Enzyme Catalysis

Biotransformations in Pure Organic Medium: Organic Solvent-Labile Enzymes in the Batch and Flow Synthesis of Nitriles

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Abstract: In recent years, there has been an increasing tendency to use biocatalysts in industrial chemistry, especially in the pharma and fine chemical sector. Preferably, enzymes or whole cells, applied as catalysts for a specific biotransformation, are utilized in aqueous reaction media since water is the natural medium for enzymes. In numerous examples of biocatalytic systems, however, a major problem is the insolubility of hydrophobic substrates in such aqueous reaction media. Apart from lipases, many enzymes are highly sensitive to organic solvents and are inactivated by an organic medium. Therefore, a change of solvent for biotransformations from water to organic solvents is usually challenging. In this study, we investigated the synthesis of nitriles by an organic solvent-labile aldoxime dehydratase in pure organic solvents, exemplified for the dehydration of *n*-octanaloxime to *n*-octanenitrile. We present a method for applications in batch as well as flow mode based on an "immobilized aqueous phase" bearing the whole cells in a superabsorber as solid phase, thus enabling the use of a purely organic solvent as "mobile phase" and reaction medium.

Introduction

In recent decades biotransformations have emerged towards a valuable tool in organic synthesis on both lab and industrial scale.[1–6] In spite of tremendous success illustrated, e.g., by numerous applications in industry, however, a general challenge related to the reaction medium remained: Typically, enzyme catalysis is conducted in aqueous medium whereas in contrast substrates and products are mostly hydrophobic.[1–8] Thus, the option to run biotransformations in purely organic medium would offer significant advantages. Besides the benefits from solubilizing hydrophobic substrates in a pure organic medium, emulsions as a result of two-phase aqueous/organic solvent systems could be avoided. Such emulsions often lead to a tedious work-up due to, e.g., difficulties in phase separation. In contrast, when using pure organic medium[9–11] the work-up could consist of a simple filtration or decantation of the organic solvent from a heterogenized biocatalyst. Such a biocatalytic process would be particularly interesting for producing bulk chemicals due to simplification of downstream-processing and minimization of the unit operation steps therein.[12] A major task for obtaining high conversions and productivities with hardly water-miscible substrates in enzyme catalysis is to overcome limitations in terms of enzyme stability when being in contact with hydrophobic reaction media. Whereas for lipases their use in solely organic media is widely known (due to their high affinity "by definition" to organic solvents), for most other enzymes general methodologies for their utilization in pure organic medium rarely exist.[3,4,9,10,13] Enzymes are often unstable in organic solvents, biphasic reaction systems consisting of water and an organic solvent and in many cases even in aqueous reaction media when containing a large portion of a water-soluble organic co-solvent.[1,5,14] Furthermore, enzymes in crude extracts and in particular in purified form might be even more unstable compared to whole-cell catalysts, which benefit from the cellular matrix protecting the enzymes. But also for whole-cell catalysts, examples are rare in which they are used in pure organic medium or biphasic systems.[15] At the same time various strategies to overcome these stability problems of biocatalysts in contact with organic solvents have been studied. One opportunity is compartmentalization of the reaction medium. This concept enables the combination of non-compatible reactions comprising biocatalytic steps and, e.g., polydimethylsiloxane (PDMS) membranes were successfully used for this purpose.[16–18] Another solution to overcome the instability problems of biocatalysts in organic media is immobilization. Besides immobilization of enzymes in crude extracts or purified enzymes themselves,[19–22] techniques of whole cell immobilization are known as well. Usually, immobilization of "free" enzymes without protection of the cells might not be sufficient to make these biocatalysts usable in pure organic solvent. In case of whole cell immobilization[15,23,24,25] some methods showed stabilizing effects and biocatalysts were found to be more stable in presence of organic solvents.[15,23] Encapsulation of whole cells in polyurethanes, as shown by von Langermann et al., turned out as a highly suitable technique to prevent contact of the biocatalyst with the organic medium, thus resulting in a significant higher stability of the catalyst in such me-

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dia.[23,26] In continuation of our ongoing study on the use of enzymes being immobilized in superabsorber materials as a technique for immobilization of "free" enzymes,[22,27,28] and our research work on aldoxime dehydratase and their application for the synthesis of nitrile bulk chemicals in the presence of aldoxime dehydratases,[29–31] we became interested if such superabsorber-immobilized whole cell catalysts can be successfully used in pure organic medium. In the following we report the development of such a solution for utilizing whole-cell catalysts being immobilized in superabsorbers in pure organic media, which also provides the perspective for a broad general applicability in biocatalysis.

