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Process optimization of enzymatic reactions to overcome enzyme deactivation by the product

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Kurzfassung

Enzymdeaktivierung durch Substrat und Produkt stellt häufig eine Limitierung für biokatalytische Prozesse dar. Daher sind solche Konzepte von allgemeiner Bedeutung, die Substrat und Produkt vom Biokatalysator trennen und dadurch die Produktivität von Bioprozessen steigern. Die Bioreduktion von *o*-Chloroacetophenon ist solch ein klassischer Fall, bei dem rasche Deaktivierung des Enzyms schon durch geringe Mengen von Substrat und Produkt im millimolaren Bereich stattfindet. Das Reduktionsprodukt, (*S*)-1-(2-Chlorophenyl)-ethanol, ist besonders toxisch für die verwendeten Biokatalysatoren und deaktiviert das eingesetzte Enzym innerhalb kürzester Zeit. (*S*)-1-(2-Chlorophenyl)-ethanol ist ein chiraler Schlüsselbaustein in der Herstellung von neuen Zellzyklusinhibitoren mit chemotherapeutischem Potential und dadurch von Relevanz für die Pharmazie. Um die Toxizität des Produktes durch ein Modellsystem mit einem kontinuierlichen Drei-Phasen-System zu überwinden, wurden in der vorliegenden Arbeit die folgenden fünf unterschiedlichen Lösungsansätze getestet: (1) Phasentrennung durch Membranen; (2) Rückhaltung des Enzyms durch Querstromfiltration; (3) Phasentrennung durch Dialyseschläuche; (4) Rückhaltung von immobilisierten Enzymen in einer Chromatographie-Säule; (5) Aufbau mit Flüssig-flüssig-flüssig-Grenzfläche. Ziel der Arbeit war es, die zuvor im Zweiphasen-Batchprozess erreichten Produktivitäten zu übertreffen. Im Grunde wurde die unterschiedliche Löslichkeit von Substrat und Produkt ausgenutzt: ein organisches Lösungsmittel wurde als Reservoir für die Substratversorgung verwendet, und ein zweites für die *in situ*-Produktextraktion. Der Biokatalysator befand sich in der dritten Phase, die wässrig war. Die höchste Produktivität von 0,87 $g_{Produkt}/g_{CDW}$ wurde mit einem Dialyseschlauchexperiment erzielt. Produktivitäten von 1,13 $g_{Produkt}/g_{CDW}$, die bereits von Eixelsberger, T. *et al*. (2013) in einem Zweiphasen-Batchprozess erreicht wurden, konnten jedoch nicht übertroffen werden. Der Prozess mit Enzymrückhaltung durch Querstromfiltration (2) wird durch schnelles Blockieren der Membran limitiert. Auch die Immobilisation von XR auf einen Träger (3) scheint bei einem Prozess, in dem das Enzym vom Produkt deaktiviert wird, nicht brauchbar. Es wird angenommen, dass die Immobilisation den toxischen Effekt des Produkts aufgrund seines gehinderten Abtransportes vom Enzym verstärkt. Hohe

Enzymstabilitäten wurden in jenen Ansätzen erreicht, in denen sich die beiden organischen Phasen in direktem Kontakt mit der wässrigen Phase befanden (3).

Schlüsselwörter: kontinuierlicher Prozess; drei-Phasen-Biotransformation; Ganzzell-Bioreduktion; asymmetrische Reduktion von *o*-Chloroacetophenone; (*S*)-1-(2-Chlorophenyl)-ethanol

Abstract

Enzyme deactivation by substrate and product is a common limitation in biocatalytic processes. Concepts that improve productivities in bioprocesses by separating substrates and products from biocatalysts are hence of general relevance. Bioreduction of *o*-chloroacetophenone constitutes the classical case with fast enzyme deactivation by substrate or product present in low-millimolar range concentrations. The reduction product, (*S*)-1-(2-chlorophenyl)-ethanol, is especially toxic to biocatalysts and deactivates free enzymes within short time. (*S*)-1-(2-chlorophenyl)-ethanol is the chiral key synthon of a novel cell cycle inhibitor with chemotherapeutic potential and is hence of pharmaceutical significance. In the present study five different settings were developed in order to overcome product toxicity in the model system by a continuous three-phase-system. The five constructions included: (1) separation of the phases with membranes; (2) retention of the enzymes by cross-flow filtration; (3) separation of phases with dialysis tubing; (4) retention of immobilized enzymes in a tube reactor and (5) a setting with a liquid-liquid-liquid interface. Aim of the study was to outperform productivities previously obtained in two-phase batch systems. Basically, we took advantage of different substrate and product solubilities: one organic solvent was used as substrate supply reservoir and the second as *in situ* extractant of the product. The biocatalyst remained in the third, aqueous phase. The highest productivity of 0.87 $g_{product}/g_{CDW}$ was obtained with the setup using the dialysis tubing. However, productivities of 1.13 $g_{product}/g_{CDW}$, as previously obtained in the two-phase batch by Eixelsberger, T. *et al.* (2013) were not reached. The process with enzyme retention by cross-flow filtration (2) is limited by rapid membrane blocking. XR immobilization onto a carrier (3) also does not seem to work in a process where the enzyme is deactivated by its product. It seems that immobilization strengthens the toxic effect of the product because of its hindered transport away from the enzyme in the particle. High enzyme stabilities were determined in the set up where the two organic solvents are in direct contact with the aqueous phase (3).

Keywords: continuous reaction; three phase biotransformation; whole cell bioreduction; asymmetric *o*-chloroacetophenone reduction; (*S*)-1-(2-chlorophenyl)-ethanol

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0 Introduction

Biocatalysis is a clear process option for the synthesis of chiral molecules (Huisman and Collier 2013). Main advantages of biocatalysts over chemical catalysts are high stereo-, chemo- and regio-selectivity (Patel 2013). Furthermore, bioprocesses are operated under environmentally friendly conditions and ensure safe handling. The main disadvantage of enzymes relates to low stabilities under process conditions i.e. deactivation caused by high temperature, high or low pH or organic solvents. Moreover, there are several reactions for which no biocatalyst has been found yet (Andreas and Bommarius 2005). Also, biocatalysts are oftentimes inhibited by substrate or product at higher concentrations, given that, in nature, enzymes work at low millimolar levels of substrate (Pollard and Woodley 2007). Development on the biocatalyst, reaction and process level is used to overcome these shortcomings by academia and industry (Meyer *et al*. 2012). Intense search for yet unexplored enzymes that perform new reactions or work under harsher conditions is going on. Molecular biotechnology is extensively used to tailor enzyme properties for biocatalytic applications (Bommarius and Paye 2013; Bornscheuer *et al*. 2012) or even engineer enzymes to enable new reactions (Jez and Penning 1998). Enzyme stabilities are increased by immobilization onto a carrier or when the whole cell is used as the biocatalyst. Several approaches to overcome substrate and product inhibition and toxicity have been previously published. For example, Carvalho *et al*. (2005) achieved an 8.3-fold increased production rate in whole cell oxidation with R. *erythropolis* by improving product tolerance levels of the cell. Vicenzi *et al*. (1997) used a resin-based substrate supply and product-removal strategy to increase the product concentration in a batch process of a whole cell reduction and scaled the optimized process up to 300 L. Jeong *et al*. (2000) overcame product inhibition by continuous product removal through liquid-liquid-extraction. However, biocatalysis is still outperformed by chemical catalysis in terms of product concentration and productivity. Hence, there is still scope for optimization, especially on the process level and in the integration into chemical synthesis (Lye *et al*. 1999; Pollard 2007; Woodley *et al*. 2008; Wohlgemuth 2007).

Generally, processes are operated in batch, continuous and fed-batch modes. The selection of operation mode is usually based on economic considerations and technical constraints (Mathys *et al*. 1999; Roberge *et al*. 2005; Roberge *et al*. 2008). Costs must be kept as low as possible and continuous processing can offer important advantages in terms of productivity and costs. Continuous processes for enzymatic hydrolysis and fermentations have been previously designed but limited studies have actually been reported from which to design or advance the technology. Thus, more information is surely needed on this subject to guide the advancement of lower cost approaches towards biocatalysis and overcome cost barriers to market entry (Brethauer and Wyman 2010). The decision between batch and continuous processes requires weighing of advantages and disadvantages against each other. Batch processes have usually simpler settings and hence reduced investment costs. Further, development cost and time for up-scaling is lower in comparison to continuous processes. This provides a clear advantage under time pressure. In the pharmaceutical industry often multipurpose-plants are used to produce API's, and they usually consist of several batch processes which can be adapted for the production of different API's. This makes new batch processes easier to integrate in such plants. Also the current quality management in the pharmaceutical industry works on the basis of clearing batches of product and not on production periods which gives an advantage to batch processes (Schaber, S. D. *et al*. 2011). On the other hand, continuous processes require usually less man hours to operate because of automation and no need to restart the process after one cycle. They oftentimes produce more product per volume and time, partly due to missing standby-times and complete usage of the reactor. Continuous processes are also more predictable to scale and often achieve better mixing and heat exchange influencing directly the assessable quality and yield (Plumb, K. 2005). Another advantage of continuous processes is that most of the time there is a better control of mass and energy flows which lead to better energy efficiency and less waste. Which reaction type will be used for a process depends therefore on parameters like reaction, size or company policy (Jiménez-González, C. *et al*. 2011; Dach, R. *et al*. 2012).

In this work, we focused on process engineering to overcome product and substrate toxicity and thereby increase the stability of the biocatalyst in processes where product and starting material deactivate the catalyst. Aim of the present study is to build up a continuous process from an already established batch process.

We used the reduction of *o*-chloroacetophenone to (*S*)-1-(2-chlorophenyl)ethanol as a model reaction (Figure 1), the enzyme system consisted of *Candida tenuis* xylose reductase (*Ct*XR) and *Candida boidinii* formate dehydrogenase (*Cb*FDH). (*S*)-1-(2-chlorophenyl)ethanol is a key intermediate for the synthesis of PLK1 inhibitors (Sato, Y. *et al*. 2009) which showed promising chemotherapeutic effects in xenograft tumor models (Santamaria, A. *et al*. 2007; Rheault, T. R. *et al.* 2010). (*S*)-1-(2-chlorophenyl)ethanol has a strong deactivating effect on the used enzyme system. Product removal is especially tricky in cases where the substrate and product show highly similar physical and chemical properties. Furthermore, (*S*)-1-(2-chlorophenyl)ethanol and *o*-chloroacetophenone are stable molecules that do not undergo chemical modification under reaction, extraction and distillation conditions. Hence, the present model is an ideal test system for our approaches to separate substrate and product in the reaction. The process we used was so far optimized for two-phase batch conditions in several steps by Kratzer, R. *et al.* (2008); Kratzer, R. *et al.* (2011); Vogl, M. *et al.* (2011); Mädje, K. *et al.* (2012); Vogl, M. *et al.* (2012); Schmölzer, K. *et al.* (2012); Gruber, C. *et al.* (2013) and Eixelsberger, T. *et al.* (2013), and achieved already reasonable results.

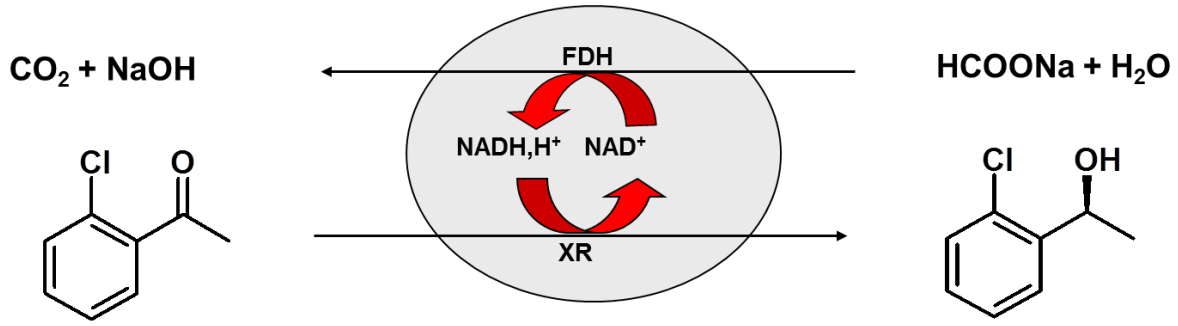


Figure 1: Reaction scheme.

The new approach should provide a continuous feed of starting material and also enable continuous harvest of the product in order to keep substrate and product concentrations under toxic limits.

1 Results and discussion

1.1 Core concept

The basic idea was a continuous process with three phases to produce a stable flow of starting material into the enzyme containing phase where it is converted to the product and removed into a refining flow (Figure 2). This should provide a low and stable concentration of both starting material and product in the aqueous phase and thereby prevent enzyme deactivation. In the following chapter, different approaches will be shown as well as problems we encountered and possible solutions for them.

