consumption per kilogram of product was further optimised.

Model-Aided Process Optimisation. When optimising a process, modeling can help to reduce experimental effort by avoiding time-consuming experiments and thus save money and resources. On the basis of initial rate experiments and a few batch experiments, a process model was developed (see also Leuchs et al.[1]). The quality of the model was tested by comparing experimental results with a prediction using the estimated parameters (Figure 5 and Table 3). Given that the model was based on initial rates and contained no further correction factors,[33] the predictions were very good. Upper and lower bounds were determined by numerical inspection of all 131072 possible combinations of errors, yielding the confidence interval for the batch and the continuous synthesis shown in

Table 3. Kinetic constants for *Lb*ADH and GDH measured with fluorescence spectroscopy at 25 °C[a]

constant	constant value	confidence interval
	*Lb*ADH	
$\dot{V}_{max,forw}$	17.50 μmol min^{-1} mg^{-1}	0.13
$KM_{2\text{-octanone}}$	0.206 mmol L^{-1}	0.017
$KP_{2\text{-octanol}}$	0.03704 mmol L^{-1}	0.00065
KM_{NADPH}	0.208 mmol L^{-1}	0.040
KP_{NADP^+}	0.212 mmol L^{-1}	0.028
$KS_{2\text{-octanone}}$	163 mmol L^{-1}	10
$\dot{V}_{max,back}$	9.96 μmol min^{-1} mg^{-1}	0.18
$KM_{2\text{-octanol}}$	0.0332 mmol L^{-1}	0.0014
$KP_{2\text{-octanone}}$	0.835 mmol L^{-1}	0.049
KM_{NADP^+}	0.0122 mmol L^{-1}	0.0021
KP_{NADPH}	0.359 mmol L^{-1}	0.030
$KS_{2\text{-octanol}}$	6065 mmol L^{-1}	4040
	GDH	
$\dot{V}_{max,forw}$	5.82 μmol min^{-1} mg^{-1}	0.053
$KM_{Glucose}$	2.73 mmol L^{-1}	0.23
KP_{GDL}	0.0258 mmol L^{-1}	0.00067
KM_{NADP^+}	0.028 mmol L^{-1}	0.0033
KP_{NADPH}	3780 mmol L^{-1}	279
$KS_{Glucose}$	62260 mmol L^{-1}	11.1 × 10^8

[a] c_{LbADH} = 1.25 mg L^{-1}; c_{GDH} = 5 mg L^{-1}; $c_{ADA\text{-}buffer}$ = 100; c_{MgCl_2} = 10 mmol L^{-1}; $c_{TEGO\ IL\ K5}$ = 100 g L^{-1}; pH = 7.0.

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Figure 5. In the batch experiment, a deviation between the experimental data and the model-based predictions was observed at higher conversions, which was most likely caused by a pH shift that was not included in the model. The beginning of the experiment was well predicted. In the continuous synthesis, the conversion and the overall trend were well represented. Thus, the model could be used to obtain reliable predictions and for further process optimisation.

The main aim of the optimisation procedure is to enhance the cofactor utilisation. In order to enhance the amount of product per amount of cofactor consumed, there are basically two promising strategies. The first one is to increase the substrate/cofactor ratio, because the stoichiometry limits the maximum possible turnover number for the cofactor (TON_{NADP^+}). The second strategy is to recycle the cofactor together with the aqueous stream.

The substrate/cofactor ratio can be increased by increasing the substrate concentration or by decreasing the cofactor concentration. As 2-octanone is a poorly water-soluble substrate, its concentration cannot be further increased without increasing IL concentration beyond a practical viscosity limit. Therefore, the cofactor concentration has to be reduced. As the reduction reaction rate strongly depends on the cofactor concentration, a decrease of the latter may lead to a reduced reaction rate. In order to estimate the effect of a decreased cofactor concentration, the process model was consulted to predict the conversion in reactors 1 and 2 as a function of the cofactor inlet concentration (Figure 6). The calculations indicated that reducing the cofactor concentration from 100 μmol L^{-1} to 50 μmol L^{-1} affected only marginally the conversion in both reactors.