Results and Discussion

We chose an aliphatic aldoxime, namely *n*-octanaloxime (**1**), as substrate because firstly, substrate **1** and product **2** are insoluble in water and secondly, based on a previous study of Asano *et al.*[32–35] recently we developed a process for transforming aliphatic aldoximes to the corresponding nitriles at high substrate loading in the presence of an aldoxime dehydratase from *Bacillus* sp. OxB-1 (OxdB) as a catalyst.[30,36] Our first approach towards conducting aldoxime dehydratase-catalyzed reactions in pure organic medium consisted of using wet *E. coli* cells with overexpressed OxdB as biocatalyst (Scheme 1) since previously[30] we utilized such whole cells in aqueous reaction media.

In our experiments, we utilized *n*-octanaloxime (**1**) at a concentration of 0.5 M in combination with various types of organic solvents (cyclohexane, methyl-*tert*-butyl ether (MTBE), toluene and dichloromethane (DCM)) as reaction medium and 33 mg of wet cell mass (wcm) of *E. coli* cells including OxdB as biocatalyst per mL of reaction medium. A control reaction was performed using potassium phosphate buffer (PPB) as reaction medium. The reactions were performed at 30 °C reaction temperature for 24 h in round-bottomed flasks and 20 mL scale.

During the chosen reaction time, the whole cell catalyst behaved very different in each solvent, as visualized in the photos shown in Figure 1. In case of cyclohexane and toluene, the cells were suspended in the solvent, but in case of DCM and especially MTBE, the cells precipitated and were not suspended in the organic medium (Figure 1). After a reaction time of 24 h, the conversion of *n*-octanaloxime (**1**) to the corresponding nitrile **2** was analyzed by GC (Table 1). In case of the control

reaction in phosphate buffer, the reaction was extracted with ethyl acetate (EtOAc) beforehand.
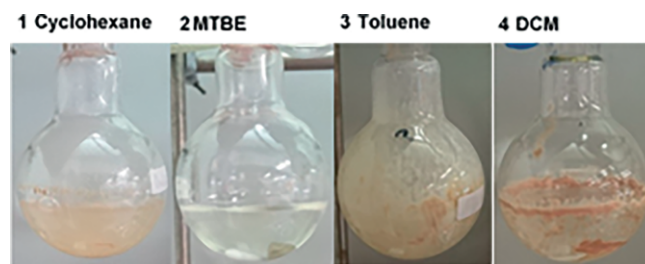

Figure 1. Pictures of reaction mixtures with OxdB in wet whole cells in different organic solvents.
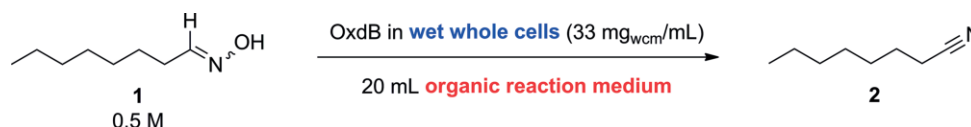
Table 1. OxdB in wet whole cells catalyzed dehydration of *n*-octanaloxime (**1**) in pure organic medium.

| #	Reaction medium/solvent	Conversion to **2** /%[a]
1	Cyclohexane	2
2	Methyl-*tert*-butyl ether (MTBE)	1
3	Toluene	2
4	Dichloromethane (DCM)	<1
5	Potassium phosphate buffer (PPB)	61[b]

[a] 33 mg$_{wcm}$/mL OxdB in whole cells were suspended in 20 mL organic solvent or PPB including 500 mM *n*-octanaloxime (**1**). The reactions were performed at 30 °C for 24 h and conversions to *n*-octanenitrile (**2**) were determined by GC analysis. [b] Conversion was determined after extraction of the reaction mixture with ethyl acetate (EtOAc). The EtOAc phase was analyzed by GC for determination of conversion.