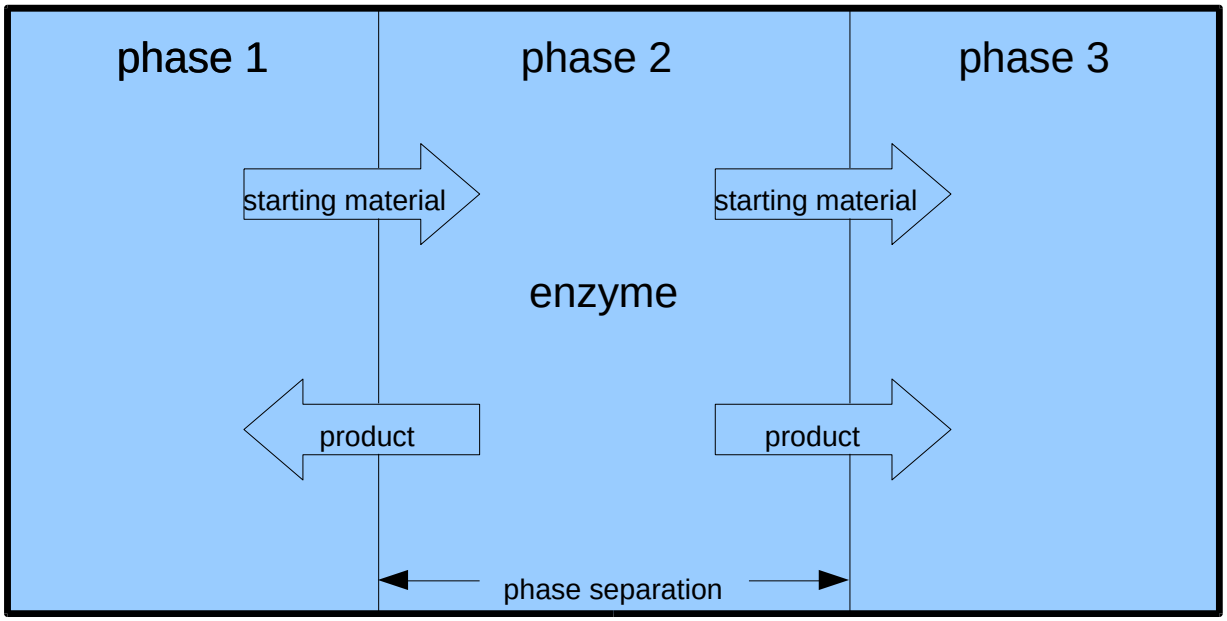


Figure 2: Core concept.

1.2 Screening for solvents

The choice of solvent affects a process not only at the catalyst and reaction level but also accounts considerably for the economics and ecologies of a process. Therefore, solvents not only need to fit physical and chemical requirements but have also to fulfill regulations in environment, health and safety provisions. Solvent choice is hence a key decision in process development. Also, there are some databases and methods with different capabilities to confine a search to a number of solvents. Still, there is no clear approach as to how to choose the right solvent but there is a rough guideline. First, the problem has to be identified; second, the search criteria has to be defined; third, the search has to be performed; and fourth, the results have to be verified (Powell, L. *et al*. 2006).

Set-up of the three-phase-system required the selection of two organic solvents: one for providing the starting material and the second for product extraction. General selection criteria for both solvents were: poor water miscibility, low toxicity, absence of halogens and low explosion risk. Widely used bulk chemicals were preferred over rare/fine chemicals due to established handling and economic considerations. Criteria specific for the solvent providing the substrate reservoir are (1) provision of a stable substrate concentration in the aqueous phase (2) low dissolving capacity for the product (ideally no dissolution of the product). The product extracting solvent should, on the contrary, (1) fully extract the product from the aqueous phase and (2) not dissolve any substrate (ideally). Furthermore, considering product isolation and solvent recycling after the bio-reduction, (3) a low boiling point is advantageous. The solvent-screening process is described in more detail in the experimental section (see 2.3 Screening for solvents) and results are shown in Figure 3 (data from solvents excluded by the pre-screening are omitted in the figure). The above described criteria for the solvent selection are best checked by comparing distribution coefficients (water to solvent) of substrate and product (Figure 3). The optimal substrate reservoir enables a low substrate concentration in the aqueous phase and does hardly extract the product from the aqueous phase, i.e. the substrate concentration in the aqueous phase remains relatively low in comparison to the product concentration (Figure 3). These features are shared by the solvents cyclohexane, *n*-hexane and *n*-heptane. Cyclohexane showed the highest difference

in the distribution coefficient for substrate and product and was hence chosen as substrate reservoir. However, cyclohexane was replaced by *n*-heptane because the later provided a higher concentration of *o*-chloroacetophenone in the H_2O. 2-Methoxy-2-methylpropane showed the highest extraction capacity for the product and was hence chosen for the product harvest.

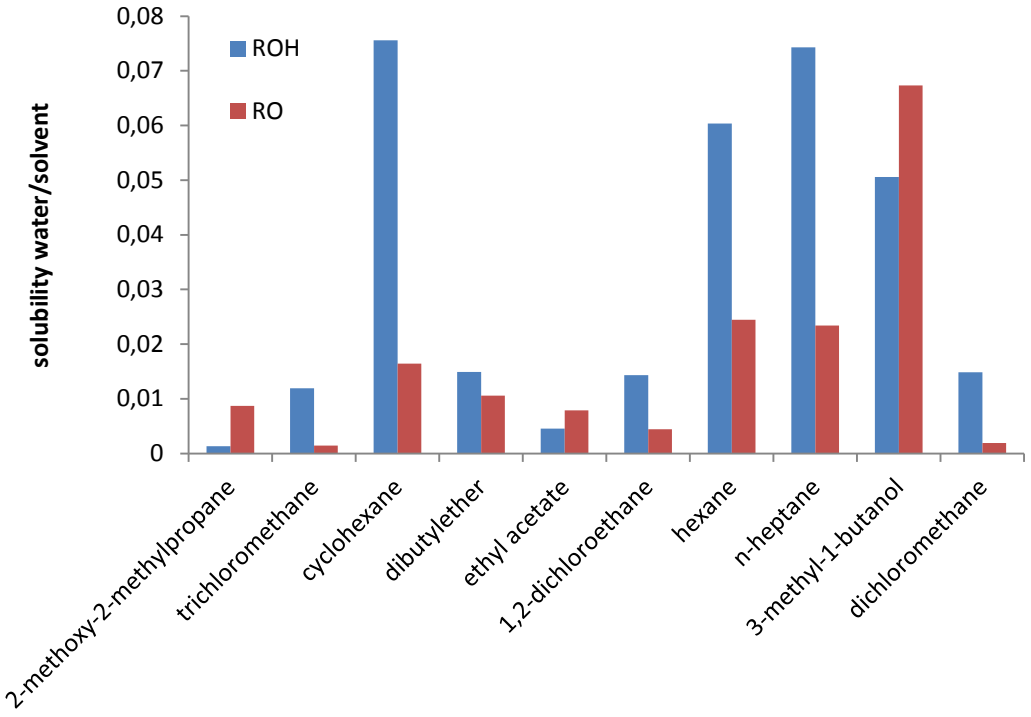


Figure 3: Distribution coefficients of (*S*)-1-(2-chlorophenyl)ethanol (ROH) and *o*-chloroacetophenone (RO) in different solvents. The solubility water/solvent was calculated as [concentration in water]/[concentration in solvent].

1.3 Reactor concepts

Due to the high diversity in the concepts of our approaches and the quantity of minor differences and findings emerging from it, it seemed to be in the interest of clarity and manageability to summarize in the section 1 Results and discussion only the reactor concepts and show the results most suitable for comparison. Most suited to compare the different approaches seemed to be the yield in product per cell dry weight [$g_{product}/g_{CDW}$] and the enzymes half-life times [$T_{1/2}$]. The more detailed description of the single reactions as well as minor findings are described in the section 3 Conversion experiments due to their more experimental nature.

1.3.1 Three-chamber-reactor

The basic concept, as shown in Figure 4, was a separation of the three phases by membranes and by the poor solubility of the organic phases in the aqueous phase. For this purpose the membrane should let pass the slightly water soluble starting material and product but retain the solvents. The starting material is provided by a solution of *o*-chloroacetophenone in *n*-heptane circulating through the first chamber. Buffer with enzyme and sodium formate is pumped through the second chamber; the consumed formic acid is restocked with a pH controlled pump adding formic acid solution. The third chamber is rinsed by 2-methoxy-2-methylpropane which extracts the (*S*)-1-(2-chlorophenyl)ethanol and is later regenerated by distillation. For the main experiments a $MgAl_2O_4$ ceramic membrane with an average pore size of 7 nm from Kerafol GmbH was used.

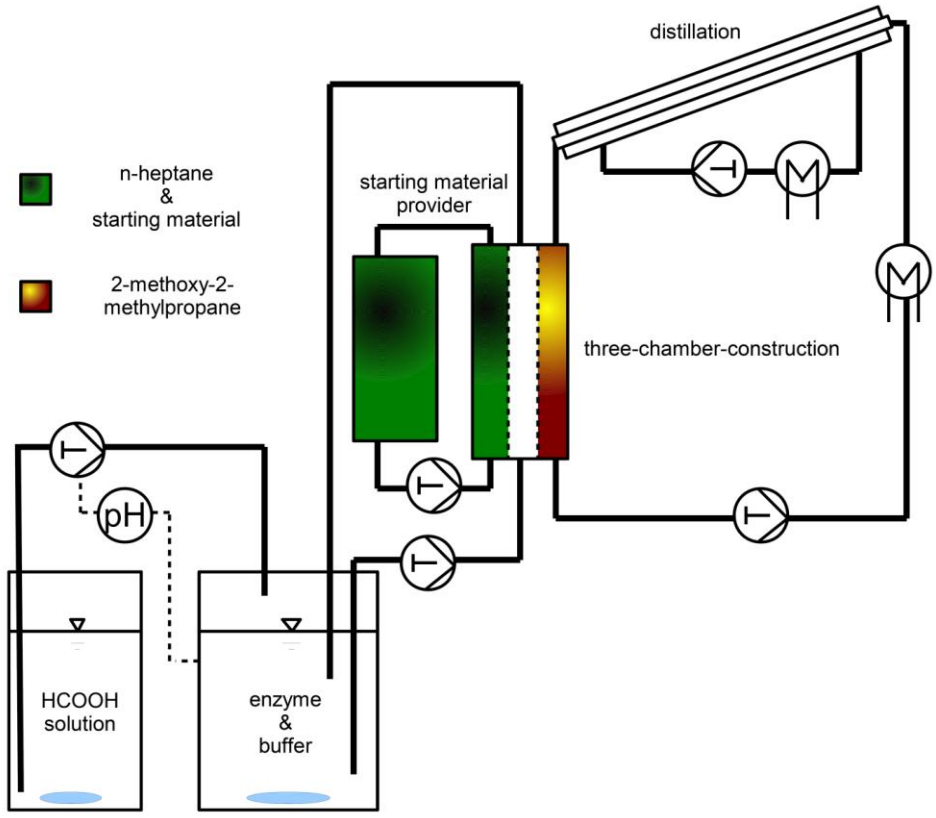


Figure 4: Scheme of the three-chamber-reactor set up.

A first prototype of the reactor missing the grids and with simplified drillings for the tubing was built out of PTFE (polytetrafluoroethylene). In the course of our experiments, a series of technical difficulties were encountered including leaky connections between tubes and reactor, rupture of ceramic membranes, leaky sealing of reactor parts and low mass transfer. Furthermore, it was difficult to evacuate the membranes in order to avoid air pockets before filling the reactor with solvent. Air pockets in the membranes would prevent any exchange of material between the chambers. Finally, in the attempts to assemble the reactor leak-tight, the housing got wrapped and the reactor was discarded. Therefore, no results can be shown for the PTFE prototype. Encountered problems could be overcome with an improved design made from steal as shown in Figure 5. But until now the steal reactor was not built for both time and cost reasons.

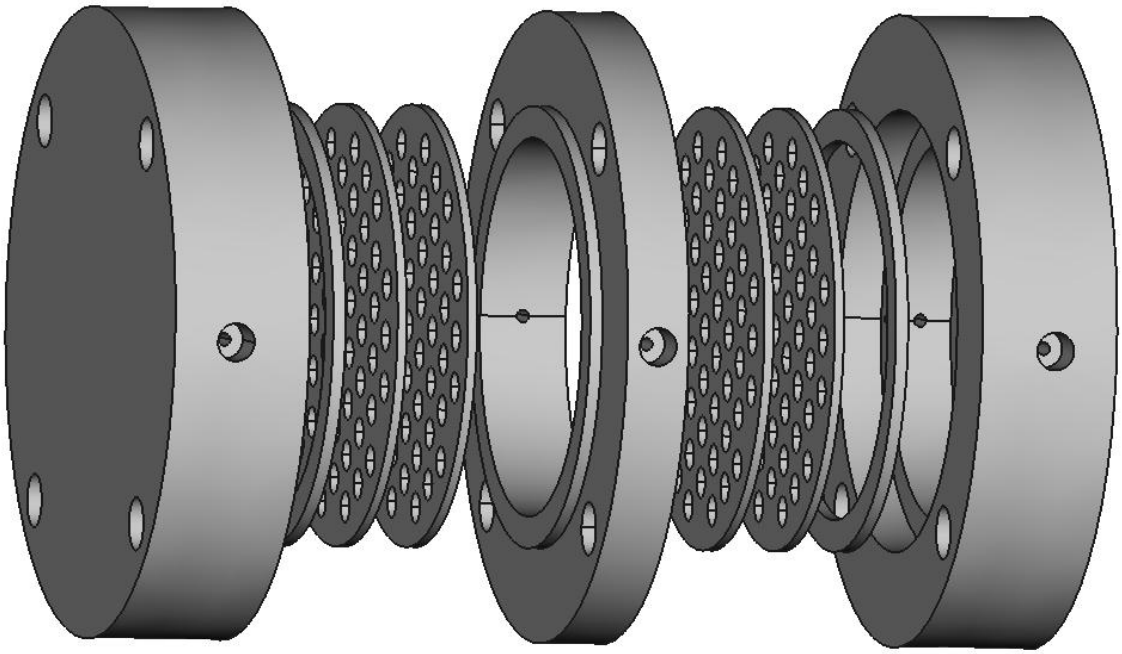


Figure 5: Final design of the three-chamber-membrane reactor.