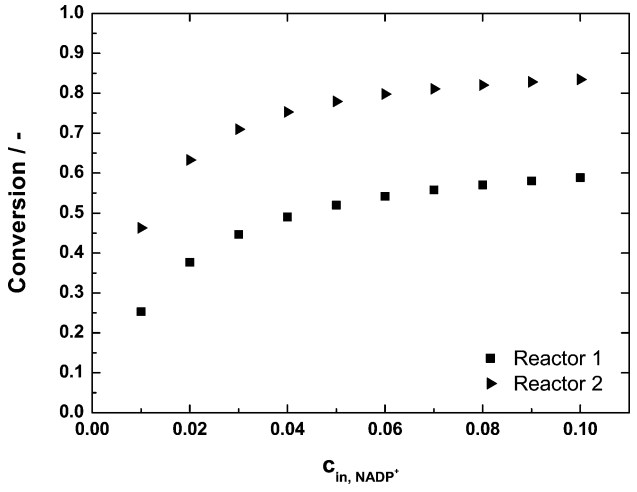

Figure 6. Estimated mean conversion in reactor 1 and reactor 2 as a function of the cofactor concentration in the feed stream. $\dot{V}$ = 4 mL h^{-1}; $c_{2\text{-octanone}}$ = 60 mmol L^{-1}; c_{LbADH} = 50 mg L^{-1}; c_{GDH} = 250 mg L^{-1}; t_{total} = 1000 h.

In view of recycling, it is known that nicotinamide cofactors possess only restricted stability in aqueous solutions, in which the phosphorylated cofactors (NADP(H)) are less stable than the nonphosphorylated ones (NAD(H)) and the reduced ones (NAD(P)H) are less stable than the oxidised ones ($NAD(P)^+$) under typical, near-neutral pH.[8,34] Parameters strongly influencing the stability of cofactors are pH and temperature. While decreasing the temperature will increase stability, a higher pH will increase the stability of the reduced cofactors, and a lower pH will enhance the stability of the oxidised cofactors.[29] In order to quantify these trends, we investigated the half-lives of the oxidised and reduced cofactors under conditions that were relevant for the process (Figure 7). The

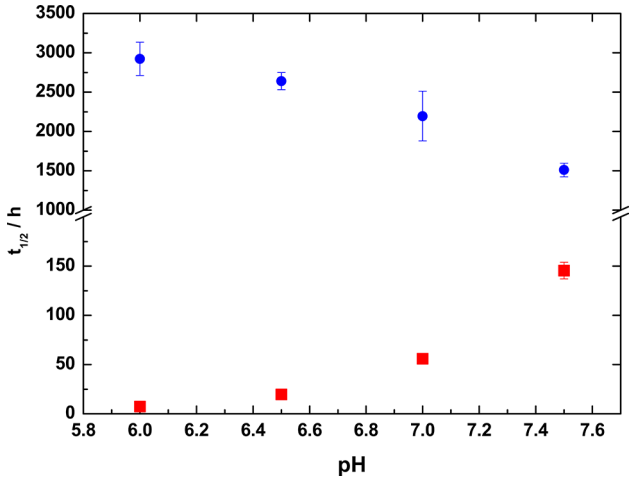

Figure 7. Half life of $NADP^+$ (circles) and NADPH (squares) as a function of pH, T = 25 °C; c_{ADA} = 150 mmol L^{-1}; c_{MgCl_2} = 20 mmol L^{-1}; pH = 6.0–7.5; values for $NADP^+$ partly extrapolated, measuring time was 1950 h.

half-life of the oxidised cofactor $NADP^+$ was longer than 1000 h in the pH range of 6.0–7.5. Thus, the stability of $NADP^+$ was not limiting in this case. The stability of NADPH varies from less than 10 h at pH 6.0 to almost 150 h at pH 7.5. Hence, in order to ameliorate the cofactor stability and thereby its recyclability, the pH has to be increased to the inlet pH of 7.5 immediately after the product stream leaves the reactor. The huge difference in the stability of NADPH and $NADP^+$ can be utilised as well to improve recyclability. By varying the *Lb*ADH/GDH ratio in reactor 2, the steady-state ratio of $NADP^+$/NADPH can be shifted to higher $NADP^+$ and lower NADPH concentrations. Thus, the overall stability of the cofactor is increased and allows for a decreased NADP(H) replacement in the recycled solution.