The use of any type of organic solvent as reaction medium directly led to a strong drop of activity, indicating the high degree of deactivation of the enzyme in these cases. Thus, we found very low conversions in the range of <1 % to 2 % of *n*-octanaloxime (**1**) to *n*-octanenitrile (**2**) when using OxdB in pure organic medium (Table 1, entries 1–4). In contrast, in the control reaction using the aqueous buffer as reaction medium a conversion of 61 % was observed (entry 4). These experiments revealed that wet cells are not applicable in pure organic solvent. Thus, as a next step we studied the use of lyophilized cells containing OxdB as a catalyst in pure organic medium (Scheme 2).

Again, we used *n*-octanaloxime (**1**) in a concentration of 0.5 M in different solvents (cyclohexane, MTBE, toluene and DCM) and at 30 °C reaction temperature for 24 h. In this case,

Scheme 1. OxdB in wet whole cells catalyzed dehydration of *n*-octanaloxime (**1**) to *n*-octanenitrile (**2**) in pure organic medium.
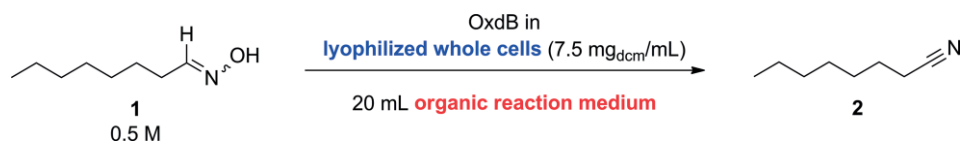
Scheme 2. OxdB in lyophilized cells catalyzed dehydration of *n*-octanaloxime (**1**) to *n*-octanenitrile (**2**) in pure organic medium.

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7.5 mg dry cell mass (dcm) per mL of reaction medium was used, corresponding to 33 mg bio wet mass. We again performed a control reaction using resuspended dry cells in aqueous buffer. As shown in Figure 2, in all solvents the lyophilized whole cells were homogeneously suspended. After 24 h reaction time, the conversion of *n*-octanaloxime (**1**) to the corresponding nitrile was analyzed by GC (Table 2). In case of the control reaction in aqueous buffer the reaction was extracted with EtOAc beforehand.
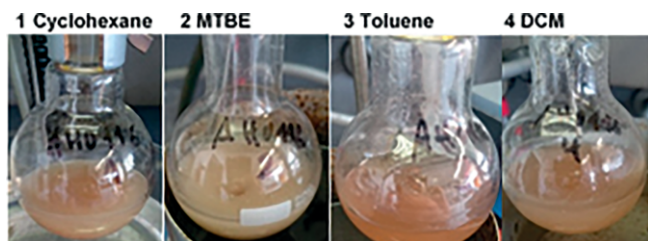

Figure 2. Pictures of reaction mixtures with OxdB in lyophilized cells in different organic solvents.

Table 2. OxdB in dry whole cells catalyzed dehydration of *n*-octanaloxime (**1**) in pure organic medium.

#	Reaction medium/solvent	Conversion to **2** /%[a]
1	Cyclohexane	<1
2	Methyl-*tert*-butyl ether (MTBE)	<1
3	Toluene	<1
4	Dichloromethane (DCM)	<1
5	Potassium phosphate buffer (PPB)	23[b]

[a] 7.5 mg$_{dcm}$/mL OxdB in whole cells were suspended in 20 mL organic solvent or PPB including 500 mM *n*-octanaloxime (**1**). The reactions were performed at 30 °C for 24 and conversions to *n*-octanenitrile (**2**) were determined by GC analysis. [b] Conversion was determined after extraction of the reaction with ethyl acetate (EtOAc). The EtOAc phase was analyzed by GC for determination of conversion.

