Selective redox biocatalysis in multiphasic enzyme reactors

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M. Sc. Bartłomiej Tomaszewski

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1. Gutachter: Prof. Dr. Andreas Schmid

2. Gutachter: Prof. Dr. Norbert Kockmann

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LIST OF ABBREVIATIONS AND SYMBOLS

Abbreviations

2LP	two-liquid phase system
ACN	acetonitrile
BSA	Bovine Serum Albumin
CLEA	cross-linked enzyme aggregate
CV	column volume
DPA	dextran polyaldehyde
EBA	expanded bed adsorption chromatography
ePTFE	expanded PTFE
ETFE	ethylene-tetrafluoroethylene
FAD	flavin adenine dinucleotide
FDH	formate dehydrogenase (E.C. 1.2.1.2)
FOR	formate salt
g_{CDW}	gram cell dry weight
g_{CWW}	gram cell wet weight
HbpA	2-hydroxybiphenyl 3-monooxygenase (E.C. 1.14.13.44)
HPLC	high performance liquid chromatography
i.d.	internal diameter
KPi	potassium phosphate buffer
MTBE	methyl *tert*-butyl ether
NAD^+	nicotinamide adenine dinucleotide, oxidised
NADH	nicotinamide adenine dinucleotide, reduced
o.d.	outer diameter
OD_{450}	optical density at 450 nm
PEEK	polyetheretherketone
PFA	perfluoroalkoxy alkane
pHBP461	plasmid carrying *hpbA* gene
PMMA	polymethyl methacrylate
PTFE	polytetrafluoroethylene
RP-HPLC	reversed phase high performance liquid chromatography
RT	room temperature
STY	space time yield
Teflon AF-2400	amorphous fluoroplastic, a copolymer of 2,2-bistrifluoromethyl-4,5-difluoro-1,3-dioxole and tetrafluoroethylene
Tefzel®	modified ETFE (ethylene-tetrafluoroethylene)
TFA	trifluoroacetic acid
TiTR	tube-in-tube reactor
TLC	thin layer chromatography
Tris	2-amino-2-hydroxymethyl-propane-1,3-diol
US*	Uwe Sauer

Symbols

Bo	Bond number
Ca	capillary number
d	diameter, m
g	gravitational acceleration, m s^{-2}
J_{N2}	flux of nitrogen

J_{O2}	flux of oxygen
k_{cat}	catalytic constant, turnover frequency, s^{-1}
K_D	partition ratio
K_i	inhibition constant, M
k_La	overall volumetric mass transfer coefficient, s^{-1}, min^{-1}
K_m	Michaelis-Menten constant, M
$logP_{o/w}$	logarithm of partition coefficient between octanol and water
Re	Reynolds number
U	activity unit, equal to 1 µmol product per minute
U	velocity, m s^{-1}
μ	viscosity, Pa s
ρ	density, kg m^{-3}
σ	interfacial tension, N m^{-1}

SUMMARY

Reactions catalysed by oxidoreductases are indispensable for chemical synthesis. However, despite obvious advantages like mild reaction conditions and regio-, chemo-, and stereoselectivity, which simplifies product work-up, their implementation in the chemical industry is lacking. Therefore, an integrated approach involving catalyst and reaction engineering and proper reactor design is necessary for commercialising oxidoreductases for organic synthesis. Rational reaction design linking kinetic characterisation of the oxidoreductase and reaction optimisation formed the basis for this thesis. The overall aim of this PhD project was the application of 2-hydroxybiphenyl 3-monooxygenase (HbpA), for preparative scale synthesis of 3-phenylcatechol with an acceptable space time yield (STY). The key challenges limiting the biocatalytic process, namely biocatalyst operational stability, supply of the hydrophobic substrate, oxygen delivery, cofactor regeneration and mass transfer were identified and addressed. Formate dehydrogenase, FDH, was used for continuous NADH regeneration. Addition of the surfactant Tween 20 stabilised the enzymes in the presence of the organic solvent and allowed to recover 100% of the respective enzymatic activity opening the door for application of the biocatalysts in an aqueous/organic two-liquid phase segmented flow microreactor. A biphasic reaction system allowed high substrate loadings whereas the microreactor ensured excellent mass transfer rates between organic and aqueous phases. Finally, using a Teflon AF-2400 membrane in a tube-in-tube fashion allowed for radial delivery of oxygen and improved oxygen availability with respect to one point oxygen delivery in an aqueous/organic/air three phase segmented flow microreactor. Thereby a successful preparative scale biotransformation reaction in a segmented flow tube-in-tube reactor was possible yielding 740 mg of product purified by chromatography and subsequent sublimation. The STY of 14.5 g L_{total}^{-1} h^{-1} reached is the highest reported for this enzyme. It is 38 times higher as compared to the batch reactions described earlier (Lutz et al. 2002). Oxygen transfer rates through Teflon AF-2400 membrane were as high as 24 mM min^{-1} i.e. 16 times higher than in traditional biotechnological processes in stirred-tank reactors. The presented reactor is a promising tool for oxygen dependent biocatalytic reactions in microreactors, and may be regarded as a basis for applications in gram scale organic biosyntheses. Furthermore, it may become a platform for other gas dependent reactions since it extends the operational boundaries beyond the scope of available reactors with respect to aqueous/organic mass transfer and gas transfer rates.

ZUSAMMENFASSUNG

Oxidoreduktasen katalysieren eine Vielzahl an interessanten Reaktionen und können bei chemischen Synthesen überaus hilfreich sein. Trotz der offensichtlichen Vorteile wie milde Reaktionsbedingungen und der sich durch die ausgezeichnete Regio-, Chemo- und Stereoselektivität ergebenden vereinfachten Produktaufreinigung, ist ihre Bedeutung für die produktive Katalyse lediglich sekundär. Für eine großflächige Verbreitung von Oxidoreduktasen in der organischen Synthese ist daher ein integrierter Ansatz notwendig, der sowohl die Entwicklung der Reaktionstechnik und des Katalysators aber auch des geeigneten Reaktorkonzeptes beinhaltet. Die Verknüpfung von kinetischer Charakterisierung eines Biokatalysators und der Optimierung der Reaktionsparameter bildete die Grundlage der vorliegenden Arbeit. Das Ziel dieser Arbeit war die Anwendung der 2-Hydroxybiphenyl 3-Monooxygenase (HbpA) zur Synthese von 3-Phenyl-Catechol in präparativem Maßstab. Von zentraler Bedeutung war hierbei die Identifizierung der Hauptlimitationen des biokatalytischen Prozesses: die Stabilität des Katalysators, die Bereitstellung des hydrophoben Substrates, die Gewährleistung der Sauerstoffzufuhr, die Kofaktorregenerierung und der Massentransfer. Der Einsatz der Formiat-Dehydrogenase (FDH) erlaubte eine kontinuierliche NADH-Regeneration. Durch die Zugabe des Tensids Tween 20 konnten HbpA und FDH in Anwesenheit organischer Lösemittel stabilisiert werden, was eine Anwendung der Biokatalysatoren in organisch-wässrigen Zweiphasen-Systemen in ‘segmented-flow‘ Mikroreaktoren erlaubte. Durch die zweiphasige Reaktionsführung wurden hohe Beladungen mit Substrat erzielt und durch die Anwendung des Mikroreaktors konnte der Massentransfer zwischen organischer und wässriger Phase maximiert werden. Die Sauerstoff-Verfügbarkeit wurde durch den Einsatz einer Teflon AF-2400 Membran in einem Tube-in-Tube Reaktor durch radiale Begasung gegenüber der traditionellen Ein-Punkt Begasung signifikant verbessert. Der Einsatz des Tube-in-Tube Mikroreaktors führte im präparativen Maßstab zu 740 mg 3-Phenyl-Catechol, isoliert mittels Chromatographie und anschließender Sublimation. Die zugrundeliegende Raum-Zeit-Ausbeute (STY) von 14,5 g L_{total}^{-1} h^{-1} ist im Vergleich zu Batch-Reaktionen (Lutz et al. 2002), 38-fach erhöht und weist somit die höchste jemals berichtete STY für diese Reaktion auf. Mit 24 mM min^{-1} ist die Sauerstoff Transferrate durch die Teflon AF-2400 Membran etwa 16-fach höher als in traditionell für biotechnologische Prozesse eingesetzte Rührkessel-Reaktoren. Das in dieser Studie untersuchte Reaktorkonzept stellt für sauerstoffabhängige, biokatalytische Reaktionen einen vielversprechenden Ansatz dar, welches als Grundlage für die Synthese organischer Komponenten im Gramm-Maßstab angesehen werden kann. Darüber hinaus kann der Tube-in-Tube Reaktor zu einer generell anwendbaren Plattform für Gas-abhängige Reaktionen entwickelt werden, da er die systembedingten Limitierungen vorhandener Reaktorkonzepte in Bezug auf organisch-wässrigen Massentransfer und Gas-Transferrate überwindet.

CHAPTER 1

INTRODUCTION

REDOX REACTIONS

Functionalisation of non-activated carbons within the paradigm of Green Chemistry

Early industrial syntheses and later development of the pharmaceutical industry was based on processes that ignored the toxic nature of their reagents, waste to product ratios, and non-renewable origins of the catalysts.[1] For changing the paradigm and making new processes environmentally friendly a set of 12 Principles of Green Chemistry was published in 1998 by Anastas and Warner.[2] Those read as follows:

1. **Prevention** - It is better to prevent waste than to treat or clean up waste afterwards.
2. **Atom Economy -** Design synthetic methods to maximise the incorporation of all materials used in the process into the final product.
3. **Less Hazardous Chemical Syntheses -** Design synthetic methods to use and generate substances that minimise toxicity to human health and the environment.
4. **Designing Safer Chemicals -** Design chemical products to affect their desired function while minimising their toxicity.
5. **Safer Solvents and Auxiliaries -** Minimise the use of auxiliary substances wherever possible; make them harmless when used.
6. **Design for Energy Efficiency -** Minimise the energy requirements of chemical processes and conduct synthetic methods at ambient temperature and pressure if possible.
7. **Use of Renewable Feedstocks -** Use renewable raw material or feedstock whenever possible.
8. **Reduce Derivatives -** Minimise or avoid unnecessary derivatisation if possible, which requires additional reagents and generates waste.
9. **Catalysis -** Catalytic reagents are superior to stoichiometric reagents.
10. **Design for Degradation -** Design chemical products so they break down into harmless products that do not persist in the environment.
11. **Real-time Analysis for Pollution Prevention -** Develop analytical methodologies needed to allow for real-time, in-process monitoring and control prior to the formation of hazardous substances.
12. **Inherently Safer Chemistry for Accident Prevention** - Choose substances and the form of a substance used in a chemical process to minimise the potential for chemical accidents, including releases, explosions, and fires.

Those principles became the basis for developing environment-friendly processes in organic chemistry as well as in biotechnology.

In the context of those principles the chemical oxidation of hydrocarbons is not a 'green process'. In organic chemistry oxidation may be defined as elimination of hydrogen as in the dehydrogenation of ethane leading to acetylene, or as a replacement of a hydrogen atom bonded to a carbon by a more electronegative atom e.g. oxygen.[3] Harsh reaction conditions are often needed in order to activate C-C or C-H bonds, which in the course of the reaction are oxidised to C-O, C=C or C-N. The selectivity of those reactions is compromised when strong acids and bases are used and in earlier times quantitative amounts of metal salts were required for an oxidative addition reaction.[4] For example the oxidation of toluene by trifluoroperacetic acid and hydrogen peroxide does not stop at 2-hydroxytoluene but continues and yields a mixture of the former as well as 2,3-dihydroxytoluene and 2,5-dihydroxytoluene[5] requiring complex product work-up. Also the very successful Suzuki-coupling reaction[6] is rather difficult in terms of the Green Chemistry principles. Heavy metal catalysts, organic solvents, and an acidic environment are necessary to couple halides and organoborane species for the synthesis of e.g. substituted biphenyls.[7] In contrast to chemical oxidation reactions, enzyme catalysed reactions run at ambient temperature and pressures, and under neutral pH. Biodegradable catalysts (enzymes or cells) are used, which are derived from renewable resources. Reactions are regio-, chemo-, and stereoselective (Figure 1-1), and no group activation, protection or deprotection is required. Biocatalytic processes involve less steps than competitive organic syntheses, hence less waste is generated and the quality of product is often superior with respect to classical processes.[1] The use of oxidoreductases (either soluble or as a whole cell biocatalyst, see later) is therefore advantageous compared to the traditional chemical approaches. Additionally, oxygenases often directly give the required regio- and chemoselectivity,[8] making them very important catalysts in chemistry. Over past decades biocatalysis has emerged as an important tool for organic chemistry.[9] The discovery of the structure of nucleic acids by Watson, Crick and Franklin in 1953, and establishment of recombinant DNA technology was a milestone for first recombinant *E. coli* producing indigo dye.[10] Chemical producers like Hoffmann – La Roche recognised the potential of the biocatalytic paths for the production of vitamins by fermentation in 1998 and turned to biocatalysis replacing their existing chemical process for vitamin B-2 (riboflavin).[11] The diversity of biocatalysts should be regarded as an aid for complicated organic synthases, and where possible should replace chemical steps, if this makes sense regarding ecological and economical aspects.[12]

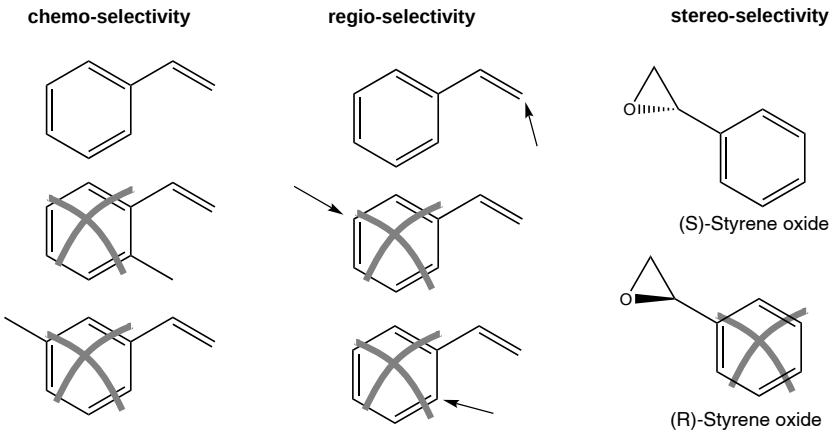


Figure 1-1: Enzyme selectivity on the base of styrene monooxygenase StyAB. Chemo-selectivity refers to the preference of an enzyme to react with one chemical compound over another. Regio-selectivity refers to the preference of the direction of the new bond formation where one bond direction is preferred over another. Stereo-selectivity refers to the preference of one enantiomer over another.[13]

ISOLATED ENZYMES VS WHOLE CELL BIOCATALYSTS

Whether a biocatalyst is applied as a whole-cell or an isolated enzyme should be based on the requirement of the process e.g. duration, available reactors, skills of the technical personnel, and in the very end on the economic feasibility (Figure 1-2).

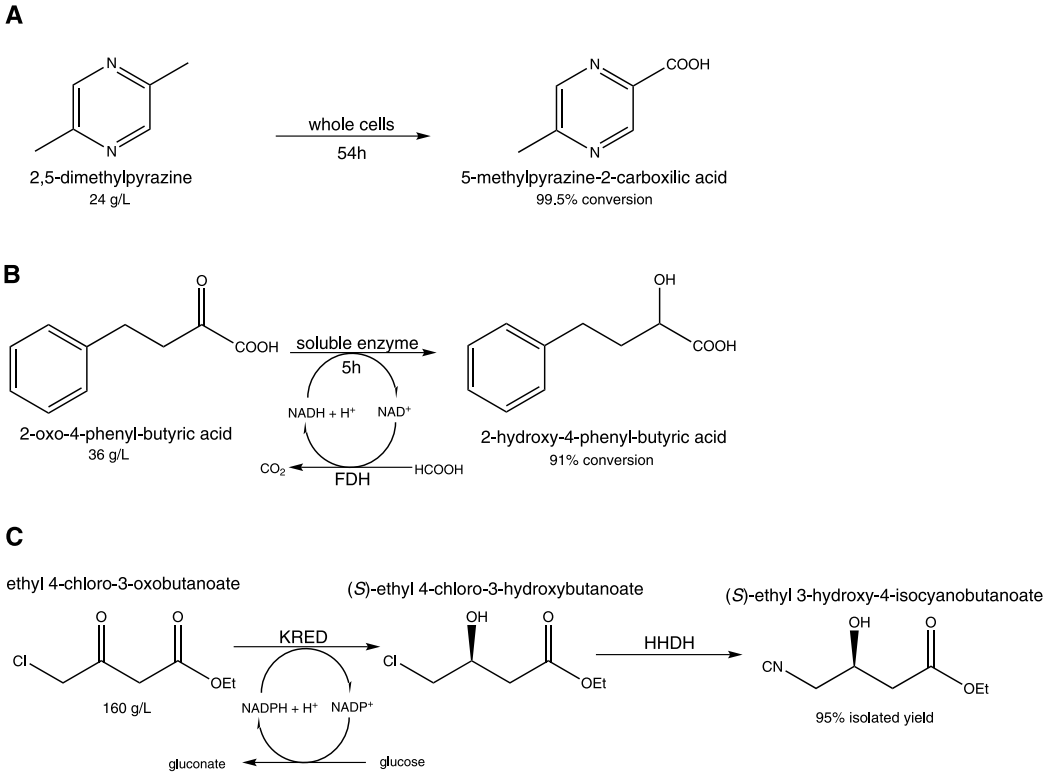


Figure 1-2: Industrial processes with oxidoreductases. A – Oxidation of 2,5-methylprrazine with whole cell catalyst *P. putida* ATCC 33015 producing xylene monooxygenase at Lonza.[14] B – Dehydrogenation of 2-oxo-4-phenyl-butyric acid with solubilised *(R)*-lactate-NAD oxidoreductase at Ciba-Geigy.[14] C – Synthesis of the atorvastatin intermediate by an isolated ketoreductase (KRED) and halohydrin dehalogenase (HHDH) developed by Codexis.[15]

Although examples for the industrial application of soluble oxidoreductases exist, those are scarce,[14] despite having industrial relevance.[16] Table 1-1 summarises advantages and disadvantages of applying either whole cells or isolated enzymes.

Table 1-1: Application of a whole-cell catalyst vs. soluble enzyme for biocatalytic redox reaction – advantages and disadvantages.

Whole cells	**Isolated enzymes**
In-vivo cofactor regeneration	Cofactor regeneration required
Renewable biocatalyst	No skills in fermentation required
Volumetric activities limited by enzyme levels in cell and by endogenous respiration[17]	High catalyst loadings possible
Limited oxygen transfer rates[17]	Oxygen used only for reaction, not for endogenous respiration
Separation and purification of the enzymes avoided[1]	Prior enzyme purification required, additional stabilisation necessary, high production cost,[18] limited enzyme availability[19]
Product/substrate toxicity of the host,[20] different growth and reaction conditions[21]	No growth inhibition or substrate/product toxification of the host, reaction conditions uncoupled from the growth
Limited substrate uptake[22]	Substrate/product transport not limited by the cell membrane
Tedious downstream processing	Separation of the product by ultrafiltration/membranes[23]

Two major issues are to be considered when working with isolated enzymes, cofactor regeneration and enzyme stabilisation. While in a whole cell enzymes are usually in a protected environment and undergo the overall protein turn-over of their host cell, they are prone to deactivation due to oxidation, and susceptible to changing pH outside the cell environment. Especially challenging is the stability issue when non-aqueous solvents are used. In this context enzyme inactivation and strategies for stabilisation will be introduced below in detail.

Cofactor Regeneration

A crucial limitation when working with isolated enzymes is the necessity of cofactor regeneration. Different strategies for regeneration of cofactors exist. The simplest method is the application of the whole cell biocatalyst, which takes advantage of the natural cellular cofactor regeneration capacity.[24] In such an approach the biotransformation reaction requiring the cofactors is coupled to the metabolism of the host delivering reducing equivalents (e.g. NADH). However, the reaction has to compete with host's metabolism and various background activities, which consume the respective cofactors. A possible solution is the application of resting, so non-growing, but metabolically active cells as an alternative to

growing cells. Often these show higher specific activities for the biotransformation because cell metabolism can be exploited more effectively for the reaction of interest.[25]

If working with isolated enzymes an additional system for cofactor regeneration needs to be introduced into the overall reaction scheme. Due to cofactors high price, it is economically not feasible to add these in stoichiometric amounts. Thus, they need to be generated *in-situ* and used in catalytic amounts.[26] Different enzymatic cofactor regeneration methods exist. Most popular enzyme coupled regeneration systems are formate dehydrogenase (FDH),[27] alcohol dehydrogenase (ADH),[28] glucose dehydrogenase (GDH),[29] and phosphite dehydrogenase.[30] The respective biotransformation reaction is coupled to a second reaction, which uses the same cofactor in its different oxidation state. These methods require stoichiometric addition of a second sacrificial substrate e.g. ammonium formate or 2-propanol for FDH and ADH respectively, which is consumed in the course of the regeneration. Advantage of those two enzymatic systems is their irreversible character when their products (carbon dioxide or acetone) are continuously removed. Using FDH in a low water system is also possible, expanding its application range.[31]

Out of the non-enzymatic regeneration methods of reduced nicotinamide cofactors it is worth mentioning the organometallic pentamethylcyclopentadienyl rhodium bipyridine complex $[Cp^*Rh^*(bpy)(H_2O]^{2+}$, which is a versatile catalyst used in electrochemical regeneration of NADH,[32] as well as in formate coupled direct regeneration of flavins ($FADH_2$),[33] The reduction of $NAD(P)^+$ to NAD(P)H is highly selective yielding only the 1,4–NAD(P)H enzymatically active form,[26] therefore, no cofactor loss occurs during the reaction. The disadvantage of that system is however its low turnover frequency (k_{cat}, s^{-1}) of 36 h^{-1} with respect to 2.4 s^{-1} for formate-driven NADH regeneration.[26]

One more interesting method that solves the cofactor requirement for oxidoreductases but does not regenerate the cofactor *in-situ* is the application of a cofactor mimics i.e. a synthetic form of a nicotinamide analogue that replaces natural cofactor. Recent advances allowed for efficient application of the mimic for the enoate reductase catalysed C=C reduction reactions.[34] Cheap analogues of NADH like 1-butyl-1,4-dihydropyridine-3-carboxamide showed excellent performance exceeding that of the natural cofactor, maintaining the same enantioselectivity as with their natural counterparts.

Further methods for solving the cofactor dependency of oxidoreductases go beyond the scope of this introduction and are covered in available reviews.[26, 35]

Among the mentioned cofactor regeneration methods the one that received most attention for the industrial application is the formate dehydrogenase coupled NADH regeneration (Figure

1-3). Examples of processes applying this cofactor regeneration method are listed in the Table 1-2.

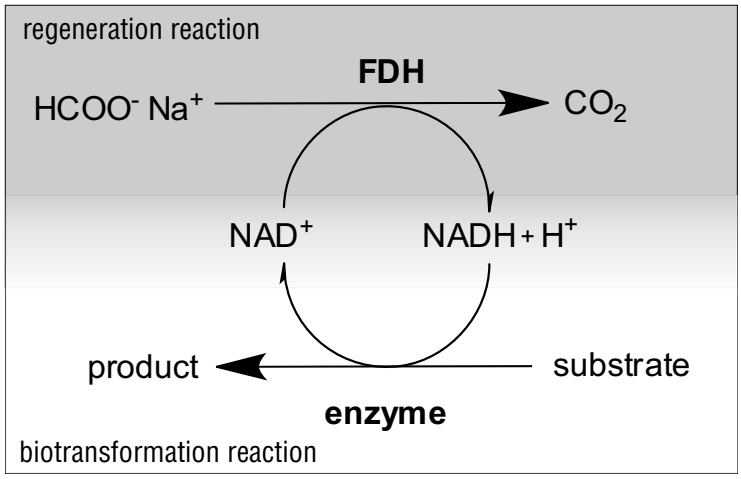


Figure 1-3: Reaction scheme of FDH catalysed cofactor regeneration. Regeneration half-reaction and biotransformation half-reaction are shown.

Table 1-2: Selection of the biotransformations utilising formate dehydrogenase for NADH regeneration.[14]

Substrate	Product	Enzyme	Conversion	Time (h)	Volume (L)	Productivity (g L^{-1} h^{-1})
1-phenyl-2-propanone (15 mM)	1-phenyl-propanol	Alcohol dehydrogenase *R. erythropolis*	72%	0.33 h	0.05	2.6
2-oxo-4-phenyl-butyric acid (200 mM)	2-hydroxy-4-phenyl-butyric acid	R-lactate-NAD oxidoreductase *S. epidermidis*	91%	4.6	0.2	6.9
trimethylpyruvic acid (500 mM)	*tert*-leucine	L-leucine-NAD oxidoreductase *B. sphaericus*	74%	2	Not specified	26.6

Application of formate dehydrogenase catalysed NADH regeneration has multiple advantages: its cheap substrate sodium formate (80.5 €/kg (>99% BioUltra)[36]) is a non-toxic salt that is widely tolerated by enzymes.[35c] There are examples however, when formate, as a monovalent salt inhibits the biotransformation enzyme. CO_2 evolves as only side product of the regeneration reaction. It is a non-toxic gas constantly leaving the reaction mixture, and thus driving the reaction to completion.[35c]

If an efficient way to produce and purify heterologous proteins in sufficient amounts exists and the cofactor problem can be solved by applying a second catalyst e.g. formate dehydrogenase or alcohol dehydrogenase, then application of isolated enzyme is an attractive alternative to a whole cell process. The section below introduces some interesting redox enzymes and lists accessible biotransformations.

INTERESTING ENZYMES FOR CELL FREE APPLICATIONS

Redox enzymes

The class of enzymes referred to as oxidoreductases (EC 1) catalyse the transfer of electrons between redox partners. Oxidoreductases may be sub-divided into four groups as proposed earlier,[37] namely oxidases, peroxidases, oxygenases/hydroxylases, and dehydrogenases/ reductases. Oxidases utilize oxygen as electron acceptor reducing it to water or hydrogen peroxide without incorporating it into the substrate (hydride transfer). They are cofactor independent and contain iron, copper or flavin in their active site.[38] Glucose oxidase catalysing oxidation of β-D-glucose to gluconic acid is an example of a flavin containing oxidase.[39] Peroxidases use hydrogen peroxide as an electron acceptor in order to regenerate its oxidised prosthetic group.[12] The majority of peroxidases are heme-enzymes, some may contain manganese, vanadium or selenium. Catalase is an example of a peroxidase catalysing breakdown of hydrogen peroxide into water and oxygen. Oxygenases/hydroxylases catalyse NAD(P)H dependent incorporation of single oxygen (monooxygenases) or two oxygen atoms (dioxygenases) into their substrate using molecular oxygen as an oxidant. Examples are styrene monooxygenase, which catalyses oxidation of styrene to styrene oxide,[24] and benzene dioxygenase catalysing dihydroxylation of benzene to *cis*-benzene glycol respectively.[40] Dehydrogenases (aka reductases) display a broad substrate spectrum and stereo-specificity towards $NAD(P)^+$ dependent reversible oxidation of primary and secondary alcohols and amines.[12] An example of an alcohol dehydrogenase catalysed reaction is oxidation of ethanol to acetaldehyde. A brief summary of different types of reactions catalysed by oxidoreductases is given in Figure 1-4.

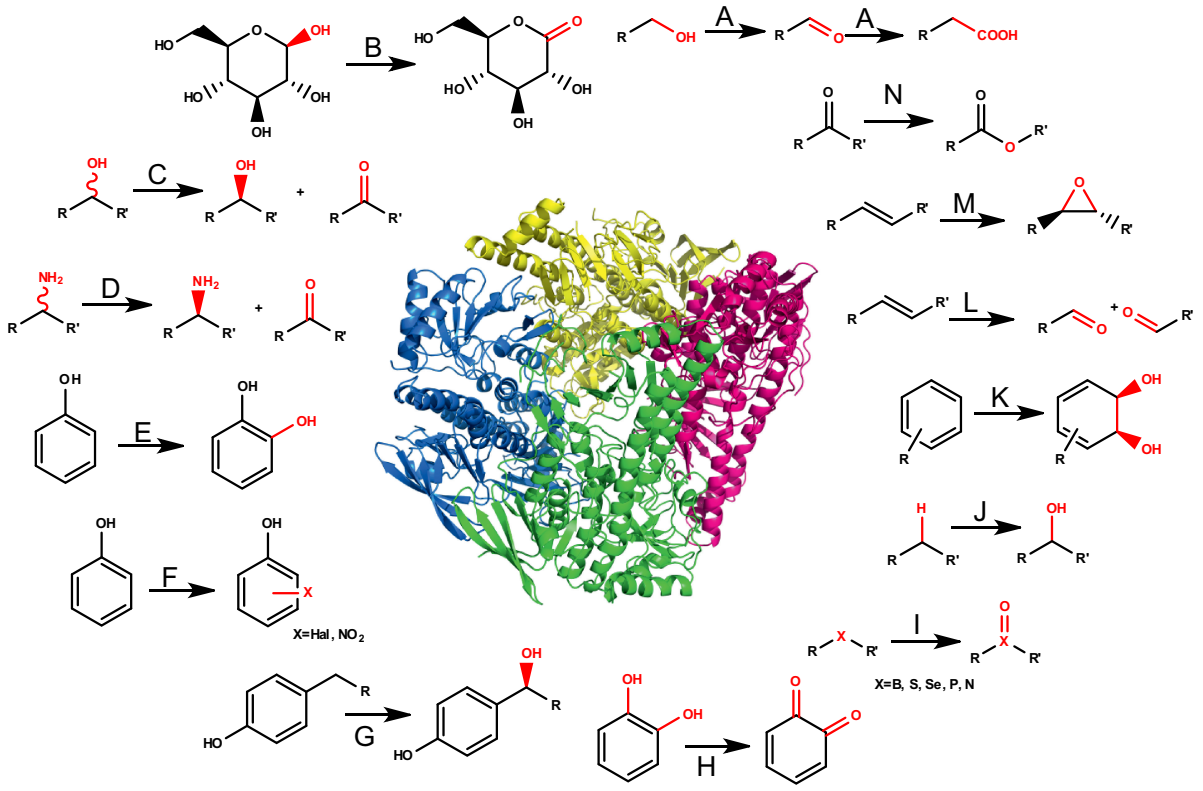


Figure 1-4: Selection of reactions catalysed by oxidoreductases (adapted after Hollmann et al. (2011)[12]). A – selective oxidation to the aldehyde/acid. B- regioselective oxidation of polyol. C – kinetic resolution of alcohols. D – kinetic resolution of amines and amino acids. E – hydroxylation of phenols. F - halogenation and nitration of phenols. G – oxidation of *p*-alkylphenols. H – oxidation of catechols. I – oxidation of heteroatoms. J – hydroxylation of non-activated C-H bonds. K – aromatic *cis*-hydroxylation. L – ozonolysis reaction. M – double bond epoxidation. N – Baeyer-Villiger oxidation. Structure depicted inside is the tetrameric form of 2-hydroxybiphenyl 3-monooxygenase.

2-hydroxybiphenyl 3-monooxygenase

2-hydroxybiphenyl 3-monooxygenase (HbpA) (EC 1.14.13.44) is a monooxygenase catalysing regioselective *ortho*-hydroxylation of 2-substituted phenols. It is a tetrameric enzyme with a mass of 256 kDa exhibiting broad substrate specificity,[41] and thus is an interesting biocatalyst for the production of 3-substituted catechols. Among many substrates (Figure 1-5) 2-hydroxybiphenyl is the most preferred one (V_{max} 3.5 U mg_{prot}^{-1}, k_{cat} 3.7 s^{-1} and K_m 2.8 µM).[42] Its reaction scheme is presented in Figure 1-6. HbpA belongs to the group of FAD dependent flavin monooxygenases[42-43] and requires NADH, molecular oxygen and the respective hydrophobic substrate for catalytic activity. The 3-substituted catechols, the reaction products, or their oxidised analogues (3-substituted quinones) are found in the structure of several naturally occurring compounds, namely miltirone, urushiol, barbatusol, and taxodione,[41a] and can serve as synthons in their synthesis.