1.3.2 Set up with enzyme retention by cross-flow filtration

The basic concept (Figure 6) was to keep the enzymes in the reaction space with constant supply of starting material and removal of product. Enzymes were retained with a cross-flow membrane (Vivaflow 50) and the product bypassed the membrane. The product was further on extracted and distilled in continuous mode. The buffer was subsequently loaded with starting material and pumped back into the reaction space.

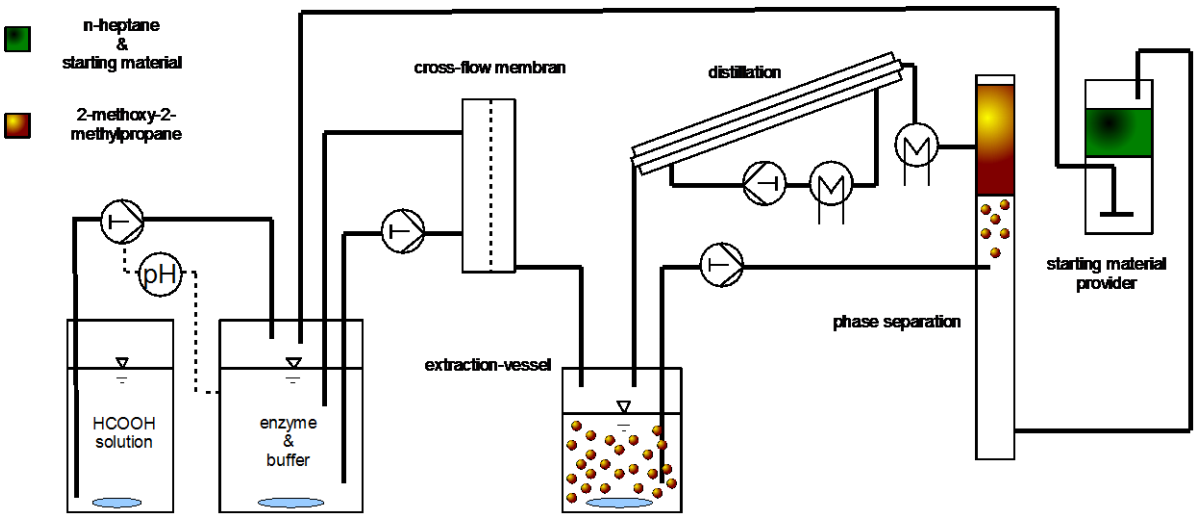
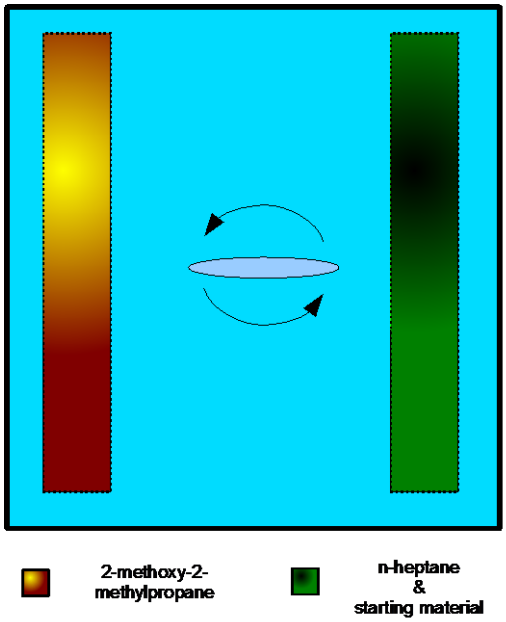


Figure 6: Scheme for the construction with holding back of enzyme through cross-flow filtration.

In the first experiment a promising high productivity of 0.46 $g_{product}/g_{CDW}$ was reached. This is 8-fold higher as compared to productivities previously obtained in aqueous batch reductions using the whole *E. coli* cells as catalyst. The constant product removal led to product concentrations below 0.7 mmol/L in the aqueous phase. Low concentrations of (*S*)-1-(2-chlorophenyl)ethan-1-ol seem to improve the stability of the enzymes and thereby allow a longer reaction time. Over the course of the experiment, enzyme precipitated and plugged the membrane resulting in a drastically reduced transmembrane flow. The EtOH, which was used to supply *o*-chloroacetophenone, was suspected as a main reason for the enzyme precipitation. In subsequent experiments we showed that EtOH concentrations over 30 % led to the total loss of XR activity and concentrations over 40 % led to the complete loss of FDH activity. We therefore avoided the addition of ethanol in the second and third

experiment. The substrate was supplied by the buffer streaming through an *n*-heptane phase enriched with *o*-chloroacetophenone (Figure 6). Furthermore, the enzymes were roughly purified to reduce membrane clogging and the flow was increased to compensate for decreased transmembrane flows. We achieved an increased g/g yield of 8-fold compared to the reaction in plain buffer. But mainly due to the membrane clogging no yields higher than 0.46 $g_{product}/g_{CDW}$ were reached.

1.3.3 Dialysis tubing approach



In this setup the organic phases were filled in dialysis tubes; the closed tubes were floating in the stirred aqueous phase (see Figure 7). For this approach no continuous process was established. The setup was simple to handle and showed good results in initial experiments at small scale.

Figure 7: Scheme of dialysis tubing approach.

In the first experiment the reaction was carried out for 90 h. After the reaction, the phases were analyzed separately (Figure 8). The results showed the distribution of *o*-chloroacetophenone and (*S*)-1-(2-chlorophenyl)ethan-1-ol in the three phases. Yields of 0.32 $g_{product}/g_{CDW}$ were obtained. After the encouraging results of the first experiment, enzyme activities of 205 U XR and 20 U FDH were used in the second experiment. Enzyme activities were monitored over the reaction time of 74 h. From the activity versus time plot a half-life of ~182 h was calculated for the XR activity. The obtained half-life is significantly longer as compared to a value of 116 h that was measured in whole cells incubated in plain buffer as reported by

Schmölzer *et al*. (2012). The half-life of FDH was estimated to ~15 h and the FDH stability was identified as a main factor limiting the productivity of the process. Nevertheless, a yield of 0.87 $g_{product}/g_{CDW}$ was reached, which is the highest value achieved in this work. In a next step, we immobilized the FDH in order to further stabilize the FDH and improve the productivity.

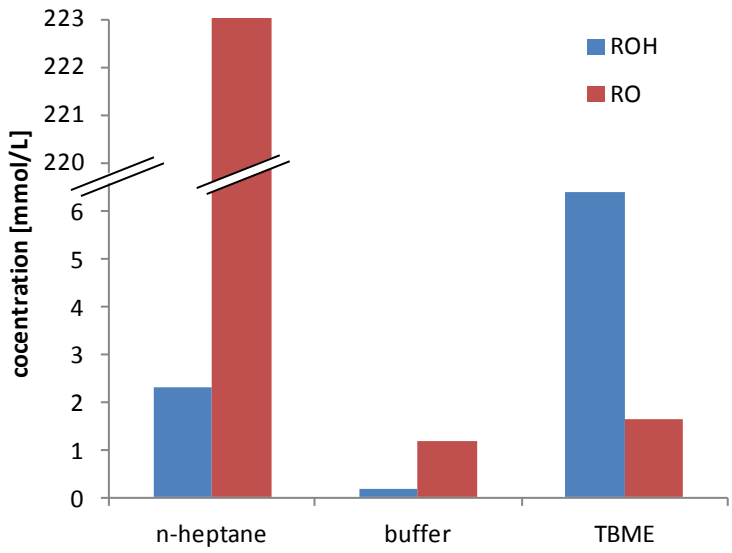


Figure 8: Starting material and product distribution in the different phases from the first experiment; TBME stands for 2-methoxy-2-methylpropane.

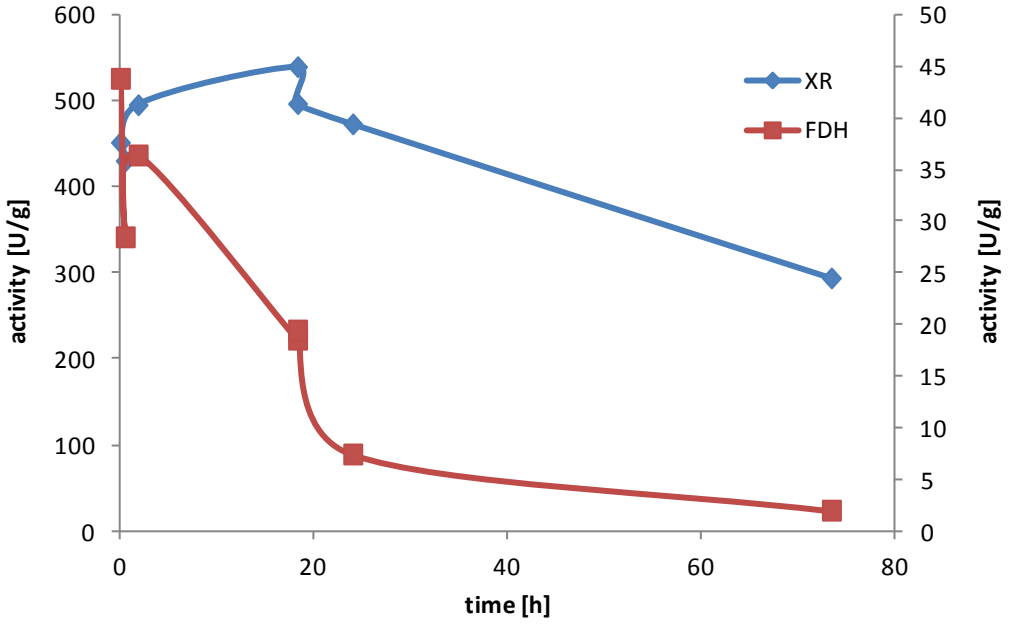


Figure 9: XR and FDH activity in the course of the second experiment.

1.3.4 Immobilized enzymes in a column

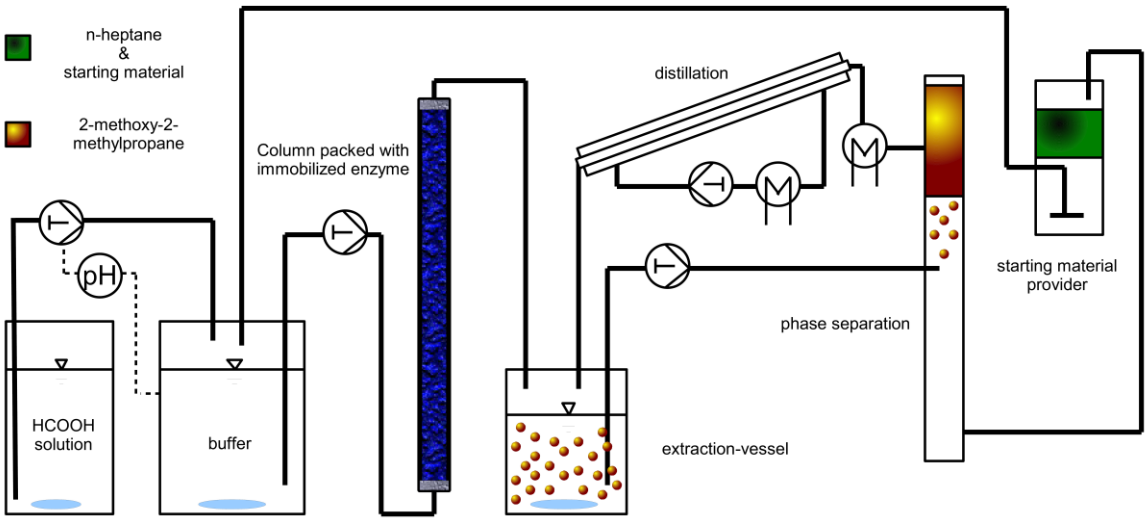


Figure 10: Scheme for the construction with immobilized enzymes packed in a column.

Immobilization of the free enzymes onto carriers enabled simple separation of the product containing reaction mixture from the enzyme as shown in the continuous process in Figure 10. Immobilized enzymes were filled into a column and integrated into the previously described system from the cross-flow experiment (1.3.2 Set up with enzyme retention by cross-flow filtration). In the first experiment with immobilized enzymes, the starting material supply by the *n*-heptane phase was not sufficient and no product formation was measured. With the flow rates we used and the area we could provide for starting material exchange, a substrate concentration of only 80-100 µM was achieved in the aqueous phase. Under these conditions FDH activity was still detected after 19 days. In the second experiment, a buffer saturated with *o*-chloroacetophenone was continuously added to the aqueous phase in order to reach the required *o*-chloroacetophenone concentration in the reaction space. An *o*-chloroacetophenone concentration between 0.4 mM and 0.9 mM was measured in the aqueous phase. However, the initially obtained reaction rate was reduced to half its speed in less than 2.5 h (Figure 20). Rest activities of XR and FDH after 40 h were determined to 0 and 74 %, respectively. Therefore, immobilization significantly stabilized the FDH but destabilized the XR. A possible explanation for the decreased XR stability might be that the formed product cannot leave the particle fast enough and thereby deactivate the immobilized enzyme.