However, in spite of a homogeneous biocatalyst suspension also in these cases a strong deactivation of the Oxd-enzyme was observed as soon as the reaction is conducted in an organic solvent, thus underlining that these enzymes are labile towards the presence of an organic medium (Table 2, entries 1–4). In detail, we found very poor conversions below 1 % of *n*-octanaloxime (**1**) to the corresponding nitrile **2** independent of the type of organic solvent as reaction medium. In contrast, the control reaction gave 23 % conversion showing that the enzyme in the lyophilized cells is still active after lyophilization.

Since we found that the use of any type of whole cells in a pure organic solvent does not lead to significant conversions, as a next step we studied this type of biotransformation running in a biphasic aqueous-organic media as this media has been utilized often in enzyme catalysis.[4,5] In these experiments, we used wet whole cells containing OxdB as a biocata-

lyst at a concentration of 33 mg$_{wcm}$/mL of total reaction medium (1:1 (v/v) aqueous/organic, total 20 mL). As aqueous phase, a phosphate buffer containing the whole cells was used. The organic phase consisted of the organic solvents cyclohexane, MTBE, toluene or DCM, containing 1 M of the substrate *n*-octanaloxime (**1**) (thus, leading to an overall concentration of 500 mM of **1**). The reactions were performed in a batch mode utilizing a round-bottomed flask with magnetic stirrer at 30 °C for 24 h (Scheme 3).
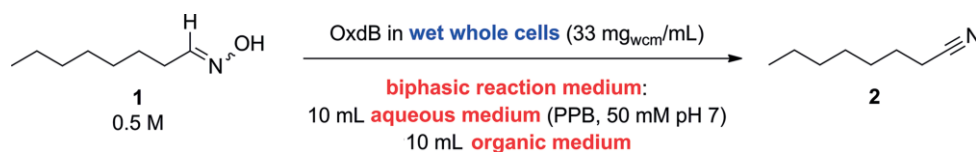
After a reaction time of 24 h, the organic phases were analyzed by GC for the determination of the conversion of *n*-octanaloxime (**1**) to *n*-octanenitrile (**2**) (Table 3). While MTBE, toluene and DCM again gave very low conversions not exceeding 4 % (Table 3, entries 2–4), the use of cyclohexane led to 9 % conversion of *n*-octanaloxime (**1**) to the corresponding nitrile **2** (entry 1), which at that time was the best result for this biotransformation in the presence of an organic solvent. Furthermore, this experiment illustrates that an aqueous phase is necessary to keep OxdB active in the cells (see comparison of experiments shown in entry 1 of the Table 1, Table 2, and Table 3). However, still such a biphasic system has a negative impact on the activity and/or stability of the enzyme.

Table 3. OxdB in wet whole cells catalyzed dehydration of *n*-octanaloxime (**1**) in biphasic systems.

#	Organic phase	Conversion to **2** /%[a]
1	Cyclohexane	9
2	Methyl-*tert*-butyl ether (MTBE)	4
3	Toluene	3
4	Dichloromethane (DCM)	<1

[a] 33 mg$_{wcm}$/mL OxdB in whole cells were suspended in 10 mL PPB and an organic solvent including 500 mM *n*-octanaloxime (**1**) was added. The reactions were performed at 30 °C for 24 and conversions to *n*-octanenitrile (**2**) were determined by GC analysis.

As a concept to overcome these limitations, we envisioned that encapsulating the whole cells in a solid and immobilized aqueous phase could make such biotransformations possible, although being conducted in a pure organic solvent as mobile phase. As such an "immobilized aqueous phase" we used superabsorber, which is basically polyacrylic acid having a very high affinity to bind water. It was already shown, that this technique is useful for the immobilization of alcohol dehydrogenases as a crude extract for ketone reduction.[22,28,37] Although such enzymes are known to be tolerant to organic solvents, we became interested to evaluate this methodology also for the entrapment of whole cells bearing enzymes which are labile against organic solvents such as the Oxd-enzyme used in this study. By means of such an immobilized aqueous phase we expected that the contact between the organic solvent and the Oxd-containing whole cells is minimized, thus leading to a stabilizing

OxdB in wet whole cells (33 mg$_{wcm}$/mL)

biphasic reaction medium:
10 mL **aqueous medium** (PPB, 50 mM pH 7)
10 mL **organic medium**

1
0.5 M

2

Scheme 3. OxdB in whole cells catalyzed dehydration of *n*-octanaloxime (**1**) to *n*-octanenitrile (**2**) in a biphasic system.