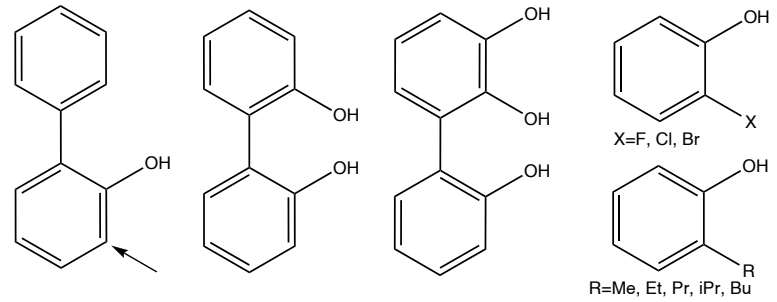


Figure 1-5: Substrate spectrum of 2-hydroxybiphenyl 3-monooxygenase. Arrow indicates the *ortho*- position at which hydroxylation takes place.[41]

To the best of our knowledge there is only one direct synthetic route that yields 2,3-dihydroxybiphenyl form 2-hydroxybiphenyl involving protection of the hydroxyl group by methoxymethyl (MOM) with a subsequent metalation, boronation, and final oxidation.[7a, 44] An alternative synthetic route starts with a 1,2 dimethoxybenzene and proceeds through the Suzuki reaction.[7b] The reported overall yield is 58% and 53% respectively which is good value for a 3-4 steps synthesis. HbpA has broad substrate spectrum,[20, 41b] and is therefore an interesting biocatalytic alternative for this *ortho*-hydroxylation reaction.

Application of isolated HbpA offers several advantages. The biocatalytic reaction may be uncoupled from the cell growth, and higher catalyst loadings are possible since the biocatalyst amount is not limited by gene expression and protein levels in the cell. All the oxygen available in the bioreactor can be used exclusively for the biotransformation and not the endogenous cell respiration. Additionally, there is no host toxification and growth inhibition by high substrate/product levels.[20] However, when applied in the isolated form, a continuous cofactor regeneration strategy needs to be implemented into the overall reaction scheme (see above).

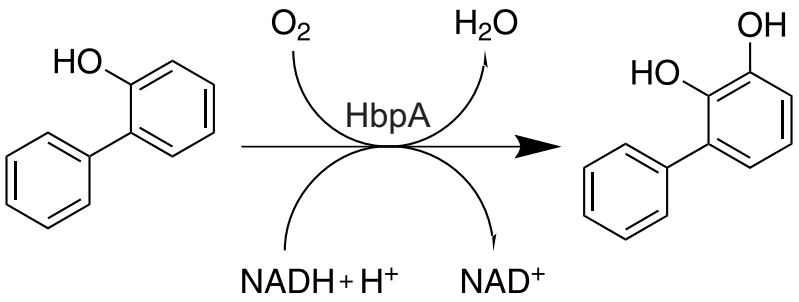


Figure 1-6: Reaction scheme of NADH dependent 2-hydroxybiphenyl 3-monooxygenase catalysed hydroxylation of 2-hydroxybiphenyl to 2,3-dihydroxybiphenyl (3-phenylcatechol).

MULTIPHASIC REACTIONS – ADVANTAGES, LIMITATIONS AND CURRENT APPLICATIONS

Introducing multiphasic systems

From the chemistry standpoint conducting reactions in pure organic solvents would be highly valued for many important reactions. Apart from simplified work-up of the reaction products, higher substrate loading would be possible and there would be no interfacial mass transfer limitations. Unfortunately, apart from a couple of examples of conducting biocatalysis in pure organic solvents, an additional aqueous phase is often required for enzymes to remain active or dissolve necessary cofactors or salts. There are two types of biphasic systems. One of them is a low water system where the organic phase constitutes for most of the volume and the residual water ensures the necessary mobility of the enzyme and proper interactions with counter-ions;[45] less than 1% water (v/v) is usually efficient.[46] The other is a conventional liquid/liquid two phase system (2LP) where aqueous and organic phases are added at different ratios and a clear interface can be distinguished.[47] Whether one or another is used depends on the type of the enzyme and the involved reactants.

Two phase systems may be beneficial for a given reaction, if either substrates and/or products are poorly soluble in the aqueous phase itself, substrates/products inhibit the enzymatic reaction, and/or synthesis is favoured over the hydrolysis.[48] A vast number of 2LP processes for biocatalysis were developed in the 1980s, using mainly lipases and proteases. There are examples of other enzymatic reactions in 2LP systems or in low water media for peroxidases, oxidases, dehydrogenases but the field was dominated by simple hydrolytic enzymes.[49] Those processes are listed and discussed by Halling (1987).[46] The reason for application of lipases instead of other enzymes was their availability, cofactor independency, solubility of their substrates in organic solvents, and the fact that they were highly active in non-aqueous media, which still poses an exception among known enzymes.[50]

Organic solvents may be categorized on the base of their $\log P_{o/w}$ value, which is a measure of their tendency to partition between the phases of different polarity,[51] and has been first introduced by Laane et al. (1987).[52] $\mathrm{Log}P_{o/w}$ is calculated as logarithm of the solubility ratio of a compound in octanol to its solubility in water. As a rule of thumb solvents with a $\log P_{o/w} < 2$ are not suited for biocatalysis as they interact with the water molecules essential for the enzyme, they tend to reside in water and therefore form a homogenous mixture (miscible solvents). Solvents with $\log P_{o/w}$ between 2 and 4 distort the water and affect the catalysis only to some extent, and the last group of solvents with a $\log P_{o/w} \geq 4$ do not interact with the

essential water coat of an enzyme and are therefore well suited for biocatalysis.[52] The $logP_{o/w}$ values of the most common solvents (over hundred) are listed therein.[52]

Beside the $logP_{o/w}$, the partition ratio, $K_{D,}$ of substrate and product between the phases should be considered when working with 2LP systems, for the enzyme has to be surrounded with the reactants for efficient catalysis. In most of the cases the hydrophobic substrates will be present mostly in the bulk organic phase[51] and enter the aqueous phase via continuous diffusion, whereas the hydrophobic products will be extracted after the reaction back into the organic phase. The organic phase thus serves as a substrate reservoir and product sink and helps to overcome problems caused by reactant solubility limits and/or substrate/product inhibition.[46] This situation is particularly favourable when the biocatalyst is inhibited by high substrate and/or product concentrations.

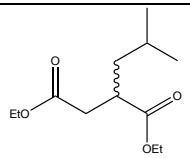
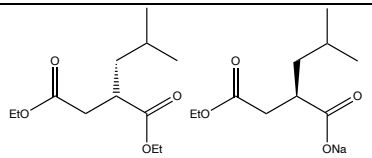
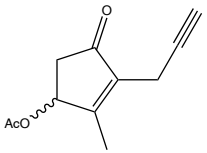
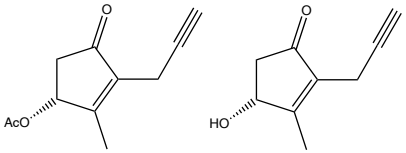
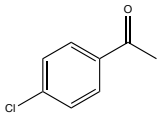
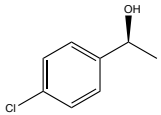
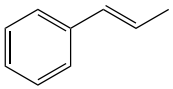
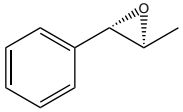
The solvent needs to be carefully selected so that it does not have an adverse effect on the biocatalyst. The interfacial effects i.e. a direct contact of the biocatalyst with the liquid/liquid interface[53] is a common shortcoming impairing enzyme stability (see below). Also the partitioning behaviour of substrates and products needs to be considered, such that the equilibrium concentration of the substrates (C_x^*), in aqueous phase is higher than the Michaelis-Menten constant value, K_m, for the enzyme to sustain catalysis, and the product equilibrium concentration in the aqueous phase should be lower than the inhibitory product concentration, K_i. Satisfying all above requirements is often difficult and usually a compromise has to be made between partially impaired biocatalyst and productivity.[48] For instance, when in a particular solvent system the substrate C_x^* is lower than the K_m value for that substrate, but the biocatalyst is stable and thus sustains longer reaction times leading to higher final conversions, the compromise of the reaction rate is acceptable. In that particular case one may increase enzyme concentration and compensate for the lower enzyme specific activity, when mass transfer between the phases is fast enough and the reaction is not mass transfer limited (see below).

Applying a 2LP for reactions catalysed by oxidoreductases is beneficial when their substrates display low water solubility. For example the epoxidation of 1,7-octadiene by *Pseudomonas oleovorans* whole-cell catalyst resulted in higher substrate conversions in the presence of a cyclohexane phase than in the respective pure aqueous system.[54] In the case of the production of iodine by immobilised lactoperoxidase the reaction suffered from a strong product inhibition. This problem was solved by a concomitant iodine extraction with an organic solvent.[55]

Biotransformations with soluble enzymes in an aqueous/organic two liquid phase system

As already mentioned, the application of soluble enzymes in an aqueous/organic two liquid phase system may have a deleterious effect on the biocatalysts. Cofactor dependent alcohol dehydrogenases and monooxygenases are often used with bovine serum albumin (BSA) as a stabilising agent because BSA is known to be a 'hard protein' that prevents interfacial inactivation of biocatalysts by replacing them at the aqueous/organic interface.[56] However, there are examples of biotransformations where no particular means of enzyme stabilisation was used and biocatalysts were applied in an aqueous/organic two liquid phase system for up to 192 h without activity loss[57] (Table 1-3). In this case, i.e. the conversion of cholic acid methyl ester to 12-ketochenodeoxycholic acid methyl ester catalysed by l2α-hydroxysteroid dehydrogenase, the reaction was limited by the mass transfer and/or equilibrium substrate concentration in the aqueous phase and not by the enzyme inactivation. Measured product formation rates were over 10 times lower in a 2LP system than in a respective single phase aqueous reaction with more hydrophilic substrate. Additionally, the high K_m value (0.47 mM) for cholic acid methyl ester, and its low equilibrium concentration in the aqueous phase in the beginning of the biotransformation (0.3 mM) limited the reaction. This example shows the importance of choosing the right organic phase with the appropriate physical properties, e.g. partition ratio of substrate/product between the phases. Most important aspects concerning the enzyme stability and maximising the mass transfer in 2LP systems will be discussed later (see below).

Table 1-3: Examples of biotransformations with soluble enzymes in aqueous/organic reaction systems.

Biocatalyst	Substrate	Product	Organic solvent	Time	remark	Ref.
Subtilisin Carlsberg *Bacillus sp.*			Substrate itself	48h	emulsion	[58]
Triacylglycerol acylhydrolase *Arthrobacter sp.*			Methanesulphonyl chloride	≥7h	-	[59]
Catalase	H_2O_2	H_2O+O_2	Potassium *tert*-butoxide	>1h	-	[14]
(S)-Alcohol dehydrogenase R. *erythropolis/* formate dehydrogenase *C. boidinii*			*n*-heptane	92h	-	[60]
l2α-hydroxysteroid dehydrogenase/ glutamate dehydrogenase (GlDH)	Cholic acid methyl ester	12-ketochenodeoxycholic acid methyl ester	Butyl acetate	192h	emulsion	[57]
β-hydroxysteroid dehydrogenase *P. testosterone/* lactate dehydrogenase	Testosterone	4-androstene-3,17-dion	Butyl acetate	50h	emulsion	[61]
P450 monooxygenase (CYP102A1)/ formate dehydrogenase *Pseudomonas sp. 101*	Cyclohexane	Cyclohexanol	Cyclohexane	100h	BSA 10 g L^{-1}, catalase 600 U mL^{-1}	[62]
Styrene monooxygenase *Pseudomonas sp. VLB120/* formate dehydrogenase *Pseudomonas sp. 101*			Dodecane	11h	emulsion, BSA 2 g L^{-1}	[63]

Enzyme stability – reasoning for stabilisation and various stabilisation methods

Although isolated enzymes are prone to O_2 oxidation, and susceptible to changing pH outside the cell environment, in this subsection only the effects of the organic solvents on the enzyme stability will be discussed. As mentioned above the presence of organic solvent in the reaction mixture may have a negative effect on the biocatalysts. Different mechanisms of biocatalyst inactivation should be distinguished from one another in order to decide for a successful stabilisation strategy. For example, hydrophobic organic species dissolved in the aqueous phase can inhibit or denature the enzyme by disrupting the hydrogen bonding of an enzyme and changing its native conformation.[64] However, interfacial inactivation, as reported for epoxide hydrolase,[65] may be more damaging to an enzyme than the presence of low concentrations of organic solvent in the aqueous phase. In this case interfacial inactivation can be minimised by applying surfactants like Tween 20 as proposed earlier.[66] However, since coverage of the aqueous/organic interface with surfactant is a dynamic process requiring time for distributing the surfactant molecules,[67] interfacial inactivation cannot be completely prevented.[68] Application of surfactants is one possibility, other techniques e.g. adsorption, covalent attachment, cross-linking, or embedding/entrapping the enzyme in a matrix are discussed below.

Enzyme immobilisation techniques

Although in the past most work on the enzyme immobilisation was done in order to render biocatalysts more suitable for application in water saturated organic solvent, e.g. to facilitate mixing and prevent inactivation in solvents with low $\log P_{o/w}$ values, or to simplify their recovery after complete reaction, here immobilisation is discussed mainly as a means of preventing interfacial inactivation in two liquid phase systems. Immobilisation techniques can be divided into carrier-bound and carrier-free methods, or entrapment. The former involves adsorption to a support or covalent attachment of an enzyme to a support material, whereas the carrier-free method does not require any support material and mainly involves direct cross-linking of the enzyme molecule. Irrespective of the method, immobilisation is carried out to create a protective environment around the enzyme and simplify its recovery and recycling. Enzyme immobilisation methods are summarised in Figure 1-7.

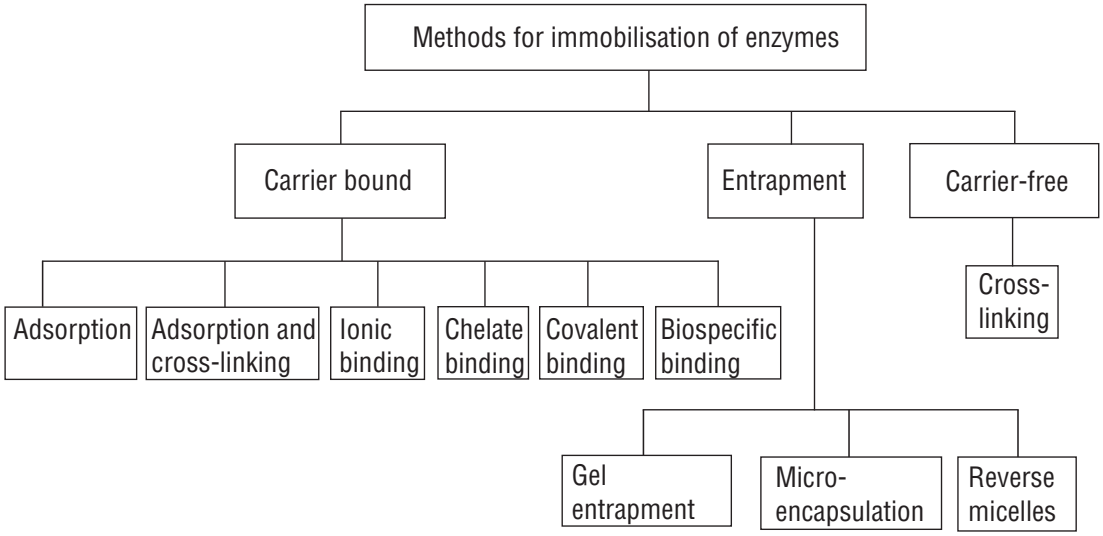


Figure 1-7: Immobilisation methods for enzymes. Adapted and modified from Přenosil et al (2000).[69]

Carrier-free immobilisation methods: Lipases were often 'stabilised' by precipitation from buffer by cold acetone, and subsequent drying with air and under vacuum, they exhibited a unique feature of functioning as a precipitate. Enzymes in the resulting powder were 'locked' in the right conformation, which means that the pH of the buffer that they were precipitated from had a great influence on their ionization state and hence their conformation. If the lipase was precipitated from the buffer with favourable pH and later assayed in the organic solvent it exhibited high activity, whereas when the pH of the buffer was not the optimal one for the reaction then it was also reflected in the subsequent assay conducted in organic solvent.[50] Those early methods of lipases preparation are similar to the methodology proposed by Cao and co-workers,[70] with the difference that the resulting powder was further cross-linked to provide the catalyst that could be used in 2LP systems. This method provided a basis for developing cross-linked enzyme aggregates (CLEAs). These catalysts were easy to prepare,[1] could be used in 2LP systems, and were easily separated from the reaction products and recycled.

Carrier-bound immobilisation methods: Another common technique for increasing operational stability of enzyme is adsorption onto an activated matrix e.g. Sepharose, glass beads or ionic binding with an ion exchange resin like DEAE. For more thorough discussion on various enzyme immobilisation techniques, see a monograph by Cao (2006).[71]

The choice of the immobilisation method needs to be driven by the enzyme and reaction design. For instance, adsorbed and covalently bound enzymes are much less leaky in aqueous solutions than non-covalently bound ones, but on the downside their activity may be impaired due to structural changes. If leaking of the enzyme is not possible because it is used in a low-water system adsorption itself may be sufficient. Another advantage of simple adsorption is

the ease of recovery of carrier-material, as immobilised biocatalyst will be released after subsequent washing steps with an aqueous solution.

Entrapment: Entrapment in the polymeric matrix is yet another method of preparing insoluble biocatalysts. It is based on the principle that a solubilised or dispersed enzyme is brought to contact with a reagent that causes a spontaneous solidification/gelation of the matrix thus, resulting in the entrapment of the enzyme.[72] An example of such method is gelation of sodium alginate caused by Ca^{2+} ions and successful immobilisation/encapsulation of glucose oxidase,[73] or entrapment of 2-hydroxybiphenyl 3-monooxygenase and formate dehydrogenase in a gelatine/chitosan matrix stabilised by crosslinking with glutaraldehyde.[74] Application of a hydrophilic polymer like alginate, gelatine or chitosan, and subsequent preparation of the immobilised biocatalyst will affect the environment around an enzyme making it hydrophilic by retaining water, therefore despite the immobilisation enzymes are entrapped in an aqueous-like environment and can be successfully applied in a 2LP system up to 50 hours.[74]

Mass transfer

The mass transfer is the transfer of a species (mass) from a region of high concentration towards the region of low concentration until the equilibrium is established between the two regions. Molecular and convective transport are distinguished.[75] The molecular transport of molecules is driven by diffusion i.e. movement of particles by Brownian motion.[76] As proposed earlier,[77] in diffusional mixing one may define the mean transport length as $l = \sqrt{2Dt}$, where l is the distance a particle moves in a time t, and D is its diffusion coefficient. With typical diffusion coefficients D in liquids of 10^{-9} m^2 s^{-1} one may calculate the time the particle requires to travel 1 mm to be 500 s. In this case diffusion would not be enough to provide sufficient mixing. Fortunately mass transfer can be additionally improved by the bulk flow of the fluid, which is called convective mass transport.[75] The overall mass transfer with respect to diffusion and convection can be given as:

Equation 1-1

$$N_x = k_L(C_X{}^* - C_X)$$

Where, N_x is the flux of the component 'x' over the interface, k_L is the mass transfer coefficient (cm s^{-1}), $C_x{}^*$ is the concentration of 'x' in the liquid phase at saturation, and C_x is the actual liquid concentration of 'x'. Mass transfer is an important issue in 2LP reaction systems,[46] independent of the reactor format applied. In those systems, interfacial surface area is often difficult to be determined experimentally therefore, the mass transfer equation of the component 'x' over the interface is often given as:

Equation 1-2

$$dC_X/dt = k_L a\ (C_X{}^* - C_X)$$

Where a is a surface area per liquid volume ($cm^2\ cm^{-3}$), hence, the product of k_L and a is called the volumetric mass transfer coefficient (k_La) and has a unit of reciprocal of time (s^{-1}). Amongst various reaction systems the k_La is higher for microchannel based systems, than for shake flasks or stirred tank reactors (Table 1-4). Within given examples, microchannel reactors profit mainly from a large interfacial area and short diffusional distances, and compared to other reactors provide higher mass transfer rates, which subsequently facilitate fast reactions, which rely on substrate/product transport across the interface, and would otherwise be mass transfer limited. More examples of mass transfer characteristics of microstructured reactors can be found in literature.[78]

Table 1-4: Mass transfer coefficients and interfacial area in typical reaction formats.

Reaction format	Interfacial area ($m^2\ m^{-3}$)	Overall mass transfer coefficient k_La (s^{-1})	Reference
Shake flask			
Baffled (40% filling)[a]	not available	0.0164±0.0021	[79]
Non-baffled (40% filling)[a]	not available	0.0085±0.0019	[79]
Baffled (10% filling)[b]	not available	0.083	[80]
Non-baffled(10% filling)[b]	not available	0.033	[80]
Stirred tank[c]	not available	0.01-0.05	[81]
Static mixer[d]	100-1000	0.1-2.5	[82]
Microchannel			
Gas-liquid microchannel[e]	3400-9000	0.3-21	[83]
Liquid-liquid microchannel[f]	590-4800	0.13-0.98	[84]

[a] gas/liquid mass transfer – 250 mL open shake flask, 100 mL liquid, 250 rpm, 37°C

[b] gas/liquid mass transfer – 125 mL shake flask, 12,5 mL liquid, 250 rpm, 37°C

[c] gas/liquid mass transfer – 1.8 L water, agitation 100-1000 rpm, aeration 1-5 $L_{air}\ min^{-1}$, different impellers

[d] gas/liquid mass transfer – horizontal pipe, 52.6 mm in diameter, with static mixers., interfacial area without a mixer was 15-40 $m^2\ m^{-3}$.

[e] gas/liquid mass transfer – rectangular channel 1 mm in depth, 0.5 mm in width

[f] liquid/liquid mass transfer, extraction of iodine from water by kerosene, capillary inner diameter 0.5-1 mm.

Application of enzymes in microstructured reactors

Microstructured reactors were introduced in the late 70' as devices for flow injection analysis (FIA) and continuous segmented flow analysis.[85] They can be regarded as precursors of immobilised enzyme reactors used for high throughput sample analysis under continuous flow

conditions. Miniaturising the dimensions of the reactors and conducting reactions in microchannels results in lower diffusional distances and better mixing within shorter time. In microchannels when two immiscible liquids flow one after another in an ordered fashion often referred to as segmented, bubble, or slug flow the internal circulations within the segment, so called Taylor vortices, which promote axial mixing, can be observed.[86] Taylor vortices can be seen in liquid/liquid, or liquid-gas flows, and arise from the shear force between the liquid and the channel wall and between two consecutive slugs (Figure 1-8). Intense internal circulations promote mixing within the slugs and are therefore responsible for large enhancement of the interfacial mass transfer rates in liquid/liquid slug flow reactors.[87] Because of two independent and controllable transport mechanisms i.e. convection and diffusion, microchannels (microreactors, microstructured reactors) provide excellent conditions for enhancing the performance and tuning of mass transfer limited reactions.[87]

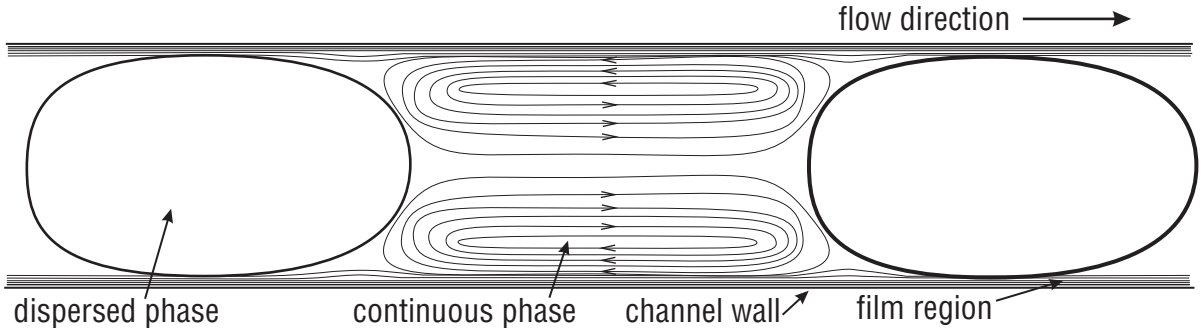


Figure 1-8: Taylor flow pattern in a two-liquid flow in a microreactor.

Mass transfer intensification and enhanced interfacial area over conventional stirred tank reactors are main advantages of microreactors[88] (Table 1-4), additionally precise control of the temperature and the reaction times is possible.[89] They enable rapid screening of reaction conditions and generation of compound libraries with minimised reagents or enzymes consumption.[90] Other advantages of running the reactions in flow are faster reaction rates, less waste generation, lower solvent usage, simplified downstream processing due to lack of emulsion formation, or possibility of running sequential reactions without the need of intermediate purification[91] (and references therein). Many examples of chemical processes in flow and examples of process intensification with respect to conventional reactors have been discussed by Ley (2012).[91] Also enzymatic flow reactors have been successfully implemented in small scale synthesis (Table 1-5). Many reviews have been published on the topic.[76, 92]

Table 1-5: Examples of two liquid phase soluble enzyme processes in microreactors. Adapted from Bolivar and Nidetzky (2013).[88]

Reaction	Enzymes	Phases	Residence time [h]	Reactor volume [mL]	STY [g L^{-1} h^{-1}]	Remark	Reference
Synthesis of isoamyl acetate	*C. antarctica* lipase B (Lipozyme CALB L) adsorbed on the interface	1-butyl-3-methylpyridinium dicyanamide/n-heptan	0.56	0.155	174	Reaction rate in liquid/liquid two phase Taylor flow in microchannel 3-fold higher than in a batch reaction. Emulsion formation observed.	[93]
Degradation of *p*-chlorophenol	*Trametes* sp. Laccase adsorbed on the interface	Isooctane/succinic buffer	0.0056	0.0005	1.26[a]	Liquid/liquid two-phase parallel flow. Conversion and efficiency increased with respect to gently shaken batch reaction.	[94]
Reduction of 1-heptaldehyde	Soluble thermophilic alcohol dehydrogenase and formate dehydrogenase	Hexadecane/potassium phosphate buffer	1.75	37.6	5.2	Liquid/liquid two-phase segmented flow.	[95]
Synthesis of cyanohydrins from respective aldehyde	Soluble (S)-*Hevea brasiliensis* hydroxynitrile lyase	MTBE/aqueous buffer	Up to 0.5	0.0016	404[b]	Liquid/liquid two-phase parallel flow. Screening of reaction parameters.	[96]
Asymmetric reduction of $\alpha_l\beta$ unsaturated activated alkenes	Soluble pentaerythritol tetranitrate reductase	Isooctane/potassium phosphate buffer	0.33	not specified	8.02[c]	Liquid/liquid two-phase drop flow.	[97]

[a] calculated for dehalogenation of *p*-chlorophenol with a 20 sec residence time at a flow rate of 1.5 µL min^{-1} resulting in 65% conversion of 100 µM solution.

[b] calculated for the conversion of benzaldehyde (230 mM) to 2-hydroxy-2-phenylacetonitrile with 2.5 min residence time and 55% conversion

[c] calculated for the reduction of 2,6,6-trimethylcyclohex-2-ene-1,4-dione (20 mM) to 2,2,6-trimethylcyclohexane-1,4-dione with 20 min residence time and 88% conversion.

The choice of the reactor system necessitates careful investigation of the respective reaction parameters. Karande *et al.* (2011)[95] proposed to use the Damköhler number which describes the relation between the mass transfer rate and the reaction rate (Equation 1-3),[95] as a decision basis.

Equation 1-3

$$Da = \frac{k_{cat}\, e_0\, \varphi}{k_L a\, C_x^*}$$

k_{cat} is the catalytic turnover frequency, e_0 is the enzyme concentration, φ is the phase ratio, k_La is the overall volumetric mass transfer coefficient, and C_x^* is the equilibrium substrate concentration in the aqueous phase. $Da = 1$ implies a reaction rate equal to the mass transfer rate, $Da < 1$ points towards a kinetically limited reaction and for $Da > 1$, the reaction is mass transfer limited and the turnover frequency of enzymes (k_{cat}, s^{-1}) is no longer used effectively. Shifting a system from the kinetic limited regime into the mass transfer controlled regime is done by increasing enzyme loading or by reducing substrate concentration in the organic phase while keeping all the other parameters constant. By assuming that $Da = 1$, one may calculate the k_La below which reaction will be mass transfer limited and decide whether conducting the reaction in microreactors makes sense or if the mass transfer will be fast enough also in a shake flask or stirred tank reactor.

Oxygen availability in microreactors

Oxygen is a critical parameter when working with oxidoreductases. There are three main strategies of delivering oxygen for the reaction in microreactors, it may be dissolved in liquids, delivered in a gas segment next to an aqueous or organic segment, or continuously perfused through a gas permeable membrane (Figure 1-9).

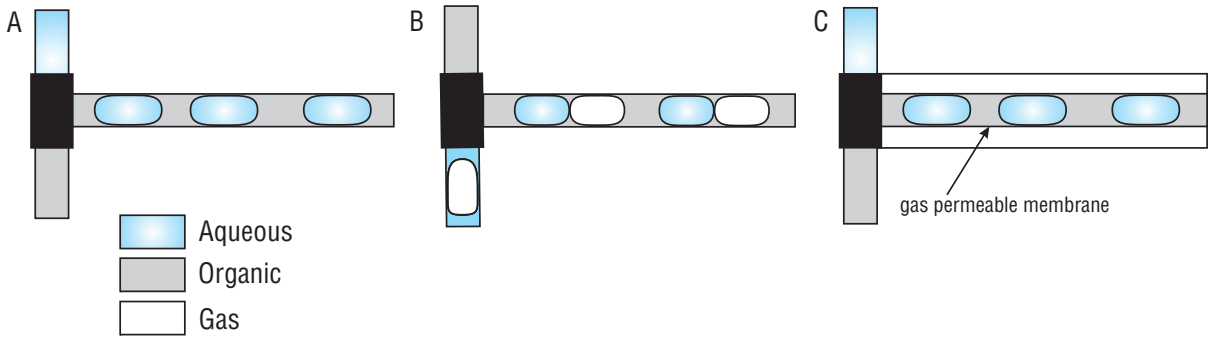


Figure 1-9: Modes of gas delivery for the reaction in microreactors. A – Gas dissolved in liquids. B – Gas delivered in a separate gas segment. C – Gas continuously delivered through a gas permeable membrane.

Depending on the chosen method, the amount of oxygen present in the reactor will be different and will affect the maximum reaction time after which all the oxygen is consumed.

The first two methods (Figure 1-9AB) are one-point-delivery methods, meaning that the amount of oxygen is finite and maximum at the point of inlet, and is continuously consumed along the reactor length, thus defining the maximum reactor length. Maximum product concentration cannot exceed the initial concentration of oxygen, therefore with a fixed reactor volume, enzyme concentration and product formation rate, the residence time is limited by the oxygen concentration. The third one (Figure 1-9C) is a continuous method where the concentration of oxygen is constant in the whole reactor as oxygen is delivered through the gas permeable membrane throughout the overall reactor length. The advantage of the third method is obvious, it provides unlimited amount of oxygen, the residence time is independent of the initial oxygen concentration allowing for higher conversions at longer residence times, and also the reaction conditions with respect to oxygen are constant in the whole reactor. When lower conversions, not exceeding initial concentration of oxygen are acceptable, and the effect of decreasing concentration of oxygen, as the reaction progresses, is negligible, it is still possible to use air (or pure oxygen) segments at an atmospheric or higher pressures for oxygen supply. The first two methods do not require specialized membranes and can be readily applied in a single channel microreactor without any modifications. Absolute concentrations of oxygen in microreactors with different modes of delivery are listed in Table 1-6.

Table 1-6: Oxygen concentration in microreactors at 30°C and total pressure of 101.325 kPa depending on the mode of delivery. Concentrations are calculated for water as aqueous phase and decanol as organic phase. Respective phase ratios are given in parentheses.

Mode of delivery	Concentration at O_2 partial pressure 21.28 kPa. (0.21 atm) [mM][a]	Concentration at O_2 partial pressure 101.325 kPa. (1 atm) [mM][b]
Dissolved in liquids, (aq:org 1:1)	0.89 *initial*	4.26 *initial*
In segments, (aq:org:gas 1:1:1)	3.34 *initial*	15.89 *initial*
Through membrane, (aq:org 1:1)	0.89 *constant*	4.26 *constant*

[a] Concentration of oxygen at 30°C and 101.325 kPa (1 atm) total pressure in water, decanol, and air is 0.22 mM, 1.35 mM,[98] and 8.44 mM respectively (with oxygen having a molar fraction of 0.21 in air and a molar volume of 24.87 L mol^{-1}).

[b] at higher total pressures exceeding 1 atm oxygen concentrations will be proportionally higher.