The effects of varying *Lb*ADH-to-GDH ratios on the $NADP^+$/NADPH ratio and the conversion in reactor 2 were predicted using the aforementioned process model (Figure 8). A low *Lb*ADH/GDH ratio in reactor 1 in combination with a high *Lb*ADH/GDH ratio in reactor 2 turned out to be beneficial for the $NADP^+$/NADPH ratio in reactor 2, allowing for high recyclability, as well as for the conversion in reactor 2, which is mandatory to conduct a reasonable synthesis and facilitate downstream processing. Unfortunately, the regime with high conversion (Figure 8 right) did not overlap with the regime of a high $NADP^+$/NADPH fraction (Figure 8 left).

Considering the results from modelling and the investigations on cofactor stability, another experiment was designed, aiming for a higher cofactor utilisation. Measures to improve cofactor productivity included a lower cofactor inlet concentration of 50 μmol L^{-1} instead of 100 μmol L^{-1}, and enzyme concentrations changed from 66 mg L^{-1} for *Lb*ADH and 280 mg L^{-1} for GDH in both reactors to 50 mg L^{-1} for *Lb*ADH and 250 mg L^{-1} for GDH in reactor 1 and 250 mg L^{-1} for *Lb*ADH and 100 mg L^{-1} for GDH in reactor 2. To make use of the higher NADPH stability at elevated pH values, the pH was adjusted directly after reactor 2 instead of fixing it after

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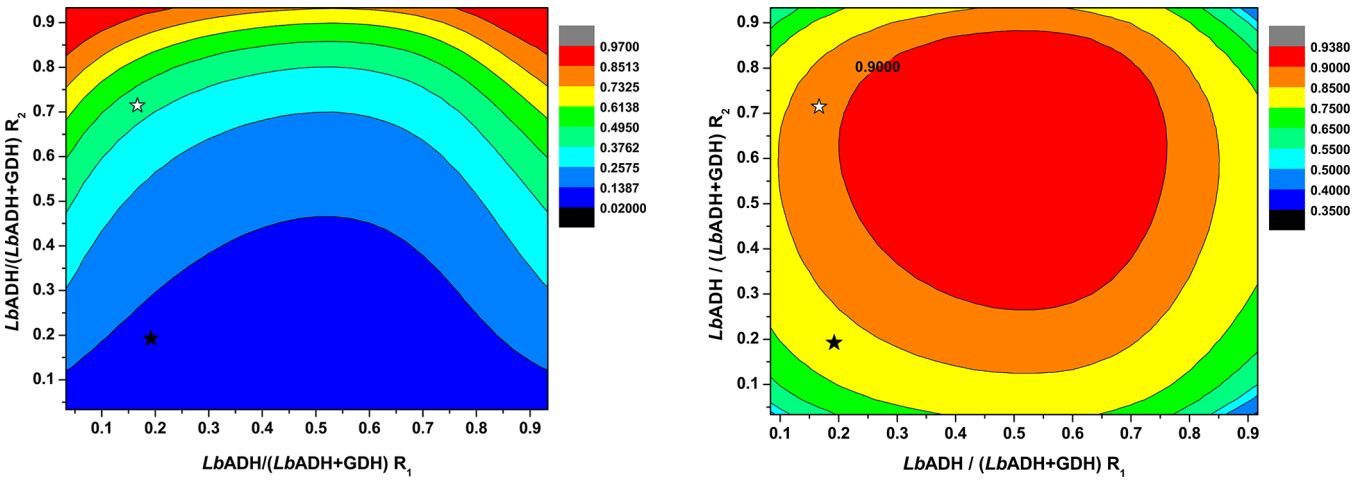

Figure 8. Estimated $NADP^+$/NADPH fraction in reactor 2 (left) and mean conversion in reactor 2 (right) as a function of the *Lb*ADH/(*Lb*ADH + GDH) fraction in reactors 1 and 2. $\dot{V}$ = 4 mL h^{-1}; $c_{2\text{-octanone}}$ = 60 mmol L^{-1}; c_{LbADH} = 10−280 mg L^{-1}; c_{GDH} = 300 − c_{LbADH} mg L^{-1}; t_{total} = 1000 h; c_{NADP^+} = 0.05 mmol L^{-1}; black star: conditions base case; white star: conditions improved synthesis.

the adsorption unit. The pH-stat unit was kept at 5 °C, and the experimental setup was optimised to reduce void volume in order to reduce cofactor residence time in the recycling loop.