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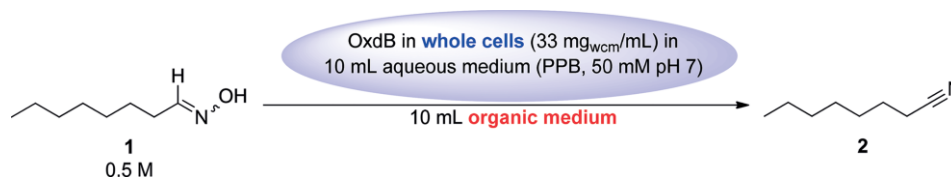
Scheme 4. OxdB in whole cells immobilized in superabsorber catalyzed dehydration of *n*-octanaloxime (**1**) to *n*-octanenitrile (**2**) using a pure organic solvent as reaction medium.

effect on the whole cells and enzymes therein, and to the opportunity to conduct biotransformations with organic solvent-labile enzymes in a pure organic reaction medium as liquid phase. Furthermore, separation of the reaction mixture from the biocatalyst would be simplified. The concept of this approach is visualized in Scheme 4.

In the resulting experiment, 33 mg$_{wcm}$ whole cells containing OxdB per mL of total reaction medium (1:1(*v/v*) aqueous/organic, total 20 mL) were dissolved in 10 mL of aqueous buffer, and the resulting aqueous phase was immobilized by addition of the superabsorber whilst stirring in a round-bottomed flask. Then, 10 mL organic phase containing 1 M *n*-octanaloxime (**1**) (0.5 M overall concentration) was added, and the reaction was performed at 30 °C for 24 h. We were pleased to find that after 24 h reaction time with cyclohexane as organic solvent an excellent conversion of >99 % of *n*-octanaloxime (**1**) to *n*-octanenitrile (**2**) was achieved (Table 4, entry 1). When utilizing toluene, a conversion of 11 % was detected and in case of MTBE (5 %) and DCM (<1 %) lower conversions were obtained (entries 2–4), thus indicating a significant impact on the type of organic solvent and the high suitability of cyclohexane as organic reaction medium.

Table 4. OxdB in wet whole cells catalyzed dehydration of *n*-octanaloxime (**1**) in the "superabsorber system".

#	Organic phase	Conversion to **2** (isolated yield) /%[a]
1	Cyclohexane	>99 (82)
2	Methyl-*tert*-butyl ether (MTBE)	5
3	Toluene	11
4	Dichloromethane (DCM)	<1

[a] 33 mg$_{wcm}$/mL OxdB in whole cells were suspended in 10 mL PPB and immobilized in superabsorber. An organic solvent including 500 mM *n*-octanaloxime (**1**) was added. The reactions were performed at 30 °C for 24 and conversions to *n*-octanenitrile (**2**) were determined by GC analysis.

These results showed that this "superabsorber approach" in combination with the right choice of organic solvent component is useful for the biocatalytic synthesis of nitriles in organic medium although using an enzyme being labile against organic solvents. We found cyclohexane to be the most suitable solvent for our purpose. In comparison to the simple use of whole cells in pure organic solvent, immobilization in superabsorber gave a much more stabilized catalyst, thus leading to an excellent conversion. This also indicates that the superabsorber stabilizes the whole cells by protecting them from direct contact with the solvent. The positive impact of cyclohexane can be rationalized by its high log P value, thus ensuring a low concentration in the aqueous super-absorber phase as well as a negligible "dry out"-effect of removing water from the superabsorber to the organic solvent. As we were interested to explore the suitability