1.3.5 Liquid-liquid-liquid reactor

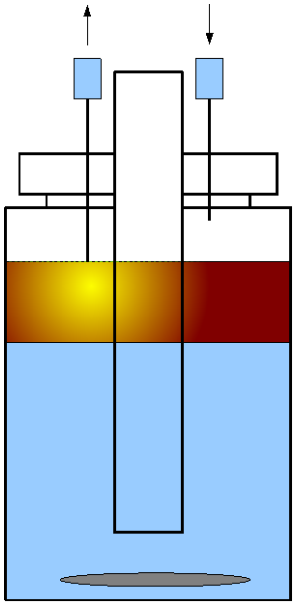


Figure 11: Scheme for the liquid-liquid-liquid reactor.

In this construction, the aqueous phase containing the catalyst is used to separate the two organic phases as shown in Figure 11. In practice, a glass tube is fixed concentrically in a glass beaker. The glass tube and the buffer separate the inner and the outer organic phases provided that the used solvents have densities less than the aqueous phase. In the inner organic phase, the *o*-chloroacetophenone is provided. After reaction in the aqueous phase, the (*S*)-1-(2-chlorophenyl)ethan-1-ol is removed from the process in the outer organic phase which is distilled and reused.

In the first experiment, crude lysate from *E. coli* Rosetta 2 (DE3) overexpressing XR and FDH was used. In the inner chamber, *o*-chloroacetophenone was provided dissolved in *n*-heptane. After 92 h, a yield of 0.26 $g_{product}/g_{CDW}$ was reached. During the reaction the activities were monitored and showed improved stabilities of XR and FDH activities (Figure 12). However, FDH stability still seemed to limit the reaction. Substrate and product concentrations were measured over time in the product removal phase (Figure 13).

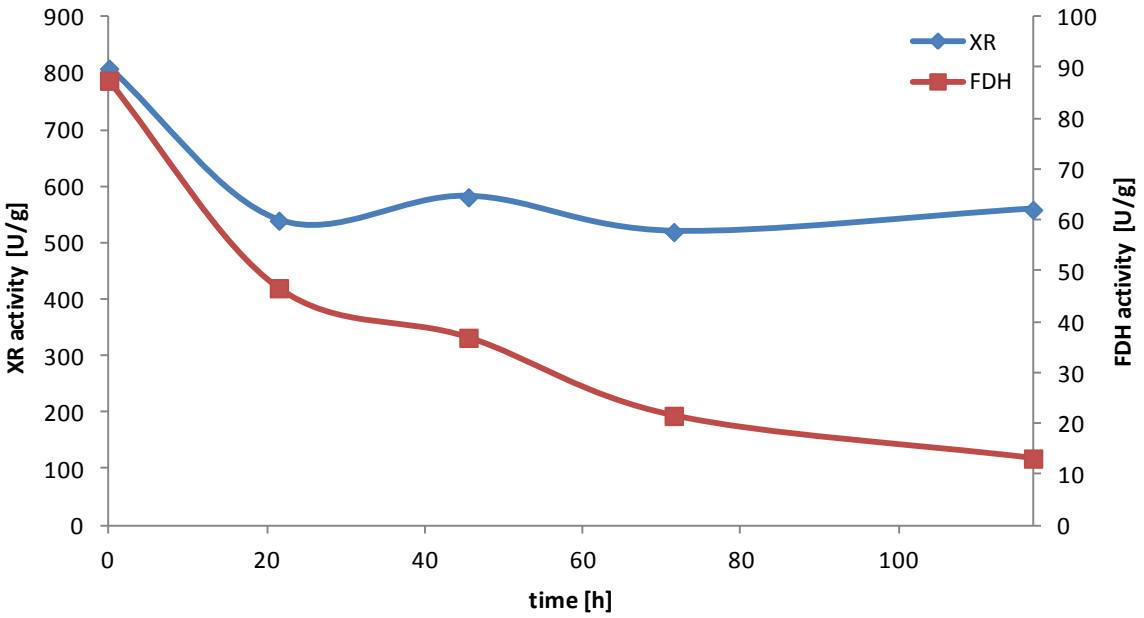


Figure 12: XR and FDH activities in the course of the first experiment.

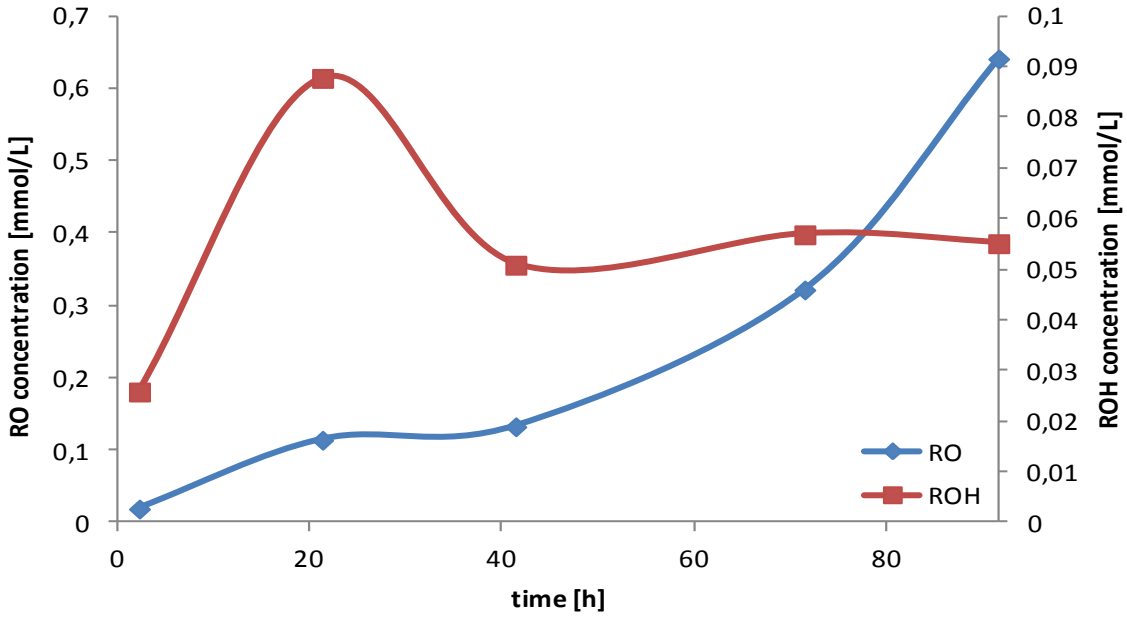


Figure 13: Concentrations of *o*-chloroacetophenone and (*S*)-1-(2-chlorophenyl)ethan-1-ol in the product removal phase over time.

In the second experiment, immobilized FDH was used instead of the free enzyme in order to increase its stability. But although the FDH was stabilized 1.5-fold as compared to the first experiment, only a yield of 0.09 $g_{product}/g_{CDW}$ was reached with this approach. The lower yield can be partially explained by loss of activity through immobilization. Furthermore, activities estimated from the reaction in the reactor seem to be lower in comparison to activities measured photometrically with the activity assay. No explanation for this behavior has been found.

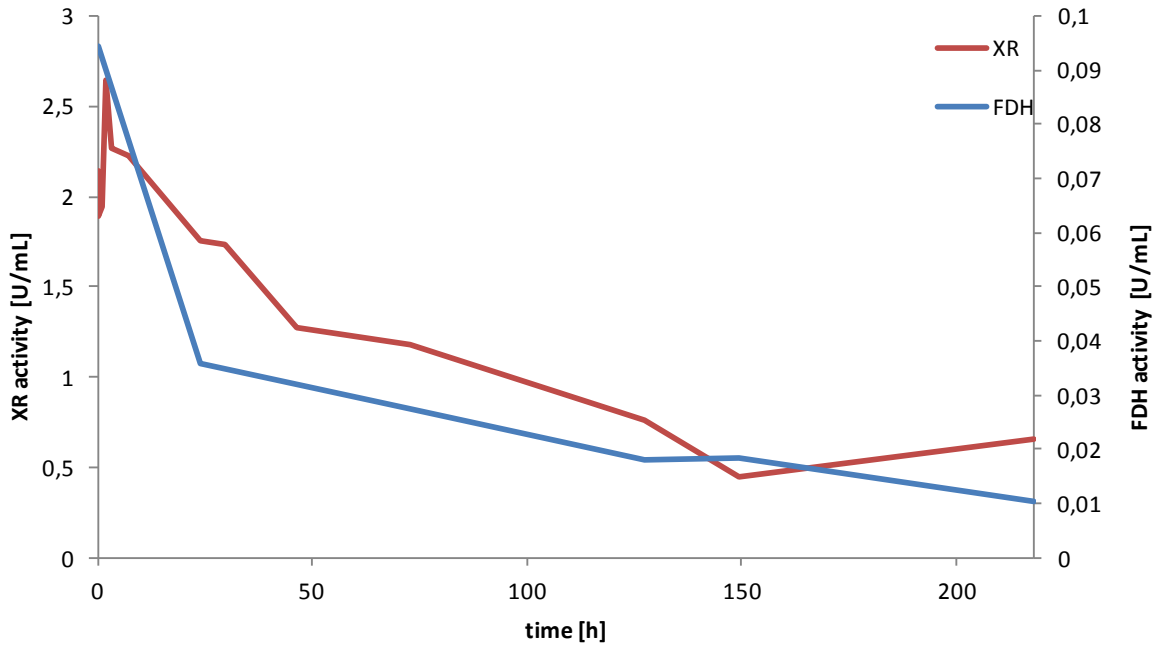


Figure 14: XR and FDH activity in the course of the experiment with immobilized FDH.

1.3.6 Summary of the results from the different approaches

In this work, several approaches for the continuous operation of a bioreduction with highly toxic product were developed. The main results of the experiments are summarized in the following Table 1 and the findings are explained in more detail afterwards.

Table 1: Summary of the main data from the different experiments (the numbers in brackets in experiment 2.1 are extrapolated from the FDH activity; RO mg and ROH mg are the amounts measured at the end of each experiment).

Experiment	*g/g*	*U/g XR*	*U/g FDH*	*U/g XR immobilized*	*U/g FDH immobilized*	*U used XR*	*U used FDH*	*h $T_{1/2}$ XR*	*h $T_{1/2}$ FDH*	*RO mg*	*ROH mg*
2.1	0.46	(1153)	(111)	-	-	(218)	21.0	-	-	43	87
2.2	0.06	680	222	-	-	476	155	-	-	8	40
2.3	0.06	62.2	2.85	-	-	43.5	2.00	24.3	5.9	37	44
3.1	0.32	-	-	-	-	-	-	-	-	3540	96
3.2	0.87	451	43.8	-	-	205	19.9	182	15.2	4512	395
3.3	-	506	50.5	8.57	2.97	4.23	1.47	-	-	-	-
4.1	-	444	38.0	130	6.14	28.3	0.89	-	-	-	-
4.2	0.06	1081	173	176	40.0	58.3	41.6	< 2	36.7	1558	80
5.1	0.26	810	87.7	-	-	243	26.3	151	39.6	965	78
5.2	0.09	424	64.2	-	8.85	210	8.49	93.7	60.8	1445	134

Although data from the cross-flow filtration experiments for yield and stability are available, a direct comparison with the other approaches does not seem reasonable. All cross-flow filtration approaches had early setbacks in flow and had to be stopped after short times. The process is limited by rapid membrane blocking and also membrane fouling is expected during longer reactions. Summarizing our results, the process with enzyme retention by cross-flow filtration seems not worth of pursuing.

XR immobilization onto a carrier also does not seem to work in a process where the enzyme is deactivated by its product. It seems that immobilization strengthens the toxic effect of the product because of its hindered transport away from the enzyme in the particle. The complete loss of XR activity after 40 h and the decrease of product flow from the columns from 0.17 mM to 0.07 mM in 2.5 h, which led to a half-life time of less than 2 h in the second experiment with both enzymes immobilized (3.4.2 Experiment 4.2), renders this approach unfeasible for continuous conversion. The most likely explanation of this 50-fold decrease of XR half-life in comparison to the other experiments is a high product concentration that accumulates due to diffusion limitation in the vicinity of the immobilized XR. To prove this theory, further experiments and especially a way to measure the product concentration in the vicinity of the enzyme would be necessary.