CHAPTER'S SUMMARY

From the above it can be seen that noteworthy work has been done in the field of enzymatic microreactors. Unfortunately most of the enzymatic processes in such systems are based on the usage of proteolytic enzymes immobilised on the microchannel walls or glass beads filling the channel and used for protein or peptide digestion.[92f] Examples listing other enzymes, some of which are used in multiphasic systems also exist.[99] However, application of soluble enzymes in the multiphase microreactors is still scarce (Table 1-5). Motivations for moving away from batch reactors towards miniaturised systems are obvious: increased reaction rates in phase transfer catalysis due to higher mass transfer rates, better control of the reaction time and reaction temperature, or minimised reactants usage.[88] Although methods for multiphasic chemical catalysis are well established,[91, 100] and advantages of running reactions in microreactors, are obvious, transferring this knowledge for biocatalysis is challenging and careful investigation of the reaction parameters (educts concentration and supply, (bio)catalyst stability, solvent selection etc.) is needed. In series of experiments a set of boundaries/constraints like e.g. suitable reactor format, educts concentrations, residence times, and minimum product concentrations, can be defined.

AIMS OF THE THESIS

This thesis aims at applying 2-hydroxybiphenyl 3-monooxygenase (HbpA) and formate dehydrogenase (FDH) in a two-liquid phase system in a segmented flow microreactor for continuous production of 2,3-dihydroxybiphenyl (3-phenylcatechol). Gram-scale preparation of the soluble HbpA and FDH by expanded bed anion exchange chromatography or ammonium sulphate precipitation, and their characterisation with respect to the storage stability, and operational stability in contact with second organic phase is shown in chapter 2.

Chapter 3 summarises the preparation of Cross-Linked Enzyme Aggregates (CLEAs) of HbpA and FDH, which were prepared in order to stabilise the biocatalysts that proved susceptible towards the aqueous/organic interfaces. Their application in the two-liquid phase system is presented therein. Also the crystal structure and the implication of the surface residues and domain mobility which might influence efficiency of CLEA methodology are discussed.

Chapter 4 is a proof of concept and shows an alternative, low water system for NADH regeneration with formate dehydrogenase and formic acid ester. This novel cofactor regeneration concept enables using high organic to aqueous phase ratios which facilitate solubilising of high amounts of usually hydrophobic substrates. The formic acid ester serves also for a continuous product removal.

Chapter 5 aims at application of soluble HbpA and FDH in a segmented flow microstructured reactor. Two different reactor concepts, namely aqueous/organic/air three-liquid phase microreactor, and tube-in-tube microreactor with a gas permeable Teflon AF-2400 membrane are compared. HbpA-catalysed reaction is discussed with respect to the cofactor regeneration, hydrophobic substrate supply and oxygen supply. Tube-in-tube microreactor is later chosen for continuous preparative gram scale synthesis of 2,3-dihydroxybiphenyl described in chapter 6, which also touches the downstream processing. Ultimately the chapter 6 gives a proof of concept of applying an ePFTE membrane for constructing TiTR for achieving complete aqueous, organic, and gas phase separation. This setup is later discussed with respect to its potential for later scale-up purposes (Chapter 7).

CHAPTER 2

PREPARATION AND CHARACTERISATION OF 2-HYDROXYBIPHENYL 3-MONOOXYGENASE (HBPA) AND FORMATE DEHYDROGENASE (FDH)

Bartłomiej Tomaszewski, Katja Buehler, Andreas Schmid

Bartłomiej Tomaszewski performed the experiments and wrote the manuscript, Katja Buehler, and Andreas Schmid coordinated and supervised the project and corrected the manuscript.

ABSTRACT

Application of isolated enzymes requires established protocols for their production and purification. Moreover providing means of enzyme stabilisation for *in-vitro* reactions is of equal importance. Here methods are presented for production and purification of two enzymes 2-hydroxybiphenyl 3-monooxygenase (HbpA), and formate dehydrogenase (FDH). These biocatalysts were characterised in terms of storage and reaction stability. Both enzymes have been produced in recombinant *E. coli* JM101, subsequently purified/enriched by means of a single step expanded bed adsorption chromatography and stored in 50% glycerol for months without losing activity. However, when applied to a two liquid phase system comprising of n-decanol and buffer, in a capillary microreactor, stability was poor. Addition of the surfactant Tween 20 stabilised the enzymes in the presence of the organic solvent and it was possible to recover 100% of the respective enzymatic activity. This set the stage for enzymatic synthesis of 3-phenylcatechol using aqueous/organic two-liquid phase segmented flow microreactors.

INTRODUCTION

Biocatalytic hydroxylation of non-activated carbons is an important tool for synthesis of valuable compounds, either intermediates or final products. Their chemical equivalents often do not exist, or require multiple synthetic steps; therefore every new application of a whole-cell biocatalyst or a soluble enzyme in this field unravels a way to a new, attractive product.

2-hydroxybiphenyl 3-monooxygenase (HbpA) is a flavin dependent monooxygenase, which catalyses synthesis of 3-phenylcatechol from 2-hydroxybiphenyl in *Pseudomonas azelaica* HBP1[41b] (Chapter 1, Figure 1-6). Synthesis of catechols is difficult in abiotic conditions and biocatalysis presents a green-alternative. HbpA has already been used for the gram scale synthesis of 3-substituted catechols in recombinant *E. coli*,[20, 41a, 101] and in soluble form in a 2-liquid phase system.[21, 102] As HbpA is dependent on the cofactor NADH, a continuous supply thereof is required. Due to cost reasons, it cannot be added in stoichiometric amounts. Therefore various strategies for the turnover of NADH have been developed of which utilisation of the intrinsic host cell metabolism is by far the easiest one. In case of isolated enzymes, application of formate dehydrogenase FDH has been widely established (see also chapter 1). Although successful, yielding gram amounts of product, previous processes[21, 102] suffered from low productivities (up to 0.46 $g\,L^{-1}\,h^{-1}$) believed to be limited by the mass transfer, catalyst loading, and/or oxygen supply.[21, 102] Therefore, application of soluble HbpA and FDH in microstructured reactors may be a possibility to relieve these bottlenecks as such systems are characterised by high mass transfer rates.[87] To evaluate the applicability of capillary microreactors for the HbpA-catalysed reaction, significant amounts of enzymes were needed. The most commonly used host for the preparation of heterologous proteins is *E. coli*. It is easy to cultivate and depending on the chosen expression system produces high levels of heterologous protein (up to 40%-50% of the total cell protein).[103] Although there are means of protein secretion from the *E. coli* host, translocated proteins are often insoluble due to the presence of the respective signal sequence,[104] therefore the majority of heterologous proteins are produced intracellularly and need to be purified from disrupted cells. The cell homogenate obtained after cell disruption contains cell debris, intact cells, and soluble cell components, all of which need to be separated to obtain a protein solution of defined purity and activity [105]. Solid particles may be removed by centrifugation or filtration, and further protein purification may be realised by chromatography, ultrafiltration,[106] or fractional precipitation with ammonium sulphate.[107] Separation of solid debris and the subsequent chromatography step may be combined in a one-step expanded bed adsorption chromatography.[105, 108] The advantage of that method is the reduction of unit operations required for the primary purification of proteins. In the obtained protein solution the target protein is usually enriched

by a factor of up to 15 times.[108] What distinguishes it from a typical packed bed chromatography is the fact that the bed is not constrained and can expand before and during sample loading (Figure 2-1). It allows cell debris to pass through the column without being trapped and clogging the column. Charged particles (e.g. proteins) interact and adsorb onto the column matrix. Cell debris and loosely bound proteins are removed in the consecutive washing step. Flow is then reduced allowing the bed to settle and the sample is eluted in a reverse (downward) flow.[105]

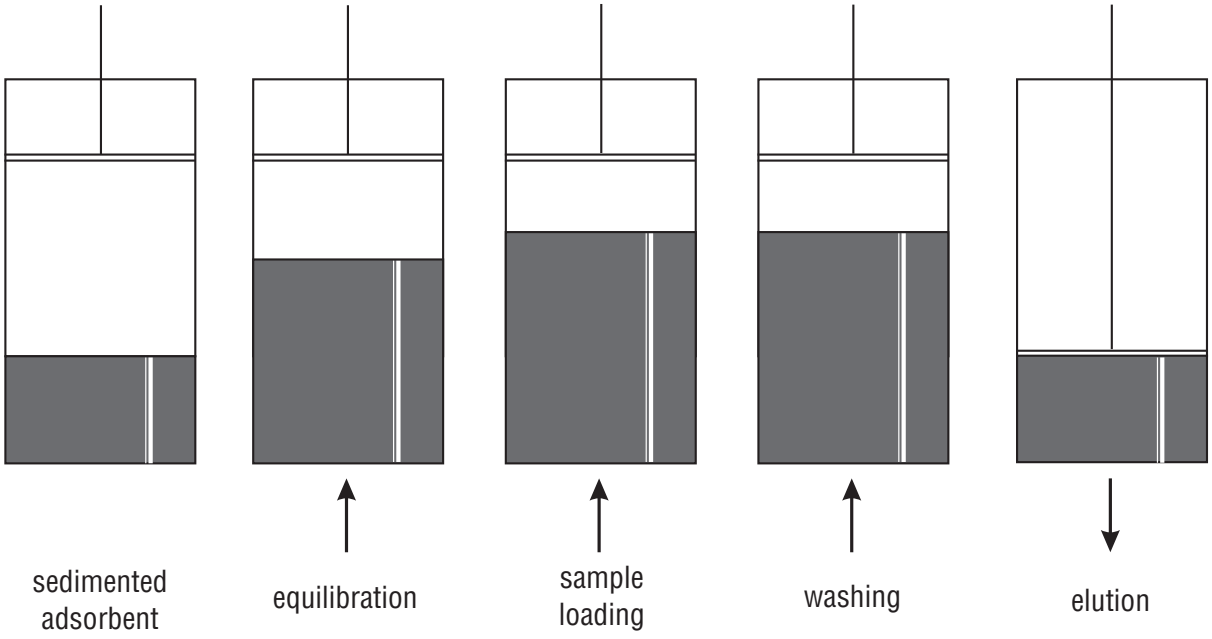


Figure 2-1: Schematic presentation of consecutive steps of expanded bed adsorption chromatography.

After appropriate selection of a column matrix and adjustment of the elution method, expanded bed adsorption chromatography may yield almost pure (95%) protein preparation, which has been shown for the purification of the formate dehydrogenase.[109]

Biocatalysts are subjected to denaturation in the environments other than the cell if appropriate precautions (stabilisation) are not taken. It is therefore important to determine the stability of the biocatalysts under reaction conditions and provide necessary means of stabilisation. Different stabilisation methods as well as the reasoning for application of enzymes in multiphasic microreactors are discussed in details in chapter 1.

In this chapter the ways to produce and purify 2-hydroxybiphenyl 3-monooxygenase (HbpA), and formate dehydrogenase (FDH) are described. Moreover the biocatalysts are characterised regarding storage and reaction stability and its performance in a 2-liquid phase system.

MATERIALS AND METHODS

Chemicals

NADH disodium salt and 1-decanol were obtained from Applichem (Darmstadt, Germany), sodium formate, NAD^+ disodium salt and FAD from Fluka (Buchs, Switzerland), 2-hydroxybiphenyl, and 2,3-dihydroxybiphenyl from Wako Pure Chemical Industries Ltd. (Osaka, Japan). All other chemicals have been bought either at Fluka, Applichem or Sigma-Aldrich and used as received.

Media

If not stated otherwise either LB supplemented with 150 mg L^{-1} ampicillin or M9 minimal medium supplemented with 0.5% glucose (v/v), 150 mg L^{-1} ampicillin, 0.001% (w/v) thiamine, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$ and 1 mL L^{-1} of US* trace elements solution, prepared as described previously,[110] was used for cultivations.

Bacterial strains and plasmids

Table 2-1: Bacterial strains and plasmids used in this work.

Strain or plasmid	Characteristic	Source or reference
Strains		
E. coli JM109	*rec*A1 *sup*E44 *end*A1 *hsd*R17 *gyr*A96 relA1 thi Δ(lac-proAB) F' [traD36 proAB$^+$ lacI^q lacZ ΔM15]	[111]
E. coli JM101	supE thi Δ(lac-proAB) F' [traD36 proAB$^+$ lacI^q lacZ ΔM15]	[111]
Plasmids		
pHBP461	pUCBM20 derivative carrying *hbpa* containing cassette (3.2 kbp) cloned into the *SalI* restriction site. Amp^R.	[101]
pBTac2 (C23S)	cloning vector with pBR322 origin carrying a modified (C23S) *fdh* gene. Amp^R, Tet^R	[112]

Cultivation and biomass production

2-hydroxybiphenyl 3-monooxygenase HbpA

For optimal HbpA production, *hbpA* was expressed in *E. coli* JM101 carrying the plasmid pHBP461.[101] 1st pre-culture was grown overnight (30°C, 200 rpm) in a Multitron orbital shaker (Infors HT, Homberg, Germany) in 5 mL LB medium and was subsequently used to inoculate 100 mL of M9 medium (2nd pre-culture) in a shake flask. Batch cultivation was started by inoculation of 1.7 L M9 medium in a 3L stirred tank bioreactor (Bioengineering AG, Wald, Switzerland) with 5% (v/v) of the 2nd pre-culture. Cells were grown in batch mode (30°C, 1000 rpm, aeration 60 L h^{-1}) until all glucose (15 g L^{-1}) was completely consumed (approx. 7-8h). Fed batch was started with glucose exponential feed (50% glucose (v/v),

80 mM $MgSO_4$) to a growth rate of μ=0.1-0.12 h^{-1}. To ensure plasmid retention, ampicillin was added once again (150 mg L^{-1}) at the beginning of the fed batch and then every 4h. At 7.5 g_{CDW} L^{-1} cells were induced with 1 mM IPTG and cultivated until finally 25 g_{CDW} L^{-1} were reached. Supernatant was removed by centrifugation at 4000 *g* for 20 min at 4°C in a Heraeus Multifuge 1 S-R bench top centrifuge (Thermo Fischer Scientific, Langenselbold, Germany) and the obtained cell paste was kept at -20°C until further use. During the growth pO_2 level was kept above 20% by controlling stirrer speed and aeration.

Formate dehydrogenase FDH

Formate dehydrogenase (FDH) C23S was produced in *E. coli* JM101 pBTac2 (C23S)[112a] similar as described above for HbpA.

Preparation of HbpA

HbpA purification protocol was adapted from previously published work.[21, 102] HbpA was enriched in a single step by expanded bed adsorption (EBA) chromatography (Labomatic Instruments, Allschwil, Switzerland) on a Streamline 50 DEAE anion exchange column (100 cm high, 5 cm inner diameter, total 2L volume) (Amersham Bioscience, Uppsala, Sweden). 140 g of *E. coli* JM101 pHBP461 cell paste was resuspended to 20% (w/v) in 10 mM Tris-HCl, pH 7.5, containing 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$ and DNaseI and left to thaw. Cell disruption occurred via one to two passages through a French Pressure Cell Press Unit (cell volume 40 mL) (Aminco SLM Instruments, Urbana, IL, USA). The resulting homogenate was spun at 15000 *g* for 40 min at 4°C in a SLA-1500 rotor on a Sorvall RC 5B PLUS Superspeed Centrifuge (Thermo Fischer Scientific, Langenselbold, Germany). Clear supernatant was collected and its conductivity was adjusted to ≤5 mS/cm with Seralpur Pro 90 CN water (Seral Druck, Ransbach-Baumbach, Germany). The cell extract was stored on ice until being loaded onto the column.

The Streamline 50 column filled with 510 mL Streamline DEAE anion exchanger material (Amersham Bioscience, Uppsala, Sweden), was equilibrated (1.5 CV of 20 mM Tris-HCl, pH 7.5) at room temperature with an upward oriented flow (200 cm h^{-1}) (Watson Marlow 502S pump) forming a stable expanded bed with a 2.2-fold expansion. Sample was loaded with the same flow rate and then the column was washed with 20 mM Tris-HCl until a clear flow-through liquid was collected. The flow rate was decreased (45 cm h^{-1}) to allow the bed to settle, and elution was started in a packed bed mode with a downward oriented flow of 120 cm h^{-1} (3 CV of 5 mM NaCl in 20 mM Tris-HCl) followed by a NaCl gradient from 5 mM to 280 mM over 3 CV until finally the NaCl concentration was kept constant (1.5 CV, 280 mM NaCl in 20 mM Tris-HCl). 100 mL fractions were collected. Due to the bound FAD,

HbpA containing fractions were identified by the 450 nm signal on UVIS-205 Absorbance Detector (Linear Instruments, Reno, NV, USA). Enzyme activity was measured using a standard spectrophotometer based assay (see below) and fractions containing the highest HbpA activity were pooled and either freeze-dried or concentrated by ultrafiltration through a 30 kDa filter paper in an Amicon Stirred Cell 8400 (Merck Millipore, Billerica, USA). The obtained HbpA concentrate was mixed with glycerol at 1:1 (v/v) ratio and stored at -20°C. The obtained protein preparation was of technical purity (approx. 40-50%). Such prepared HbpA could be used for at least a year without losing activity.

Preparation of FDH

Formate dehydrogenase (FDH) of technical purity was purified similarly as HbpA, modifying the EBA chromatography elution step. After the first 2 CV of 20 mM Tris-HCl, the NaCl concentration was set to 160 mM for 1.5 CV, followed by 1 CV of 220 mM NaCl and finally 1 CV of 280 mM NaCl. Enzyme detection was monitored by recording the 280 nm signal. Enzyme activity was measured using a standard spectrophotometer based assay (see below) and fractions containing the highest activity were pooled, ultrafiltered through a 30 kDa filter paper, mixed with glycerol at 1:1 (v/v) ratio and stored like HbpA. No activity loss was observed at these storing conditions for at least a year.

Purification of HbpA and FDH by precipitation with ammonium sulphate

An alternative purification method for HbpA and FDH by ammonium sulphate precipitation was also established. It was quicker than the EBA chromatography and minimised consumption of the buffers used for the elution from the DEAE column. For example, 1.08 g of *E. coli* JM101 pHBP461 cell paste was resuspended in potassium phosphate buffer (50 mM, pH 7.5, up to 4 mL). A sample was passed once through the French Press, and the resulting cell lysate was centrifuged (40 min, 4°C, 12300 *g*) in a Sorvall RC 5B PLUS Superspeed Centrifuge (Thermo Fischer Scientific, Langenselbold, Germany). 3 mL of the supernatant was removed and combined with 2.09 mL of saturated ammonium sulphate solution (51.4% w/v, 0°C) for reaching 41% saturation at 0°C. It was mixed by inverting and incubated on ice for 30 min. A sample was centrifuged and 4.75 mL of the supernatant was recovered. Similarly, 1.25 g of *E.coli* JM101 pBTac2 (C23S) cell paste was resuspended in potassium phosphate buffer (50 mM, pH 7.5, up to 4 mL). A sample was passed once through the French Press, and the resulting cell lysate was centrifuged (40 min, 4°C, 12300 *g*) in a Sorvall RC 5B PLUS Superspeed Centrifuge. 3 mL of the supernatant was removed and combined with 3.95 mL of saturated ammonium sulphate solution (51.4% w/v, 0°C) for reaching 56% saturation at 0°C. It was mixed by inverting and incubated on ice for 30 min.

Sample was centrifuged and 6 mL of supernatant was recovered. Alternatively if the initial mass of the cell pellet was greater, instead of adding a saturated solution of ammonium sulphate, one may add it in a solid form, for example to bring 3 mL of unsaturated solution to 41% saturation (at 0°C) 0.71 g of salt has to be added, or for reaching 56% saturation (at 0°C) in 3 mL sample 1.02 g of solid ammonium sulphate has to be added.[107]

For preparative scale purification the resulting supernatant was diluted at least 3 times with 100 mM KPi buffer (pH 7.5) and ultra-filtered through a 30 kDa filter paper in an Amicon Stirred Cell 8400. The obtained enzyme concentrate was mixed with glycerol at 1:1 (v/v) ratio and stored at -20°C. HbpA and FDH activities of such prepared glycerol stocks were 109.7±2.3 U mL^{-1} and 50.8±0.98 U mL^{-1} respectively. This protein preparation was exclusively used for the tube-in-tube ePTFE membrane reactor.

HbpA-activity assay

HbpA activity was determined by measuring the consumption of NADH (ε = 6220 M^{-1} cm^{-1}) at 340 nm using a Cary 300 Bio UV-vis spectrophotometer (Varian, Darmstadt, Germany). 1 unit of enzyme activity (U) refers to 1 µmol of NADH consumed in 1 min at 30°C. A standard assay was conducted as follows: 960 µL of 100 mM KPi buffer (pH 7.5) supplemented with 12 µL of 25 mM NADH and 10 µL of 0.8 mM FAD was mixed in a 1.5 mL PMMA cuvette and thermostated at 30°C for 5 minutes, 10 µL of enzyme solution (ca. 0.03 U) was added and the endogenous NADH oxidation in the absence of a substrate was determined. Subsequently the reaction was started by the addition of 8 µL of 25 mM methanolic solution of 2-hydroxybiphenyl. The reaction was monitored for 1 min. Substrate specific activity was corrected for endogenous NADH oxidation.

FDH activity assay

Determination of FDH activity was done similar to the spectrophotometric method described for HbpA activity. A standard assay was conducted as follows: 840 µL of 100 mM KPi buffer (pH 7.5) supplemented with 100 µL of 2 M sodium formate was mixed in a PMMA cuvette thermostated at 30°C for 5 minutes. Subsequently 10 µL of enzyme solution (ca. 0.03 U) and 50 µL of 10 mM NAD^+ were added to start the reaction. The reaction was monitored for 1 min.

Protein determination

Protein concentrations were determined with the Quick Start Bradford Protein Assay (Bio-Rad, Munich, Germany), using BSA as a protein standard. Protein concentrations were measured in the range of 0 to 1 mg mL^{-1} in a 1 mL assay format.

Protein stability in batch or continuous flow mode

HbpA and FDH glycerol stock solutions were diluted with 50 mM KPi buffer up to the required protein concentration or volumetric activity. Depending on the experiment enzyme solutions were either incubated in 2 mL reaction cups on a thermoshaker (Eppendorf Thermomixer Comfort, Hamburg, Germany), pumped into the PTFE microcapillary reactor by means of a 4 channel Ismatec Reglo pump (Ismatec, Glattbrugg, Switzerland) with a 1.14 mm i.d. Tygon tube, or circulated therein. After respective time a sample was taken and its activity was measured in a standard UV activity assay.

For the experiments in a two liquid phase system decanol was used as organic phase at a ratio of 1:1 aqueous to organic phase. For the experiments conducted in the flow system the ratio was kept equal by pumping the aqueous and organic phase at equal flow rates.

Stability in organic solvents

Glycerol stocks of HbpA and FDH were dissolved in 50 mM KPi buffer pH 7.5 for reaching a volumetric activity of 10.2±0.05 and 8.9±0.4 U mL^{-1} for HbpA and FDH respectively (measured before addition of the organic solvent). 0.6 mL of that solution was transferred into a 2 mL reaction cup and subsequently an equal volume of a respective organic solvent i.e. either heptan, methylcyclohexan, S-(-)-limonene, octane, octanol, *o,m,p*-xylene mixture, perfluorodecalin, or decanol was added. Reaction cups were incubated on a thermoshaker (Eppendorf Thermomixer Comfort, Hamburg, Germany) at 1200 rpm and 30°C. 100 µL samples of aqueous and organic phase were taken at 15, 30 and 60 min after a short spin (10 sec and 10000 rpm). After sampling the mixing was resumed. Aqueous samples were diluted 3 times with KPi buffer and stored on ice until activity measurements as described above.

RESULTS

Growth of *E. coli* pHBP461 and production of HbpA

For choosing the right host for the production of HbpA, two *E. coli* strains (JM101 and JM109) carrying the pHBP461 plasmid were grown on different carbon sources (glucose or glycerol) with or without induction with IPTG (Figure 2-2).

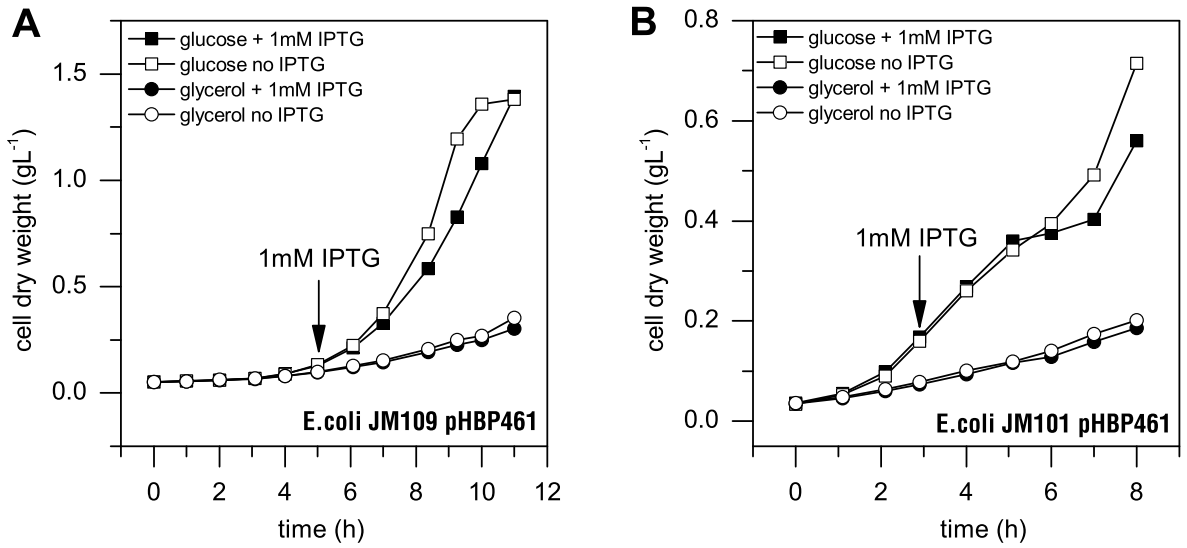


Figure 2-2: Growth curves of *E. coli* pHBP461 on different carbon source with or without induction with 1mM IPTG. A – *E. coli* JM109 pHBP461. B – *E. coli* JM101 pHBP461. Cell dry weight was estimated by correlating optical density measurements at 450 nm with cell dry weight. Correlation factor was 0.166 g L^{-1} for OD=1.

Both strains showed better growth on glucose. The induction with IPTG resulted in slightly reduced growth rates but did not affect final biomass concentrations reached (Table 2-2). Validation of the protein yield by SDS-PAGE (Figure 2-3) revealed higher HbpA levels in *E. coli* JM101. In this strain cultures grown on glycerol clearly had higher expression rates than when grown on glucose, however, under these conditions pHBP461 was leaky and expression of *hbpA* was strong with or without IPTG (Figure 2-3B).

Table 2-2: Summary of the growth experiment with *E. coli* strains carrying pHBP461.

E coli strain	Specific growth rate μ [h^{-1}]	*E coli* strain	Specific growth rate μ [h^{-1}]
JM109 pHBP461 glucose (0.5% v:v) 1mM IPTG	0.41	JM101 pHBP461 glucose (0.5% v:v) 1mM IPTG	0.47
JM109 pHBP461 glucose (0.5% v:v) no IPTG	0.47	JM101 pHBP461 glucose (0.5% v:v) no IPTG	0.48
JM109 pHBP461 glycerol (0.6% v:v) 1mM IPTG	0.19	JM101 pHBP461 glycerol (0.6% v:v) 1mM IPTG	0.21
JM109 pHBP461 glycerol (0.6% v:v) no IPTG	0.21	JM101 pHBP461 glycerol (0.6% v:v) no IPTG	0.21

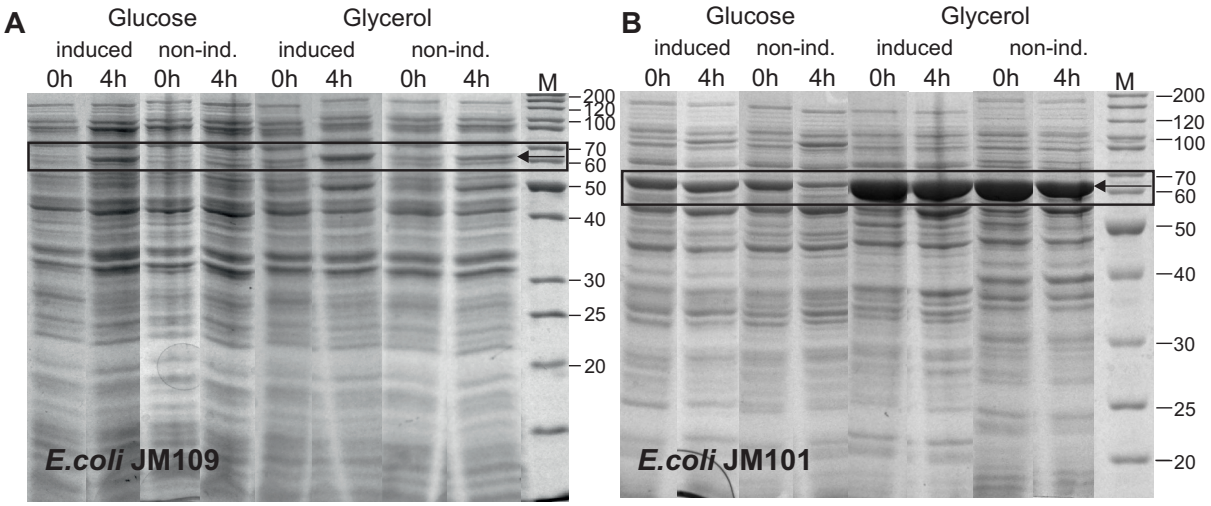


Figure 2-3: Production of HbpA during the growth of *E. coli* (pHBP461) in a shake flask on M9 minimal medium with glucose (0.5% w/v) or glycerol (0.6% w/v) as a carbon source. Protein samples were analysed by SDS PAGE under reducing and denaturing conditions, stained with Coomassie Brilliant Blue. Panel A – *E. coli JM109.* Panel B – *E. coli JM101* Induction with 1 mM IPTG at time point 0. Arrows indicate monomer of HbpA (64kDa). Lanes M – molecular marker (Fermentas PageRuler SM0661, VWR, Darmstadt, Germany).

Due to catabolite repression, the cultures grown on glucose did not show high protein levels. By changing the carbon source this effect was circumvented. Due to these findings *E coli* JM101 pHBP461 was chosen as host organism and in the shake flask experiments glycerol was supplied as carbon source, although at the expense of a reduced growth rate (Table 2-2).

Biomass production in 3L KLF bioreactor

For the large scale production of the biocatalysts, *hbpA* and *fdh* were expressed in *E. coli* JM101 strains carrying respective plasmid (pHBP461 for *hbpA* or pBTac2 for *fdh*) and cultivated in a 3L bioreactor as described in the materials and methods section. Growth curves of the respective cultivations are presented in the Figure 2-4. Both cultures showed exponential growth over the whole fed-batch phase, and their growth rates did not decrease after induction with 1 mM IPTG. Glucose feed was adjusted to keep the growth rate low at μ=0.1 h^{-1} to allow for high HbpA and FDH production (Figure 2-5). Exponential glucose-limited feed with additional IPTG induction resulted in higher protein levels than with glycerol as a carbon source. Because glucose was immediately consumed after addition, its concentration in the bioreactor was low (not detectable by conventional glucose-strip test) exerting no catabolite repression on *hbpA* or *fdh* expression. Nevertheless, induction with IPTG was required to render the *lac* operon active by binding to the repressor protein and permitting transcription.[113]

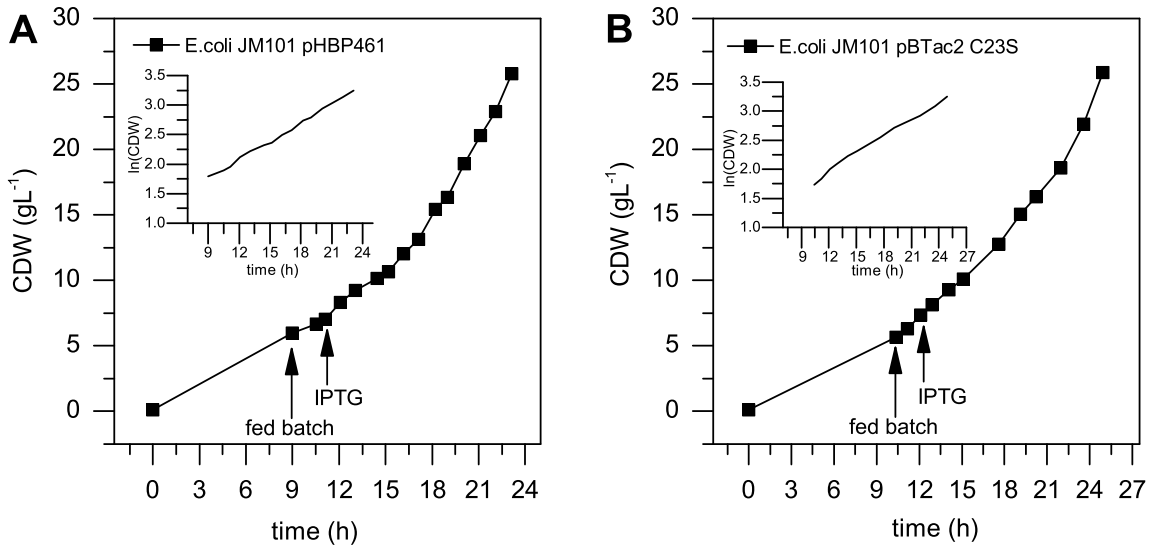


Figure 2-4: Production of biomass in a 3L KLF bioreactor on M9 minimal medium with exponentially fed glucose. Batch phase: 30°C, 1000 rpm, aeration 60 L h^{-1}, 15 g L^{-1} glucose, pH 7.5 – 7-8 h. Fed-batch: 30°C, glucose exponential feed adjusted to specific growth rate μ=0.1-0.12 h^{-1}, induction at 7.5 g L^{-1} with 1 mM IPTG, stirring and aeration controlled to maintain pO_2 above 20%. All the other conditions as described in the respective materials and methods section.