Evaluation of the Improved Process. A continuous experiment was conducted, applying the conditions elaborated in the previous section. The enzyme ratio in the second reactor and the cofactor inlet concentration were changed (Figure 9).

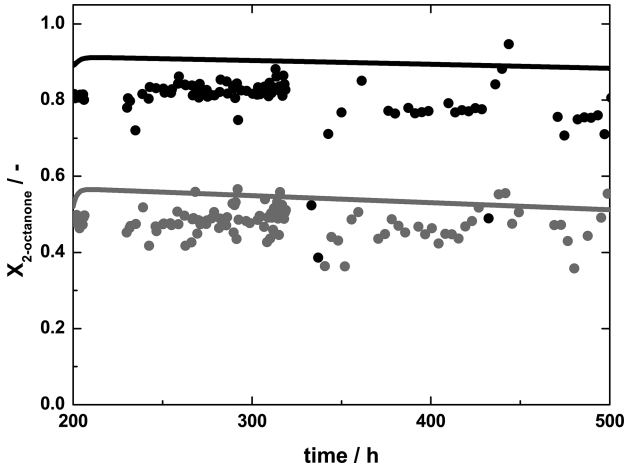

Figure 9. Improved continuous production of (*R*)-2-octanol; $x_{2\text{-octanone}}$ = conversion of 2-octanone in reactor 1 (grey) and reactor 2 (black); grey line = model prediction reactor 1; black line = model prediction reactor 2; conditions: $\dot{V}$ = 4 mL h^{-1}; $c_{2\text{-octanone}}$ = 60 mmol L^{-1}; c_{LbADH} = 50 mg L^{-1} (R1) ; c_{GDH} = 250 mg L^{-1} (R1); c_{LbADH} = 250 mg L^{-1} (R2) ; c_{GDH} = 100 mg L^{-1} (R2); t_{total} = 350 h; c_{NADP^+} = 0.05 mmol L^{-1}.

The conversion was stable for more than 350 h. Compared to the base case synthesis and recycling, a further reduction of the *E*-factor to 17 (PMI = 18) was achieved. Although the run time of the new experiment was shorter, the TONs for both enzymes were still in the range of several million, and the enzyme contribution to the overall cost was within the above-mentioned range of 5%.[12] The cofactor turnover number per pass through the cascade was improved from 539 to 918. In addition to this improvement, roughly 25% of the cofactor could be recycled. This was also reflected by the much lower overall cost of €52/kg for the improved synthesis (−65% compared to base case) (Figure 4). The costs per kilogram of product in our case exceed €15/$kg_{product}$ given in Tufvesson et al.[12] as a minimum selling price for a fine chemical. However, as the costs of the substrates 2-octanone and glucose already lie in the same range (Figure 10), €15/$kg_{product}$ is not achievable in this case. Typically, enantiopure alcohols achieve higher prices, judging from related examples.

Future Perspectives. The overall system for the continuous synthesis of enantiomeric pure alcohols has a high level of complexity and leads to a multiparameter optimisation problem. In order to guide the process towards an economically viable one, a sensitivity analysis was carried out (see Table 4).[35] In the study of Degenring et al.[35] conversion and enantiomeric ratio were the evaluation targets. In the present investigation, the enantioselectivity of the enzyme was not an issue. In all cases, the measured ee was always ≥99.5% for long-chain aliphatic alcohols, which was in line with previous findings.[1,19−21,33]

The cost analysis revealed that cofactor utilisation was the bottleneck of this process. Thus, the target key figures were X_{R2}, $g_{NADP^+\ consumed}/g_{2\text{-octanol}}$, and STY, as well as the overall cost. Modifications of the process that needed to be addressed were improved enzyme stability or activity, different enzyme and cofactor concentrations, as well as higher flow rates and higher solubility of the substrates. These modifications fit into three groups: Group 1 involved changes on a reaction engineering basis, such as increased flow rate, increased enzyme concentrations, or elevated cofactor concentration. Group 2 consisted of changes on a molecular biology basis such as improved enzyme activity or stability, whereas group 3 contained changes that would require a reconsideration of the process itself. An increased substrate concentration fell in group 3 due to the restricted solubility of the substrate. A higher IL concentration would improve substrate solubility, but at the same time, the viscosity of the solution would increase (data not shown) which leads to practical limitations in the ultrafiltration step. Moreover, a higher IL concentration also renders the downstream processing more challenging due to different partition behavior (higher solubility in the aqueous IL-containing phase). Thus, another solubiliser should be considered when a higher substrate concentration is required. The effect of improving or increasing all these parameters by a