of this method to operate at higher substrate loading as a desired criterion for a technical process, we investigated substrate concentrations of 500 mM, which corresponds to a substrate loading of approximately 75 g/L reaction medium. We were pleased to find that the biocatalyst remains stable in the superabsorber system over several hours and that quantitative conversions were obtained in the biotransformation. The time-course of this reaction with the superabsorber-immobilized whole cells using cyclohexane as solvent as well as a photo of this reaction mixture is shown in Figure 3.
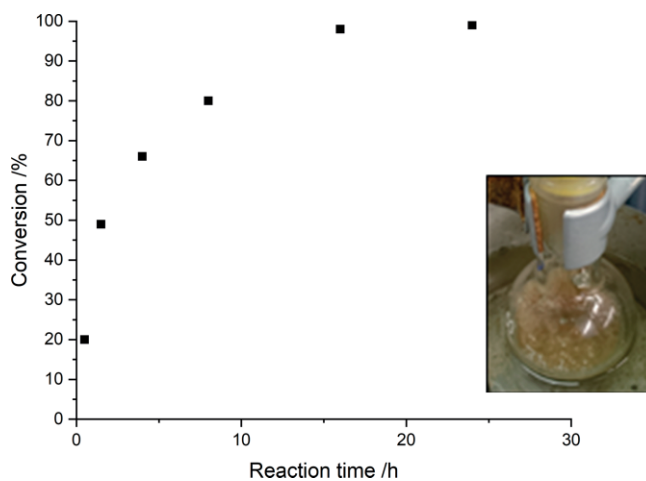

Figure 3. Picture and time-course measurement of the "superabsorber-system" with cyclohexane as organic phase.

The time-course measurement indicates that the reaction further proceeds even after 8 h reaction time. This result shows that the enzymes within the whole cell-catalyst maintain their activity (at least to a large extent) in the superabsorber when using cyclohexane as organic solvent. In order to get further insight into the stability of the OxdB biocatalyst under the chosen reaction conditions, we performed a stability assay of OxdB whole cells in superabsorber and in biphasic reaction medium using cyclohexane as organic solvent. After different incubation times of the OxdB(-immobilisate) in an organic solvent or biphasic system, *n*-octanaloxime (**1**) was added and the conversion to *n*-octanenitrile (**2**) was determined after 15 min. The results are shown in Figure 4.

As shown in Figure 4, the activity of OxdB-containing whole cells immobilized in superabsorber does not decrease when being incubated for one hour. After 5 hours of incubation in cyclohexane, still approximately 80 % activity was left. In the case of the biphasic reaction medium, OxdB showed already lower activity without incubation beforehand. The activity is approximately 60 % of the activity of superabsorber-immobilized cells

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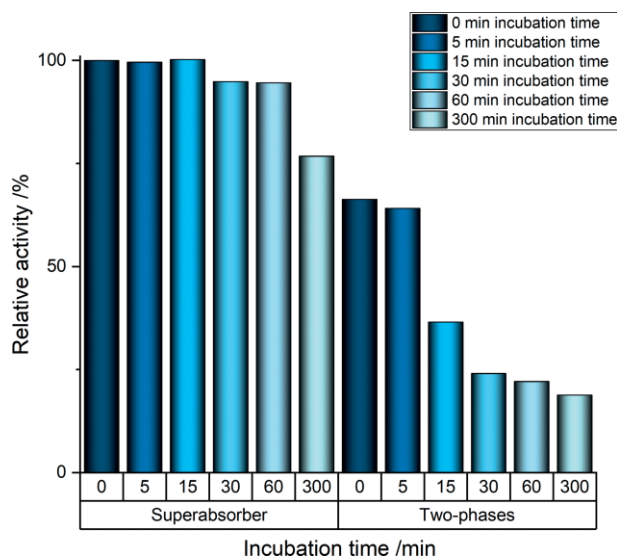European Journal
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Figure 4. Stability assay of OxdB whole cells immobilized in superabsorber in cyclohexane and OxdB whole cells in a biphasic system consisting of buffer and cyclohexane. The superabsorber-approach without previous incubation in cyclohexane was set as 100 %.

without incubation. The activity drops very fast in case of the biphasic approach, indicating again that the superabsorber-immobilization protects the biocatalyst against the organic solvent.