Most reasonable to compare are the experiments from the dialysis tubing approach and the liquid-liquid-liquid reactor due to the enzyme stabilities and the running time of the experiments which lie in a comparable range. Both methods have in common that the two organic phases are in direct contact with the aqueous phase. Basically, high enzyme stabilities were determined in both set ups and promising productivities were obtained in the dialysis tubing approach. The decreased stability of FDH in the dialysis tubing experiments can be assumed to be the result of the use of TBME and *n*-heptane as solvent instead of only *n*-heptane, which was used in the liquid-liquid-liquid reactor. Compared to *n*-heptane with 2.2 mg/L water solubility at 25°C, TBME is with 42 g/L water solubility at 20°C far more abundant in water than *n*-heptane, and therefore more likely to interact with the enzyme and to denaturate it. A further and probably more important reason why TBME could decrease the stability of the enzyme is its polarity and its log P of 0.94, which is expected to enable the TBME to exchange with the water bound to the surface of the enzyme and thereby denaturating it. The change of solvent could also be a reason for the lower yields in the liquid-liquid-liquid reactor. When the yield of experiment 5.1 is compared to experiment 5.2, the decrease correlates with the loss of activity due to the immobilization, whereas the stability in the presence of *n*-heptane as sole solvent is only increased slightly and thereby

does not affect the productivity significantly. Experiments with direct contact of aqueous to organic solvents and experiments with the dialysis tubes seem most promising. An optimization regarding substrate concentration and enzyme loading would be necessary to reveal the full potential of both setups.

To achieve further improvements in our concepts, one of the next steps would have to be to carry out stability essays of immobilized FDH in presence of different solvents, especially TBME. For the choice of solvent also ethyl *tert*-butylether would be an option because of its similar structure to TBME and its smaller water solubility. Although it is a bulk chemical, it was not possible to obtain a relevant amount for a reasonable price. Another approach worthy of pursuing seems to be the use of TBME as second solvent in experiments similar to 5.1 and 5.2. The most promising approach appears to be an improvement of the two methods using membranes, hence the three-chamber-reactor or the dialysis tubing approach. For the dialysis tubing approach, the next step should be the establishment of a continuous process. Finally, for the three-chamber-reactor it seems reasonable that the construction of the improved prototype should yield better results.

2 General experiments

2.1 Chemicals, materials and strains

Racemic (*S*)-1-(2-chloroacetophenyl)ethanol was from Alfa Aesar GmbH Co KG (Karlsruhe, Germany). NADH (sodium salt; ≥ 98 % pure) and NAD^+ (free acid; ≥ 97.5 % pure) were obtained from Roth (Karlsruhe, Germany). All other chemicals were purchased from Sigma-Aldrich/Fluka (Gillingham, Dorset, U.K.) or Roth (Karlsruhe, Germany), and were of the highest purity available.

50 mL and 15 mL tubes were purchased at Sarstedt (Wr. Neudorf, Austria). 2 mL and 1.5 mL Eppendorf tubes were bought from Eppendorf Austria GmbH (Wien, Austria). Vivaflow 50 cross-flow cassettes with different size exclusions were purchased at Satorius Stedim Biotech GmbH (Göttingen, Germany). 15 kD ceramic membranes were purchased at Kerafol GmbH (Stegenthumbach, Germany). The first prototype of the three-chamber-membrane reactor was made of PTFE and manufactured at the in-house workshop. The Infors HT bioreactor was purchased from Infors AG (Bottmingen, Switzerland) with a 7.5 L vessel and had a working volume of 6 L. It was fitted with a twin 6-blade disc ("Rushton") turbine impeller, temperature, pH and pO_2 measurement and control.

The microorganism used for the simultaneous production of *Ct*XR (from *Candida tenuis* CBS 4435) and *Cb*FDH (from *Candida boidinii* ATCC 18810) was *E. coli* Rosetta 2 (DE3). The parent strain carries a pRARE2 plasmid with tRNAs for seven rare codons (Merck, 2011). The *E. coli* Rosetta 2 (DE3) used in this work additionally harbors the pET-Duet-1 and pRSF-1b plasmids from which the former carries genes for *Ct*XR and *Cb*FDH (pETDuet_XR_FDH), while the latter carries the *Cb*FDH gene (pRSF_FDH) (Mädje *et al.* 2011). pET-Duet-1 encodes for ampicillin resistance, pRSF-1b for kanamycin resistance and the pRARE2 for chloramphenicol resistance. The *E. coli* BL21 (DE3) strain used for the production of *Cb*FDH was transformed with a pRSF-1b plasmid which carries a gene for *Cb*FDH and encodes for a kanamycin resistance (Mädje *et al*. 2011). For the production of His-tagged *Ct*XR an *E. coli* JM109 was used harboring a pQE-30 plasmid which carries the gene for *Ct*XR with the His-tag on the N-terminus and encodes for an ampicillin resistance (Pival *et al*. 2008).

2.2 Cell cultivation

Cultivation of biomass was done in shaking flasks and in the bioreactor. Optimized growing and expression conditions were used as previously reported by Eixelsberger *et al.* 2013.

2.2.1 Fermentation in shaking flasks

Pre-cultures were grown in 250 mL shaking flasks with 50 mL LB medium (Table 2) at 37°C and 100 rpm. Main-cultures were grown in 1 L shaking flasks with 250 mL LB medium at 37°C and 100 rpm. At an optical density of 0.5-0.6 (OD_{600nm}) cultures were cooled to 18°C and protein production was induced by addition of isopropyl-ß-D-thiogalactopyranosid (IPTG). After 24 h of induction, the cells were harvested and centrifuged with 5000 rpm at 4°C. Cell-pellets were stored in the freezer at -18°C for further use.

Antibiotics and IPTG solutions were prepared as 1000-fold concentrated stocks in water (Table 3) and sterile-filtered. The strains and the needed antibiotics are shown in Table 4.

Table 2: Composition of LB medium.

Component	g/l
Pepton	10
NaCl	5
Yeast extract	5

Table 3: Stock solutions of antibiotics.

Antibiotic	Stock factor	Producer	Solvent	Concentration [g/l]	[g/mol]	Concentration [mmol/l]
Amp (ampicillin sodium salt)	1000	Roth K029	H_2O	115	371.39	310
Kan (kanamycinsulfate)	1000	Roth T832	EtOH	50	582.58	86
Cam (chloramphenicol)	1000	Fluka 23275	H_2O	34	323.14	105
IPTG	1000	Roth CN08	H_2O	60	238.30	252
Carbenicillin disodium salt	1000	Roth 6344	H_2O	100	422.36	237

Table 4: Strains used in the present study.

Strain	Antibiotics	Expression	Origin
E. coli Rosetta 2(DE3) pRSF_FDH pETDuet_XR_FDH	Cam Kan Amp	*Cb*FDH *Ct*XR and *Cb*FDH	Mädje 2012
E. coli BL21 (DE3) pRSF_FDH	Kan	*Cb*FDH	Mädje 2012
E. coli JM109 pQE-30 with *Ct*XR	Amp	*Ct*XR_His-tag	Pival, Klimacek, Nidetzky 2008

2.2.2 Cultivation in the bioreactor

E.coli Rosetta 2 (DE3) harboring pETDuet_XR_FDH and pRSF_FDH was used for cultivation in the bioreactor. Ampicillin was replaced by carbenicillin because of its higher stability towards spontaneous and enzymatic hydrolysis as previously described (Eixelsberger *et al*. 2013). Pre-cultures were grown in LB medium as described above.

The protocol for the cultivation in the 7.5 L bioreactor was adapted from Eixelsberger *et al*. (2013) and the bioreactor was operated according to the manual. Media components were divided into media parts A, B, C, D, E and thiamin solution. Parts B, C and D were sterilized with an autoclave, part A was sterilized *in situ* (in the assembled bioreactor). Part E and thiamine were sterile-filtered. Media composition is shown in /g for FDH.

Table 5 and Table 6).

The pH-electrode was calibrated prior to sterilization using two buffer solutions (pH = 4.01 and pH = 7.0), while the pO_2-electrode was calibrated just before the cultivation started. For the 0 %-value, the medium was saturated with N_2 and for the 100 %-value with air.

For the cultivation all parts of the media, the thiamine and the antibiotics were combined under sterile conditions. The cultivation was started by adding 200 mL of pre-culture with an OD of ~1.4. The cultivation was done at a temperature of 25°C until the cells reached an OD of 1.44. Then the temperature was reduced to 18°C and 0.5 mM IPTG was added. The pH was controlled and kept constant between 6.94 and 7.00 by addition of sterile H_3PO_4 (1 M) and sterile KOH (2 M). The pO_2-level was controlled via a cascade regulation of stirrer speed and air flow rate and was kept at 60-70 % air saturation. After 60 h 0.6 g carbenicillin was added to prevent plasmid loss. After 116 h the glucose was depleted (detection with a Diabur-Test 5000 from Roche) and the fermentation was stopped. Cells were harvested by centrifugation using a Sorvall RC-5B centrifuge with a FAS-10C rotor at a speed of 5000 rpm

for 60 min. The cell-pellet was frozen at -70°C, freeze-dried with a Christ α 1-4 lyophilizer from Braun Biotech International and stored at -20°C. The fermentation profile is shown in Figure 15. The fermentation yielded 32.4 g dried cell mass with an activity of 1153 U/g for XR and 111 U/g for FDH.

Table 5: Media composition.

Part	Component	Concentration (g/L)
A	K_2HPO_4	4.0
	KH_2PO_4	4.0
	$Na_3C_6H_5O_7 \cdot 2H_2O$	2.67
	Na_2HPO_4	2.8
	Polypropylene glycol	0.1[a]
B	$(NH_4)_2SO_4$	3.5
	NH_4Cl	3.0
	$MgCl_2 \cdot 6H_2O$	0.83
C	$CaCl_2 \cdot 2H_2O$	0.15
D	Glucose$\cdot H_2O$	30
E	Trace element solution	1.0[a]

[a](ml/L)

Table 6: Trace element solution: All compounds were dissolved in 5 M HCl.

Component	Concentration (g/L)
$FeSO_4 \cdot 7H_2O$	4
$MnSO_4 \cdot H_2O$	1
$AlCl_3 \cdot 6H_2O$	0.55
$CoCl_2$	0.4
H_3BO_3	0.1
$CuSO_4 \cdot 5H_2O$	0.15
$ZnSO_4 \cdot 7H_2O$	0.2
$Na_2MoO_4 \cdot 2H_2O$	0.2

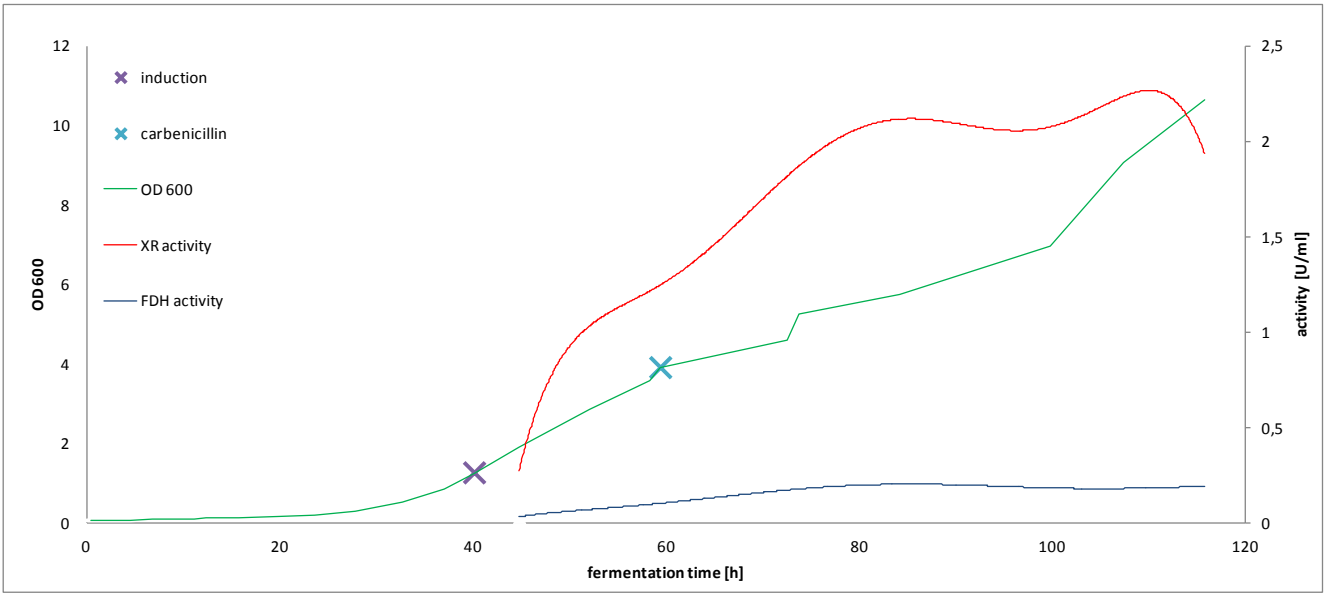


Figure 15: Fermentation profile: Temperature was reduced from 25°C to 18°C when an OD of 3.9 was reached. Additional carbenicillin was added after 60 h to prevent depletion.

2.3 Screening for solvents

2.3.1 Pre-screening

We started with a list of common solvents provided by the German page of Wikipedia (09/18/2012) and reduced the number of solvents from 59 to 10 in several steps by applying the following criteria and experiments:

a) Water solubility [+ ≤1%, 1%< to ≤10% +-, <10% -]
b) Probability of reaction with product or starting material [if not = +, if = -]
c) Availability in the laboratory [if = +, if not = -]
d) Difficulties at the experimental distribution coefficient determination [no problems = +, problems = -]

The results are shown in Table 7; solvents fulfilling all requirements are highlighted in green.