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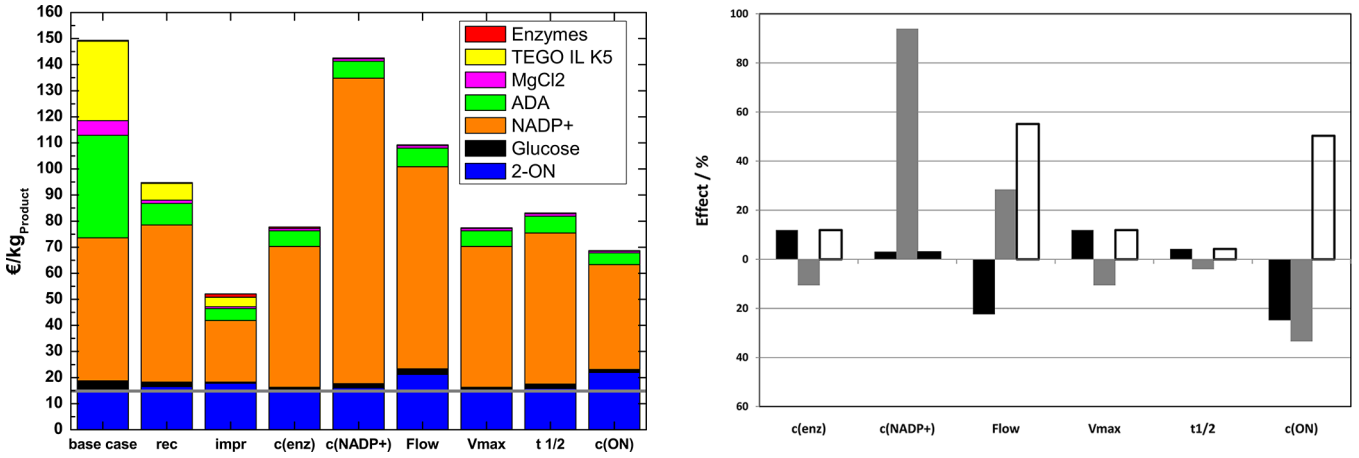

Figure 10. (Left) Cost analysis based on 250 mL of substrate solution without recycling, afterwards 90% recycling for the base case, the recycling case, and the improved synthesis as well as for selected hypothetical improvements; grey line = stoichiometric minimum €/$kg_{product}$ based on substrate costs; conditions here: c_{LbADH} = 50 mg L^{-1}; c_{GDH} = 250 mg L^{-1}; $\dot{V}$ = 4 mL h^{-1}; c_{NADP^+} = 0.1 mmol L^{-1}; $c_{2\text{-octanone}}$ = 60 mmol L^{-1}; $c_{TEGO\ IL\ K5}$ = 100 g L^{-1}; c_{ADA} = 100 mmol L^{-1}; c_{MgCl_2} = 20 mmol L^{-1}; pH = 7.0. (Right) Sensitivity analysis of the process (mean values based on the prediction of a 1000 h experiment). Improvements and aggravations compared to the base case. black bars = conversion in reactor 2; grey bars = $g_{NADP^+\ consumed}/g_{2\text{-octanol produced}}$; white bars = STY_{total}.

Table 4. Comparison between the base case (including recycling) and the improved synthesis

	base case		improved	
	reactor 1	reactor 2	reactor 1	reactor 2
$X_{2\text{-octanone}}$/% *	75.8	96.9	50.5	83.5
$TON_{LbADH}/10^6$ a	45.6	12.8	12.3	1.62
$TON_{GDH}/10^6$ a	7.7	2.9	0.945	3.12
TON_{NADP^+} (per pass)/$^-/_+$	411	539	588	918
STY/mmol L^{-1} d^{-1} (g L^{-1} d^{-1})*	291 (37)	124 (16)	194 (25)	124 (16)
ee/%		>99.5		>99.5

factor of 2 is demonstrated in Figure 10. Only one parameter was considered at a time to avoid excessive extrapolation of the model.