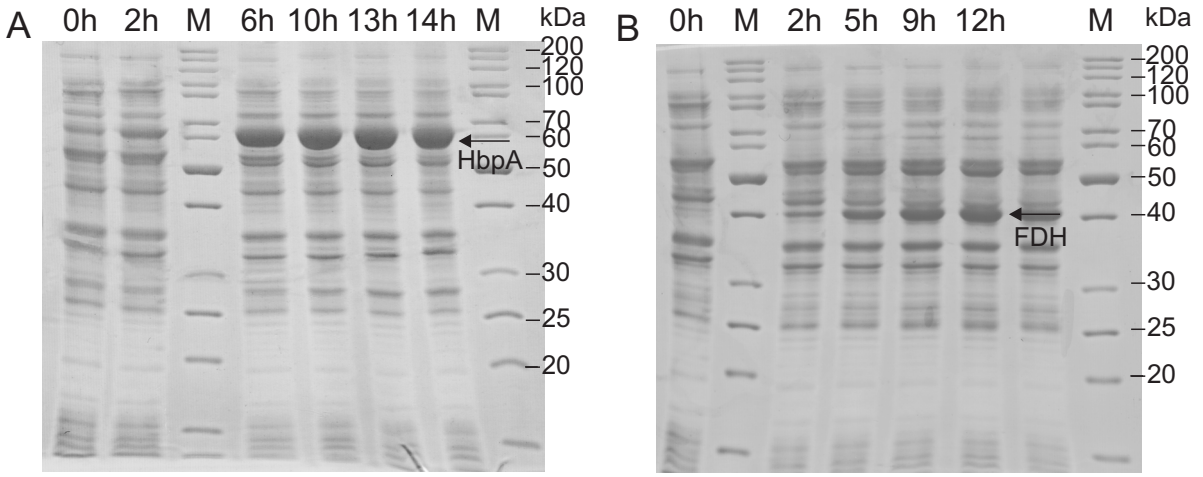


Figure 2-5: A – Production of HbpA during the growth of *E. coli* JM101 pHBP461 on M9 minimal medium in a 3L KLF bioreactor. Time above the lanes indicates time after starting the fed batch with glucose (50% w:v, 80 mM $MgSO_4$). Induction with 1mM IPTG at 2h. B - Production of FDH during the growth of *E. coli* JM101 pBTac2 C23S on M9 minimal medium in a 3L KLF bioreactor. Time above the lanes indicates time after starting the fed batch with glucose (50% w:v, 80 mM $MgSO_4$). Induction with 1 mM IPTG at 2h.

Purification of HbpA by expanded bed anion exchange chromatography

HbpA was produced on a gram scale from *E. coli* JM101 (pHBP461) cell paste prepared as described in materials and methods. The overall purification procedure was similar to the one previously published.[102] HbpA eluted in a broad first peak (Figure 2-6). Pooled fractions were yellow in colour because of the bound flavin cofactor. SDS-PAGE gel conducted after filtration through the 30 kDa filter paper revealed that HbpA was the main protein in the final

sample (Figure 2-7). Typical purification table (Table 2-3) summarises all purification steps. From the initially used 140 g cell pellet (cell wet weight) 11.7 kU of HbpA were prepared with an overall specific activity of 1.5 U mg^{-1}.

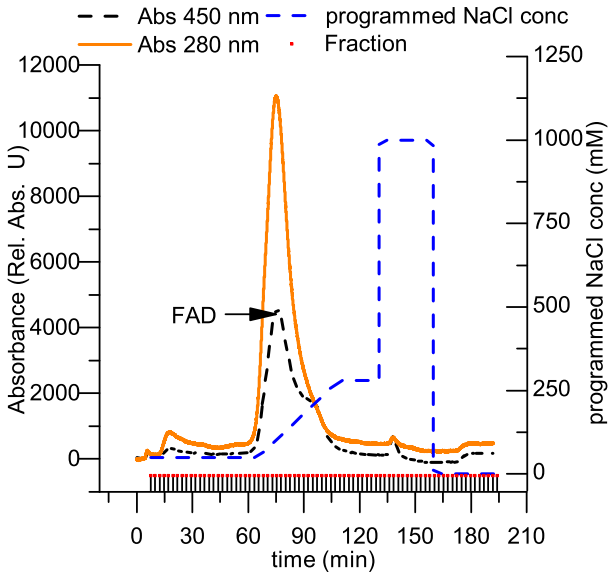


Figure 2-6: Typical chromatogram from the purification of HbpA on the Streamline 50 DEAE expanded bed anion exchange column (100 cm high, 5 cm inner diameter, total 2L volume) (Amersham Bioscience, Uppsala, Sweden).

Table 2-3: Typical purification table of HbpA isolation using expanded bed anion exchange chromatography. 140 g *E. coli* JM101 pHBP461 cell pellet (g_{CWW}) was used.

Sample	Volumetric activity [U mL^{-1}]	Protein concentration [mg mL^{-1}]	Specific activity [U mg^{-1}]	Total activity [U]
Centrifugation	22.35	-	-	8958
AEX fraction pool	7.85	-	-	10952
Ultrafiltration (30kDa cut-off)	60	40.1	1.50	11670

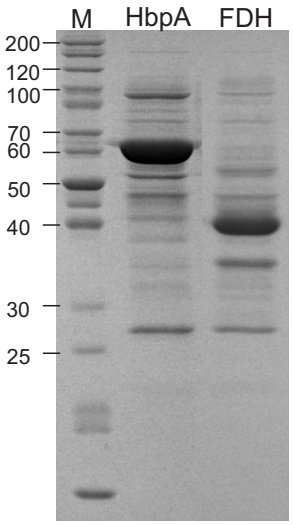


Figure 2-7: Typical 12% SDS-PAGE gel of HbpA and FDH after EBA chromatography. 10 µg protein sample/well have been loaded. Protein weight marker (Fermentas PageRuler SM0661, VWR, Darmstadt, Germany) is shown in lane M, concentrated samples of the most active fractions of HbpA and FDH from expanded bed anion exchange chromatography filtered through 30 kDa cut-off filter paper are shown in lanes HbpA and FDH respectively.

Optimisation of FDH purification on expanded bed anion exchange chromatography

FDH was purified from *E. coli* JM101 pBTac2 (C23S) cell paste prepared as described in materials and methods. The French press was preferred over the bead mill for it offered better cell opening efficiencies (Figure 2-8A). By stepwise optimisation of the NaCl gradient it was possible to elute the loaded proteins in three distinct peaks from the column (Figure 2-8C). Subsequent elongation of the first step (160 mM NaCl) to 1.5 CV instead of initially used 1 CV resulted in better peak separation and almost no FDH activity was detected in the following peaks (Figure 2-8D). Some FDH activity was detected in the fractions 2 and 3 as a result of the pump break-down that initially failed to operate continuously and lead to the dissociation of the proteins once switched back on.

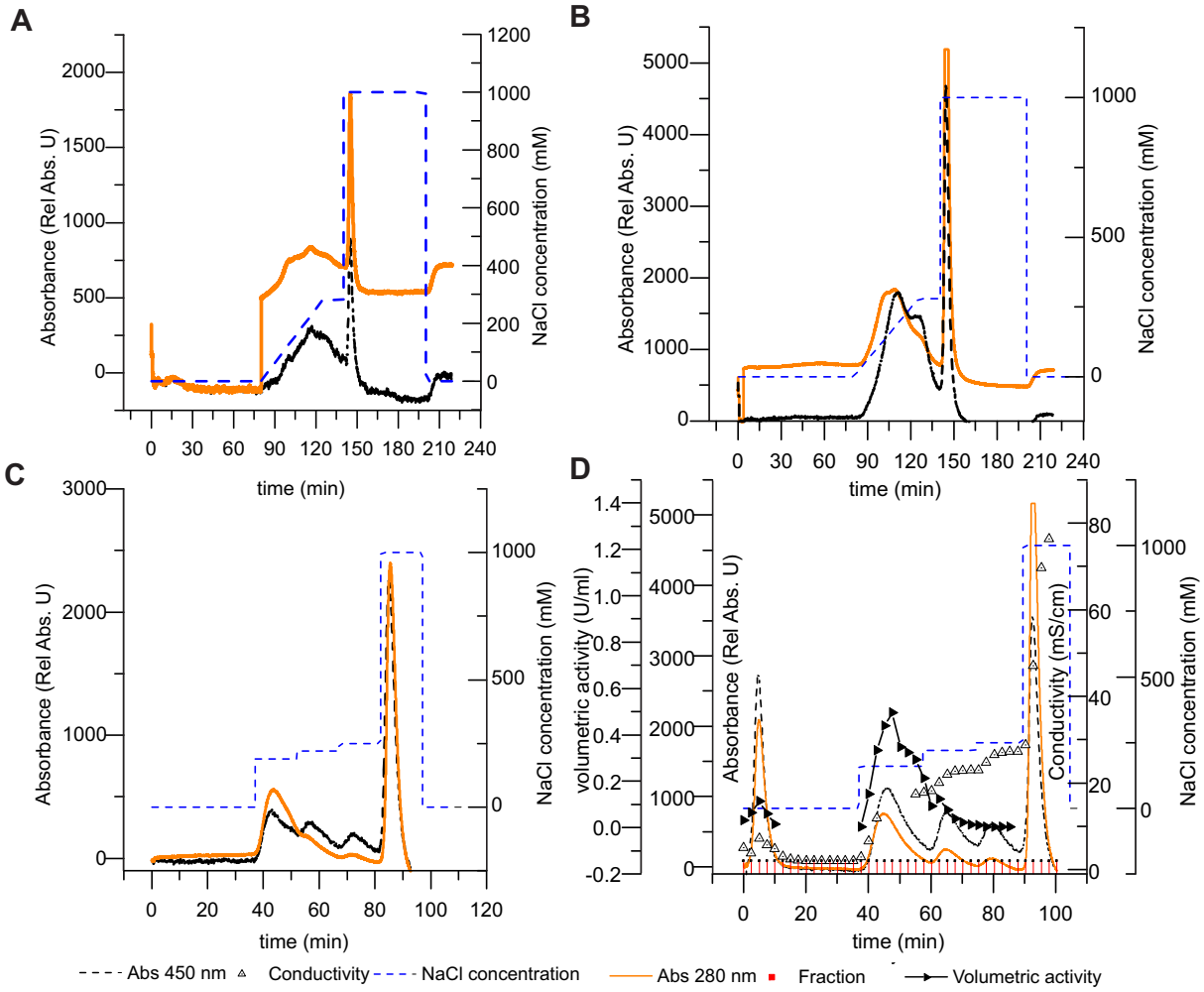


Figure 2-8: Optimisation of FDH purification by expanded bed anion exchange chromatography. A – cell disruption done by bead mill, 40 g cell pellet, gradient elution (5-280 mM NaCl over 3 CV). B – cell disruption method changed to French Press, 40 g cell pellet, gradient elution (5-280 mM NaCl over 3 CV). C – Step-wise elution profile with 1 CV at 160 mM NaCl, 1 CV at 220 mM NaCl, and 1 CV at 280 mM NaCl, 10 g cell pellet, French Press. D – Step-wise elution profile with extended first step 1.5 CV at 160 mM NaCl and then 1 CV at 220 mM NaCl, and 1 CV at 280 mM NaCl, 30 g cell pellet, French Press.

After successful optimisation of the elution protocol 270 g_{CWW} of the cell pellet was used at once to prepare 5.8 kU of FDH with an overall 69% yield (Table 2-4). After ultrafiltration FDH was the most abundant protein in the sample, as confirmed by SDS-PAGE analysis (Figure 2-7).

Table 2-4: Typical purification table of FDH isolation using expanded bed anion exchange chromatography. 270 g of *E. coli* JM101 pBTac2 (C23S) cell pellet (g_{CWW}) was used.

Sample	Volumetric activity [U mL^{-1}]	Protein concentration [mg mL^{-1}]	Specific activity [U mg^{-1}]	Total activity [U]	Purification factor
Cell extract	9.9	34.2	0.29	8877	1
Centrifugation	11.4	30.94	0.37	9125	1.3
Flow through	0.36	-	-	533	-
AEX fraction pool	9.88	9.25	1.02	4940	3.5
Ultrafiltration (30kDa cut-off)	65±1.7	48.2±1.7	1.3±0.04	6500	4.5

Purification by ammonium sulphate precipitation

This simplified purification protocol allowed for quick preparation of technical pure proteins (50-70% estimated on the base of SDS PAGE analysis) without using time consuming chromatography. Resulting protein samples were of comparable purities to the one prepared by EBA chromatography (Table 2-5). The SDS-PAGE analysis showed an efficient purification with respect to the cell extract sample. The overall yield based on the unit recovery was 60% for HbpA and FDH. For long term storage, proteins can be concentrated by ultrafiltration and stored in 50% glycerol as samples originating from EBA chromatography.

Table 2-5: Typical purification table of HbpA and FDH for the ammonium sulphate precipitation method.

Sample	Volumetric activity [U mL^{-1}]	Protein concentration [mg mL^{-1}]	Specific activity [U mg^{-1}]	Volume [mL]	Total activity [U]	Purification factor
HbpA						
Cell extract	44.4±1.0	29.0	1.53	4	178	1
Centrifugation	44.3±0.7	28.2	1.57	3.5	155	1.03
Precipitation	22.3±0.3	11.6	1.92	4.75	106	1.25
FDH						
Cell extract	21.5±1.3	29.5	0.73	4	85.8	1
Centrifugation	22.7±0.9	28.6	0.79	3.75	84.9	1.08
Precipitation	8.7±1.1	6.1	1.43	6	52.3	1.96

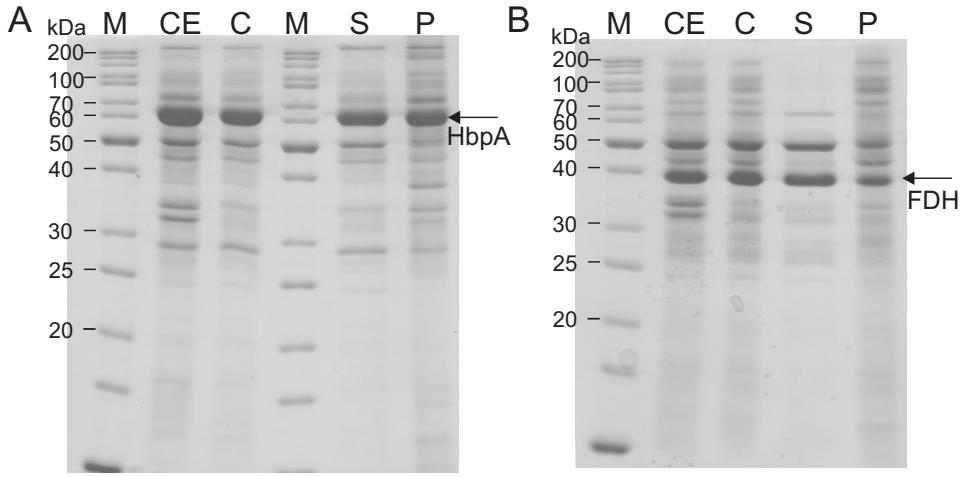


Figure 2-9: SDS PAGE gel of proteins after purification of HbpA (panel A) and FDH (panel B) by ammonium sulphate precipitation. Lane M – molecular marker (Fermentas PageRuler SM0661), CE – cell extract, C – sample after centrifugation, S – supernatant after precipitation, P – resuspended pellet.

HbpA stability in a single aqueous phase

Storage stability at 30°C

To assess storage stability of HbpA at different protein concentrations (2 mg mL^{-1} to 5 mg mL^{-1}) samples of 1 mL prepared in KPi buffer were incubated in 2 mL reaction cups at 30°C without shaking with periodical sample removal for activity assessment. HbpA showed high stability over 70 hours, displaying only minor differences between the samples with different protein concentrations. Overall activity loss was lower than 20% for the least concentrated sample and only 6% for the sample containing 5 mg mL^{-1}.

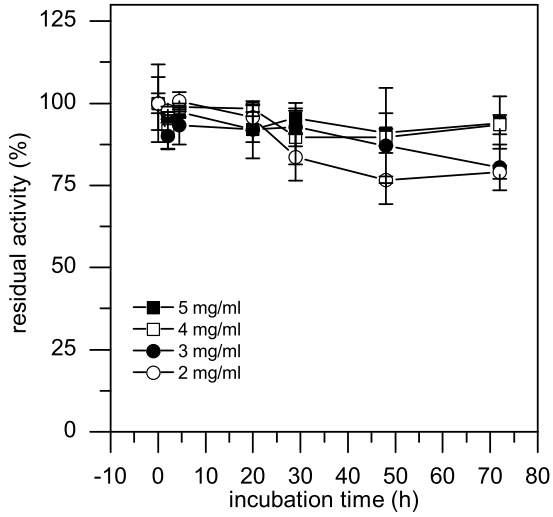


Figure 2-10: Time course of inactivation of HbpA at different protein concentrations upon incubation at 30°C without stirring (batch mode). Initial volumetric activities were 15.0 U mL^{-1} and 6.4 U mL^{-1} for 5 mg mL^{-1} and 2 mg mL^{-1} respectively. Error bar represent standard deviation of 3 consecutive measurements.

Stability in microreactors in flow

In the following experiments HbpA and FDH stability was evaluated under continuous flow conditions in a 9-m-long polymeric PTFE tube with an i.d. of 0.25 mm. The respective protein samples were circulated through the tube and samples were removed regularly to determine enzyme activity. At higher concentrations of HbpA (5 mg mL^{-1}) no decrease in activity was observed over 50 hours of circulation. Only in diluted protein samples (1 mg mL^{-1}) the interfacial effect between solid and liquid phase has an impact (not shown). Compared to the reference sample (without circulation) almost 50% activity was gradually lost in 24 hours. FDH was stable irrespective of the protein concentration in the sample.

Stability of HbpA and FDH in a 2 liquid phase system

In the following enzyme stability was assessed in the presence of an organic phase used as substrate carrier.

Batch mode

Aqueous/organic interfacial effects were first studied in batch mode where the surface to volume ratio is lower than in microreactors,[78] therefore, slower inactivation was expected. At the interface of the hydrophobic organic solvent proteins are prone to be inactivated, as due to their amphiphilic nature they will unfold and denature as described previously.[65-66] First, residual activity of HbpA and FDH was tested after orbital shaking with an organic solvent. Various solvents were checked (see materials and methods section). At high catalyst loadings (9-10 U mL^{-1} equivalent to 6-7 mg mL^{-1}) no significant inactivation was observed after 15 or 30 min of shaking, therefore residual enzyme activity was again tested after a total incubation time of 60 min (Figure 2-11A). White precipitate in various amounts was observed at the interface after orbital shaking and phase separation with every solvent (Figure 2-11B). It was assumed, that this precipitate was denatured enzyme, which may be correlated to the degree of enzyme activity loss. Amongst all solvents tested only octanol and decanol were able to solubilise 2,3-dihydroxybiphenyl. Given the fact that the enzymes had comparable stabilities in octanol and decanol, decanol was the solvent chosen as an organic phase for further studies.

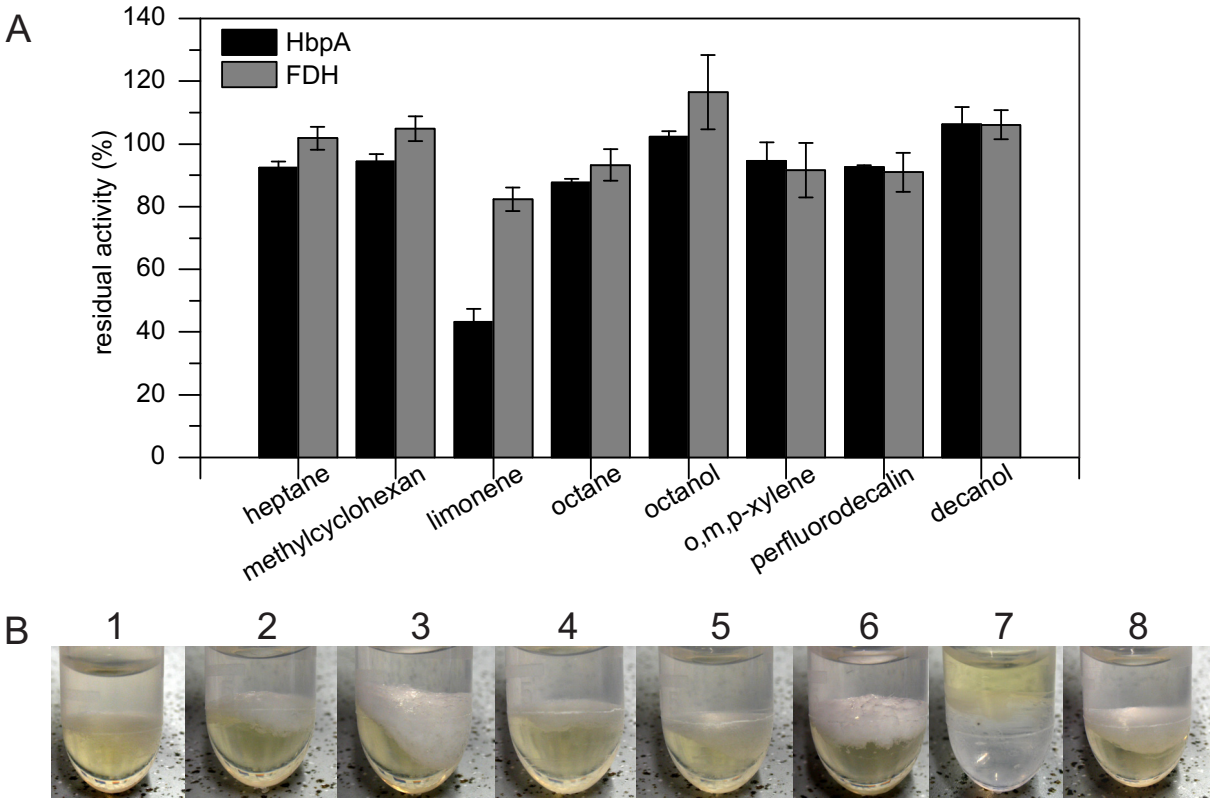


Figure 2-11: A – Influence of the different organic solvent on the residual activity of the HbpA and FDH upon mixing in the two liquid phase system. Activities were measured after 1h of mixing at 1200 rpm at 30°C in a spectrophotometric assay and compared to the enzymes' initial activities. The error bar represent the standard deviation of 2 consecutive measurements. B – Enzyme precipitate present at the interface of an aqueous and organic phase. Respective organic solvents are 1 – heptan , 2 – methylcyclohexan, 3 – limonene, 4 – octane, 5 – octanol, 6 – *o,m,p*-xylene 7 – perfluorodecalin, and 8 – decanol. Initial volumetric activities were 10.2±0.05 and 8.9±0.4 U mL^{-1} for HbpA and FDH respectively.

Subsequent experiments with reduced enzyme loadings (low protein concentrations) in a two-liquid phase system with decanol as organic phase, showed that the lower the enzyme concentration, the faster the inactivation of the respective sample. However, the absolute amount of denatured protein was always in the range of 30 to 63 µg mL^{-1} irrespective of the protein concentration loaded (Table 2-6). For FDH, results were similar to those of HbpA, 110 µg of protein denatured in comparison to 63 µg in the HbpA sample, which comprises 37% of total activity loss.

Table 2-6: Inactivation of HbpA and FDH in moderate to low protein concentrations in two-liquid phase system upon orbital mixing at 1400 rpm (batch mode).

			HbpA		FDH
Initial	protein concentration [µg mL^{-1}]	500	100	50	500
	volumetric activity [U mL^{-1}]	1.55	0.45	0.09	0.43
after 30 min	activity loss	21%	93%	99%	37%
	relative protein loss [µg mL^{-1}]	105	93	49.5	185
	absolute protein loss [µg]	63	56	30	110

Continuous mode – stability in microreactors

Next to the above described batch assays the experiments were repeated in continuous mode in the microcapillary tube with an extended protein concentration range. Again, the same trend as in batch mode was observed, meaning the relative activity loss (percentage of the total protein that becomes inactivated) was greater in more diluted samples for both HbpA and FDH. In addition the inactivation was faster in narrow (0.5 mm i.d.) capillaries as compared to the wider (1.0 mm i.d.) ones (Figure 2-12). Residence time in the microreactor was only 1 min and the rate of inactivation was greater than observed in the experiments conducted in batch mode. For an efficient biotransformation it is required to extend the residence times. It will result in even lower residual activity at the reactor outlet. To extend the catalyst's stability an efficient and simple stabilisation approach is necessary.

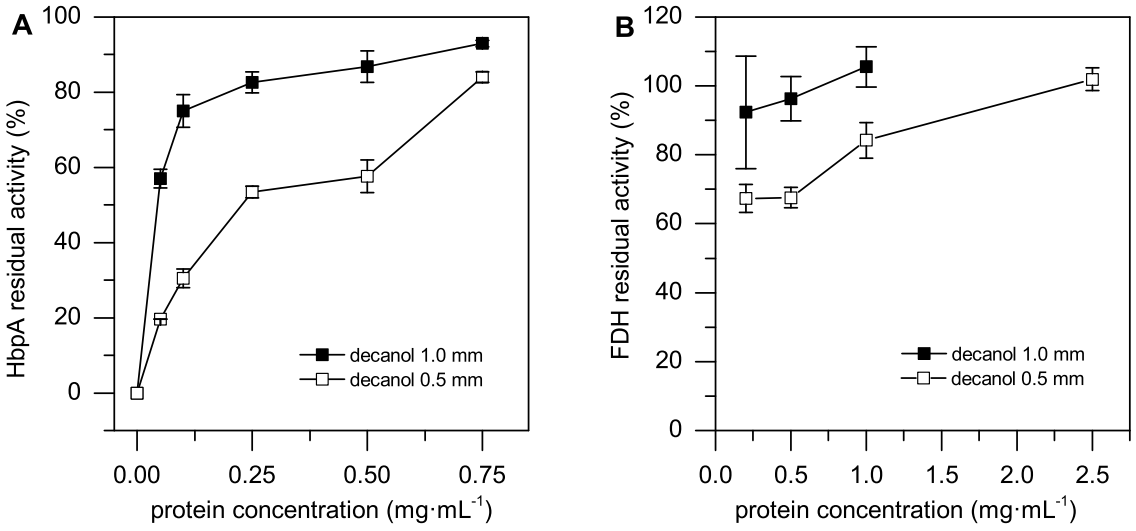


Figure 2-12: Residual activity of HbpA (panel A) and FDH (panel B) at different protein concentrations after contacting enzyme solution with an organic solvent (decanol) for 1 min in flow in 1 mm or 0.5 mm i.d. PTFE capillary. For 0.5 mm i.d. capillary the flow rate of aqueous and organic phase was 0.05 mL min^{-1} each, for 1 mm i.d. capillary the flow rate of aqueous and organic phase was 0.2 mL min^{-1} each for a respective residence time of 1 min. Initial volumetric activities for HbpA were 2.8, 1.9, 0.9, 0.35, and 0.2 U mL^{-1} for 0.75, 0.5, 0.25, 0.1, and 0.05 mg mL^{-1} respectively, while for FDH 2.3, 1.0, 0.5, and 0.2 U mL^{-1} for 2.5, 1.0, 0.5 and 0.2 mg mL^{-1} respectively. Error bar represent standard deviation of two consecutive measurements.

Effect of surfactant on enzyme residual activities in microreactors

The effect of different surfactants on the HbpA residual activity was studied in microreactors. Diluted protein samples of HbpA (0.1 mg mL^{-1}) containing Triton X-100 or Tween 20 were pumped through the microreactor in the presence of a second organic phase (decanol). Tween 20 and Triton X100 had a stabilising effect on HbpA. However Tween 20 proved to be more efficient than Triton X-100 and at 0.15 mg mL^{-1} allowed for 80% activity recovery in the narrow PTFE capillary (0.5 mm) whereas close to 100% HbpA activity was recovered at 0.6 mg mL^{-1} (Figure 2-13).

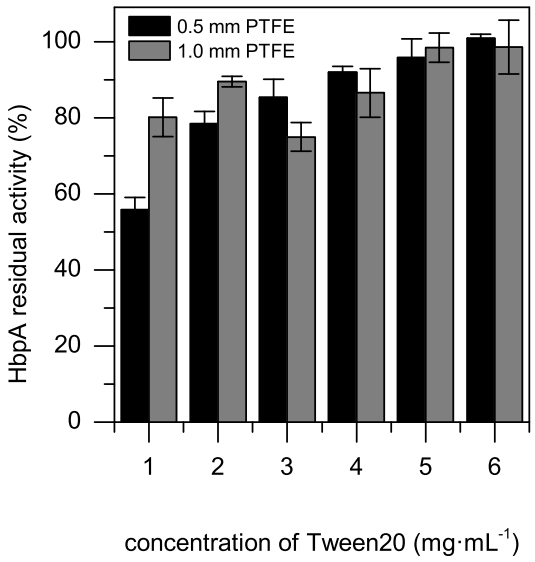


Figure 2-13: Effect of increasing concentration of Tween 20 on HbpA residual activity in flow in 0.5 and 1.0 mm internal diameter PTFE capillary. HbpA concentration was 0.1 mg mL^{-1} and initial volumetric activities were 0.33 to 0.38 U mL^{-1}. Error bar represent standard deviation of at least two consecutive measurements.

Consequently, influence of 0.15 mg mL^{-1} Tween 20 on the reaction with HbpA and FDH was tested. More than 80–90% of FDH activity and 70–100% HbpA activity was recovered as compared to 30 to 50% activity recovery of FDH and HbpA when no surfactant was used (Figure 2-14).

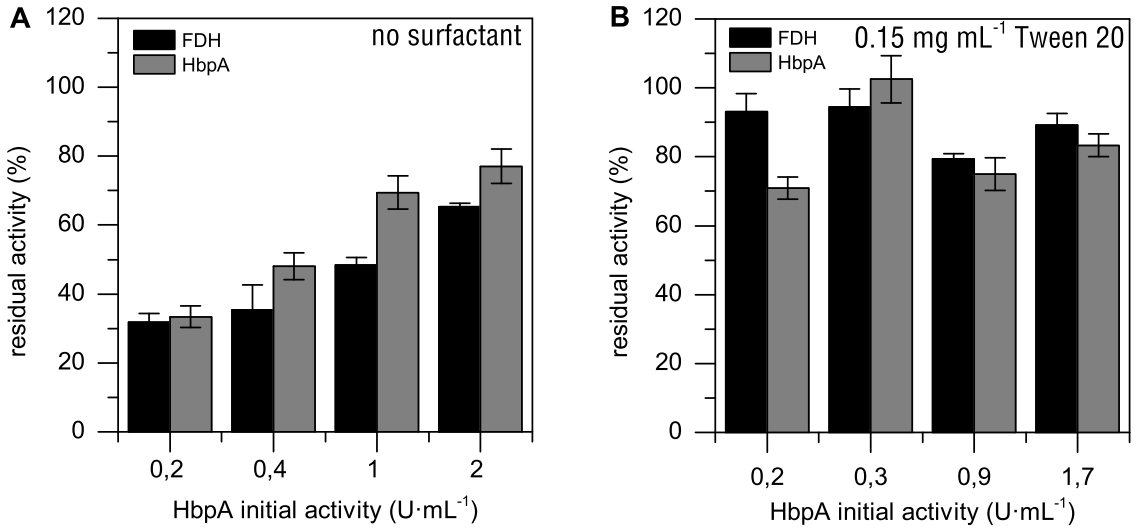


Figure 2-14: Residual activity of HbpA and FDH after biotransformation in PTFE capillary (5.3 m long, 0.5 mm i.d.). A - No surfactant. B - Aqueous phase supplemented with 0.15 mg mL^{-1} of Tween 20. Flow rate of aqueous and organic phase was equal to 0.05 mL min^{-1} each. Residence time reached was 8 to 9 min. Ratio of the volumetric activity of FDH to HbpA was equal to 1.5. Error bar represent standard deviation of at least two consecutive measurements HbpA and FDH specific activities were 3 and 1 U mg^{-1} respectively.