Table 7: Solvent check list.

Solvents	Boiling point [°C]	density [g/cm^3] at 20 °C	a	b	c	d
propanone	56.2	0.7889	-			
acetonitrile	81.6	0.7857	-			
phenylamine	184	1.0217	+	-		
methoxybenzene	155.4	0.9961	+	+	+	-
benzene	80.1	0.87565	+	+	+	-
benzonitrile	190.7	1.0102 (15 °C)	+	+-	-	
bromobenzene	156	1.4950	+	+	-	
butan-1-ol	117.3	0.8098	+-	+	?	
2-methoxy-2-methylpropane	55.3	0.74	+	+	+	+
dihydrofuran-2(3H)-one	204–206	1.13	-			
quinoline	238	1.0929	+	+	-	
chlorobenzene	132	1.1058	+	+	-	
trichloromethane	61.7	1.4832	+	+	+	+
cyclohexane	80.7	0.7785	+	+	+	+
dibutylether	142.5	0.764	+	+	+	+
2-(2-hydroxyethoxy)ethan-1-ol	244.3	1.1197 (15 °C)	-			
ethoxyethane	34.5	0.7138	+-	+	?	
N,*N*-dimethylacetamide	165	0.9366 (25 °C)	-			
N,*N*-dimethylformamide	153	0.9487	-			
dimethyl sulfoxide	189	1.1014	-			
1,4-dioxane	101	1.0337	-			
acetic acid	117.9	1.0492	-			
acetic anhydride	139.5	1.0820	-			
ethyl acetate	77.06	0.9003	+-	+	+	+

ethanol	78.3	0.7893	-			
1,2-dichloroethane	83.5	1.2351	+	+	+	+
ethane-1,2-diol	197	1.1088	-			
1,2-dimethoxyethane	84	0.8628	-			
methanamide	210.5	1.1334	-			
n-hexane	68	0.6603	+	+	+	+
n-heptane	98	0.684	+	+	+	+
isopropyl alcohol	82.3	0.7855	-			
methanol	64.7	0.7914	-			
3-methyl-1-butanol	130.5	0.8092	+-	+	+	+
2-methylpropan-2-ol	82.5	0.7887	-			
dichloromethane	40	1.3266	+-	+	+	+
butan-2-one	79.6	0.8054	-			
1-methyl-2-pyrrolidone	202	1.03	-			
N-methylformamide	183	1.011 (19 °C)	-			
nitrobenzene	210.8	1.2037	+	+	-	
nitromethane	100.8	1.1371	-			
pentane	36	0.6262	+	+	-	
Petroleum ether						
piperidine	106	0.8606				
propan-1-ol	97.2	0.8035				
4-methyl-1,3-dioxolan-2-one	241.7	1.2069				
pyridine	115.5	0.9819	-			
methanedithione	46.3	1.2632	+	+	-	
tetrahydrothiophene 1,1-dioxide	285	–	-			
tetrachloroethene	121	1.6227	+	+	-	
tetrachloromethane	76.5	1.5940	+	+	?	
oxolane	66	0.8892	-			
methylbenzene	110.6	0.8669	+	+	+	-
1,1,1-trichloroethane	74.1	1.3390	+	+	-	
trichloroethene	87	1.4642	+	+	-	
triethylamine	89.3	0.7275	-			
2-[2-(2-hydroxyethoxy)ethoxy]ethanol	278.3	1.1274 (15 °C)	-			
triethylene glycol dimethyl ether	222	–	-			
water	100	0.9982	-			

2.3.2 Determination of the distribution coefficient

Solutions of *o*-chloroacetophenone and (*S*)-1-(2-chlorophenyl)ethan-1-ol were prepared by pre-dissolving 50 µL of the pure substance in 5 mL EtOH and further dissolving this in 95 mL H_2O. For each solvent we prepared three samples by mixing 5 mL solvent with 5 mL of the solution containing starting material, product or water. The organic-aqueous mixtures were allowed to stand and equilibrate for 30 h. For each solvent the concentrations of *o*-chloroacetophenone or (*S*)-1-(2-chlorophenyl)ethan-1-ol were determined spectrophotometrically in the aqueous phases. Corresponding H_2O-probes were equally analyzed and served as blank references in each case. Concentrations of *o*-chloroacetophenone and (*S*)-1-(2-chlorophenyl)ethan-1-ol were measured at fixed wavelengths of 243 and 213 nm, respectively. Rough distribution coefficients were calculated from analyte concentrations as shown in Table 8 and Figure 3.

Table 8: Distribution coefficient in different solvents.

Substance	**cons. H2O/ cons. LM**		**Log (cons. LM/ cons. H_2O)**	
	ROH	RO	ROH	RO
2-methoxy-2-methylpropane	0.0013	0.0087	2.9	2.1
chloroform	0.012	0.0015	1.9	2.8
cyclohexane	0.076	0.016	1.1	1.8
dibutylether	0.015	0.011	1.8	2.0
ethyl acetate	0.0045	0.0079	2.3	2.1
1,2-dichloroethane	0.014	0.0045	1.8	2.4
n-hexane	0.060	0.024	1.2	1.6
n-heptane	0.074	0.023	1.1	1.6
3-methyl-1-butanol	0.051	0.067	1.3	1.2
dichloromethane	0.015	0.0019	1.8	2.7

In the first experiments cyclohexane was used to supply the starting material and 2-methoxy-2-methylpropane was used to separate the product. Later on cyclohexane was replaced by *n*-heptane.

2.4 Analytical methods

2.4.1 Activity measurements

XR and FDH activities of the free enzymes were determined on a Beckman DU 800 spectrophotometer at 25°C by following the depletion or formation of NADH at 340 nm (ε_{NADH} = 6.22 $cm^{-1}mM^{-1}$). Composition of assays is listed in Table 9. For immobilized enzymes, the reaction was carried out in Eppendorf tubes and stopped at several time points by centrifugation and separation of immobilisate from the solution. All XR activities shown were measured with xylose.

Table 9: Activity assays.

Solution	Concentration [mM]	Used Volume [µL]	End Concentration [mM]
		***Ct*XR**	
Xylose in K_2HPO_4Buffer pH = 6.2	700 100	480	700 100
NADH in H_2O	15	10	0.30
Sample		10	
		***Cb*FDH**	
HCOONa in K_2HPO_4Buffer pH = 6.2	200 100	480	200 100
NAD^+ in H_2O	100	10	2
Sample		10	

2.4.2 Measurement of the reaction products

Measurement of the reaction products was either carried out by high performance liquid chromatography (HPLC) or by gas chromatography (GC).

2.4.2.1 HPLC Measurements

Samples were either directly measured or in adequate dilutions. Standards were prepared by pre-dissolving *o*-chloroacetophenone or (*S*)-1-(2-chlorophenyl)-ethanol in ethanol to a final ethanol concentration of 5 % in H_2O. Analyte concentrations in standards were in the range between 4 mM and 0.2 µM. A Merck HitachiLaChrom HPLC system with a Chiralpak AD-RH column from Daicel was used. The eluent was 50 % acetonitrile and 50 % H_2O, the column temperature was 40°C and for detection an UV detector at 210 nm was used.

2.4.2.2 GC Measurements

Prior to analysis hydrophobic molecules were extracted by mixing aqueous samples 1+1 with *n*-heptane. After shaking by hand for 1 min, the organic phase was collected and injected in the GC. Standards were prepared in *n*-heptane from 80 nM to 160 mM. Samples were analyzed at an HP 5890 Series II plus gas chromatograph equipped with a Chrompack WCOT fused Silica 25 m x 0.25 mm coating CP Chirasil-DEX CB DF=0.25 µm. The column inlet temperature was 220°C and a constant column temperature of 130°C was used. (*R*)- and (*S*)-1-(2-chlorophenyl)-ethanol were separated on the column and detected with a flame ionization detector (FID) at a temperature of 275°C.

2.5 Immobilization

2.5.1 XR immobilization

A *Ct*XR variant with N-terminally fused His-tag was immobilized by metal ion affinity onto Chelating Sepharose^{TM} Fast Flow beads from Amersham Biosciences. The His-tagged enzyme was produced in *E. coli* JM109 according to Pival *et al*. 2008 and used as crude lysate for the immobilization.

10 mL Chelating Sepharose^{TM} Fast Flow beads were loaded with Cu^{2+} ions by the following protocol: The beads were washed three times with 10 mL H_2O, shaken with 10 mL 0.2 M $CuCl_2$ solution for 20 min, rinsed with H_2O four times and finally washed with a salt solution containing 0.02 M NaAc and 1 M NaCl (pH = 4.0) four times. The enzyme from 1 - 2 g wet cell mass in 100 mM K_2HPO_4 buffer (pH = 6.2) and the activated beads in salt solution were combined and NaCl was added until a concentration of 1 M NaCl in an end-volume of 10 mL was reached. The mixture was shaken for 40 min, then washed with 1 M NaCl solution four times and four times with 100 mM K_2HPO_4 buffer (pH = 6.2).

2.5.2 FDH immobilization

The *Cb*FDH was immobilized onto glyoxyl agarose beads. Amino groups of the enzyme were covalently bound to the glyoxyl groups onto the beads. The enzyme was produced in BL21 (DE3) according to Mädje *et al*. 2012 and used as crude lysate. 1 g of glyoxyl agarose 6 BC2 (Bolivar *et al.* 2006) and 1 - 2 g of wet cells were combined and gently stirred in 10 mL of 100 mM $NaHCO_3$ (pH 10.05) for 20 min. After this, 10 mg $NaBH_4$ was added and stirred for another 30 min in order to reduce imine- to amine-functionalities.

3 Conversion experiments

3.1 Three-chamber-reactor

Although several experiments were performed with the prototype of the three-chamber-reactor, crafted in our in-house workshop, no meaningful experimental data are available. Due to construction flows, difficulties were encountered regarding the tight connection of the tubings. Another problem was that the membrane stabilization was not sufficient enough to prevent membranes from breaking. The integrity of the system was compromised by the breaking of the membranes which could only be confirmed after the experiments. Therefore, the resulting data of several experiments could not be used. Furthermore, due to sealing difficulties, it was challenging to evacuate the prototype in order to prevent air pockets from forming in the membranes.

3.2 Enzyme retention by cross-flow filtration

3.2.1 Experiment 2.1

500 mL buffer solution (100 mM K_2HPO_4, pH 6.1) containing 300 mM HCOONa, 0.5 mM NAD^+, 10.5 U/mL FDH activity (as 2 mL cell lysate) and 218 U/mL XR activity was used as aqueous phase. Product extraction was carried out with TBME (starting volume 500 mL). Starting material was dissolved in ethanol to 32 mM and was added continuously with a rate of 0.2 mL/min. For cross-flow filtration a Vivaflow 50 cross-flow cassette with 30.000 MWCO PES from Satorius Stedim Biotech GmbH was used. The pump was used at its maximum speed of ~180 mL/min; the achieved transmembrane flow was determined to 12.5 mL/min with buffer. When the enzyme was added, the transmembrane flow decreased to 1.5 mL/min after 3 h due to membrane blocking. To prevent overconcentration, the *o*-chloroacetophenone solution was diluted 1/10. Nevertheless, after approximately ~12 h the aqueous phase became slightly turbid and the enzyme started to precipitate. We suspected the high ethanol concentration as a main reason for enzyme denaturation. The enzyme stability in the presence of ethanol was tested in order to overcome fast enzyme denaturation and subsequent membrane clogging. Assuming that the applied FDH activity of 21 U is equal to 190 mg of dry cells, a productivity of 0.46 $g_{product}/g_{CDW}$ can be roughly estimated. However, only 87 mg of (*S*)-1-(2-chlorophenyl)ethan-1-ol was found in the ether phase corresponding

to a low recovery of 24 %. Concentrations of *o*-chloroacetophenone and (*S*)-1-(2-chlorophenyl)ethan-1-ol in the extraction-vessel and of ethanol in the reaction-vessel were measured during the reaction (Figure 16).

