Overcoming Work-Up Limitations of Biphasic Biocatalytic Reaction Mixtures Through Liquid-Liquid Segmented Flow Processes

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Biphasic biocatalytic reactions have gained much attention in the field of enzyme-catalysed synthesis. As most components being of relevance for the pharmaceutical industry are hydrophobic, often biphasic reaction media turned out to be the solvent system of choice. However, in spite of successful reaction courses practical difficulties in the downstream-processing, in particular extremely difficult phase separations due to emulsification and precipitation, represent a challenge to overcome in process development. In this work, we report our studies on the benefits of a simple flow set-up being capable to minimise such work-up limitations. In detail, a segmented flow system based on a biphasic MTBE/buffer mixture was successfully applied for two types of enzymatic reductions of a hydrophobic ketone in the presence of an alcohol dehydrogenase (ADH) as an enzyme class being known for their excellent enantioselectivity and successful utilization in the synthesis of a range of active pharmaceutical ingredients. The applicability of this flow system was demonstrated with two different enzymes as well as different substrates. Besides an ADH from Lactobacillus brevis, an ADH from Ogataea minuta was utilized for the reduction of acetophenone and 2,2,2-trifluoroacetophenone, respectively.

A range of impressive achievements in the field of biocatalysis have been made during past decades with respect to finding new enzymes, reactions and modification of enzymes using various techniques from different fields, such as protein engineering and immobilization.[1,2] In addition, process development represents an important task when it comes to industrial applications. An emerging research theme in this field are continuously running syntheses, which nowadays gained more and more interest also for enzyme catalysis.[3–5] This trend has been certainly stimulated by recent recommendations of the U.S. Food and Drug Administration (FDA) as well as the European Medicines Agency (EMA) for continuous manufacturing, which were added to their regulatory guidelines.[6] Often, the use of biphasic reaction systems offers advantages over the use of monophasic reactions.[6] Since typically enzymes are less stable in organic reaction media, but pharmaceutical compounds often tend to be hydrophobic, a compromise must be found. One of the favoured solutions is the use of an aqueous/organic biphasic media, which turned out to be the reaction medium of choice for numerous biotransformations. By leaving the enzyme in its native aqueous environment and adding a second, organic phase as substrate/product reservoir, the enzyme stability should be less affected compared to the use of a pure organic solvent. Thus, high substrate loadings can be reached by means of a biphasic system while avoiding strong deactivation of the enzyme.[2,7] Furthermore, downstream processing can be designed efficiently by means of phase separation.[8] However, most examples of biphasic biocatalytic reactions so far are limited to batch processes.[9] At the same time, although this approach looks promising in theory, it raises practical challenges and concerns. As many scientists might have suffered on their own, formation of emulsions being difficult to separate is a major issue when working with biphasic biocatalytic reaction mixtures.[10]

In a pioneering work on the use of an alcohol dehydrogenase (ADH; being an important enzyme class for the biocatalytic synthesis of pharmaceuticals) for processes running in a flow mode, Schmid and Buehler et al. recently investigated the stability of a thermostable ADH in a segmented flow system.[5a] The same group also investigated this ADH-catalysed reduction with a focus on mass transfer-limited reactions concluding that mass transfer-limited reactions benefit from this type of setup.[9b] As a further benefit of such a segmented flow process, a better phase separation was mentioned by the authors of this
Furthermore, flow systems turned out to provide successful solutions for many types of phase-separation challenges.\cite{1b}

Inspired by this pioneer work\cite{2} of Schmid and Buehler and the success of flow systems in general for solving phase-separation challenges and due to the importance of an efficient downstream process for technical purpose (besides achieving an efficient reaction course and, e.g., high substrate loading), we became interested to study to which extend enzymatic ketone reductions running in a flow mode can benefit from such a segmented flow-setup due to an improved work-up. In particular we were interested to study the differences of phase-separations when conducting such biotransformations in a batch-mode and segmented flow-mode, respectively, exemplified for different enzymes and substrates.

Herein we report a simple, yet robust segmented flow process simplifying and minimising work-up efforts and unit-operation steps. We focused on favoured solvents and ketone substrates as model compounds for pharmaceutically relevant building blocks. As such a model reaction for an ADH-catalysed reduction, at first the reduction of acetophenone (1) to (R)-1-phenylethanol ((R)-1) in the presence of an alcohol dehydrogenase from \textit{Lactobacillus brevis} (LB-ADH)\cite{3} was chosen as this enzyme has been shown to be relatively tolerant towards various organic solvents.\cite{4b} As the required cofactor NADPH is an expensive compound, a cheap and efficient glucose dehydrogenase (GDH)-based cofactor regeneration system\cite{5} was applied. This cofactor regeneration system is based on the in situ-reduction of NADP$^+$ through oxidation of glucose to gluconolactone and a subsequent irreversible ring opening to gluconic acid (see Scheme 1).