Flow stability

The flow stability in above presented experiments was dependent on the concentration of the protein and the surfactant in the aqueous phase. When no enzyme was added, despite apparent pulsation caused by the nature of the peristaltic pumps, the flow was stable and segments

could be distinguished at the microreactor outlet. The same holds true for low protein concentration; here some precipitate was seen but the segmented flow was observed throughout. Increasing protein concentration destabilised the flow. It could be stabilised by adding Tween 20 (0.15 mg mL^{-1}), but even with the surfactant that covered the interface, forcing the proteins to reside in the bulk of the liquid, at higher protein concentrations the nature of the flow changed yielding pear shaped segments and segments' coalescence. The flow characteristic summary is given in Table 2-7.

Table 2-7: Flow characteristic of the segmented two liquid phase flow in the microreactor (0.5 mm i.d.) with and without surfactant at different initial enzyme volumetric activities. Peristaltic pumps were employed for those experiments. Flow rate of aqueous and organic phase was equal to 0.05 mL min^{-1} each. Residence time reached was 8 to 9 min. FDH to HbpA unit ratio was equal to 1.5.

Prot conc[a] (mg mL^{-1})	no surfactant	Prot conc[a] (mg mL^{-1})	Tween 20 (0.15 mg mL^{-1})
0.4	stable segmented flow with some precipitate	0.4	very stable flow, equal segments, at the whole length of the tubing visible small bubbles like in a sparkling wine
0.7	unstable flow	0.55	stable flow, some segments merged at the beginning and carried load of precipitated protein, more bubbles than previously, transparent precipitate
1.8	unstable flow	1.6	pear shaped segments, segments can still be distinguished at the end of the tube, some long merged segments visible, precipitate present
3.7	unstable flow	3.1	flow unstable, lots of pear shaped segments, precipitate present but less than when no surfactant applied; flow more stable than for the same sample without the surfactant

[a] sum of the concentration of HbpA and FDH protein sample.

DISCUSSION

HbpA and FDH have been successfully purified by means of single step expanded bed adsorption chromatography. Initially two *E. coli* hosts for the HbpA production were considered. *E. coli* JM109 and *E. coli* JM101 carrying plasmid pHBP461 were previously used and proved efficient for HbpA production.[41a, 42, 101-102] Here, the choice was based on the protein synthesis on different carbon sources in M9 medium. Despite similar growth characteristic (Table 2-2), high HbpA titres were only observed in the JM101 strain (Figure 2-3), which was eventually chosen as host organism. *E. coli* JM109 is recA1^{-}, which minimises recombination of foreign DNA with that of a host, yielded only low protein levels and was disregarded later.

Glucose catabolite repression was previously reported for *hbpA* expression under the control of the *lac* promoter,[20] but fermentations were possible with an exponential glucose-limited feed and concomitant IPTG induction (Figure 2-5A).

Expanded bed adsorption chromatography was chosen for simple preparation of technical pure HbpA and FDH. According to literature this chromatography method allows for simple and cost efficient purification of an enzyme directly from crude cell extract.[105, 108] Despite the fact, that EBA can be operated directly with crude cell extract, in this study crude cell extracts were centrifuged prior to loading. Thereby, the extent of the bed expansion was reduced and the following washing step was shortened. One centrifugation step at 15000 *g* was enough to keep the extent of the bed expansion below 5-10% when loading the sample at a constant flow rate of 200 cm h^{-1}. Fractions containing enriched HbpA were of an overall purity of approximately 50% based on the specific activity and the SDS PAGE gel analysis. The purity reached is similar to reported values for HbpA.[102] Although the purification protocol of FDH was optimised with respect to cell breakage and elution profile from the chromatography column, the purity and specific activity of the final protein preparation (1.3 U mg^{-1}) are much lower than literature values reporting a specific activity of 5.5 U mg^{-1} of a pure protein.[112a] However, based on the SDS PAGE gel analysis (Figure 2-7), the FDH was 40-50% pure with an overall 4.5-fold purification (Table 2-4), which was sufficient for its later application.

Pure proteins are often prone to fast inactivation. Addition of proteic feeders (e.g. pepsin or BSA) is a means to increase protein stability in special applications like CLEA preparation[114] or two-liquid phase biotransformations.[63, 65] Natural impurities originating from the cell extract may fulfil a similar role, as long as they do not interact with the catalytic activity of the respective enzyme (e.g. by an excessive consumption of reducing equivalents), and may be regarded as ‘natural proteic feeders’, which prolong catalytic activity of a catalyst and prevent its denaturation.

Throughout this chapter the protein preparations used for characterisation purposes originated from different fermentation batches, therefore discrepancies in their specific activities are present. Surprisingly HbpA stored in the 50% glycerol showed unexpected gradual specific activity increase. Initially the glycerol stock’s specific activity was 2.3 U mg^{-1} and increased over the two month to more than 3 U mg^{-1}. This was also observed for subsequent HbpA glycerol stocks similarly. There was no precipitate present in the glycerol stocks and the activity increase resulted from higher volumetric activities assayed. No further study was however taken to explain this phenomenon.

Characterisation of the enzymes revealed lower stability in the presence of organic solvent (n-decanol) in microreactors where surface-to-volume ratios were higher than in conventional reactions. Addition of the surfactant Tween 20 stabilised the enzymes in the presence of the organic solvent and allowed to recover 100% of the respective enzymatic activity (depending on the concentration of the surfactant applied).

Next to the catalytic activity the operational stability is important. As shown in the Table 2-7 the flow stability in a microreactor system was improved when surfactants were used; ordered flow patterns were observed and the amount of precipitate was reduced. The surfactant beneficial effect may be attributed here to the dynamic surface coverage of the interface by the surfactant's molecules,[66a, 67, 115] this phenomenon, with an emphasis on the interfacial effects, is discussed in more details in chapter 7. Surfactants are therefore useful for stabilising the flow by uniform interface coverage, thereby having a positive impact on the operational stability, as well as on the catalytic activity of the respective enzymes.

At the same time surfactants lower the surface tension of the segments. In systems driven by peristaltic pumps, where the flow is subjected to pulsations, lower surface tension leads to segments' coalescence. Especially two liquids forming consecutive segments having different viscosities (e.g. water and n-decanol) resulting in different translational velocities of segments in a microchannel are prone to this phenomenon. Coalescence is a rapid process facilitating mixing of two coalescing segments,[92b] it causes reorganization of the segment interface and enzyme denaturation because no protection is provided until the full surface coverage is renewed.

CONCLUSIONS

In this chapter efficient ways to produce biocatalysts have been presented. *E. coli* JM101 was chosen as host for enzyme production. HbpA and FDH were purified to technical purity by means of expanded bed adsorption chromatography and could be stored without activity loss in 50% glycerol. They showed excellent stability in single phase experiments. Their stability was however impaired when an organic solvent was used in a two liquid phase system. By using a surfactant, which covered the interface between organic and aqueous phase, enzyme denaturation was partially prevented. Different ways of biocatalyst stabilisation for application in a two liquid phase system are discussed in the next chapter.

ACKNOWLEDGMENTS

We would like to thank Prof. Martina Pohl (Jülich) for kind supply of plasmid containing FDH gene, Jochen Lutz for his help with enzyme preparation and purification.

CHAPTER 3

FUNCTIONAL COMBI-CLEAS OF FDH AND HBPA APPLIED IN A TWO-LIQUID PHASE SYSTEM

Bartłomiej Tomaszewski, Katja Buehler, Frank Hollmann, Jochen Lutz and Andreas Schmid

Bartłomiej Tomaszewski performed the experiments. Bartłomiej Tomaszewski, Katja Buehler and Frank Hollmann wrote the manuscript. Katja Buehler, Frank Hollmann, Jochen Lutz and Andreas Schmid coordinated and supervised the project and corrected the manuscript.

ABSTRACT

Two component cross-linked enzyme aggregates (Combi-CLEAs) consisting of 2-hydroxybiphenyl 3-monooxygenase (HbpA) and formate dehydrogenase (FDH) were applied in a two-liquid phase system for the hydroxylation of 2-substituted phenols with concomitant cofactor regeneration. Replacing glutaraldehyde with dextran polyaldehyde as crosslinking agent lead to activity recoveries of up to 40% for single CLEA and 15% for Combi-CLEA preparations. Addition of the surfactant Tween 20 had a positive impact on CLEA stability and handling, and allowed for consecutive reaction cycles of the immobilised catalysts on the 100 mL scale.

INTRODUCTION

Immobilised enzymes are important catalysts for organic synthesis.[116] Particularly their activity and stability in the presence of organic solvents are advantageous for preparative applications. Various stabilisation techniques for isolated proteins such as entrapment in matrices,[72] covalent binding,[117] and cross-linking[69, 118] are available. The cross linking of enzymes is an immobilisation method, which has been introduced by Quiocho and Richards (1964).[119] They reported using glutaraldehyde for carrier-free covalent cross-linking of enzyme crystals (CLECs) intended for diffraction studies. Sheldon and co-workers simplified the CLEC concept towards cross-linked enzyme aggregates (CLEAs).[70] This method has ever since been widely used mainly for immobilisation of hydrolases.[120] CLEAs were also successfully prepared from tyrosinase,[121] laccases and peroxidases.[122] However, monooxygenase CLEAs, to the best of our knowledge, have not been reported yet. The enzyme used in this study is 2-hydroxybiphenyl 3-monooxygenase (HbpA) (EC 1.14.13.44). It is a tetrameric enzyme that catalyses regioselective *ortho*-hydroxylation of 2-substituted phenols. This enzyme exhibits a broad substrate specificity[41] and thus, is an interesting biocatalyst for the production of 3-substituted catechols. HbpA belongs to the group of FAD dependent flavin monooxygenases[42-43] and requires NADH, molecular oxygen, and the respective substrate for catalytic activity. Therefore an efficient regeneration system like the well-known formate dehydrogenase (FDH) for the continuous supply of reducing equivalents [123] is essential for effective *in vitro* applications of HbpA. In addition, substrate and product pairs for oxygenases most often display low water solubility, which may be facilitated by adding an organic solvent to the reaction mixture forming a two liquid phase system (2LP). 2LP systems typically comprise a water immiscible organic solvent and an aqueous phase. Hydrophobic substrates dissolve in the organic phase and enter the aqueous phase via continuous diffusion, whereas the hydrophobic products are extracted into the organic phase (Figure 3-1A). The organic phase thus serves as a substrate reservoir and product sink and helps to overcome problems caused by reactant solubility limits and/or substrate/product inhibition.[48] However, due to the ambivalent nature of proteins, they act as surface active compounds, adsorb to the phase boundary and denature.[66a, 124] To extend enzyme stability and allow for repeated-batch or continuous operations of a given process, it is thus most often necessary to stabilise the biocatalyst.

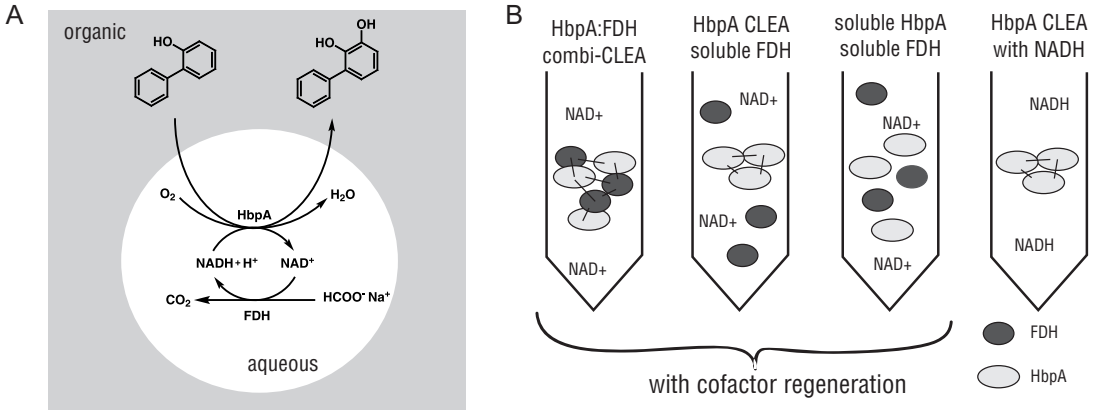


Figure 3-1: A – 2 liquid-phase (2LP) biotransformation – organic phase serves as a substrate reservoir and product sink. B – Possible 2LP biotransformation setups with HbpA and FDH, with or without cofactor regeneration. Bar between the enzymes indicate crosslinking event.

In this study HbpA:FDH Combi-CLEAs were evaluated for the *ortho*-hydroxylation of 2-hydroxybiphenyl in a 2LP system. The performance of the CLEA based approach was compared to soluble enzyme. Respective data are presented and the suitability of the monooxygenase Combi-CLEAs for catalysis is discussed.

MATERIALS AND METHODS

Chemicals

β-Nicotinamide adenine dinucleotide disodium salt, sodium formate and 2-hydroxybiphenyl have been obtained from Fluka (Buchs, Switzerland), β-Nicotinamide adenine dinucleotide reduced disodium salt, 1-decanol were from Applichem (Darmstadt, Germany), 2,3-dihydroxybiphenyl were from Wako Pure Chemical Industries Ltd. (Osaka, Japan). All other chemicals have been bought at Fluka, Applichem or Sigma-Aldrich at the highest purity required.

Enzyme production and purification

The production and purification of HbpA and FDH was based on the procedures described in chapter 2. The enzymes were of technical purity (FDH 1.21 U mg^{-1} approx. 22% pure, HbpA 1.37 U mg^{-1} approx. 40% with respect to the maximum reported velocity of a pure protein) and were used directly as 50% (v:v) glycerol stocks stored at -20ºC. Enzyme activity was measured using a standard spectrophotometer described in chapter 2.

HbpA-CLEAs and HbpA:FDH Combi-CLEAs

If not stated otherwise CLEAs were prepared accordingly: 125 µL of protein solution (either HbpA for CLEAs of HbpA or an enzyme mixture of HbpA and FDH for Combi-CLEAs) was mixed with 250 µL or 839 µL of 50% (w/v) $(NH_4)_2SO_4$ solution to a final saturation of 61% or 80% at RT respectively (100% saturation equals 54.12% (w/v) $(NH_4)_2SO_4$[107]). The solution

was mixed on a thermoshaker (30 min, 600 rpm, RT) and subsequently supplemented with DPA (0.2 M) to a final cross-linker concentration in the range of 12 to 50 mM. The mixture was stirred at RT for 3h at 600 rpm. Subsequently 100 mM solution of sodium cyanoborohydride was added to a final concentration of 10 mM and stirred again at RT for 30 min at 900 rpm. Then an equal volume of water was added and the preparation was stirred again at RT for another 30 min at 900 rpm. CLEAs were eventually collected by centrifugation (10 min, 16200 *g*, RT), the resulting precipitate was washed thrice with 800 µL of 50 mM potassium phosphate buffer (KPi) (pH 7.5) (vortexed and centrifuged (10 min, 16200 *g*, RT) between every washing step). CLEAs were finally resuspended in 500 µL of buffer and kept at 4°C for further use. The procedure is summarised in the Table 3-1. If not stated otherwise Combi-CLEAs mentioned in the text refer to the HbpA:FDH Combi-CLEA prepared as described below.

Table 3-1: Summary of CLEA preparation conditions.

	HbpA:FDH Combi-CLEA	HbpA-CLEA
HbpA	62.5 µL glycerol solution[a] (3.8±0.3U, 2.7±0.1 mg)	125 µL glycerol solution[a] (7.5±0.5U, 5.5±0.2 mg)
FDH	62.5 µL glycerol stock[b] (3.9±0.2U, 3.3±0.2 mg)	-
$(NH_4)_2SO_4$ (50% w/v)	839 µL (for 80% saturation)	250 µL (for 61% saturation)
DPA (0.2M)[c]	41.8 µL (8.4 mM)	41.8 µL (20 mM)
$NaCNBH_3$ (100mM)	235 µL (for 19 mM)	47 µL (for 10 mM)
H_2O	800 µL	470 µL (1:1 ratio)
Temperature	23°C	23°C

[a] HbpA glycerol stock solution of 164.1±11 U mL^{-1}, 119.4±4 mg_{prot} mL^{-1}, specific activity 1.37 U mg^{-1}_{prot}, diluted to 60.4 U mL^{-1} prior to use.
[b] FDH glycerol stock solution of 63.1±2.5 U mL^{-1}, 52.1±2.7 mg_{prot} mL^{-1}, specific activity 1.21 U mg^{-1}_{prot}
[c] Combi-CLEAs have been prepared using an enzyme:cross-linker ratio of 1:2050, while for HbpA-CLEAs a ratio of 1:1010, because the ratio was based on the amount of HbpA only.

Residual CLEA activity

Residual CLEA activity was given as the ratio of the total activity of the CLEA to the activity of the respective soluble enzyme used for its preparation expressed in percents. Activity was measured as described above. Specific activities are based on the concentration of the soluble enzyme under the assumption that all proteins precipitate during CLEA preparation.

Determination of the apparent K_m values

0.083 U mL^{-1} HbpA (0.06 mg_{prot}), 0.083 U mL^{-1} FDH (0.07 mg_{prot}) - for soluble enzymes reaction, or 70 µL of Combi-CLEA solution (prepared from 0.53 U mL^{-1} HbpA (0.38 mg_{prot}) and 0.53 U mL^{-1} FDH (0.44 mg_{prot})) - for HbpA:FDH Combi-CLEA reaction, were mixed

with 50 µL of 200 mM sodium formate, 50 µL of 1 mM NAD^+, 5 µL of 8 µM FAD in a small reaction cup and filled with 50 mM KPi up to 500 µL. Above prepared mixtures were combined with 500 µL of decanol containing varying concentrations of 2-hydroxybiphenyl (1-150 mM). Substrate concentration in the aqueous phase was calculated on the base of the partition ratio K_D for 2-hydroxybiphenyl between organic and aqueous phase of 480. Reaction was conducted on the Eppendorf thermo mixer comfort (Eppendorf, Hamburg, Germany) at 30°C and 1400 rpm for 1 h. For sampling (15, 30, 60 min), agitation was paused and samples were left to settle for 2 min for phase separation. After 15 µL of the upper organic phase were removed, agitation was resumed, withdrawn samples were immediately centrifuged (1min, 16200 *g*, RT) and 10 µL of organic phase was transferred to 990 µL acetonitrile (supplemented with 0.1% v/v TFA) solution for RP-HPLC analysis. Initial reaction rates were plotted against the initial substrate concentration in the aqueous phase. Apparent Km values were calculated by using a Michaelis-Menten fitting function of the Origin 8.6 software (OriginLab Corporation, Northampton, MA, USA).

Synthesis of dextran polyaldehyde (DPA)

In this work dextran polyaldehyde was synthesised in-house according to the protocol given in.[125] Shortly, 1.65 g of dextran (mean molecular mass 100-200 kDa) was dissolved in 50 mL of Seralpur Pro 90 CN water (Seral Druck, Ransbach-Baumbach, Germany) followed by 3.85 g of sodium periodate. This mixture was stirred at RT for 90 min. Subsequently it was dialyzed 4 times against (>2h each time) 5 L of Seralpur water using a 12.2 kDa cut-off dialysis sleeve. The liquid was transferred to a glass vial and kept at 4°C.

Standardisation of DPA

The degree of substitution of formyl groups in DPA was determined according to the method described in.[126] Shortly, 0.25 M hydroxylamine solution (pH 4) was prepared by dissolving 3.5 g of dried reagent and 0.6 mL of methyl orange (0.1% solution) in a final volume of 200 mL Seralpur water.

Three DPA samples (approx. 100 mg, exactly 3 mL) were dried to a constant mass in a freeze-dryer. 25 mL of hydroxylamine solution was added to every dried DPA sample and for 4 h at RT to dissolve. The pH dropped from 4 to 1.6, indicated by a colour change from orange to pink. Samples were later titrated with 0.096 M NaOH solution. The end of titration was indicated by the colour change matching that of a blank (i.e. hydroxylamine solution with no DPA added), pH values with respect to the volume of added base were recorded. The volume of NaOH solution needed to neutralise all HCl liberated in the reaction of aldehyde

with hydroxylamine could be calculated from the point of inflection of the respective titration curves (Figure 3-2B). Aldehyde substitution was calculated according to the Equation 3-1.[127]

Equation 3-1: Calculation of the molarity of the DPA solution

$$vol\ in\ mL_{NaOH} \times N_{NaOH} \times 10^{-3}\ mol \times \frac{10^3}{vol\ in\ mL_{\ DPA\ solution}} = \frac{mol\ of\ aldehyde}{1\ litre_{DPA\ solution}}$$

HbpA activity determined by product formation – RP-HPLC analysis

Monitoring of the HbpA catalysed biotransformation reaction was achieved by determining the concentration of the product 2,3-dihydroxybiphenyl. Measurements were done using a Shimadzu UFLC system equipped with a UV-Vis SPD-M20A detector or Hitachi Elite LaChrom HPLC system (Merck Hitachi, Darmstadt, Germany) equipped with a Hitachi Elite LaChrom L-2450 Diode Array Detector. All measurements were performed either on an XTerra RP 18 3.5 µM column (Waters, Dublin, Ireland) or on a CC Nucleosil 100-5 C18 HD column (Macherey Nagel, Düren, Germany). An isocratic method at a total flow rate of 1 mL min^{-1} 50:50 (% v/v) ACN-water (both supplemented with 0.1% TFA) was used for elution. The product 2,3-dihydroxybiphenyl as well as the substrate 2-hydroxybiphenyl were detected at 2.69 and 3.19 min retention time respectively on an XTerra RP column and 4.89 and 7.39 min retention time respectively on the CC Nucleosil column. Results were analysed using the LC Solution software. Concentrations of 2,3-dihydroxybiphenyl were calculated as a peak area ratio of 2-hydroxybiphenyl to 2,3-dihydroxybiphenyl.

Protein determination

Protein concentrations were determined with the Quick Start Bradford Protein Assay (Bio-Rad, Munich, Germany), using BSA as a protein standard. Protein concentration measured in the range of 0 to 1 mg mL^{-1} in a 1 mL assay format.

Two phase biotransformation at a 2 ml scale

Biotransformations were conducted in a 2LP system (Figure 3-1A). Unless stated otherwise standard reaction conditions were as follows: 5 µL of 0.8 mM FAD, 50 µL of 2 M sodium formate and either soluble enzyme, single CLEA or Combi-CLEAs (respective amounts are specified in the experiments) were topped to 475 µL with KPi buffer and mixed with 500 µL of 2-hydroxybiphenyl solution (5-50 mM) in decanol in a 2 mL reaction cup. The reaction was started by adding 25 µL of 10 mM NAD^+. The tubes were thermostated at 30°C in an Eppendorf thermo mixer comfort (Eppendorf, Hamburg, Germany) with a constant agitation of 1400 rpm. For sampling, agitation was paused and samples were left to settle for 2 min for phase separation. After 15 µL of the upper organic phase were removed, agitation was

resumed, withdrawn samples were immediately centrifuged (1 min, 16200 *g*, RT) to allow phase separation and 10 µL of organic samples were transferred to 990 µL acetonitrile (supplemented with 0.1% v/v TFA) solution for RP-HPLC analysis.

Two phase biotransformation at a 200 ml scale

To validate the performance of CLEAs on a bigger scale they were tested in 200 mL 2LP biotransformations. Reactions were conducted in a RALF stirred tank bioreactor with a working volume of 300 mL (Bioengineering AG, Wald, Switzerland) where pH, temperature, aeration and stirring speed were controlled. Initial composition of the aqueous phase was as follows: 200 mM formate, 8 µM FAD and 1 mM NAD^+ have been added to initiate the reaction; organic phase was a 20 mM decanolic solution of 2-hydroxybiphenyl. The reaction mixture was supplemented with Tween 20 to a final concentration of 0.2 mg mL^{-1}, phase ratio 1:1. Initially 10.6 U (7.7 mg) of soluble HbpA and 10.9 U of soluble FDH and an equivalent of HbpA (49.2 mg) and FDH for CLEA reaction were used. For analysis, an emulsion sample of 300 µL was withdrawn without interrupting the mixing. The sample was immediately centrifuged (1 min, 16200 *g*, RT) to allow phase separation and 10 µL of the organic sample was mixed with 990 µL acetonitrile containing 0.1% TFA and analysed on the HPLC.

SDS-PAGE analysis

SDS-PAGE samples were prepared by mixing respective protein samples with an equal volume of loading buffer. Samples were vortexed and kept on a thermoshaker at 95°C and 600 rpm for 7 min. 10 µL of the sample are eventually loaded on to a 12% SDS PAGE acrylamide gel prepared and operated according to the standard procedure given in.[128]

RESULTS

Optimising CLEA preparation

To prepare an active CLEA catalyst one has to find optimal precipitation as well as crosslinking conditions. Ammonium sulphate was chosen as a precipitant (Figure 3-2A). DPA was applied as a crosslinker. A molar ratio of 1:2050 enzyme to crosslinker was chosen as a compromise between decreasing residual activity and increasing leaching stability[122a, 129] (Figure 3-3AB). Extending the crosslinking time did not have any effect on CLEAs' performance (Figure 3-3C). Unless stated otherwise HbpA:FDH Combi-CLEAs with a 1:1 HbpA to FDH activity unit ratio were used throughout the studies.

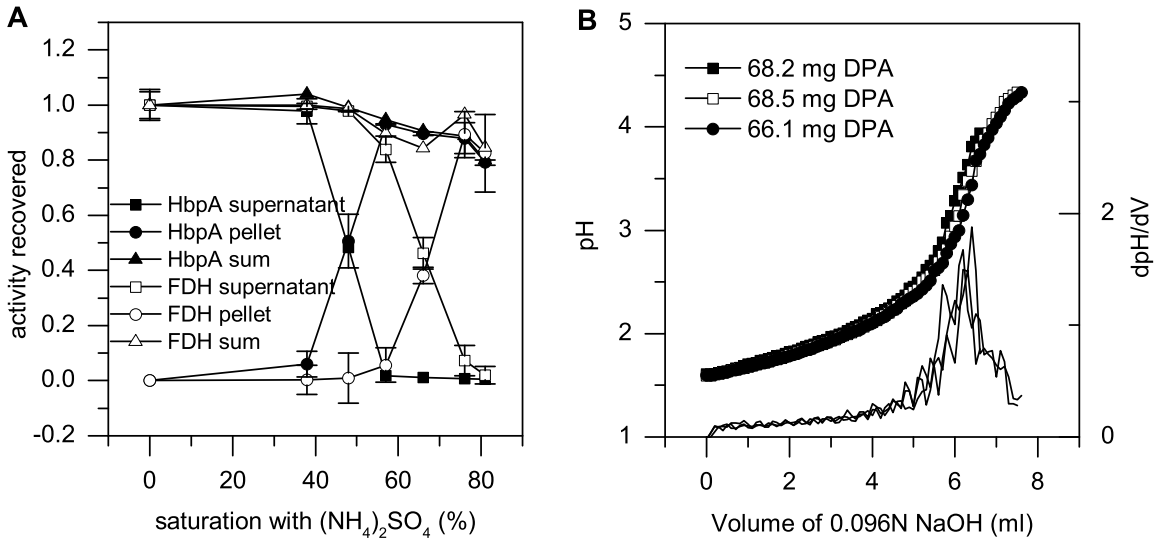


Figure 3-2: A - Precipitation curves for HbpA and FDH. Residual activities in the pellet and the supernatant were determined using the standard spectrophotometric assay described in the method section. 1.0 refers to an activity of 8.2 U mL^{-1} in the case of HbpA and 3.2 U mL^{-1} for FDH, respectively. After precipitation, supernatant and pellet were separated; the pellet was resuspended in KPi buffer (50 mM, pH 7.5) and the residual HbpA and FDH activity was measured in both fractions. B - Titration curves for determination of aldehyde substitution in oxidised dextran polyaldehyde. Masses of the samples taken for investigation are indicated on the graph. Maxima of first derivatives plotted indicate the point of inflection of the titration curves (6.2, 6.3 and 6.5 respectively) – this point was also determined by the methyl orange colour shift from pink to yellow as compared to blank sample.

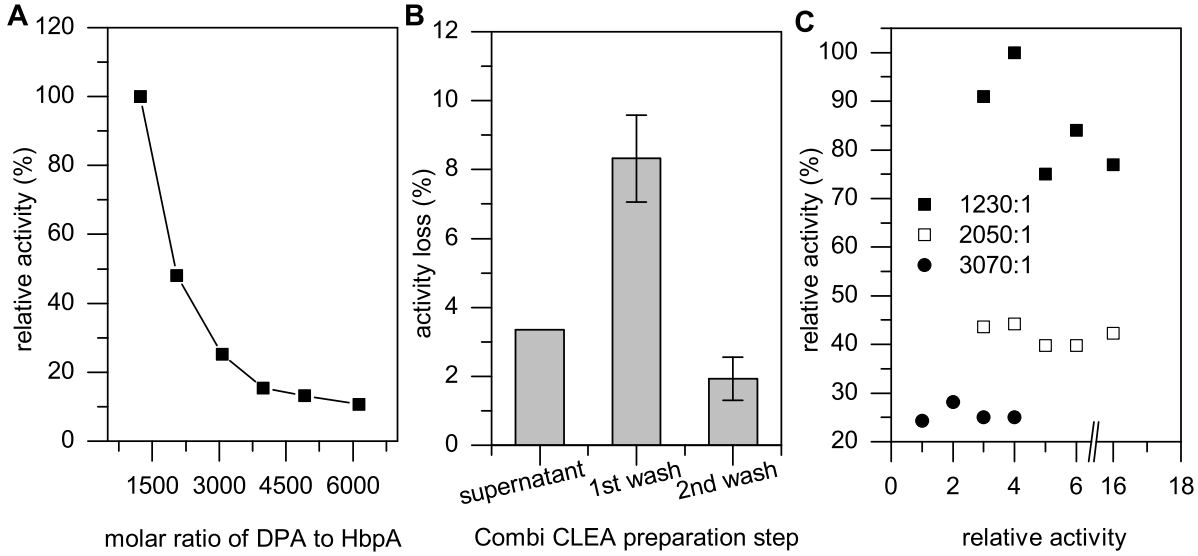


Figure 3-3: Influence of preparation conditions on CLEA activity and leaching. A – Influence of the molar ratio of dextran polyaldehyde (DPA) to HbpA on Combi-CLEA activity. Total cross-linking time 3 h. B– Leaching of the catalyst during consecutive preparation steps as a percentage of the total activity present in Combi-CLEA (80% precipitant saturation level, 1:2050 HbpA to crosslinker ratio). 'Supernatant' refers to supernatant collected after crosslinking and reduction with $NaCNBH_3$. '1st wash' and '2nd wash' are consecutive washes; for washing sample was resuspended in potassium phosphate buffer, left at RT for 10 min and spun down; supernatant was collected and activity determined in a 2LP biotransformation. C - Influence of the cross-linking time and DPA to HbpA molar ratio on the Combi-CLEA activity recovery.

SEM micrographs and CLEA pictures

SEM micrographs of Combi-CLEAs presented in Figure 3-4A and C show rather uniform particle size distribution (130 to 220 nm in diameter) regardless of the type of the crosslinker used. Only a slight variation of the particle size was observed in case of 12 mM DPA. In this case the single aggregate particle sizes varied from 80 to 250 nm in diameter, which represents the overall size distribution of all CLEAs prepared in this study. However, one has to keep in mind, that prior to image acquisition the samples had to be freeze-dried, which might alter the structure of the aggregates and thus the SEM images might not truly reflect the CLEAs arrangement in the aqueous environment which can be seen on Figure 3-5.