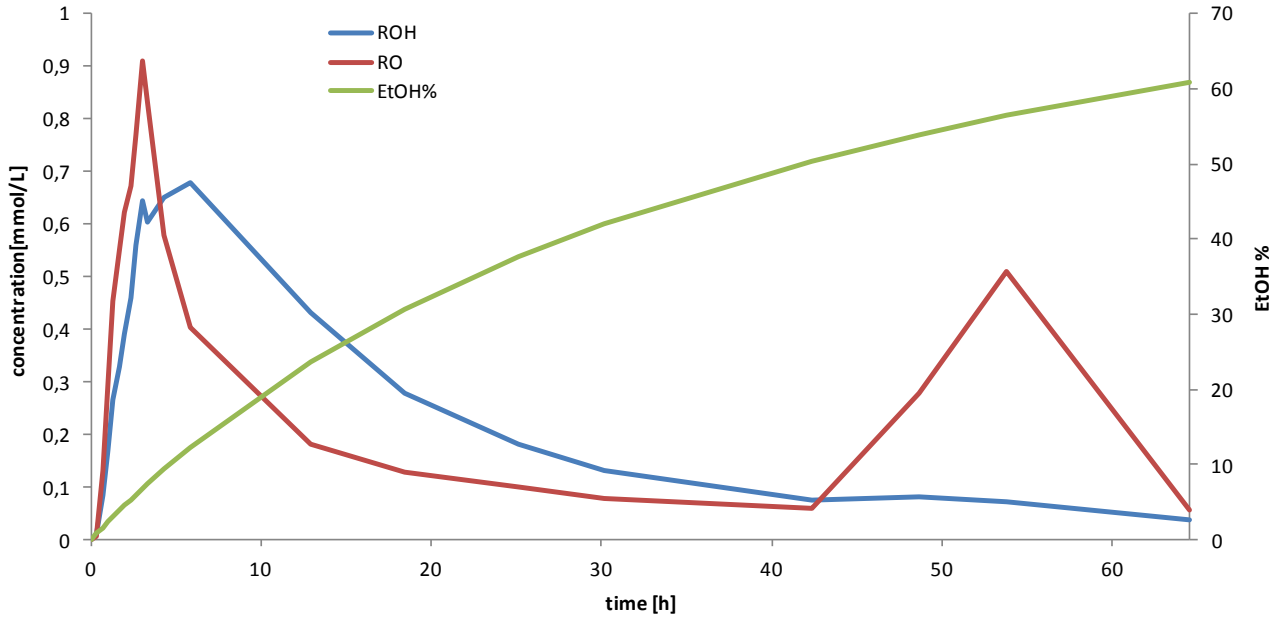


Figure 16: Concentrations of *o*-chloroacetophenone and (*S*)-1-(2-chlorophenyl)ethan-1-ol in the extraction-vessel and of ethanol in the reaction-vessel during the reaction.

3.2.1.1 Follow-up experiments of experiment 2.1

Stabilities of XR and FDH in the presence of ethanol

100 µL of crude lysate (1.7 U/mL FDH and 7 U/mL XR) were mixed with 900 µL of aqueous ethanol solutions. Samples were withdrawn after short shaking and measured with standard activity assays. Results show high losses of XR activity for ethanol concentrations above 30 % and high losses of FDH activity for ethanol concentrations above 40 % as seen in Figure 17.

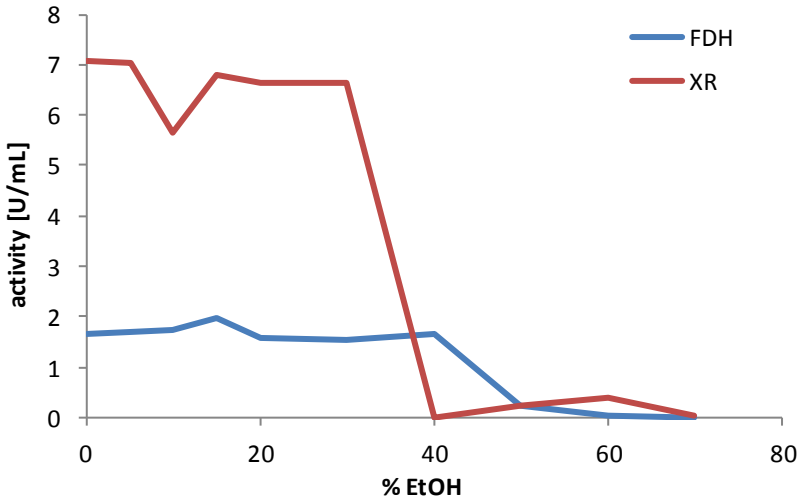


Figure 17: Activity of XR and FDH after short incubation with different EtOH concentrations

Basic purification of the cell-free lysate

The centrifuged lysate was additionally filtered through Minisart filters from Satorius Stedim (0.45 µm or 0.20 µm) in order to reduce membrane blocking. Activities were measured before and after filtration. The filtration had no observable effect on the activity.

Alternative starting material delivery

After noticing the short-term instability of the enzymes in the presence of ethanol, a new way of starting material delivery was designed. For this, a flow of aqueous phase dropped through the *n*-heptane phase containing the substrate as shown in Figure 6. The substrate enriched aqueous phase was subsequently added to the reaction mixture in a flow rate that was approximately adjusted to the transmembrane flow.

3.2.2 Experiment 2.2

1.5 g of dried cells (cultivated in the 7.2 L bioreactor described in section 2.2.2 Cultivation in the bioreactor) were mixed with 15 mL of buffer (100 mM K_2HPO_4, pH 6.1) and disrupted with a FrenchPress. Cell debris was removed by centrifugation at 13 000 rpm for 60 min. Then the lysate was filtered through a 0.20 µm Minisart filter as described under 3.2.1.1 Follow-up experiments of experiment 2.1. For cross-flow filtration a Vivaflow 50 cassette with 30.000 MWCO PES was used. 7 mL of the obtained cell lysate were added to 500 mL buffer

(100 mM K_2HPO_4, pH 6.1) containing 300 mM HCOONa and 0.5 mM NAD^+. The pump was used at its maximum speed with a flow rate of about 180 ml/min resulting in a transmembrane flow of 12.5 ml/min. After 2 h, the pH titration with 5.5 M formic acid was stopped due to the appearance of white precipitant at the acid entrance. After ~15 h reaction time, the tubing broke due to mechanical stress caused by the peristaltic pump and the buffer leaked out. The remaining buffer phase and the ether phase were analyzed. 40 mg of (*S*)-1-(2-chlorophenyl)ethan-1-ol were produced by 700 mg of dry cells equal to a productivity of 0.06 $g_{product}/g_{CDW}$ (as corrected for the loss of buffer). Concentrations of *o*-chloroacetophenone and (*S*)-1-(2-chlorophenyl)ethan-1-ol were measured in the extraction-vessel during the reaction (Figure 18).

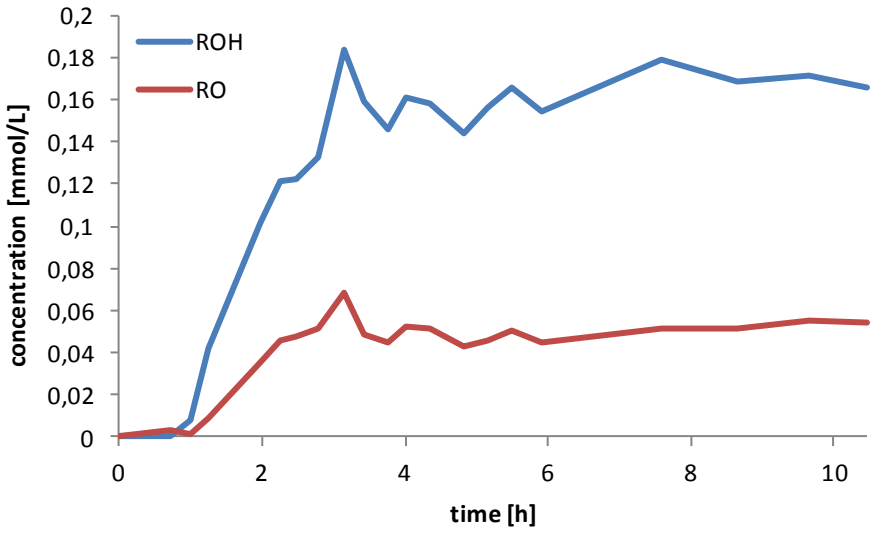


Figure 18: Experiment 2.2 Concentrations of *o*-chloroacetophenone and (*S*)-1-(2-chlorophenyl)ethan-1-ol measured in the extraction-vessel during the reaction.

3.2.2.1 Basic purification

In order to roughly purify the lysate from components of higher molecular weight, it was filtered through Vivaspin tubes of 10, 30, 50 or 100 kDa cut off (SatoriusStedim Biotech). Activities were measured in all permeates and supernatants. Neither XR nor FDH activities were found in permeates. After a washing step, the recovery of 50 % was found with the 50 kDa filter in the supernatant.

Alternatively, the lysate was treated with $(NH_4)_2SO_4$ and $MgCl_2$ to precipitate high molecular DNA/RNA and protein. For this purpose, different percentages of saturated $(NH_4)_2SO_4$-solution (pH 6.5) and $MgCl_2$ in an amount equal to the dry cell mass were added. The $MgCl_2$

was used for precipitating DNA/RNA (Palmiter, R. D. 1974) and had no influence on the activity of the enzymes. The saturated $(NH_4)_2SO_4$-solution was used to precipitate proteins other than XR and FDH. However, no separation of XR and FDH from other proteins by fractionated protein precipitation with $(NH_4)_2SO_4$ was achieved. XR and FDH precipitated with the bulk of host proteins (Figure 19).

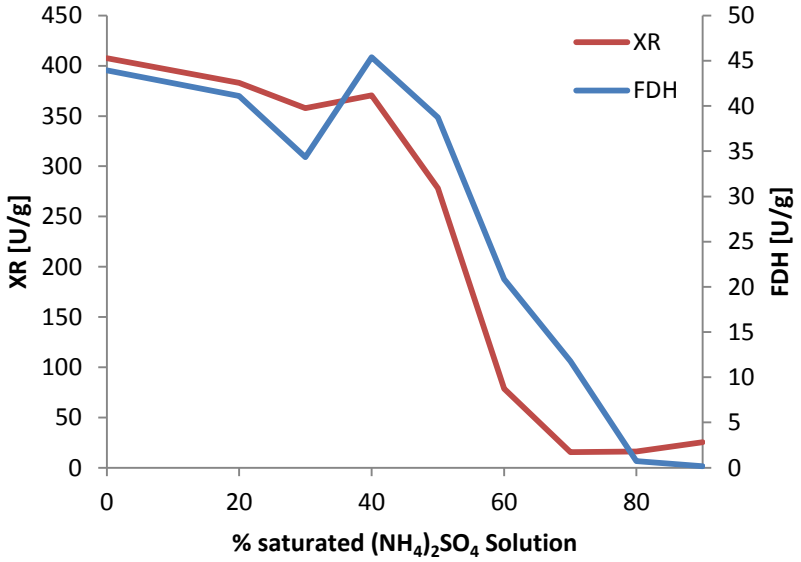


Figure 19: XR and FDH activities measured in the supernatant of $(NH_4)_2SO_4$-precipitated lysate.

3.2.3 Experiment 2.3

1.5 g of dried cells (cultivated in the 7.2 L bioreactor described in section 2.2.2 Cultivation in the bioreactor) were mixed with 15 mL of buffer (100 mM K_2HPO_4, pH 6.2) and disrupted with 7.5 g of glass beads (diameter 0.5 mm). For this, a sequence of 30 s vortexing and 30 s cooling was repeated ten times. 7 mL of the obtained cell lysate were added to 500 mL buffer (100 mM K_2HPO_4, pH 6.2) containing 300 mM HCOONa and 1.0 mM NAD^+. 1.5 g $MgCl_2$ were added and cell debris and precipitated DNA/RNA were centrifuged at 5 000 rpm for 60 min. A Vivaflow 50 cassette with 30.000 MWCO PES was used for the continuous cross-flow filtration. A Verder 2006 pump was used with a flow of ~400 mL/min. In the time span between 4 h and 10 h reaction time the tube broke. In an attempt to rescue the experiment, the buffer was collected and reused after filtration through a 0.45 µm Minisart filter. Tubes were renewed, 200 mL buffer (100 mM K_2HPO_4, pH 6.2) and 200 mg NAD^+ were added. After 42 h, as a precaution, the tubing was renewed. The ether which accumulated in the *n*-heptane phase was removed through distillation.

After 65 h reaction time, additional *o*-chloroacetophenone was added. For 700 mg of dried cell, 44 mg (*S*)-1-(2-chlorophenyl)ethan-1-ol were found in the ether and buffer phases. A quasi productivity of 0.06 $g_{product}/g_{CDW}$ was calculated. An additional problem of this experiment was the initial low activity probably due to insufficient cell disruption.

3.3 Cell retention using dialysis tubing

3.3.1 Experiment 3.1

3 mL cell lysate was mixed to 500 mL phosphate buffer (100 mM K_2HPO_4, pH 6.2) containing 300 mM HCOONa and 1.0 mM NAD^+. The lysate was prepared by mixing 0.5 g dried cells with 2.5 g glass beads (diameter 0.5 mm) and 5 mL buffer (100 mM K_2HPO_4, pH 6.2). After ten cycles of 30 s vortexing and 30 s cooling, 0.5 g $MgCl_2$ were added and the mixture containing cell debris and precipitated DNA/RNA was centrifuged at 5 000 rpm for 60 min. Two dialysis tubes were placed into the aqueous phase, one contained 95 mL *n*-heptane and 3 mL *o*-chloroacetophenone, the other contained 100 mL *tert*-butylmethylether. After 90 h of stirring, the phases were collected and analyzed by HPLC (Figure 8, Table 10). For 300 mg of dried cells 96 mg (*S*)-1-(2-chlorophenyl)ethan-1-ol were obtained in the aqueous and the two organic phases equal to a productivity of 0.32 $g_{product}/g_{CDW}$.

Table 10: Starting material and product distribution in the three phases of experiment 3.1.