The aqueous phase was prepared by dissolving the ADH as well as the cofactor NADPH and its in situ-cofactor regeneration system consisting of GDH and glucose in phosphate buffer. The substrate was dissolved in methyl tert-butyl ether (MTBE), thus representing the organic counterpart to the buffer solution. These two immiscible solutions were combined in a Y-shape mixer, thus resulting in a continuous stream of uniform segments. These segments were then inserted into a PFE coil reactor (inner diameter 0.8 mm), in which the biotransformation takes place. The set-up of this flow process is shown in Figure 1. Afterwards, the reaction mixture was collected and immediately quenched with HCl solution to avoid further reaction in the collection vial. It should be added that when the reaction was quenched to investigate the conversion after a certain reaction time, precipitation and emulsification occurred again due to the magnetic stirring in the collection vial.

The flow reaction shows two major practical advantages over the analogous reaction conducted in batch mode: first, in case of the flow reaction, an additional air-liquid interface, occurring in batch reactions due to stirring, is avoided. Second, mixing is changed from a vigorous stirring to a gentler mixing, thus resulting in less protein aggregation and precipitation due to reduced shear forces compared to the batch mode. As shown in Figure 2, the phase separation after 1 h reaction time for the batch process compared to the one for the flow process after 2 h residence time (which represents the equivalent in flow processes to the reaction time in batch processes) shows a better phase separation for the flow process (right) in spite of

**Scheme 1.** ADH-catalysed reduction of acetophenone (1) to 1-phenylethanol ((R)-1) and trifluoroacetophenone (3) to the corresponding alcohols ((R)-2 and (R)-4) with GDH based cofactor regeneration system.

**Figure 2.** Phase separation of the reaction mixture compared for batch after 1 h reaction time (left) and developed flow process after 2 h residence time (right) using LB-ADH.
doubled reaction time. The batch reaction (left) led to significantly worse separation even after resting for only 1 h.

Furthermore, after 3 h reaction/residence time (see Figure 3) with threefold increased amount of catalyst and cofactor regeneration system concentration, in the batch process emulsification and precipitation prevent a clear phase separation. In contrast, for the developed flow process the phase boundary is clearly visible (Figure 3), and, thus, phase separation can be done easily.

When comparing the conversions obtained for the model reaction in a flow and batch mode, respectively, no differences between flow and batch process could be observed after a certain reaction time (Figure 4). As the mixing, and therefore the mass transfer, in segmented flow process is reported to be significantly increased compared to batch processes, a mass transfer limitation appears to be unlikely for the investigated reaction, thus being in accordance with the literature.[5]

Whereas the reduction of acetophenone (1) in a batch process gave a conversion of 32% to alcohol (R)-2 after 1 h reaction time, a very similar conversion of 33% was obtained in a flow mode under comparable reaction conditions (residence time of 1 h and catalyst loading per overall volume of 1 U·mL⁻¹ for a 50 mM substrate solution when considering the total volume). In this case, the enzyme loading in terms of amount of biomass per reaction volume corresponds to approximately 30 μg of protein per mL of reaction mixture. As both, batch and flow, processes perform similar, the enzyme activity per mmol of substrate is identical.

To investigate the robustness and reliability of the developed flow process, the biotransformation running in the "segmented flow mode" was repeated using the same reaction conditions and we were pleased to find that the average conversion (after 1 h equilibration time) turned out to be very similar to the first experiment with conversions of 33% and 32%, respectively (Figure 5). It should be added that in general, the equilibration time in our flow experiments was adjusted to two times of the residence time ("equilibration time") in order to ensure a stable reaction system.

After having characterized the reaction, we were also interested to increase the conversion of this segmented flow process. We were pleased to find that by increasing the biocatalyst loading per overall volume to 3 U·mL⁻¹ and the residence time to 3 h, the conversion was significantly im-
proven achieving a maximum conversion to (R)-2 of 95% (Figure 6). After an initial equilibration time, in which the conversion was not yet stable, the system equilibrated to an average of 93% conversion related to the formation of the desired product (R)-2 with only a minor deviation of ±2% between the collected fractions. An extended run and a recycling of the aqueous phase containing the biocatalysts and cofactors represents a task for further research in the future.