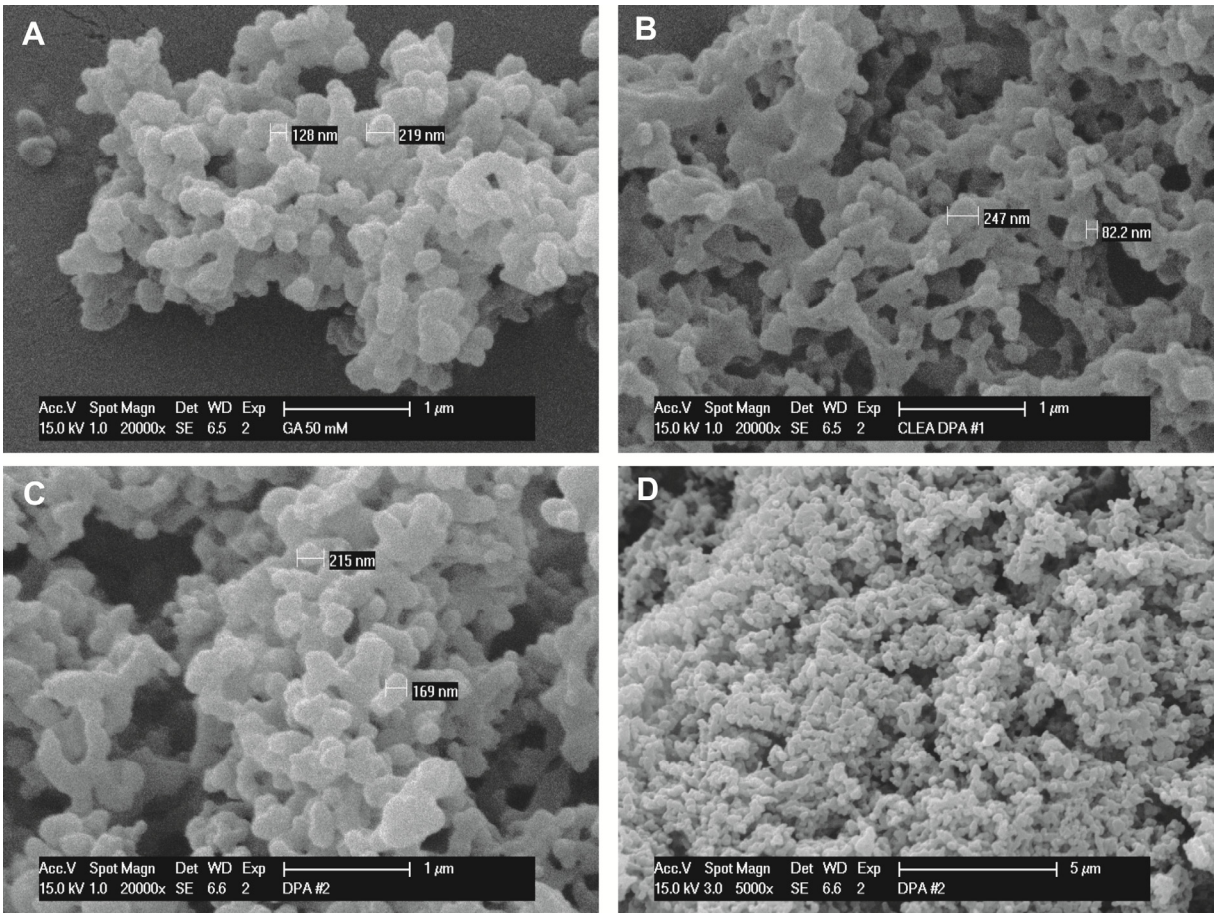


Figure 3-4: SEM images illustrating Combi-CLEAs. A – 50 mM glutaraldehyde used as a crosslinker; B – 12.5 mM DPA used as crosslinker; C – 20 mM DPA used as a crosslinker. D – Image C at a 4 fold lower magnification. Precipitant saturation level 61%. The molecular weight of HbpA tetramer is 256 kDa. According to Erickson (2009)[130], assuming that the protein is a perfect sphere, the radius and the volume of that protein will be 4.2 nm and 310 nm^3 respectively. Meaning that, for example, a 169 nm diameter CLEA-sphere presented in C could contain approx. 8000 HbpA tetramers according to the relation $(r_{CLEA\text{-}sphere}/r_{HbpA})^3$.

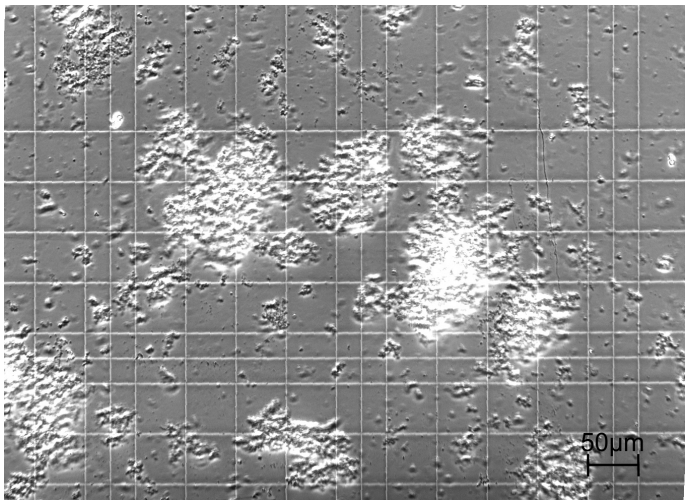


Figure 3-5: Light microscope images of Combi-CLEAs right after reduction step. The sizes of particles vary from sub micrometre range to hundreds micrometres. Precipitant saturation level 80%, HbpA to crosslinker molar ratio 1:2050.

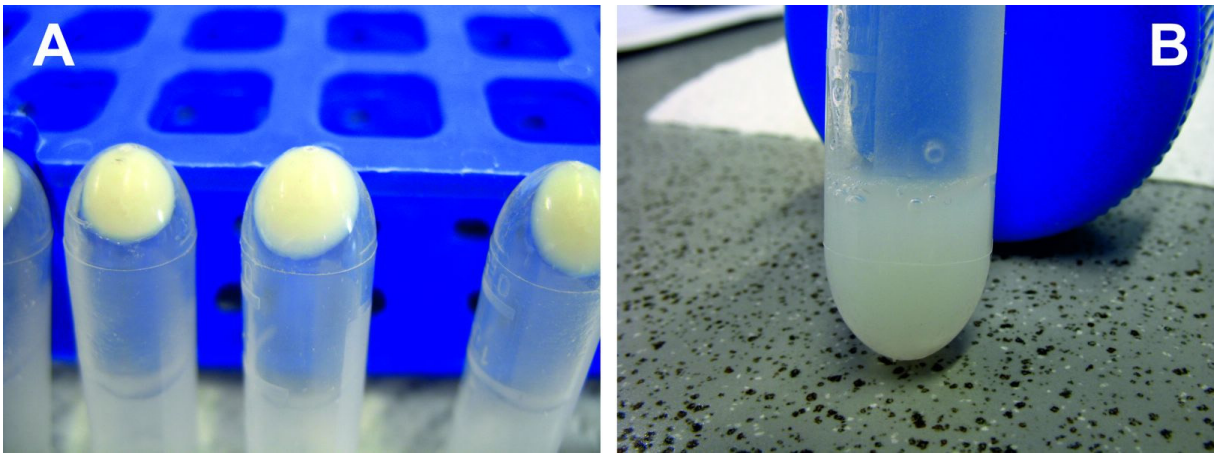


Figure 3-6: A – Combi-CLEA pellet after 3rd wash and final centrifugation; B – Combi-CLEA resuspended and homogenized in 500 µL KPi buffer (50 mM, pH 7.5)

Activity of CLEAs and Km values

Combi-CLEAs were investigated and compared to the soluble enzyme under otherwise identical conditions. As shown in Table 3-2 the specific activity of Combi-CLEAs accounted for almost 15% of the soluble enzyme and increased to 40% in the presence of 10 mM NADH. Combi-CLEAs were equally active as HbpA CLEAs supplemented with soluble FDH. Lower activities with soluble FDH in comparison to externally supplemented NADH could be explained by diffusion limitation which in the presence of stoichiometric amounts of NADH was less pronounced. Additionally the apparent Km values of the Combi-CLEAs and soluble enzymes were calculated to be 5.2 and 4.2 µM for soluble enzymes and Combi-CLEAs respectively.

Table 3-2: Characterisation of CLEAs in 2LP biotransformation.

Sample	*average initial rate*[b] *[µmol min^{-1}]*	*spec activity [U mg$_{prot}^{-1}$]*	+/-
CLEA[a] + 10 mM NADH	0.042	0.110	0.008
CLEA[a] + soluble FDH (0.55 U)	0.018	0.046	0.009
Combi-CLEA	0.016	0.041	0.001
Soluble HbpA and soluble FDH	0.108	0.280	0.009

[a] CLEA prepared with only HbpA

[b] rate calculated for 1 mL reaction volume with a phase ratio of 1:1.

In the case of a successful recycling of CLEAs 15% activity might still be acceptable from the practical point of view. Therefore the recyclability of the CLEAs was further evaluated.

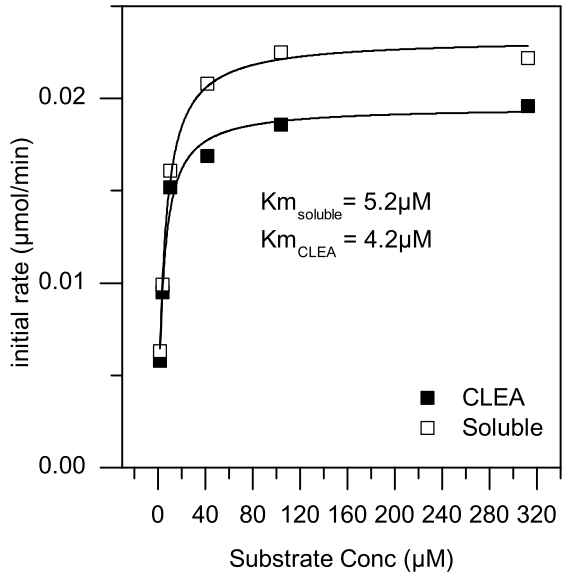


Figure 3-7: Determination of the apparent Km value. Initial reaction rates with different substrate concentrations for soluble enzymes and for Combi-CLEAs. Reaction conditions are described in the material and method section.

Stability and reusability of Combi-CLEAs

During 2LP biotransformations Combi-CLEAs tend to accumulate at the phase boundary making recycling very difficult. Tween 20 proved to be an efficient agent preventing enzyme accumulation at the interface, therefore all subsequent experiments were performed in the presence of 0.2 mg mL^{-1} Tween 20.

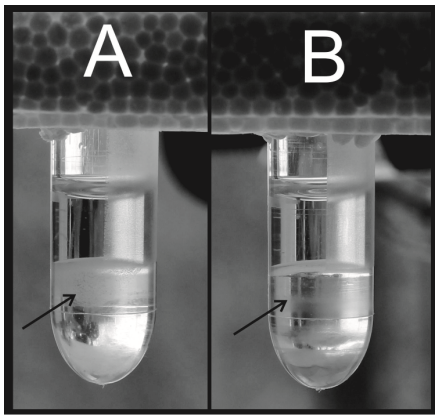


Figure 3-8: Image of Combi-CLEAs after a standard 2LP biotransformation without (panel A) and with (panel B) Tween 20. A fine precipitate is visible in A while sample B shows a clear phase boundary.

Repeated batch 2LP system biotransformation on a 200 mL scale

To compare productivities and the stability of Combi-CLEAs over several batches with that of the soluble enzymes, a biotransformation was conducted with an increased amount of Combi-CLEAs (Figure 3-9). The initial specific activities of Combi-CLEAs and soluble enzymes measured previously in the 2 mL reaction tubes were 0.04 U mg_{prot}^{-1} and 0.28 U mg_{prot}^{-1} respectively. Therefore 7 times more Combi-CLEAs than soluble enzyme have been used to match the initial reaction rates of both setups.

The space time yield reached in the first batch with soluble enzymes and Combi-CLEAs was 0.24 g L^{-1} h^{-1} and 0.13 g L^{-1} h^{-1} respectively, matching up with previously published values for the soluble enzymes.[21] On a long term basis Combi-CLEAs showed higher stability than the soluble enzyme. Already during the 2nd cycle, Combi-CLEAs performed significantly better, than the soluble counterparts. Overall the product titres achieved with soluble enzyme and with CLEAs are comparable reaching 2.5 and 2.7 mmol of 2,3-dihydroxybiphenyl respectively after three consecutive cycles. Despite being applicable in a 2LP system HbpA:FDH Combi-CLEAs do not supersede soluble enzymes without additional mechanical reinforcement. However it was possible to stabilise and preserve HbpA:FDH Combi-CLEA activity up to 90 hours.

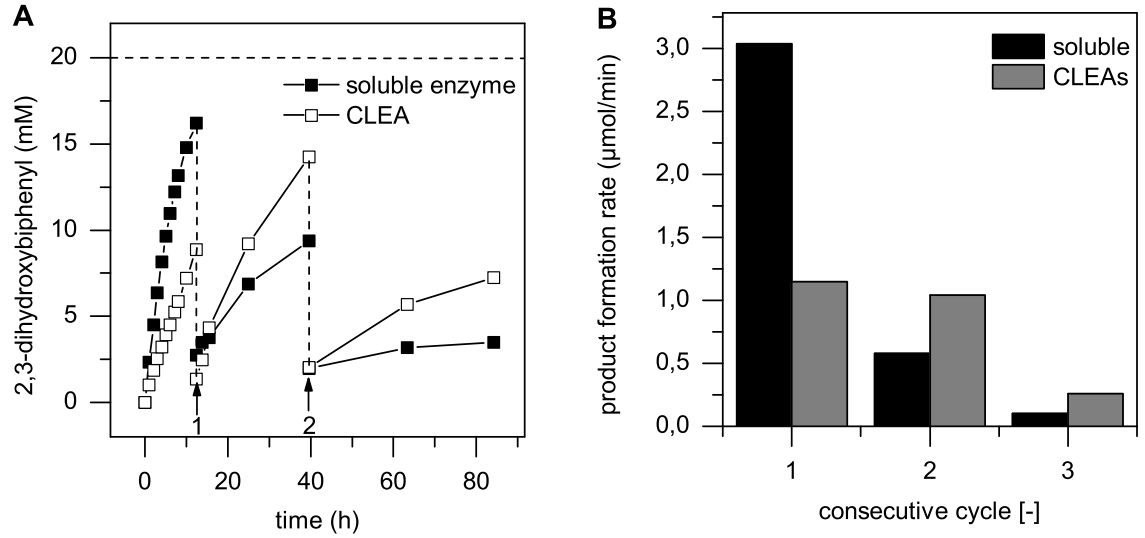


Figure 3-9: A – Comparison of Combi-CLEAs and soluble HbpA/FDH performance for the conversion of 2-hydroxybiphenyl (20 mM in decanol) in a repeated batch experiment at a 200 mL scale in a bioreactor in the presence of 0.2 mg mL^{-1} Tween 20. Arrows 1 and 2 indicate the start of a new batch by changing the organic phase. At the beginning of each new batch the aqueous phase was supplemented with 0.5 mM of NAD^+ and 0.2 mg mL^{-1} of Tween 20. The 3rd batch was additionally supplied with 100 mM of formate. B – Product formation rates for the consecutive batches reveal a stabilising effect of CLEAs with respect to the soluble enzymes. Values are calculated for the total volume of aqueous and organic phase of 200 mL with a 1:1 phase ratio.

DISCUSSION

The main motivation for the preparation of CLEAs is the application of these immobilised enzymes in organic solvents.[121a, 131] However, a detailed characterisation of such application and the associated problems is, to the best of our knowledge, not existing so far. Still most of the work in this field is done on hydrolases which are comparably simple enzymes,[132] and often function in nearly anhydrous organic solvents without any stabilisation.[50]

FDH and HbpA are multimeric enzymes.[42, 133] As reported previously poly-functional cross-linking molecules like dextran polyaldehyde stabilise multimeric enzymes covalently attached to a modified solid supports like Sepabeads or Toyopearl.[134] Wilson and co-workers reported that preparing CLEAs of multimeric enzymes instead of immobilizing them on solid supports before cross-linking, is more suited for quaternary structure stabilisation.[135]

Recently obtained crystal structure of the HbpA in its *apo* form (no FAD bound) and with bound FAD suggests that both structures differ by the presence of mobile domain that moves to accommodate the FAD. Cross-linking of HbpA might therefore impose the presence of one form (e.g. *apo*) over another leading to enzyme being catalytically inactive. The analysis of the structure revealed also the presence of the lysine side chains in the vicinity of the active site suggesting that crosslinking event might impair substrate/product diffusion (Figure 3-10).

To achieve the same initial reaction rates seven times more Combi-CLEAs than soluble enzyme – with respect to the enzyme used for Combi-CLEA preparation - was used. Despite that, at 200 mL scale biotransformation, HbpA:FDH Combi-CLEAs continuously lost activity under process conditions, although much slower than the soluble enzyme (Figure 3-9) revealing a stabilising effect of the applied preparation method. However, recycling of the CLEAs was initially challenging because CLEAs were accumulating in the interface between aqueous and organic phase. Similar observations have been reported for multimeric nitrile-hydratase CLEAs[136] in which case CLEA activity was rather low as compared to the soluble counterparts and the CLEAs were difficult to handle during recycling. Other examples report rather high residual activities e.g. for laccase or tyrosinase CLEAs, which also showed increased stability in methanol, acetone and *tert*-butanol.[121a, 137] But in these examples, either the CLEAs have only been incubated in the solvents, while the reaction was monitored in an aqueous system, or the reaction environment comprised hydrophilic organic solvent with a very low water content without an organic-aqueous phase boundary[70, 120] making a direct comparison of the various approaches difficult.

Combi-CLEAs have also been prepared from peroxidase and glucose oxidase,[131] but also in this case overall enzyme performance could not compete with the soluble counterparts. Hyper activation of CLEAs prepared of carboxylic acid esterases have also been reported,[138] authors

explained it by the presence of favourable conformational changes that resulted from the aggregated state, but also here the respective CLEAs completely lost activity after the second catalytic cycle.

Addition of Tween 20 had a severe impact on our reaction system. Interestingly, it seemed to stabilise the product 2,3-dihydroxybiphenyl, which usually turned yellowish probably due to oxidation and/or polymerisation, while in the presence of Tween 20 the organic phase remained colourless and transparent. It was also beneficial for stabilising both, soluble and immobilised enzymes, minimising the amount of CLEAs sticking to the phase boundary and facilitating their recycling. The latter is the key advantage of using the CLEAs, however, although the soluble enzyme could not be properly recycled (only the organic phase was exchanged between the different batches (Figure 3-9)) it was still possible to synthesise the same amount of product by both approaches, but the Total Turnover Number (TTN) for the soluble enzyme was nearly 6-fold higher as the TTN for Combi-CLEAs (Table 3-3).

Table 3-3: Total Turnover Numbers for soluble and Combi-CLEA enzyme in the 200 mL repeated batch biotransformation.

	total prot [mg]	HbpA amount [µmol][a]	total product [µmol]	TN (μmol_{prod} sek^{-1} μmol_{enz}^{-1})			TTN (μmol_{prod} μmol_{enz}^{-1})
				1st batch	2nd batch	3rd batch	
soluble enzymes	7.7	0.084	2497	0.602 (6h) (100%)	0.115 (11h) (19%)	0.02 (24h) (3.3%)	29649
Combi-CLEA	49.2	0.53	2777	0.036 (10h) (100%)	0.032 (3h) (90%)	0.008 (24h) (22%)	5160

[a] HbpA content in enzyme mixture was calculated on the base of percentage peak area at 210 nm after gel filtration chromatography. SDS PAGE gel confirmed the calculated purity of approx. 70% of total protein. The given value represents molar amount of the monomer of HbpA.

Despite lower turnover numbers (TN) the apparent Km values for 2-hydroxybiphenyl measured in a 2 mL 2LP system and calculated for both, soluble enzymes and Combi-CLEAs, were similar (5.2 and 4.2 µM respectively) and very promising.

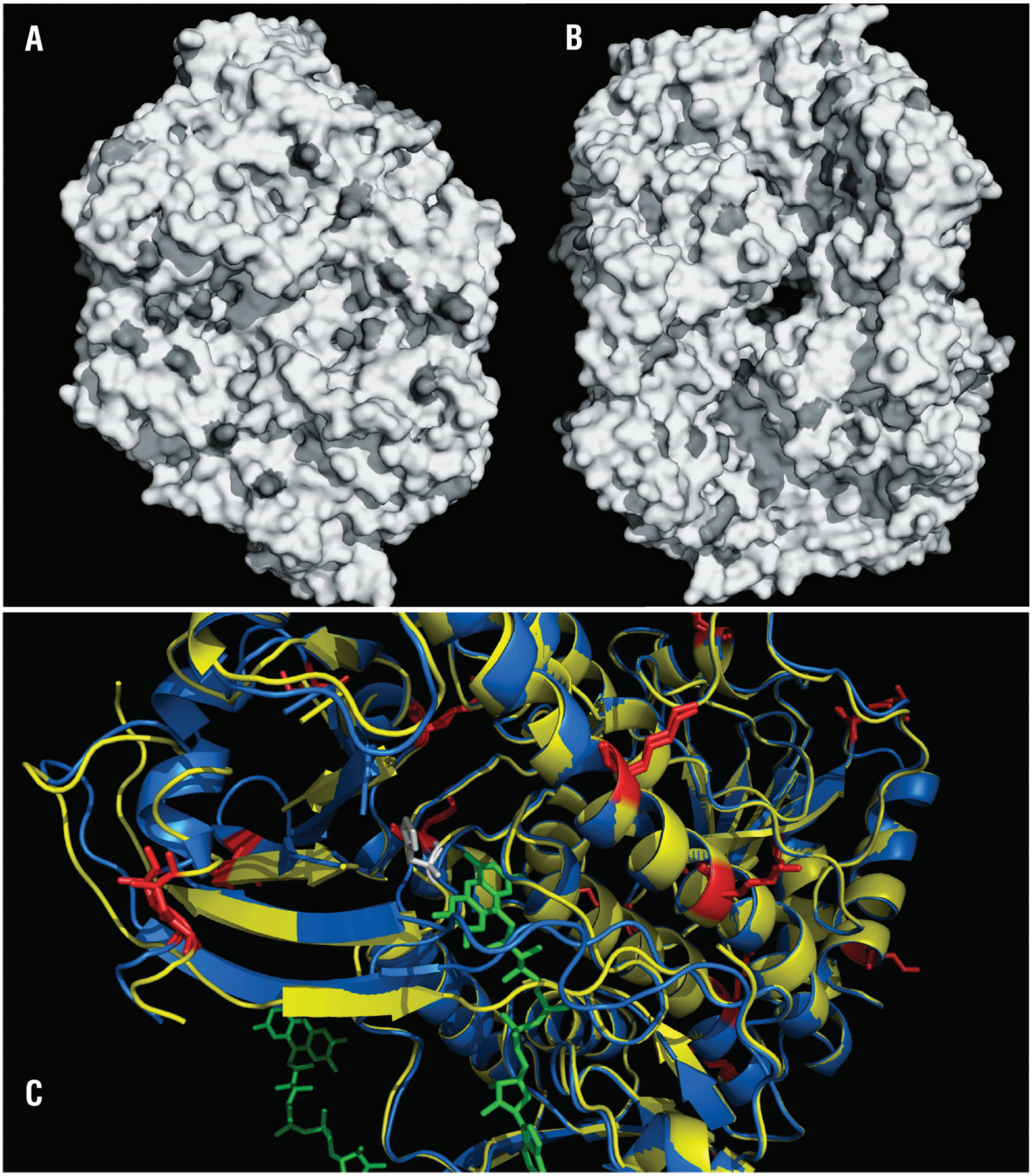


Figure 3-10: A and B - *Apo* structure of the HbpA tetramer with lysine residues in dark colour. C – *apo* (blue) and FAD bound form (yellow) superimposed. Lysine residues are in red, FAD in green, His48 in white. (copyrights Bartłomiej Tomaszewski)

The extent of stabilisation of the enzymes in Combi-CLEA preparations was not sufficient to allow for more multiple repeated batches in STR (200 mL). Even though the mixing applied was not excessive (400 rpm) and adjusted only to allow for emulsion formation in the whole reaction volume, CLEAs disintegrated and when they were eventually recovered after the 3rd batch formed a yellowish 'film' that did not resemble the initial material used (fine white particles). Due to the activity loss and low reaction rates better stabilisation that will preserve the structure integrity and CLEAs mechanical properties is necessary. As reported previously for tyrosinase CLEAs a simple entrapment of the immobilised enzyme in a calcium alginate gel extended its stability to ten cycles without activity loss, whereas only 5 cycles were possible without entrapment.[121b] Similar observations have been published for penicillin G acylase CLEAs, which have been encapsulated in a rigid polymeric matrix composed mainly of polyvinyl alcohol (LentiKats).[139] However, additional coating of the CLEAs will again have a negative impact on mass transfer rates of substrates and products and have to be carefully evaluated for the specific reactions. One could also think about using an alternative reactor system that does not produce high shear forces in order to extend the mechanical stability of the CLEAs.

As shown in Table 3-4 Combi-CLEAs preserved nearly 15% of the initial enzymatic specific activity. When CLEAs were prepared solely with HbpA, the residual enzyme activity was close to 40% in the presence of stoichiometric amounts of NADH, which is significantly higher than the activity recovery for commercially available *Candida antarctica* lipase B (CALB) or *Thermomyces lanuginosus* lipase (TLL) CLEAs (both in the range of 1%).

Table 3-4: Comparison of activity recoveries between commercially available CLEAs and HbpA:FDH Combi-CLEAs.

	Candida antarctica B lipase[a] $U\ mg_{prot}^{-1}$	*Thermomyces lanuginosa* lipase[a] $U\ mg_{prot}^{-1}$	HbpA-FDH[c] $U\ mg_{prot}^{-1}$
Max. spec. activity (pure or technical purity enzyme)	535 $U\ mg^{-1}$	2 780 $U\ mg^{-1}$	0.28 $U\ mg^{-1}$
CLEA or Combi-CLEA	10 $U\ mg^{-1}$ [b]	50 $U\ mg^{-1}$ [b]	0.041 $U\ mg^{-1}$
Activity recovery %	1.8	1.8	14.6

[a] Substrate tributyrin, 25°C, pH 7.0, gum arabicum stabilised emulsion.[140]

[b] Tributyrin 5 vol% in 40 mM Tris buffer, pH 7.5, 25°C, CLEA Technologies, (CLEA-ST) (www.cleatechnologies.com 2012).

[c] Determined in 2 mL scale reaction tubes in 2LP biotransformation with 20 mM substrate in decanol as presented in Table 3-2.

CONCLUSIONS

Initial low activity recoveries of HbpA:FDH Combi-CLEAs were improved by increasing precipitant concentrations and handling issues were addressed by Tween 20 supplementation. Although CLEAs are reported as easy to prepare and broadly applicable,[118] the technique is also considered not to be suitable for all the enzymes.[141] Without additional modification e.g. gel entrapment or encapsulation CLEAs' mechanical properties are not yet good enough for application in STR in industrial scale biotransformation.[142] However, findings presented in this study show that CLEAs are not restricted to intrinsically stable enzymes like lipases but may also be applied to much more complex enzymatic systems. This work presents a promising basis for further development of this cheap and simple immobilisation method.

ACKNOWLEDGMENTS

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CHAPTER 4

HYDROPHOBIC FORMIC ACID ESTERS FOR COFACTOR REGENERATION IN AQUEOUS/ORGANIC TWO-LIQUID PHASE SYSTEMS

Bartłomiej Tomaszewski,[⊥] Ekaterina Churakova,[⊥] Katja Buehler, Andreas Schmid, Isabel Arends, Frank Hollmann

[⊥] both authors contributed equally

Bartłomiej Tomaszewski and Ekaterina Churakova performed the experiments. Bartłomiej Tomaszewski, Ekaterina Churakova and Frank Hollmann wrote and corrected the manuscript. Katja Buehler, Andreas Schmid, Isabel Arends and Frank Hollmann coordinated and supervised the project and corrected the manuscript.

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ABSTRACT

Hydrophobic formic acid esters have been established as alternative co-substrates for the formate dehydrogenase (FDH)-catalysed regeneration of reduced nicotinamides. With this approach challenges related to the ionic nature of the commonly used formate salts, particularly their exclusive water-solubility, can be overcome. Octyl formate was demonstrated to serve as organic phase solubilising hydrophobic reagents as well as serving as a source of reducing equivalents to enable FDH-catalysed regeneration of NADH. This system was used to drive a monooxygenase-catalysed hydroxylation reaction. Phase transfer limitations appear to be the overall rate-limitation of the biphasic reaction system.

INTRODUCTION

Formate dehydrogenase (FDH, E.C. 1.2.1.2) is certainly one of the most widely applied cofactor regeneration systems[12, 26, 143] used to promote NADH-dependent reductions,[144] and oxidations.[21, 63, 102, 145] Especially the quasi-irreversibility of the formate-driven NAD^+ reduction reaction (yielding volatile CO_2 as by-product) makes it an attractive cofactor regeneration system.

One drawback of the FDH regeneration system is its dependence on highly polar formic acid and/or formates as sacrificial electron donors to drive the regeneration reaction. As a result, classical FDH-based reaction systems are limited to mainly aqueous media. Considering that the majority of the (industrially) relevant substrate/product couples are rather hydrophobic, purely aqueous reaction systems suffer from poor substrate loadings and inherently poor STYs. One possible solution to this dilemma is the use of aqueous/organic two-liquid phase systems (2LPs) wherein the bulk of reagents is dissolved in the water-immiscible organic phase and partitions into the, biocatalyst-containing, aqueous phase for transformation according to their partition ratio.[21, 63, 102, 146] However, large volumetric ratios of organic phase to aqueous phase (which are desirable envisaging high volumetric productivities) will necessitate very high formate concentrations in the aqueous layer to attain stoichiometric amounts of the sacrificial reductant. The resulting high ionic strength in the aqueous phase can pose a significant challenge to activity and stability of the dissolved biocatalysts.

Inspired by a recent contribution by Frohlich et al.[31] who reported FDH-activity on formate esters, we hypothesized that hydrophobic formic acid esters may serve as sacrificial electron donors to promote NADH-dependent reactions in 2LPs. Hence, hydrophobised formates can be furnished in the apolar organic phase in equimolar concentrations without impairing the biocatalyst(s) due to high ionic strengths in the aqueous layer. Ideally, the formic acid esters themselves form the organic phase thereby further reducing the solvent ballast. Provided the alcohol part of the formic acid ester is a hydrophobic alcohol its accumulation in the organic phase in the course of the reaction should not alter the (hydrophobic) properties of the organic phase too much.

To test our hypothesis we chose 2-hydroxybiphenyl 3-monooxygenase (HbpA, E.C. 1.14.13.44) as a model enzyme.[20-21, 32, 41a, 42, 102] As organic phase we chose octyl formate since both the ester and 1-octanol are sufficiently hydrophobic to serve as second, water-immiscible phase. Overall, a biphasic reaction setup as shown in Figure 4-1 was envisaged.

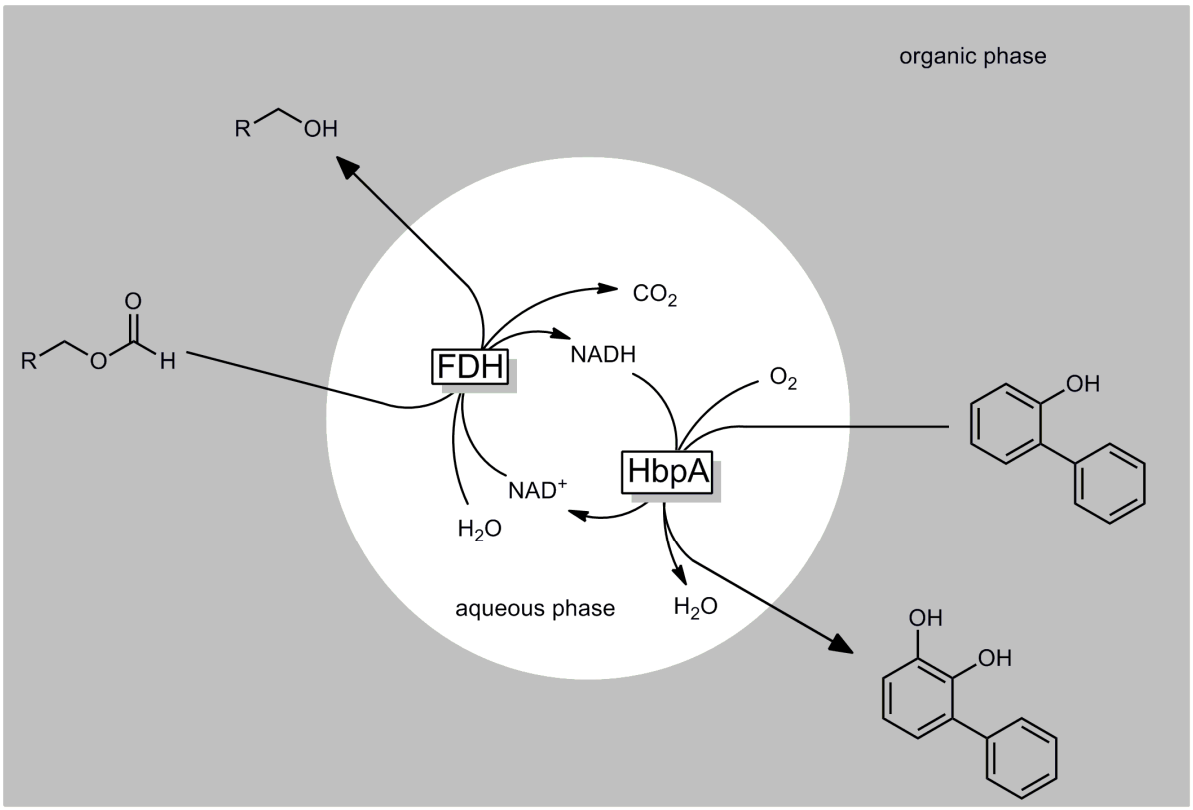

Figure 4-1: The 'hydrophobised formates concept' to promote a NADH-dependent oxyfunctionalisation reaction. Formic acid esters, such as octyl formate, form the organic phase also serving as substrate reservoir and product sink. Formate dehydrogenase (FDH) mediates the oxidative hydrolysis of the formic acid esters into CO_2 and alcohol while regenerating NADH.[31] The latter is consumed in the course of the HbpA (2-hydroxybiphenyl 3-monooxygenase)-catalysed hydroxylation of 2-hydroxybiphenyl.