When judging the effects of all the modifications, processes should be favored that increase the conversion in reactor 2, increase STY and lower the ratio of $g_{NADP^+\ consumed}/g_{2\text{-octanol produced}}$ compared to the base case. This could be achieved by increasing the enzyme concentration, the enzyme V_{max}, and the enzymes' half-life. Among these, the enzyme half-life has the least effect on the target key figures. The effects of the increased V_{max} and increased enzyme concentration are the same.

Assuming the same price for the native enzymes and the improved enzymes, nearly no differences were observed in the overall cost. Thus, to improve the process a higher enzyme concentration is advisable, whereas expensive, time-consuming, and economically risky molecular biological improvements are not warranted in this case.

A higher cofactor concentration increased the conversion and STY slightly, particularly elevating the amount of cofactor needed per kg of product. Hence, increasing the cofactor concentration is not advisable, since better results are obtained with reduced cofactor concentrations.

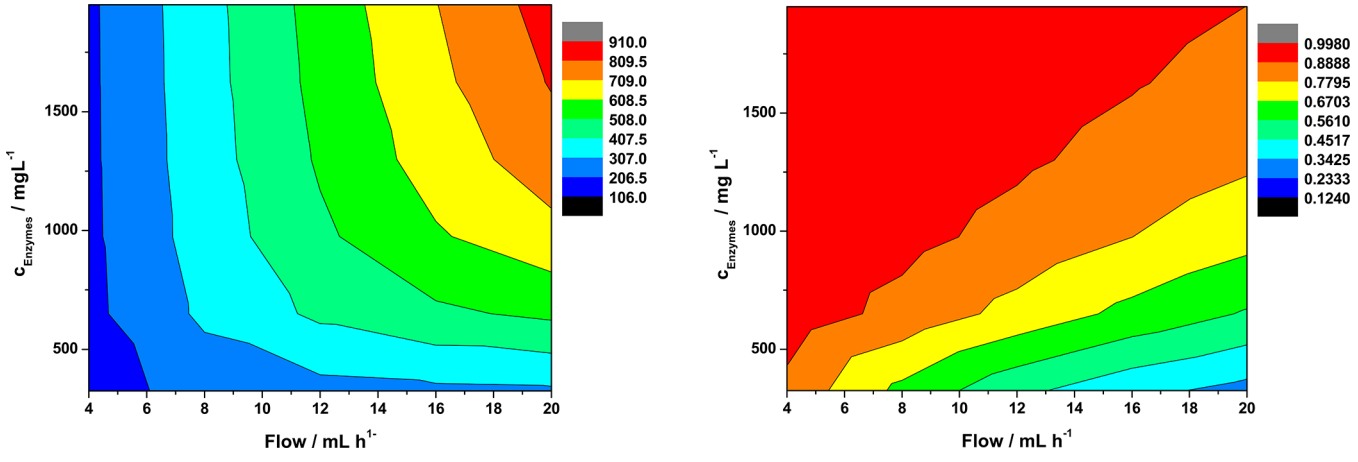

Figure 11. Space-time-yield (average R1 and R2, left) and conversion (after R2, right) as a function of total enzyme concentration and flow rate (mean values based on the prediction of a 1000 h experiment). Conditions: $c_{LbADH,\ R1}$ = 50 mg L^{-1}; $c_{GDH,\ R1}$ = 250 mg L^{-1}; $c_{LbADH,\ R2}$ = 250 mg L^{-1}; $c_{GDH,\ R2}$ = 100 mg L^{-1} and multiples of the enzyme concentrations; $\dot{V}$ = 4–20 mL h^{-1}; c_{NADP^+} = 0.01–0.1 mmol L^{-1}; $c_{2\text{-octanone}}$ = 60 mmol L^{-1}; $c_{TEGO\ IL\ K5}$ = 100 g L^{-1}; c_{ADA} = 100 mmol L^{-1}; c_{MgCl_2} = 20 mmol L^{-1}; pH = 7.0.

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Doubling the flow or the substrate concentration generates the same results when considering the three target key figures (X_{R2}, $g_{NADP^+\ consumed}/g_{2\text{-octanol produced}}$, and STY). The cost analysis confirmed that a higher flow rate made the product more expensive due to the lower cofactor utilisation. Therefore, a higher flow rate is only advisable if the enzyme concentration is adjusted at the same time to maintain a high level of conversion. The effect of applying a higher flow rate together with higher enzyme concentrations on STY (average R1 and R2) and conversion (after R2) is predicted using the process model (Figure 11).