Having such an efficient biotransformation in batch mode now in hand and stimulated by the impressive recent achievements and increasing importance of flow processes,[38–45] our next goal was to develop a flow process for this superabsorber-based synthesis of n-octanenitrile (2). Recently, biocatalysis in continuous processes getting more and more popular due to high reproducibility and productivity of these processes.[43] Thus, we were interested in the behavior and performance of our Oxd-superabsorber immobilizates in flow reactions. We chose a packed bed reactor bearing the superabsorber-immobilized whole cells, thus enabling an easy separation and re-use of the catalyst. Toward this end, the reactor was loaded first with a small cotton layer (5 mm) and then with the superabsorber granulate, followed by addition of 1 mL suspension of 66 mg_{wcm}/mL of enzyme. After all liquid was absorbed by the superabsorber, a second cotton layer (5 mm) was added and the reactor was sealed. In Figure 5 the set-up of this reactor is shown.

Figure 5. Picture of the packed bed reactor charged with superabsorber gel.

The flow process then was conducted by means of a syringe pump, and a solution of 100 mM of n-octanaloxime (1) in cyclohexane was pumped through the packed bed reactor. Due to the limited solubility of 1, the concentration was reduced to

100 mM in comparison to 500 mM in batch experiments in order to ensure a completely dissolved substrate which is needed for running the reaction in such a flow-reactor without blocking effects. The reaction was performed at room temperature with a residence time of 30 min. After an equilibration time of two residence times, a high conversion of >95 % for the formation of n-octanenitrile (2) was obtained for at least 3 h reactor run time for this flow system (Figure 6). Although within the chosen reaction time some loss in activity was observed, thus underlining at least to a low extent a deactivation effect of the organic solvent on the enzyme, this superabsorber-based whole cells immobilization method in combination with a pure organic solvent also turned out to be robust and suitable to be operated in a flow mode.
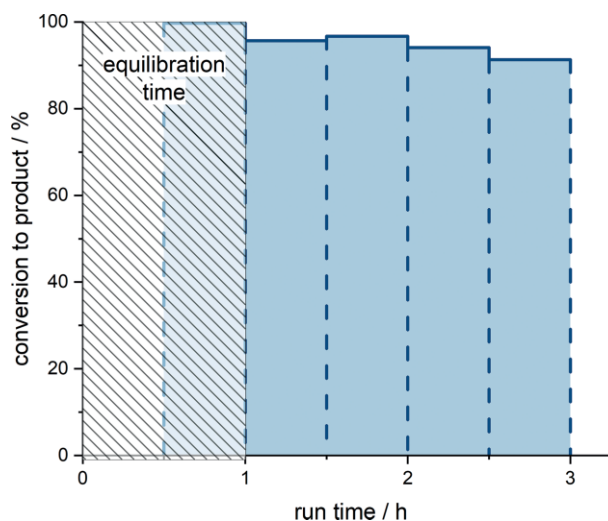

Figure 6. OxdB in wet whole cells catalyzed dehydration of n-octanaloxime (1) using a superabsorber packed bed approach.

Conclusion

In conclusion, a biocatalytic process running in pure organic medium has been reported in which an enzyme being labile towards this organic solvent is used. The process concept is based on the encapsulation of the enzyme as a whole-cell catalyst in a superabsorber as a "solid aqueous phase" in combination with an organic solvent with a high log P value as "mobile phase". This approach has been exemplified for the aldoxime dehydratase-catalyzed dehydration of n-octanaloxime (1) with formation of n-octannitrile (2), leading to excellent conversion of >99 %. In contrast, very low or no conversion was observed when using other solvents or other methods such as the direct use of whole-cell catalysts in pure organic medium or biphasic solvent mixtures. Furthermore, an efficient flow-setup with a packed-bed reactor containing superabsorber-immobilized whole cells was developed, which turned out to be also very suitable for the biotransformation leading to high conversion and low catalyst deactivation.

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