MATERIALS AND METHODS

Cultivation of biomass and enzyme purification

hbpA (encoding 2-hydroxybiphenyl 3-monooxygenase, HbpA) was expressed in *E. coli* JM101 carrying the plasmid pHBP461 as previously described,[101] and purified using established methods.[21, 102] *fdh* (encoding formate dehydrogenase, FDH) was expressed in *E. coli* JM101 carrying the plasmid pBTac2 (C23S) following a published procedure.[112a]

UV spectrophotometric activity measurements

The activities of both HbpA and FDH were determined using a spectrophotometric assay following the depletion (HbpA) or formation (FDH) of the characteristic UV-absorption band of NADH at 340 nm (ε = 6220 M^{-1} cm^{-1}). The experiments were performed using 1.5 mL disposable UV cuvettes (polystyrene) at 30°C on a Shimadzu UV-2401 PC spectrophotometer with a Julabo F12 refrigerated/heating circulator. All experiments were performed at 30°C. The following buffers and stock solutions were used: buffer (50 mM KPi pH 7.5), NADH (25 mM in buffer), NAD^+ (10 mM in buffer), HbpA (3.98 mg mL^{-1} in buffer), FDH

(1.74 mg mL^{-1} in buffer), $NaHCO_2$ (2 M in buffer, pH adjusted to 7.5), 2-hydroxybiphenyl (25 mM in methanol), FAD (0.2 mM in buffer).

HbpA assay: HbpA was diluted in buffer to a final concentration of 0.04 mg mL^{-1} and supplemented with FAD (0.002 mM final). The assay was started by addition of NADH (0.3 mM final) to determine the non-substrate related NADH oxidation rate (background activity). After one minute, 2-hydroxybiphenyl (0.2 mM final) was added to determine the total NADH oxidation rate. The specific 2-hydroxybiphenyl hydroxylation activity of the HbpA preparation (1.66 U mg^{-1}) was obtained by subtracting the background activity from the total activity.

FDH-assay: FDH was diluted in buffer to a final concentration of 0.017 mg mL^{-1} and supplemented with NAD^+ (1 mM final) and $NaHCO_2$ (100 mM final). The specific NAD^+ reduction activity of the FDH-preparation used was 1.06 U mg^{-1}. A spectroscopic determination of the FDH activity towards octyl formate proved to be very difficult due to its poor solubility in aqueous media (diffusion limitation and/or formation of optically not transparent emulsions upon mixing) and the slow hydrolysis of octyl formate leading to the formation of aqueous formic acid.

Stability measurements

The stabilities of HbpA and FDH under reaction conditions were determined by incubating 5.4 U mL^{-1} of each enzyme at 30°C for 30 hours in a 2 mL reaction tube containing a biphasic mixture (phase ratio 9:1 organic to aqueous) consisting of 100 µL of KPi buffer (50 mM, pH 7.5) and 900 µL of octyl formate, in the absence or presence of Tween 20 (0.2 mg mL^{-1}). Tween 20 was chosen as surfactant to facilitate the formation of stable emulsions with minimal shear force.[146c] At intervals, 10 µL samples of the aqueous phase were withdrawn and analysed using the photometric assay described above.

Coupling of FDH catalysed NADH regeneration to the HbpA reaction.

Unless mentioned otherwise, the coupled reaction of FDH and HbpA was performed in 2 mL tubes at 30°C and mixed at 1200 rpm. The reaction mixtures consisted of a biphasic mixture of KPi buffer and octyl formate with the ratio 1:9 (v/v). The organic phase contained 2-hydroxybiphenyl in a concentration range of 10-500 mM and the aqueous phase contained 5.4 U mL^{-1} HbpA, 5.4 U mL^{-1} FDH, 2 mM NAD^+, and 20 µM FAD. The reaction mixture was supplemented with 0.2 mg mL^{-1} Tween 20. We have refrained from adding catalase to the experiments since the cell crude extracts (originating from aerobic *E. coli* cultivations) presumably contained endogenous catalase activity. Aliquots of 10 µL of the organic phase

were withdrawn at intervals, diluted with 990 µL of acetonitrile containing 0.1% of TFA (v/v) and analysed by RP-HPLC.

The time course of 2,3-dihydroxybiphenyl formation was determined under the aforementioned conditions at 10 mL scale (50 mL tubes) with an initial ratio between the organic and aqueous phase of 9:1 (v/v). The final phase ratio was approximately 8.84:1 (v/v) after 16 samples of 10 µL.

In one experiment the effect of lipase addition was investigated under these conditions: The coupled reaction of FDH and HbpA was performed as described in a standard procedure in 50 mL tubes using NAD^+ (0.2 mM) and 2-hydroxybiphenyl (500 mM). To the reaction assay (V_{total} = 10 mL), 0.1 g of immobilised Lipase B (CALB) from *Candida antarctica* (Novozym 435) was added.

It should be mentioned here that all experiments had been performed in duplicates (unfortunately not always sampling at identical times). Therefore, no error bar are given in the figures. Nevertheless, the deviation between the single experiments was maximally 14%.

Determination of reactants using HPLC analysis

The progress of the reactions was measured by RP-HPLC on a Shimadzu LC-20 system equipped with a Shimadzu SPD-20A Photo Diode Array detector using a Waters XTerra column (RP18, 3.5 µM, 4.6×150 mm). The temperature was controlled at 40°C by a Shimadzu CTO-20AC column oven. As eluent an acetonitrile/water (containing 0.1% TFA) (40/60) mixture was used isocratically. The flow rate was set to 1.1 mL min^{-1} and the detection wavelength to 210 nm. Retention times were 2.94 min for 2,3-dihydroxybiphenyl, 3.67 min for 2-hydroxybiphenyl and 6.47 min for octyl formate. 1-Octanol was not quantified due to its low response in the detection method (UV). Data were processed using the LC solution software. The final concentrations were calculated based on standard curves obtained using 2-hydroxybiphenyl and 2,3-dihydroxybiphenyl standards in the concentration range of 10-500 mM.

RESULTS

In a first set of experiments we examined the influence of increasing formate concentrations on the activity of both HbpA and FDH (Figure 4-2).

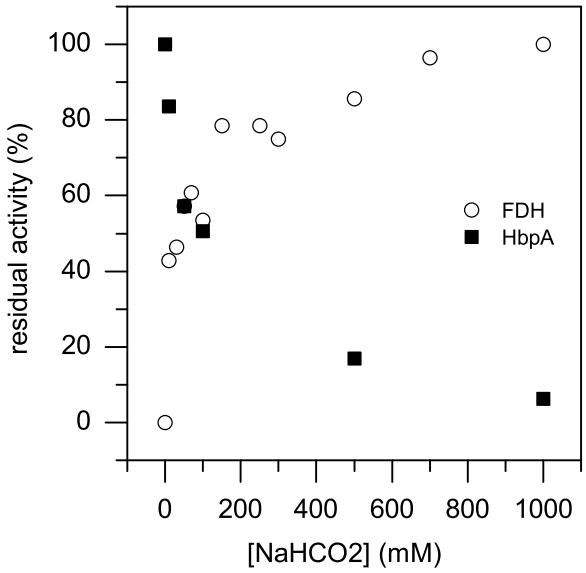

Figure 4-2: Influence of [$NaHCO_2$] on the activity of HbpA (■) and FDH (○).General conditions: KPi buffer 50 mM, pH 7.5, V = 1 mL, T = 30°C, (■): [HbpA] = 0.066 U mL^{-1} = 0.04 mg mL^{-1}, [2-hydroxybiphenyl] = 0.2 mM, [NADH] = 0.3 mM, [FAD] = 20 µM; (○) [FDH] = 0.018 U mL^{-1} = 0.017 mg mL^{-1}, [NAD^+] = 1 mM.

Interestingly, both enzymes showed an opposite behaviour with increasing sodium formate concentrations. Whereas FDH was apparently not inhibited by the presence of up to 1 M sodium formate, HbpA activity decreased significantly. Already in the presence of 100 mM of sodium formate HbpA activity was reduced by 50%. This is in line with previous observations made with HbpA.[42] Suske et al. have observed a significant influence of monovalent anions (such as azide and chloride) on the stability of the intermediate hydroperoxyflavin,[147] which may also account for the apparent inhibition observed with formate. Also, a negative influence of the increasing ionic strength on the structural integrity of the biocatalyst may be hypothesised. Further studies will be necessary to fully understand the nature of the apparent inhibition of HbpA by sodium formate. Overall, we concluded that HbpA catalysed hydroxylation reaction (being inhibited by already comparably low sodium formate concentrations) is a suitable model system to evaluate the 'hydrophobised formates concept'.

Next, we went on to test the feasibility of the 'hydrophobic formates concept'. Under arbitrarily chosen reaction conditions (see Figure 4-3, caption) we were pleased to observe stable hydroxylation activity over at least 3 weeks wherein the desired product 2,3-dihydroxybiphenyl continuously accumulated in the organic phase (Figure 4-3). In the absence of either HbpA, FDH or NAD^+, no conversion was observed.

The high robustness of the overall reaction was astonishing as control experiments in the absence of substrate and NAD^+ under otherwise identical conditions to the reaction shown in

Figure 4-3 indicated half-life times of 16 h and 24 h for HbpA and FDH, respectively. Under these conditions also a significant acidification of the aqueous layer (ranging between pH 4 and pH 6) was observed. We attribute this to slow, spontaneous hydrolysis of octyl formate and subsequent accumulation of formic acid in the aqueous medium, leading to an inactivation of the biocatalysts. Under reaction conditions (i.e. in the presence of a NAD^+) formic acid is consumed by the NADH regeneration reaction and therefore does not accumulate maintaining the overall pH value constant. In fact performing the coupled reaction in the presence of a lipase (accelerating the hydrolysis of octyl formate), only trace amounts of product were observed accompanied with a pH drop to less than 2 (data not shown).
Overall, provided the consumption of formic acid proceeds faster than its formation via hydrolysis, a pH-stable system can be achieved.

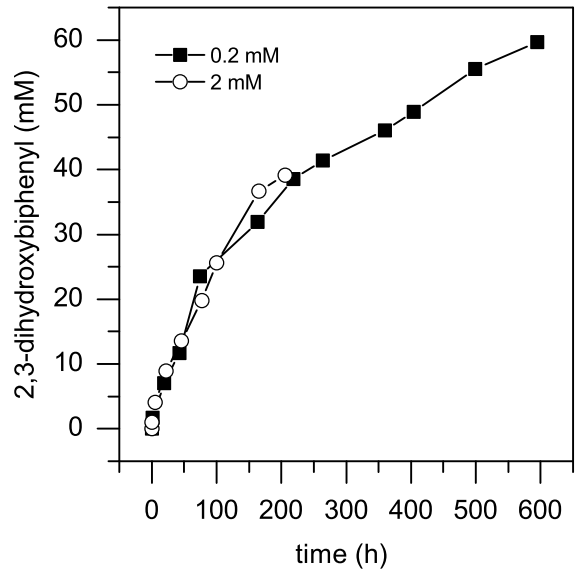

Figure 4-3: Time-course of 2,3-dihydroxybiphenyl formation in a 2LP system using octyl formate as a second organic phase. General conditions: octyl formate : KPi = 9:1 (v/v); V_{total}= 10 ml, T = 30°C; aqueous concentrations: [HbpA] = 5.4 U mL^{-1} = 3.6 mg mL^{-1}, [FDH] = 5.4 U mL^{-1} = 5.1 mg mL^{-1}, [NAD^+] = 2 mM (○) or 0.2 mM (■); organic concentrations: [2-hydroxybiphenyl] = 500 mM.

It should be mentioned here that the productivity of the reaction system (shown in Figure 4-3) significantly fell back behind the values expected from the enzyme activities applied. An initial product formation rate of a bit more than 1 mM h^{-1} (in the organic phase, corresponding to approx. 9 mM h^{-1} in the aqueous layer) was determined. This corresponds to a formal specific HbpA activity of 0.15 U mL^{-1}, which is significantly lower than (approx. 3%) the theoretical HbpA activity of 5.4 U mL^{-1} determined in independent spectrophotometric experiments. Obviously, the same calculation also applies for the formal activity of FDH. We hypothesise that the phase transfer rate (of 2-hydroxybiphenyl and/or octyl formate) into the aqueous layer was overall rate limiting thereby accounting for the low formal specific enzyme activities (*vide infra*). On average (over the whole period), the productivity reached was 0.1 m Mh^{-1}_{org}. Nevertheless, the reaction yielded the desired 2,3-dihydroxybiphenyl at 12%

yield with very promising turnover numbers (TN=final amount of product divided by amount of catalyst used) for NAD^+ and HbpA of 3680 and 18880, respectively. Also the extraordinary high robustness of the reaction (steadily accumulating the product over at least 3 weeks) is worth pointing out.

Despite these promising features, the preparative value of this reaction is impaired by the rather sluggish reaction rates. To clarify this, we systematically varied the concentrations of all reagents and catalysts to identify the overall rate-limiting component. Very interestingly, varying the concentration of either HbpA, FDH or NAD^+ did not significantly influence the overall rate of the system; within experimental error, all rates were identical. Exemplarily the influence of varying [NAD^+] is shown in Figure 4-3 (open circles 2 mM and black squares 0.2 mM). Similar results were also obtained when changing the biocatalyst concentration.

As the catalysts concentrations had no apparent effect on the overall reaction rate, we suspected that diffusion limitation over the interface might be overall rate-limiting. If so, then, amongst other factors, the surface area and the concentrations of the reagents in the organic phase influence the diffusion rate. In fact, changing the concentration of both octyl formate and 2-hydroxybiphenyl had a clear impact on the reaction rate (Figure 4-4 and Figure 4-5).

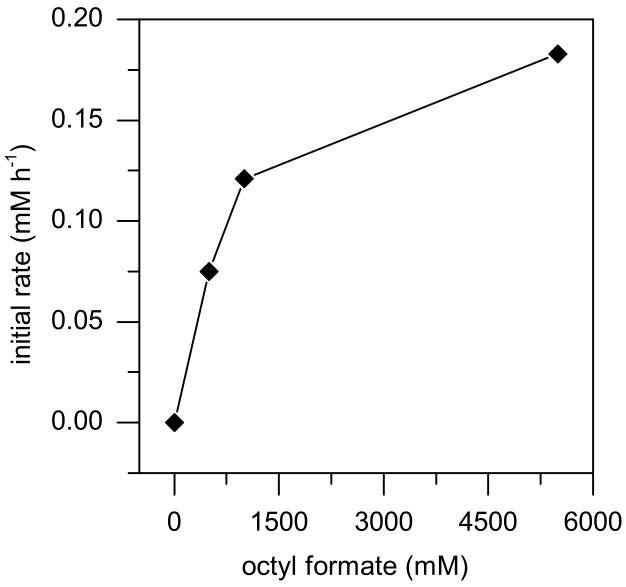

Figure 4-4: Influence of octyl formate concentration on the rate of 2,3-dihydroxybiphenyl formation (determined in the organic phase). General conditions: organic phase: KPi = 9:1 (v/v), aqueous conditions: [HbpA] = 5.4 U mL^{-1}, [FDH] = 5.4 U mL^{-1}, [NAD^+] = 2 mM; organic phase: different ratios of 1-decanol to octyl formate were used resulting in the molar octyl formate concentrations shown; [2-hydroxybiphenyl] = 10 mM.

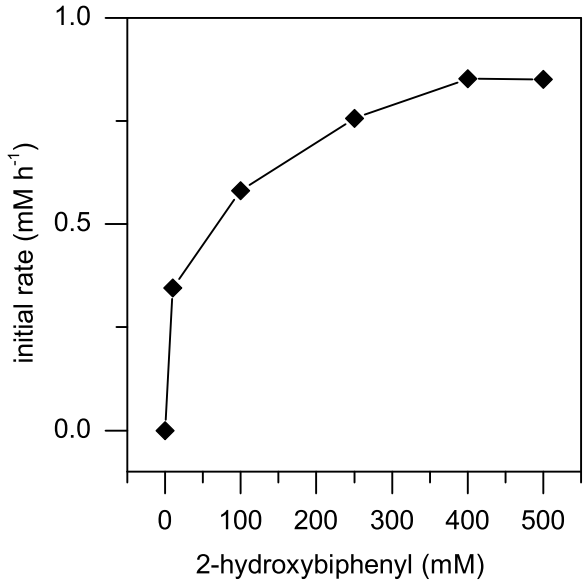

Figure 4-5: Influence of 2-hydroxybiphenyl concentration on the rate of 2,3-dihydroxybiphenyl formation (determined in the organic phase). General conditions: octyl formate : KPi = 9:1 (v/v). Aqueous conditions: [HbpA] = 5.4 U mL^{-1}, [FDH] = 5.4 U mL^{-1}, $[NAD^+]$ = 2 mM.

In both series a saturation-type behaviour of the reaction rate on the concentration of 2-hydroxybiphenyl and octyl formate in the organic layers was observed. We like to interpret these findings in terms of phase-transfer limiting the overall rate of the reaction system. Hence, the interfacial area of aqueous and organic layers should also have a significant influence on the overall rate.[146c] Indeed, changing the reaction setup from gently shaken to vigorously mixed (i.e. performing the reaction in baffled Erlenmeyer flasks) almost doubled the reaction rate albeit at the expense of a significantly reduced stability of the production system: under the mechanically very demanding conditions the production enzymes denatured rapidly leading to an almost complete loss of activity after 1 h.

DISCUSSION

For many biocatalytic reactions water is not the ideal solvent as hydrophobic reactants are only poorly solubilised. The resulting low reactant concentrations, combined with sometimes tedious reaction work-up, render biocatalytic procedures unpractical. Therefore it is not astonishing that a growing number of publications aim at ‘water-free’ or at least minimal water-content biocatalysis. In case of hydrolytic enzymes, this now can be considered to be state of the art,[148] and more and more examples for the emerging oxidoreductases (especially using alcohol dehydrogenases) can be found in the literature.[49, 146b, 149] For production systems necessitating diffusible nicotinamide cofactors (for example monooxygenases where a NADH-regenerating enzyme is inevitable), the so-called two-liquid phase system (2LP) approach may be a good compromise as here a hydrophobic organic solvent serves as substrate reservoir and product sink enabling overall high substrate loadings. In addition, 2LPs can also render biocatalytic reactions more efficient by controlling the aqueous concentrations of substrates and products. Thereby, inhibitory effects of excess reagents can

be minimised as well as undesired side reactions such as the non productive uncoupling of NADH oxidation from substrate hydroxylation as observed e.g. with HbpA.[42, 147] However, high substrate loadings are difficult to achieve using formate salts as stoichiometric reductants as the resulting high ionic strengths in the aqueous layer may significantly impair the biocatalysts' activity and stability. In the current contribution we have demonstrated the principal feasibility of using hydrophobised formic acids (by means of hydrophobic formic acid esters) in the 2LP approach. Thereby, hydrophobic formic acid esters not only serve as substrate reservoir and product sink but also as source of reducing equivalents.

The major limitation of the current reaction setup is the sluggish phase transfer kinetics of both 2-hydroxybiphenyl and octyl formate. Possibly also O_2 availability contributes to the overall slow reaction rate. Increasing the phase transfer rate, especially via emulsification will be in the focus of our further investigations. Here, the major challenge to be addressed is to reduce the mechanical stress induced by the emulsification process and to protect the biocatalysts e.g. *via* immobilisation.

Despite the early stage of development of this system and the many challenges still to be faced *en route* to becoming a truly practical reaction system we believe that hydrophobised formates represent an interesting future pointing development in FDH-based redox biocatalysis.

CONCLUSIONS

In this contribution we have demonstrated the principle applicability of the 'hydrophobised formates concept' to promote NADH-dependent redox reactions. Hydrophobic esters of formic acid can serve as organic phase in biocatalytic 2LP reactions, and as source of reducing equivalents at the same time; thereby paving the way towards low-water content redox biotransformations. The current setup is limited by poor mass transfer rates over the interface, which will be addressed in the future e.g. by conducting the reaction in microreactors to reduce phase transfer limitation.

CHAPTER 5

REGIOSELECTIVE BIOCATALYTIC AROMATIC HYDROXYLATION IN A GAS/LIQUID MULTIPHASE TUBE-IN-TUBE REACTOR

Bartłomiej Tomaszewski, Richard C. Lloyd, Antony J. Warr, Katja Buehler, Andreas Schmid

Bartłomiej Tomaszewski performed the experiments wrote the manuscript. Richard Lloyd, Antony Warr, Katja Buehler, Andreas Schmid, coordinated and supervised the project and corrected the manuscript.

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ABSTRACT

Microreactors provide higher mass transfer rates than do conventional batch reactors. A tube-in-tube microreactor was used for the NADH-dependent *in vitro* conversion of 2-hydroxybiphenyl to 3-phenylcatechol that was catalysed by 2-hydroxybiphenyl 3-monooxygenase (HbpA). A biphasic reaction system allowed high substrate loadings, whereas the microreactor ensured excellent mass transfer rates between organic and aqueous phases. Oxygen was supplied continuously *via* membrane aeration over the whole reaction compartment. The productivities achieved in the tube-in-tube microreactor were 38 times higher than those in previously described batch reactors and almost 4 times higher than for the same reaction in a microreactor in which aqueous, organic and air phases were delivered through consecutive segments. This setup is a promising concept for oxygen-dependent biocatalytic reactions in microreactors, and is developing as a basis for applications in gram-scale organic biosyntheses.

INTRODUCTION

Miniaturised enzymatic reaction systems were introduced in the late 1970s as devices for flow injection analysis and continuous segmented flow analysis.[85] These systems can be regarded as predecessors of microreactors used for high-throughput sample analysis under continuous flow conditions. Microreactors provide high mass and heat transfer rates[87] and allow better control of temperature and reaction times than do conventional stirred tank reactors (STRs).[89, 99] They enable rapid screening of reaction conditions and generation of compound libraries with minimal reagent or catalyst use.[90] Flow reactors with chemical catalysts have been used for laboratory and industrial purposes.[91, 150] Synthetic applications of enzymes in microreactors have also been reported.[76, 92a, 92c, 92e, 94-95, 99, 151]

Enzymatic redox reactions often profit from the introduction of multiple phases as gas/liquid, liquid/liquid, or gas/liquid/liquid, to supply low-soluble hydrophobic substrates and/or to overcome substrate and product inhibition by partitioning the reactants in different phases. In such a reaction environment, interfacial mass transfer could play a key role in increasing reaction rates. In a recent study, the enzymatic reaction performance was maximised by applying aqueous/organic two-liquid phase segmented flow.[95] A segmented flow is formed when two immiscible liquids are delivered to the microreactor to form elongated capped bubbles (also called slugs, plugs, or segments). Surface tension and viscous forces dominate in such a reactor, providing good local mixing (circulation within segments), which is beneficial to the interfacial mass transfer[152] and rapid equilibrium establishment. However, the use of aqueous/organic/air three-phase segmented flow for maximising the interfacial mass transfer between aqueous/air and aqueous/organic phases is limited owing to flow stability and controllability issues. In addition, the delivery of oxygen in segmented flow fashion coupled to high oxygen mass transfer (k_La 0.01-0.5 s^{-1})[78] and a high oxygen consumption rate results in the depletion of this compound within seconds to minutes, which again limits the residence time and thus effects the respective conversion. To overcome flow instability and oxygen limitations but still benefit from the high mass transfer rates achieved in microreactors, a tube-in-tube reactor (TiTR) (Figure 5-1) is an interesting concept. A TiTR consists of a gas permeable Teflon AF-2400 membrane tube placed in an outer polymeric tube; thus the respective gas is delivered directly from a pressurised tank through the inner membrane to the liquid phase(s). Gas is therefore separated from the liquid reaction components, which simplifies reactor operation, stabilises the flow, and allows for continuous supply of oxygen throughout the length of the system.

The tube-in-tube concept has been used for gas-dependent chemical syntheses in flow reactors.[153] A similar device has been proposed for safer and scale independent hydrogen

supply for STRs.[154] Although efforts have been made to develop TiTRs for chemical syntheses, a rational design of such a reactor format for enzymatic reactions, to our knowledge, has not been reported.

Herein, 2-hydroxybiphenyl 3-monooxygenase (HbpA) was used as a model enzyme for regio- and chemoselective oxidation of 2-hydroxybiphenyl with molecular oxygen to produce 3-phenylcatechol. Formate dehydrogenase (FDH) was chosen for NADH recycling.[27, 155] *n*-Decanol was used as water immiscible organic phase to overcome low solubility of the non-polar substrate and product.[21, 102] The inner tube in TiTR constituted aqueous/organic segmented flow for maximising the aqueous/organic mass transfer, whereas bubble-free continuous oxygen supply and continuous carbon dioxide removal to prevent a pH shift were obtained through the outer tube.[156] Overall, four concepts – biocatalysis in a water/organic two-liquid phase system, enzymatic cofactor regeneration, bubble-free aeration, and microreactors – were integrated for the efficient synthesis of 3-phenylcatechol in a TiTR.

Different factors affecting HbpA activity, such as NADH cofactor regeneration, hydrophobic substrate supply, and product removal, and their effect on final product titre and hydroxylation rate are discussed as a case study and proof of concept.

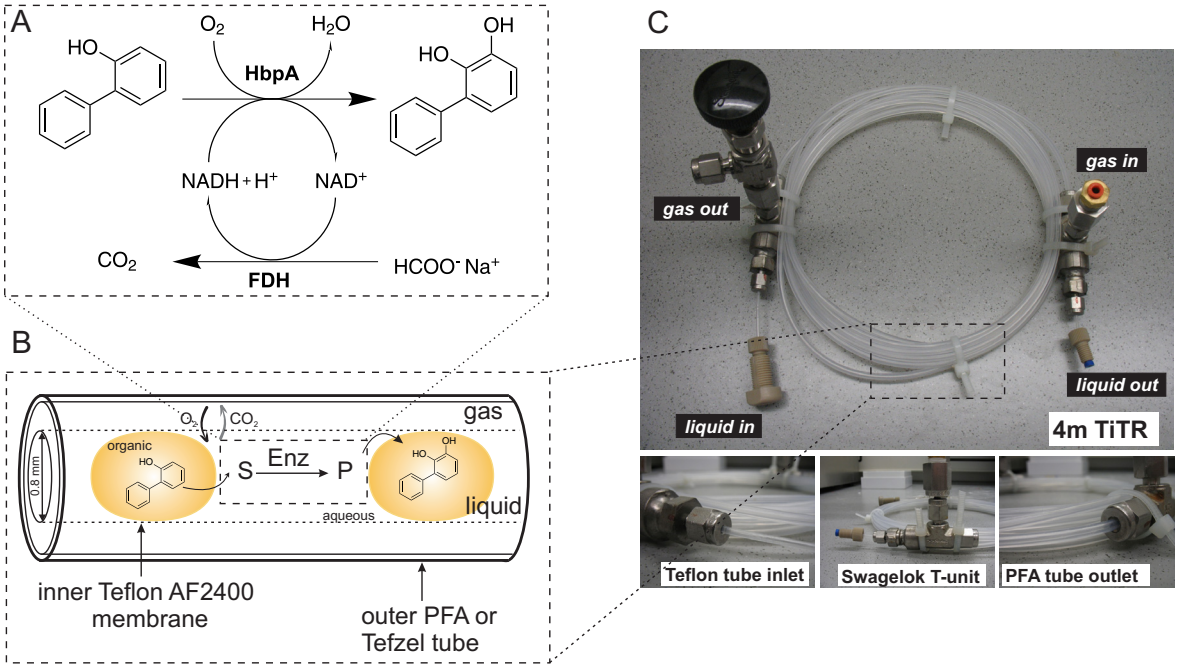

Figure 5-1: A – HbpA-catalysed hydroxylation of 2-hydroxybiphenyl to 3-phenylcatechol with concomitant NADH regeneration by FDH. B – Schematic representation of a TiTR with aqueous/organic two-liquid segmented flow; S=reagents, P=products. C – Scheme of a 4 m TiTR used herein.

MATERIALS AND METHODS

Chemicals

NADH disodium salt and *n*-decanol were obtained from Applichem (Darmstadt, Germany); sodium formate, NAD^+ disodium salt and flavin adenine dinucleotide (FAD) from Fluka (Buchs, Switzerland); 2-hydroxybiphenyl from Sigma-Aldrich (Taufkirchen, Germany) and 3-phenylcatechol from Wako Pure Chemical Industries Ltd. (Osaka, Japan). All other chemicals were purchased either from Fluka, Applichem or Sigma-Aldrich and used as received.

Assembly and operation of the TiTR

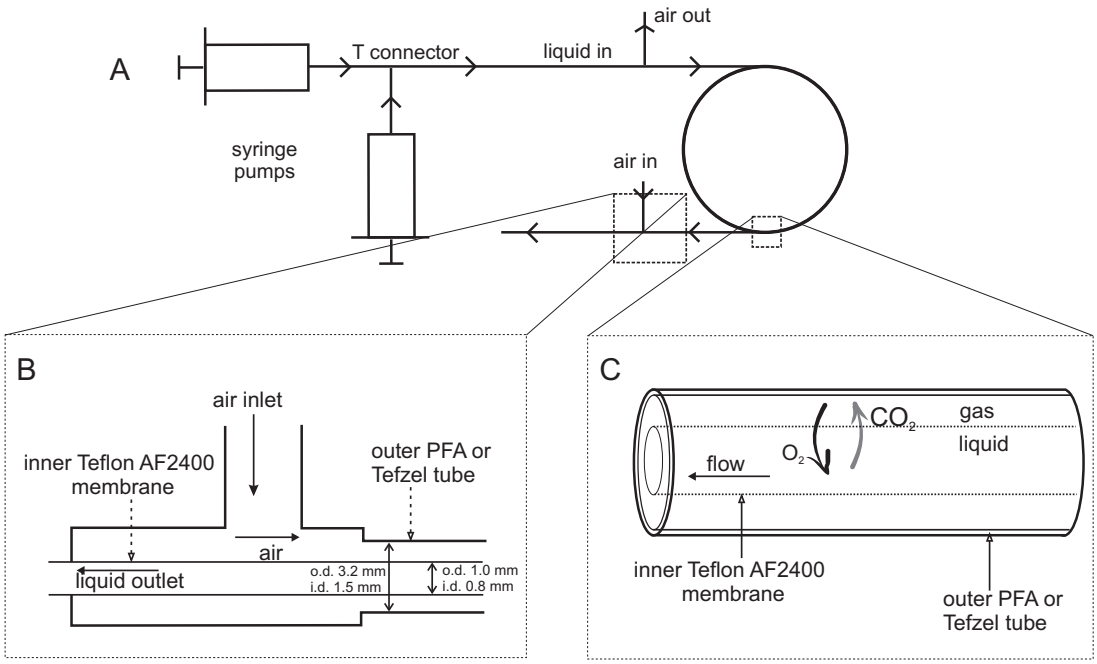

Figure 5-2: A – Schematic representation of the TiTR setup. Two syringe pumps are used to deliver the aqueous and organic liquid that are united in a four-way V100T valve or T-shaped PEEK capillary connector and continue inside a coiled Teflon AF-2400 membrane in a segmented fashion up until the outlet, at which they are collected and subsequently analysed. B – Simplified diagram of the air-line connection; o.d.=outer diameter, i.d.=inner diameter. C – Close-up of the TiTR system with a Teflon AF-2400 membrane placed inside Tefzel or PFA tube. Gas is delivered in a countercurrent fashion and flows outside the Teflon AF-2400 membrane. Along the way gas diffuses into the inner tube and saturates the liquids with oxygen, whereas formed carbon dioxide is continuously removed.