Before these conditions can be used for a larger-scale synthesis, some research should focus on the filtration step. We used polyethersulfone (PES) membranes which normally allow for higher flow rates compared to a regenerated cellulose membrane. Ionic surfactants like TEGO IL K5 can interact with such PES-membranes (see manufacturers' data sheet) which can lead to an accelerated fouling of the membrane. Experimentally, at a constant flow of 8 mL h^{-1} a constant increase in the reactor pressure was observed, rendering continuous operation impossible. Hence, to increase the synthesis further in view of STY, some effort has to be put into the improvement of the filtration step.

All the cost analyses and modeling did not include downstream processing. Thus, solvent consumption during product elution was not included in the calculations. As the performance of the adsorber depends on the solubility of the substrates/products in the aqueous solution, comparing syntheses with the same IL concentrations will not falsify the results. However, preliminary results showed, that the *n*-heptane/ethanol mixture could be reused after distillation for eluting the substrates/products from the column. When solvents are recycled by distillation, generally a loss of 10% can be assumed.[11] Thus, even though *n*-heptane is a relatively ecotoxic solvent, its release to the environment can largely be avoided.

Apart from the changes in the existing process, a totally new synthesis route could be considered, for example the biphasic synthesis as described in Leuchs et al.[21] Methyl-*tert*-butyl-ether (MTBE) is very suitable for biphasic synthesis in combination with *Lb*ADH.[36] In this context, it is mandatory to use 2-propanol for cofactor regeneration in continuous synthesis due to the low solubility of glucose in MTBE.

Sheldon[10] proposed the use of atom efficiency for a quick assessment of the environmental impact of an alternative process when compared to an existing benchmark process, which has been used frequently.[11,27] In contrast to the *E*-factor, which includes conversion, byproducts, and coupled products, the atom efficiency can easily be calculated even before the process is tried at the laboratory scale. Atom efficiency is the molar mass of the desired product divided by the sum of the molar masses of all products. It is based on stoichiometry only. The overall reaction equation for the existing monophasic approach is:

$$CH_3COC_6H_{13} + C_6H_{12}O_6 \rightarrow CH_3CHOHC_6H_{13} + C_6H_{10}O_6$$

The overall reaction equation for the alternative biphasic approach is:

$$CH_3COC_6H_{13} + CH_3CHOHCH_3 \rightarrow CH_3CHOHC_6H_{13} + CH_3COCH_3$$

Assuming high turnover numbers of the catalysts (enzymes) and cofactors in both cases, the atom efficiency is 42% for the monophasic approach (40% if hydrolysis of GDL to gluconic acid is taken into account) and 69% for the biphasic approach.

At first glance, the biphasic approach seems to be favorable, but it is known that, for this kind of transfer hydrogenation, thermodynamics play an important role.[21,37−39] With a molar ratio of 1:1 = 2-octanone/2-propanol, a conversion of 38% is expected.[21] With a 3.3-fold excess, as used in the present study (glucose/2-octanone), a conversion of 68% is expected when using 2-propanol as the hydride donor compared to full conversion when using glucose/GDH as the hydride donor. To reach an industrially relevant conversion, a large excess of 2-propanol would be required.[40] In view of atom economy and efficiency, the selective addition of a hydrogen molecule per molecule of substrate would be the most efficient method.[41] Here, the use of suitable hydrogenases for cofactor regeneration is a biocatalytically favorable alternative.[42] In chemical catalysis the asymmetric hydrogenation using ligand-modified noble metal catalysts is very successful.[43−45] A major drawback of this synthesis route is the low enantioselectivity when it comes to the reduction of aliphatic nonfunctionalised ketones.[43]

CONCLUSION

The continuous synthesis of (*R*)-2-octanol could be improved to decrease the chemical cost basis by 65%. Modelling and simulation allows the optimisation of this complex process. Further targets of optimisation can be revealed by careful extrapolation and sensitivity analysis. We demonstrated that an enzyme's affinity to NADP(H) instead of NAD(H) is not a knockout criterion for an industrial process. This is important, given the fact that molecular biological methods that change an enzymes' affinity to NAD(H) instead of NAD(P)H are not always applicable, as is the case for the *Lb*ADH, our enzyme of choice. Improvements by reaction engineering may be more cost and time effective.

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Notes

The authors declare no competing financial interest.

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