As a next step we became interested to explore another enzymatic reduction for such a flow process utilizing a different enzyme (used at a different protein loading) in combination with another ketone component. In detail, we chose an alcohol dehydrogenase from Ogatea minuta (OM-ADH) as a further recombinant biocatalyst, which has been recently reported and characterized in terms of process stability.[14,15] This enzyme was then used for the reduction of 2,2,2-trifluoroacetophenone (3) to (R)-(−)-α-(trifluoromethyl)benzyl alcohol ((R)-4). This substrate was chosen since the OM-ADH showed a higher specific activity towards 2,2,2-trifluoroacetophenone (3) compared to acetophenone (1). The segmented flow reaction was then conducted at a substrate concentration per overall volume of 100 mM of 2,2,2-trifluoroacetophenone (3) dissolved in MTBE as organic solvent. Under the initial reaction conditions and after the equilibration time, a constant conversion to the desired alcohol (R)-4 of 48% was achieved (Figure 7A).

In order to improve the conversion, we increased the enzyme loading from a biocatalyst amount per overall volume of 5 U mL⁻¹ (1.6 mg mL⁻¹ lyophilised enzyme) to 10 U mL⁻¹ (3.2 mg mL⁻¹ lyophilised enzyme) along with an increase of the residence time up to 2 h. Applying these optimized reaction conditions then resulted in a significantly increased conversion to (R)-4 of approximately 70% conversion (Figure 7B).

As for the work-up after the biotransformation, compared to the previously discussed LB-ADH-based reduction the phase separation is less clear. However, it also must be taken into account that the biomass loading is significantly increased when using OM-ADH as biocatalyst, which then causes such an emulsifying effect.

At the same time, it is noteworthy that also in this case of OM-ADH-catalysed ketone reduction the phase separation is still tremendously better for the developed flow process compared to the conventional batch process as indicated by the photos in Figure 8. Whereas an emulsion with no clear phases results from the batch reaction (Figure 8A), still separable phases are obtained for the reaction mixture of the flow process (Figure 8B).

In conclusion, we reported a comparison of phase separation of biphasic reaction mixtures when conducting enzymatic ketone reductions in batch and flow mode. Toward this end, two different types of biotransformation have been investigated based on the use of two different alcohol dehydrogenases (ADHs) as well as two different ketones. It turned out that while
Experimental Section

Typical Procedure for the Synthesis of (R)-1-Phenylethanol ((R)-2) in a Segmented Flow Process Utilizing an Alcohol Dehydrogenase from Lactobacillus brevis:

In a glass vial, an alcohol dehydrogenase from Lactobacillus brevis (LB-ADH; 10 U mL⁻¹) a glucose dehydrogenase (GDH; 6 U),[14] glucose solution (cglucose 150 mM, 300 μL in KPi (100 mM, pH 7)), NADPH (cNADPH 0.1 M, 20 μL 10 mM aqueous solution) and potassium phosphate buffer (to 1 mL, 100 mM, pH 7) were mixed and transferred into a syringe (1 mL, S.G.E. gas tight, 47.7 mm ID). Acetophenone (1, cacetophenone 50 mM, 11.7 μL, 0.1 mmol) was mixed with MTBE (resulting in a total volume of 1 mL) and transferred into a syringe (1 mL, S.G.E. gas tight, 47.7 mm inner diameter). Both syringes were attached to a syringe pump and pumped (0.25 mL h⁻¹ flow rate) via a Y-mixer (0.51 mm ID) in a coil reactor (PFE, reactor volume: 1 mL; flow rate: 0.5 mL h⁻¹). The reaction time was extended from 1 and 2 h by lowering the flow rate to 0.25 mL h⁻¹ with increment of OM-ADH (20 U mL⁻¹; 3.6 mg mL⁻¹) and GDH (20 U mL⁻¹). The reaction mixture was quenched by addition of an aqueous solution of HCl (0.5 mL, 1 M), followed by extraction with ethyl acetate (1 mL) for GC analysis.

Figure 8. Phase separation of the reaction mixture compared for batch after 2 h reaction time (A) and developed flow process after 2 h residence time (B) using OM-ADH (20 U mL⁻¹ referring to the overall volume).

Conversions of the processes in batch and flow mode are similar under comparable conditions (exemplified for the reduction using the ADH from L. brevis), emulsification and precipitation are strongly suppressed when carrying out the biocatalytic reactions in a flow mode, thus significantly simplifying and minimising work-up efforts for biphasic biocatalytic reaction systems. It is further noteworthy that the flow systems can be operated in a stable fashion for at least several hours. Currently, further process optimization as well as extension of this type of segmented flow process technology to other ADH-catalysed reductions and other types of biotransformations running in an aqueous-organic biphasic solvent system are in progress. Further tasks of future work are an extended run as well as recycling of the aqueous phase containing the enzymes and cofactor.

Conflict of Interest

The authors declare no conflict of interest.

Keywords: alcohol dehydrogenases · enzyme catalysis · downstream processing · flow chemistry · work-up

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