	(*S*)-1-(2-chlorophenyl)ethan-1-ol [mmol/L]	*o*-chloroacetophenone [mmol/L]
n-heptane	2.29	223.16
buffer	0.17	1.19
TBME	6.38	1.64

3.3.2 Experiment 3.2

4.6 mL cell lysate was mixed into 1 L phosphate buffer (100 mM K_2HPO_4, pH 6.2) containing 300 mM HCOONa and 1.0 mM NAD^+. The lysate was prepared by mixing 1.0 g of dried cells with 10 mL buffer without HCOONa. The cells were disrupted by ultrasound with a Vibra-Cell™ ultrasonic processor (600 W / 20 kHz) at 80 % input. 3 cycles of 30 s ultra sonication and 30 s cooling where used for cell disruption; afterwards, the lysate was centrifuged at 13 000 rpm. Two dialysis tubes were placed into the aqueous phase, one contained 95 mL *n*-heptane and 5 mL *o*-chloroacetophenone, the other contained 100 mL *tert*-butylmethylether. After 143 h of stirring, pumping was stopped. The *tert*-butylmethylether phase was evaporated at the end of the experiment. *o*-Chloroacetophenone and (*S*)-1-(2-chlorophenyl)ethan-1-ol were remaining in dialysis tube previously containing *tert*-butylmethylether. Because there was no *tert*-butylmethylether phase left, the buffer was washed with 200 mL *tert*-butylmethylether. Phases were collected and measured by HPLC. Enzyme activities were measured over time and are shown in Figure 9. For 450 mg of dried cells, 395 mg (*S*)-1-(2-chlorophenyl)ethan-1-ol were obtained in the aqueous and the two organic phases equal to a productivity of 0.87 $g_{product}/g_{CDW}$.

3.3.3 Experiment 3.3

For experiment three, we tried to co-immobilize FDH and XR on a Sepharose 6C2 Matrix activated with glyoxyl agarose 6BC2. However, nearly all of the XR and FDH activity was lost in the immobilization step.

3.4 Immobilized enzymes in a column

3.4.1 Experiment 4.1

The column was packed with beads prepared for experiment 2.5.1 i.e. beads with immobilized XR equal to 28 U and beads with immobilized FDH equal to 0.9 U. 1 L of phosphate buffer (100 mM K_2HPO_4, pH 6.2) containing 300 mM HCOONa and 1.0 mM NAD^+ was used. Flow rates through the column and through the extraction vessel were adjusted to 4 and 10 mL/min, respectively. Prior to addition of the *o*-chloroacetophenone, the FDH activity in the column was measured. The absorption at 340 nm of the aqueous phase was

measured before and after the column. From the absorption difference, a FDH activity of 0.35 U was calculated which was only 40 % of the starting activity. Furthermore, a low concentration of the substrate (80-100 µM) prevented conversion. Hence, product formation was not observed. However, after 19 days still some FDH activity was detected.

3.4.2 Experiment 4.2

The column was filled with FDH and XR immobilized onto sepharose and glyoxyl agarose beads, respectively. The total FDH activity on the beads was determined to 41.6 U, which is 23 % of the activity from the lysate of 1.0 g dried cell mass and a XR activity of 58.3 U, which is 16 % of the activity from lysate of 0.33 g dried cell mass. The starting material was predissolved in the reaction buffer (pH = 6.2 300 mM HCOONa, 100 mM K_2HPO_4, 1.0 mM NAD^+) saturated with *o*-chloroacetophenone. For continuous supply, the saturated buffer was added with a flow of 1 mL/min to the buffer-storing vessel. From there, the mixture was pumped with 5 mL/min through the column (Figure 21). The flow from the extraction vessel was 6 mL/min. After 40 h, the reaction was stopped and the activity of the immobilized enzymes was measured. The activity of the FDH was still at 74 % of the starting value but the activity of the XR was not detectable any more. Therefore, the lifetime of the FDH was increased significantly by the immobilization whereas the lifetime of the XR was significantly decreased. Concentrations after the column where monitored to follow the reaction progress and are shown in Figure 20. For 1.4 g of dried cells, 80 mg (*S*)-1-(2-chlorophenyl)ethan-1-ol were obtained in all three phases, equal to a productivity of 0.06 $g_{product}/g_{CDW}$.

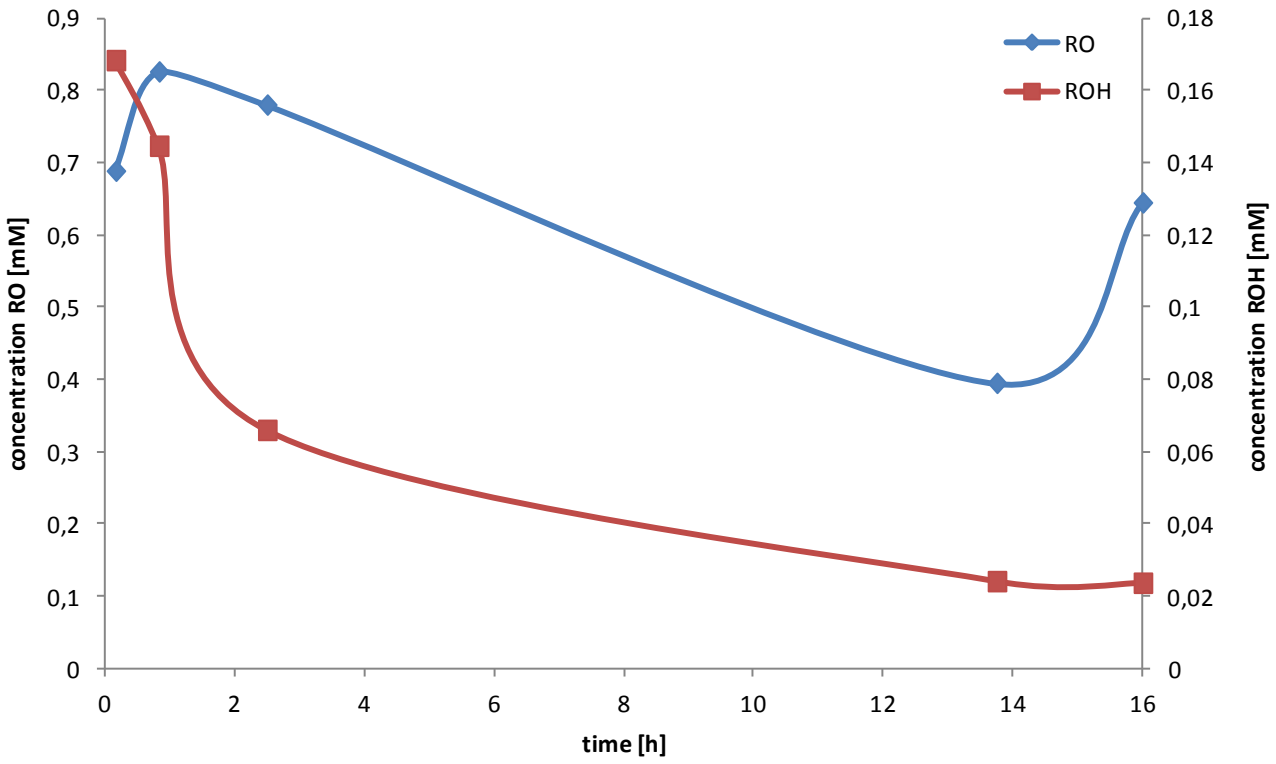


Figure 20: *o*-Chloroacetophenone and (*S*)-1-(2-chlorophenyl)ethan-1-ol concentrations measured in samples that were taken after the column in experiment 4.2.

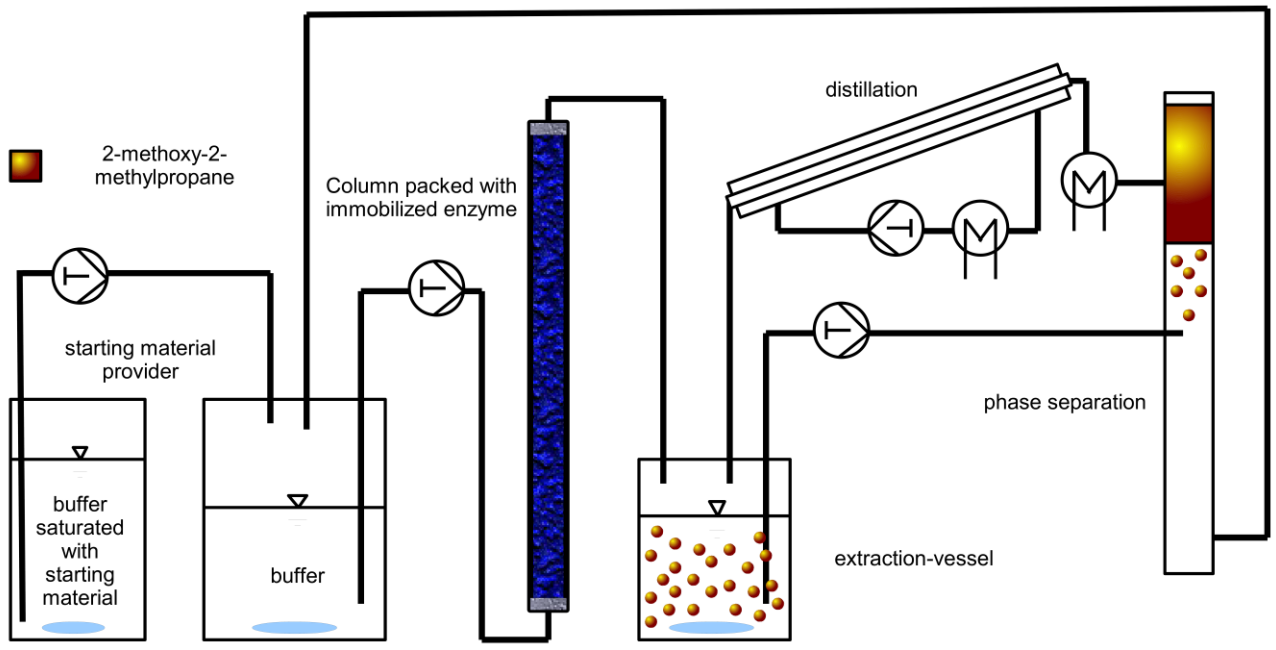


Figure 21: Scheme 2 for the construction with immobilized enzymes packed in a column.

3.5 Liquid-liquid-liquid reactor

3.5.1 Experiment 5.1

0.3 g dried cells (*E. coli* Rosetta 2 (DE3) overexpressing XR and FDH) were dissolved in 80 mL standard buffer prior to disruption by ultrasound with a Vibra-Cell™ ultrasonic processor (600 W / 20 kHz) at 80 % input. 3 cycles of 30 s ultra sonication and 30 s cooling were used for cell disruption, afterwards the lysate was centrifuged at 13 000 rpm. The aqueous phase was filled into the reaction chamber. The *o*-chloroacetophenone was provided in the inner organic phase. The neat *o*-chloroacetophenone is heavier than water; therefore, it was diluted with *n*-heptane to a concentration of about 30 %. The outer organic phase (*n*-heptane) was circulated by distillation and subsequent vapor condensation. Samples for the analysis of starting material and product concentration were taken from the organic phase before distillation (Figure 13). Samples for the analysis of enzyme activities were taken from the aqueous phase in the reactor (Figure 12). From 300 mg of dried cells, 78 mg (*S*)-1-(2-chlorophenyl)ethan-1-ol were obtained in the whole system, equal to a productivity of 0.26 $g_{product}/g_{CDW}$. Although the productivity of this experiment was not the highest in this work, it could have potential when the concentration of *o*-chloroacetophenone would be increased in the buffer phase. Also, we clearly showed that in this set up the XR is stable for a long time ($T_{1/2}$ = 151 h). Here, the instability of the FDH is most probably not due to the product but rather due to the enzyme's sensitivity to solvents. For further experiments it was therefore tried to stabilize the FDH by immobilization.

3.5.2 Experiment 5.2

In 90 mL of standard reaction buffer we had a total of 8.5 U FDH activity immobilized on 1.65 g of carrier from 0.96 g dried cell mass. The 209 U XR activity in form of free enzyme were obtained from 0.50 g dried cell mass. The outer organic phase (*n*-heptane) was circulated by distillation and subsequent vapor condensation. Before the start of the reaction with ketone, the absorption of the buffer at 340 nm was recorded over time. The measured FDH increase correlated only to 6.4 % of the introduced amount of activity. After 2 h the reaction was started by adding a mixture of 0.5 mL *o*-chloroacetophenone and 1 mL *n*-heptane in the inner cylinder. This step was repeated after 73 h, 127 h, and 150 h to supply enough *o*-chloroacetophenone for the reaction. Product and starting material concentration were measured in the *n*-heptane flow to the distillation. XR activity was measured from the buffer after centrifugation of the sample. The precipitant was used for measuring the activity of the immobilized FDH.

For 1.45 g of dried cell weight, 134 mg (*S*)-1-(2-chlorophenyl)ethan-1-ol were measured in the whole system, which means a productivity of 0.09 $g_{product}/g_{CDW}$. In this approach, the activity of FDH in the reactor was significantly under the expected value. This could be due to old NAD^+ and the lower NAD^+-concentration compared to the assay. The highest NADH-concentration we can confirm in our reactor correlates to a NAD^+-concentration of at least 19 % of the concentration used in the assay. For improved results of this experiment, it would be necessary to monitor the measured parameters more closely, especially the starting material flow. Also, it is of utmost importance to explain why the actual activity of the FDH in the reactor does not match with the activity of the assay.

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