The setup of the TiTR is shown in Figure 5-2. A 1-m-long Gastropod® reactor (0.5 mL volume inside the Teflon AF-2400 membrane) was purchased (Cambridge Reactor Design, Cambridge, UK), whereas the 4-m-long (2 mL volume) version was assembled on-site with commercially available interconnects (Swagelok, Kings Langley, UK). The inner tube of the TiTR was made of Teflon AF-2400. The gas permeability of this membrane for oxygen and CO_2 is 990 and 2800 Barrer [1 Barrer=3.348×10^{-19} kmol m $(m^2\ s\ Pa)^{-1}$], respectively. For a 2-m-long Teflon AF-2400 membrane (1 mL volume) (i.d.: 0.08 cm; o.d.: 0.1 cm) these values

translates to 20.8 μmol min^{-1} (0.41 μmol min^{-1} cm^{-2}) or 39.9 mg h^{-1} oxygen at 0ºC and an oxygen partial pressure difference of 20.8 kPa (15.6 cmHg converted to SI units), that is, when the pressure around the tube is equal to atmospheric pressure and liquid in the tube is oxygen free. The membrane was kept in a polymeric tube such as PFA tube (with 10-20 times lower oxygen permeability[157]) for 4 m TiTR; or Tefzel tube (with approx. 170 times lower oxygen permeability[158]) for the 1 m TiTR.

This assembly allows the precise control of the pressure of the delivered gas. Two Chemyx Nexus 6000 syringe pumps (KR Analytical, Sandbach, UK) were used to pump aqueous and organic phase streams. Liquids were united in a four-way V100T valve (i.d.: 1 mm; UpChurch Scientific, Göhler HPLC, Chemnitz, Germany), which formed a stable oil-in-water segmented flow pattern as illustrated in Figure 5-3. Air was delivered to the outer membrane directly from the pressurised air tank at approx. 60 g h^{-1} (as determined by using EL-FLOW mass flow meter/controller, Bronkhorst Ltd, Newmarket, UK).

Enzyme production and purification

The production and purification of HbpA and FDH was performed as previously published,[21, 102] and as described in chapter 2. The enzymes were of technical purity (FDH: 1.3 U mg^{-1} approx. 25% pure; HbpA: 1.5 U mg^{-1} approx. 40% with respect to the maximum reported specific activity of a pure protein) and were used directly as 50 vol% glycerol stocks stored at -20ºC. Both enzymes catalysed irreversible oxidation reactions. The pH range in which HbpA demonstrated good activity (>80%) was pH 7.2-7.8, with an optimum at pH 7.5,[42] which was also the optimal pH for FDH activity.[109, 112a] Therefore, this pH was the pH of choice for the biotransformation reaction. Cofactor stability (NADH and NAD^+) was confirmed at this pH.[159] KPi buffer (100 mM) was chosen to provide stable pH during the biotransformation at all residence times. Nevertheless, the carbon dioxide was removed continuously, which made the medium acidification unlikely.

HbpA activity assays

HbpA-activity determined by NADH consumption in an UV spectrophotometer

HbpA activity was determined by measuring the consumption of NADH (ε = 6220 M^{-1} cm^{-1}) at 340 nm using a Cary 300 Bio UV-Vis spectrophotometer (Varian, Darmstadt, Germany). 1 U of enzyme activity referred to 1 μmol of NADH consumed in 1 min at the 30°C. A standard assay was conducted as follows: KPi buffer (960 μL of 100 mM, pH 7.5) supplemented with NADH (12 μL of 25 mM, 0.3 μmol) and FAD (10 μL, 2 mM, 0.02 μmol) was mixed in a 1.5 mL PMMA cuvette, which was thermostated at 30°C for 5 min. Then, HbpA solution (10 μL, approx. 0.03 U) was added and mixed with a pipette; and the background activity, that is, a

futile endogenous NADH oxidation in the absence of a substrate (oxidase activity), was determined. Subsequently the reaction was started by the addition of methanolic solution of 2-hydroxybiphenyl (8 µL, 25 mM, 0.2 µmol). The reaction was monitored for 1 min. Substrate specific activity was corrected for endogenous NADH oxidation.

HbpA activity determined by product formation via RP-HPLC analysis

The HbpA-catalysed biotransformation reaction was monitored by determining the concentration of the product 3-phenylcatechol in RP-HPLC. Measurements were performed either with a Hitachi Elite LaChrom HPLC system equipped with a Hitachi Elite LaChrom L-2450 Diode Array Detector (Merck Hitachi, Darmstadt, Germany) or Gilson HPLC system equipped with a Gilson 151 UV/Vis detector. All measurements were performed at 40°C on a C_{18} based XTerra RP 18 3.5 µM column (Waters, Dublin, Ireland). ACN-water [65:35, v/v; both supplemented with 0.1% v/v TFA] was used for isocratic elution at a total flow rate of 1 mL min^{-1}. For sample preparation, the reactor effluent (100 µL) was collected directly in a tube containing ACN (400 µL, 0.1% v/v TFA). The tube was immediately shaken, which led to the precipitation of enzyme and buffer salts, and then centrifuged (4 min, 4ºC, 14900 rpm) in a benchtop centrifuge. The supernatant (100 µL) was transferred to a HPLC vial containing ACN (900 µL, 0.1% v/v TFA); the solution was mixed and analysed directly. The product 3-phenylcatechol and the substrate 2-hydroxybiphenyl were detected between 2.5 and 2.7 and between 3.1 and 3.4 min retention times, respectively. Results were analysed using the LC Solution software or Gilson Uni-Point software. The concentrations of 3-phenylcatechol were calculated as a peak area ratio of 2-hydroxybiphenyl to 3-phenylcatechol, which was possible owing to the equal response of both compounds at 210 nm. The application of 244 nm was also possible, if required. The validity of the analysis method was confirmed by using standard curves prepared for substrate/product samples premixed at different ratios and analysed accordingly (Appendix Table A2 and Figure A8). Unless stated otherwise, every measurement point represented an average of three consecutive samples and error bars represented standard deviation. All STYs, product concentrations and rates were calculated for the reaction total volume (aqueous and organic phase).

FDH-activity determined by NADH formation in an UV spectrophotometer

FDH activity was determined similarly as was HbpA activity. A standard assay was performed as follows: KPi buffer (840 µL, 100 mM, pH 7.5) supplemented with sodium formate (100 µL, 2 M, 0.2 mmol) was mixed in a PMMA cuvette, which was thermostated at 30°C for 5 minutes. Subsequently, the FDH solution (10 µL, approx. 0.03 U) and of NAD^+

solution (50 µL, 10 mM, 0.5 µmol) were added to start the reaction. The reaction was monitored for 1 min.

Biotransformation in 1 mL scale batch reaction

Small scale batch experiments were conducted in a 2LP setup and reaction conditions were as follows: HbpA (9.5 U mL^{-1}), FDH (9.85 U mL^{-1}), FAD (20 µM), sodium formate (100 mM), NAD^+ (1 mM), it was adjusted to a final volume of 500 µL with KPi buffer (50 mM, pH 7.5) and mixed with equal volume of a solution of 2-hydroxybiphenyl (200 mM) in *n*-decanol containing Tween80/Span80 mixture (0.043 mg mL^{-1}; hydrophile-lipophile balance index 6 according to[160] for an W/O emulsion) in a 2 mL reaction tube. Reaction tubes were left open to facilitate oxygen supply. The tubes were thermostated at 30°C in an Eppendorf thermo mixer comfort (Eppendorf, Hamburg, Germany) with a constant agitation of 1200 rpm for 1 h with a regular sampling and subsequent HPLC analysis.

Biotransformation in microreactor

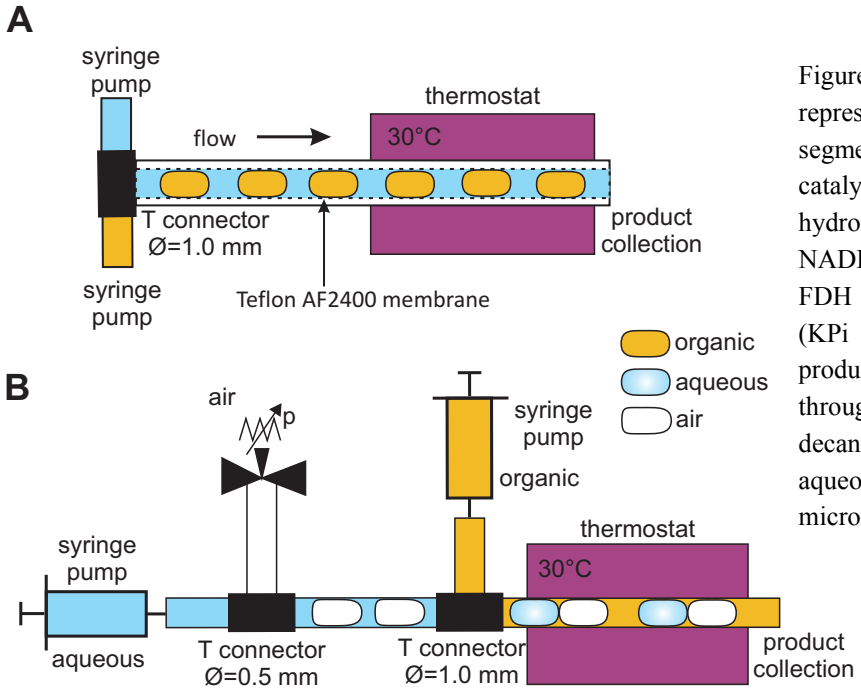

Figure 5-3: A – Schematic representation of the liquid/liquid segmented flow setup: HbpA-catalysed 2-hydroxybiphenyl hydroxylation with concomitant NADH regeneration catalysed by FDH occurs in the aqueous phase (KPi buffer). Substrates and products are extracted and delivered through the organic phase (*n*-decanol). B – Scheme of the aqueous/organic/air three-phase microreactor system.

Unless stated otherwise, all biotransformation reactions were performed in the TiTR. Residence time was controlled through total flow rates. Relative flow rates were kept constant by maintaining the aqueous to organic phase ratio of 1:1. The aqueous phase was complemented by adding 50% glycerol solution of HbpA and FDH (for final volumetric activities of 1-20 U mL^{-1}), sodium formate (100 mM), NAD^+ (1 mM), FAD (20 µM), and Tween 20 (0.6 mg mL^{-1}) for facilitating flow stability and preventing enzyme inactivation, and the reaction mixture was adjusted with KPi buffer (100 mM, pH 7.5) to the respective volume for achieving the required volumetric activities. The aqueous phase was filtered

through a 0.22 µm Millex-CP syringe filter with a polyethersulfone membrane to remove any particles that would have otherwise deposited in the reactor and resulted in flow instabilities later. 2-Hydroxybiphenyl (5-1000 mM in the organic phase) in *n*-decanol formed a separate organic phase. If required, the initial saturation of both the aqueous and organic phases with oxygen could be performed before the reaction by bubbling air through the aqueous phase (before adding enzymes) and the organic phase for 15 min. Unless stated otherwise, the reactor was thermostated at 30°C in a water bath. Product formation rates were calculated for the total working volume of the reactors as specified in the captions of Figure 5-7 and Figure 5-8 (i.e. the sum of the aqueous and organic phase).

Additional experiments were conducted in an aqueous/organic/air three phase microreactor constructed of PTFE (length: 13.3 m; i.d.: 1 mm) (Figure 5-3B). Instead of Tween 20, the organic phase was supplemented with a mixture of Span80/Tween80 (0.043 mg mL^{-1}; (hydrophile-lipophile balance index 6 according to[160] for a water-in-oil segmented flow); HbpA (8.67 U mL^{-1}), FDH (8.57 U mL^{-1}), other components were used as described above. The aqueous and organic phase flow rates were each equal to 0.4 mL min^{-1}. Air was merged with the aqueous phase in a T-shaped capillary connector (UpChurch Scientific, Göhler HPLC, Chemnitz, Germany), and its flow was regulated by manually controlling the air inlet pressure for achieving equal phases ratio (approx. 300 kPa overpressure – 3 bar converted to SI units –was needed to introduce an air segment); the average residence time was 8.5 min.

Enzyme inhibition study

The effect of sodium formate, 3-phenylcatechol, and NAD^+ on HbpA activity was studied by using a standard spectrophotometric assay, as described above. Either an aqueous sodium formate solution (2 M), an aqueous NAD^+ solution (20 mM), or the methanolic solution of 3-phenylcatechol (21 mM) was added to match the required final concentration and adjusted to the final volume with KPi buffer (100 mM, pH 7.5).

Effect of high NAD^+ and NADH concentrations on HbpA activity

The effect of varying NAD^+ and NADH concentrations (1-50 mM) was studied in a 1 mL-scale batch reaction supplemented with FAD (20 µM), sodium formate (100 mM), Tween 20 (0.6 mg mL^{-1}), completed with HbpA (6.9-9.2 U mL^{-1}) and FDH (5.6-7.5 U mL^{-1}) in KPi buffer (500 µL). 2-Hydroxybiphenyl (500 µL, 200 mM) in *n*-decanol was added to the above mixture in a 2 mL reaction tube. The reaction was started by adding the required cofactor amount (1-50 mM). The tubes were thermostated at 30°C in an Eppendorf Thermomixer comfort (Eppendorf, Hamburg, Germany) with a constant agitation of 1200 rpm. After 1 h, the reaction was stopped by addition of ACN (1 mL, 0.1% TFA v/v) and the

reaction mixture stirred for 1 min under the continued reaction conditions. Respective phases were separated by subsequent centrifugation (1 min, 16200 *g*, RT), and the upper phase (10 µL) was transferred to the ACN solution (990 µL, 0.1% TFA v/v) for direct RP-HPLC analysis.

Determination of partition ratio K_D for 2-hydroxybiphenyl and 3-phenylcatechol in *n*-decanol

Solutions of 3-phenylcatechol (200 mM) or 2-hydroxybiphenyl (1000 mM) were prepared in *n*-decanol and were combined with equal volume of water. Samples were vortexed for 30 sec and subsequently incubated in a thermoshaker (1400 rpm, 30 min, RT) to achieve equilibrium. Afterwards, the phases were separated by centrifugation (13300 *g*, 2 min, RT) and diluted in ACN before the measurement. The concentration of the respective compounds were determined spectrophotometrically at 286 nm for 2-hydroxybiphenyl and 288 nm for 3-phenylcatechol in ACN. Concentrations were determined from the standard curves.

RESULTS

The hydroxylation reaction catalysed by HbpA depends on an efficient supply of three unique substrates, namely, the cofactor NADH, the hydrophobic substrate undergoing hydroxylation and molecular oxygen. A detailed investigation of the kinetics and inhibition of HbpA by the product 3-phenylcatechol was performed by Suske et al.[42] and was taken as a basis for the study presented herein. The cofactor dependency of HbpA necessitated the addition of FDH and formate for NADH regeneration. The impact of this regeneration system on HbpA activity was studied to determine optimal reaction parameters for maximising reaction performance.

Cofactor regeneration system affects the hydroxylation reaction performance

The effect of the cofactors on HbpA activity was evaluated by varying their concentrations with or without the regeneration enzyme system in a batch reaction. With a stoichiometric amount of NADH (no cofactor regeneration), a low final product titre was achieved (Figure 5-4A), indicating a strong inhibition of HbpA by the cofactor, which was not observed in the presence of the regeneration system. Thus, HbpA is inhibited by high concentrations of NAD^+, which accumulated owing to the consumption of NADH in the hydroxylation reaction, whereas in the presence of FDH, the NAD^+ pool was low owing to its direct reduction to NADH (Figure 5-4A). Furthermore, as the product titre remained constant with the increase in the cofactor amount, the reaction rate was found to be independent of the NADH concentration and the reaction operated at zero order or maximum reaction velocity because

of the low K_m values for this compound (HbpA: NADH 0.027 mM;[42] FDH: NAD^+ 0.044 mM[112a]).

The effect of sodium formate, the substrate for NADH regeneration, on HbpA activity was investigated by varying the quantity of formate added to the reaction (Figure 5-4B). This substrate strongly inhibited HbpA as its activity negatively correlated with the supplied formate concentration. In addition, a futile NADH oxidation (background activity) was observed, which resulted from NADH oxidase activity of HbpA. On the basis of these findings, 100 mM of formate was defined as a standard condition to minimise its inhibitory effect on HbpA activity and still be sufficiently higher than the K_m value for FDH for this substrate (5.5 mM).[112a] Thus, it is essential to set a trade-off between HbpA inhibition and FDH reaction rate based on the formate concentration in order to maximise the overall reaction performance.

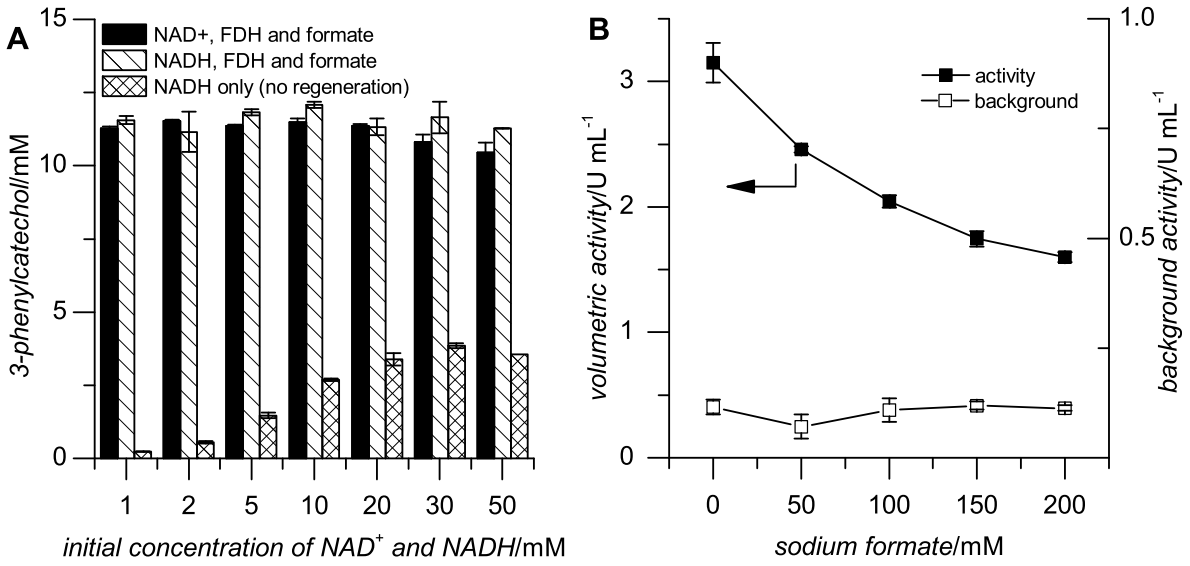

Figure 5-4: A - Effect of varying concentrations of NADH and NAD^+ on HbpA activity. Reactions were performed at 1 mL-scale in reaction tubes on a thermoshaker at 30°C and 1200 rpm for 1 h. An aqueous to organic phase ratio of 1:1 was used. The reaction mixture contained HbpA (6.5-9.2 U mL^{-1}), FAD (20 µM), KPi buffer (100 mM, pH 7.5), and 2-hydroxybiphenyl (200 mM) in *n*-decanol. In the case of reactions with cofactor regeneration, FDH (5.6 -7.5 U mL^{-1}), and sodium formate (100 mM) were also added. B - Effect of different concentrations of sodium formate on HbpA activity. Activities were measured by using a spectrophotometric assay by monitoring the NADH absorbance of at 340 nm (see *Enzyme inhibition study* in the experimental section). Background activity was defined as the consumption of NADH in the absence of substrate (2-hydroxybiphenyl).

Tween 20 stabilises HbpA activity under aqueous/organic segmented flow conditions in the TiTR

To exploit the TiTR system for biocatalytic reactions, it is crucial to determine enzyme stability under reactor operating conditions. Significant enzyme inactivation in an aqueous/organic segmented flow system has been reported, which mainly results from the

large aqueous/organic interfacial area. This problem was solved by addition of Tween 20, a surfactant.[66a] Herein, HbpA and FDH stability under aqueous/organic segmented flow conditions in the TiTR was investigated in the presence and absence of Tween 20. In terms of flow stability, a stable oil-in-water flow pattern was observed in both cases. The final substrate conversion measured for 10 min of residence time was 7.6±0.4% without surfactant and 9.6±0.5% in the presence of 0.6 $mg \cdot mL^{-1}$ of Tween 20. Spectrophotometric measurements revealed 20% loss of volumetric HbpA activity in the absence of surfactant, which accounted for the lower conversions observed without surfactant addition. Thus, Tween 20 (0.6 $mg \cdot mL^{-1}$) was added to prevent enzyme inactivation in the following experiments.

Enzymatic reaction performance in the TiTR

The efficient use of HbpA depends on the optimal supply of three unique substrates: NADH, hydrophobic 2-hydroxybiphenyl, and oxygen. For the interfacial transfer of substrates, the contacting patterns of various phases are important. This section discussed the use of different contacting patterns such as dispersionless contact for oxygen transfer by using a membrane barrier and aqueous/organic segmented flow for the supply of 2-hydroxybiphenyl.

Oxygen transfer through dispersionless contact: improvement of flow stability and oxygen availability

We initially focused on the synthesis of 3-phenylcatechol in an aqueous/organic/air three-phase segmented flow reactor. However, owing to difficulty in obtaining a stable flow as well as technical difficulties in delivering air, the investigation of this three-phase segmented flow was abandoned. Then, HbpA activity (turnover rate) was investigated in the TiTR system under the aforementioned conditions and the effect of continuous oxygen supply in TiTR was compared with that in a setup in which oxygen was delivered only through the aqueous phase. This was achieved by flushing the outer tube with either compressed air (first setup) or nitrogen (second setup). The beneficial effect of the continuous oxygen supply throughout the reactor is depicted in Figure 5-5. In the first setup, the product concentration increased steadily with the increase in the residence times, whereas in the second setup, the product concentration reached a plateau after 6 min residence time when all available oxygen was consumed (Figure 5-5). This result was an initial validation of the TiTR concept for the HbpA-catalysed hydroxylation, which formed the basis for further characterisation and optimisation of reaction conditions in the TiTR system.

Cofactor turnover does not limit reaction performance in the TiTR

The regioselective hydroxylation of 2-hydroxybiphenyl depends on the availability of NADH (Figure 5-1A) and therefore FDH-catalysed NADH regeneration needs to be fast enough to provide sufficient reducing equivalents for HbpA. Thus, the two reactions must operate at rates sufficient to keep the cofactor pool above their K_m values. When the volumetric activity of HbpA was kept constant at 3.7 U mL^{-1}, the relative FDH concentration was varied in the range of 1-20 U mL^{-1}, and the respective 3-phenylcatechol formation rate was determined. The final levels of reaction product remained constant at all FDH concentrations tested, and no distinct optimum value could be observed (Figure 5-6), which suggested that the HbpA reaction was not limited by the FDH-catalysed NADH regeneration.

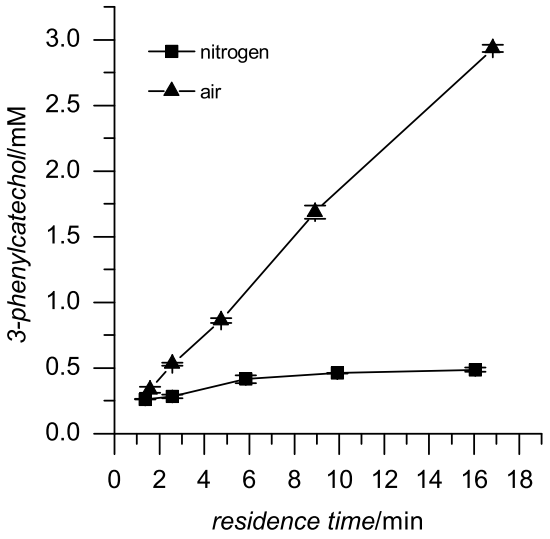

Figure 5-5: The product concentration profile obtained in a 1 m TiTR in relation to the residence time. First setup (air): outer tube flushed with air (triangles); second setup (nitrogen): tube flushed with nitrogen (squares). The aqueous to organic phase ratio was equal to 1. The total flow rates were 0.05–0.8 mL min^{-1} and respective residence times were 16.8–1.4 min. Reactor working volume was 0.8 mL. Reaction conditions used were: HbpA (4 U mL^{-1}), FDH (4 U·mL^{-1}), sodium formate (100 mM), NAD^+ (1 mM), FAD (20 µM), Tween20 (0.6 mg mL^{-1}), KPi buffer (100 mM, pH 7.5), 2-hydroxybiphenyl (50 mM) in *n*-decanol, temp 19°C.

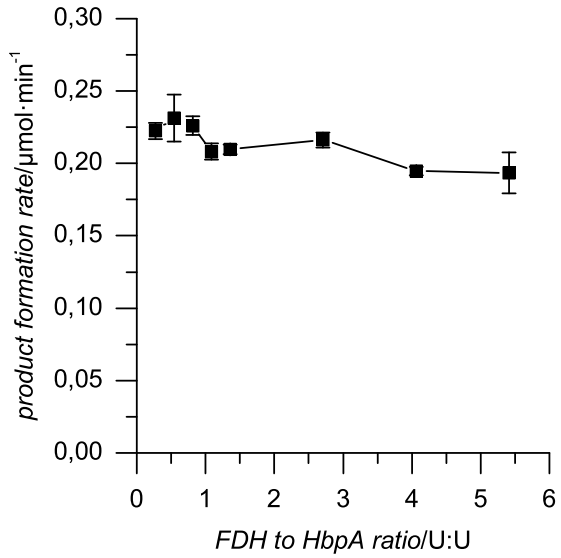

Figure 5-6: The effect of changing the FDH to HbpA unit ratio on the 2-hydroxybiphenyl hydroxylation rate. The HbpA loading was kept constant at 3.7 U mL^{-1} and FDH loading was varied accordingly. The aqueous to organic phase ratio was equal to 1. Total flow rate was 0.2 mL min^{-1} with an effective residence time of 4.92-5.58 min. Reactor working volume was 0.8 mL. Standard reaction conditions used were sodium formate (100 mM), NAD^+ (1 mM), FAD (20 µM), Tween 20 (0.6 mg mL^{-1}), KPi buffer (100 mM, pH 7.5), 2-hydroxybiphenyl (50 mM) in decanol, temp 20°C.

Substrate and product mass transfer affects the reaction performance in the TiTR

To reveal the key limiting factor that affected the reaction performance, the mass transfer of 2-hydroxybiphenyl from the organic to the aqueous phase and its effect on the reaction rate were studied. The important parameters that affect substrate mass transfer in the segmented flow are substrate concentration, flow velocity and phase ratios.[95] In aqueous/organic two-liquid phase biotransformation reactions, the substrate and product are located mainly in the organic phase owing to their partition ratios, K_D. For 2-hydroxybiphenyl and 3-phenylcatechol, K_D values between the organic and aqueous phase were determined to be 480 and 380 respectively (e.g., 200 mM of 2-hydroxybiphenyl in *n*-decanol results in an aqueous concentration of 0.41 mM). The increase in substrate concentration in the organic phase resulted in higher substrate availability in the aqueous phase, which affected the HbpA activity (Figure 5-7A). A plot of product formation rates (μmol min^{-1}) versus the initial substrate concentrations is presented in Figure 5-7A. Each single line represents an experiment with a constant flow rate and increasing concentration of 2-hydroxybiphenyl in the organic phase. In each case, the hydroxylation rate increased steadily with the increase in the concentration of 2-hydroxybiphenyl until it reached a maximum and then plateaued. A maximum rate of 3.8±0.4 μmol min^{-1} (productivity of 17.7±1.8 g L^{-1} h^{-1}) was calculated at an initial substrate concentration of 200 mM in the organic phase and a total flow rate of 0.8 mL min^{-1}. The visible trend resembles apparent Michaelis-Menten kinetics. The increase in the product formation with flow rates at substrate concentration below 200 mM reveals that the reaction was substrate mass transfer limited, which means that above 200 mM, the reaction must operate at zero order and the product formation rate should be independent of the flow rates. However, the product formation rate differed with the applied flow rates. This could owe to a slower product mass transfer from the aqueous to the organic phase, which resulted in concentrations of 3-phenylcatechol close to the K_i value (0.0016 mM) of HbpA in near vicinity of the enzyme. However, the effect of lower substrate availability was more pronounced than product inhibition (Appendix Figure A7).

Another parameter that affects substrate mass transfer is the aqueous to organic phase ratio, which correlates with the available surface area as segments change their size. Thus, the substrate transfer over the phase boundary is affected.[161] In addition, the enzyme to substrate ratio is affected. Higher phase ratios increase enzyme to substrate ratios, which may enhance substrate conversion but simultaneously move the reaction into a mass transfer controlled regime.[95] The effect of different aqueous to organic phase ratios on product formation was studied by changing the individual flow rates while keeping the total flow rate constant at 0.4 mL min^{-1} and thus obtaining the same residence times. In the experiment, the aqueous phase

had to account for at least 50% of the total reactor volume for the formation of a stable flow pattern because of the required capillary wetting. Although the product concentration in the organic phase increased with higher phase ratios, the total amount of product decreased because the product formation rate was lower. The highest amount of product was reached at a phase ratio of 1:1, whereas the highest conversion and product concentration in the organic phase was achieved at a phase ratio of 1:7 (Figure 5-7B). Thus, there is a trade-off between *percentage conversion* and *reaction rate,* which later affects downstream processing and biocatalyst costs. Furthermore, it is essential to know whether full conversion is not limited by the thermodynamics such as in the case of some alcohol dehydrogenases. Therefore, it is recommended to accomplish full conversion in the batch experiment before performing continuous reactions in flow reactors. For HbpA-catalysed reaction full conversion was possible and the reaction was not limited by substrate/product equilibrium (Appendix Figure A3).

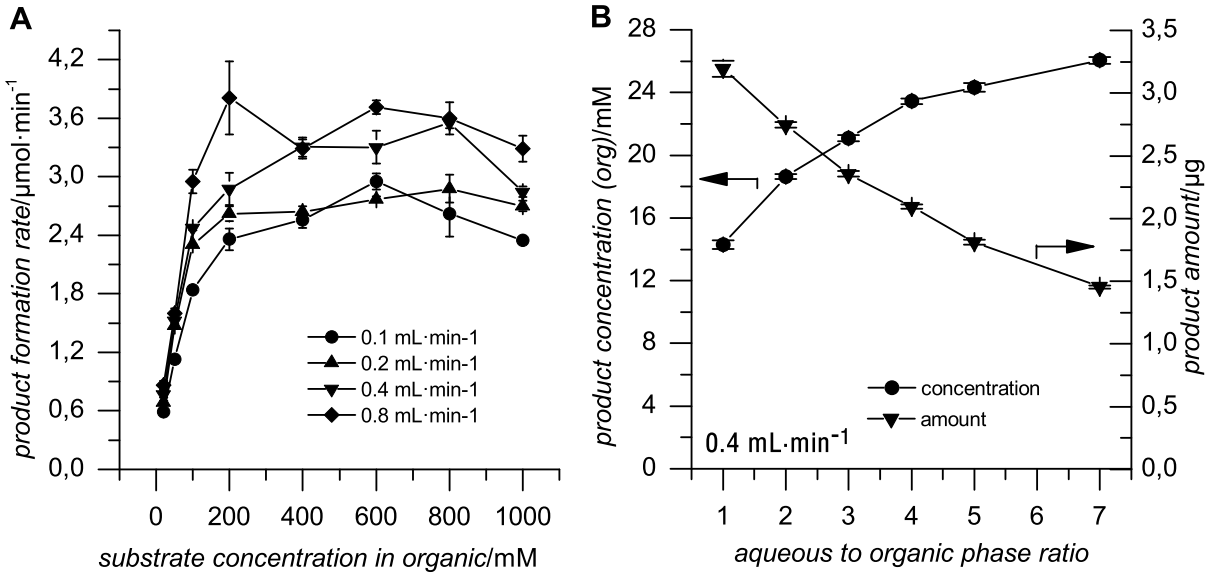

Figure 5-7: A - Effect of 2-hydroxybiphenyl concentration in the organic phase on the hydroxylation rate in a 4 m TiTR under reaction conditions. Total flow rates were 0.1-0.8 mL min^{-1}, and respective residence times varied between 23 and 3 min. Aqueous to organic phase ratio: 1:1; total reactor volume: 2.4 mL. B - Effect of the phase ratio on the final product concentration (organic phase) and product amount (considering two phases and total reactor volume of 2.4 mL) Total flow rate: 0.4 mL min^{-1}, residence time: 5 min 40 s, initial substrate concentration in the organic phase: 200 mM, total reactor volume: 2.4 mL. Reaction conditions: HbpA (9.2 U mL^{-1}), FDH (7.5 U mL^{-1}), sodium formate (100 mM), NAD^+ (1 mM), FAD (20 µM), Tween 20 (0.6 mg mL^{-1}), KPi buffer (100 mM, pH 7.5).

Longer residence time by recirculating the organic phase in the TiTR increased conversion

Although high product formation rates were achieved in the TiTR, conversions were below 10% at a phase ratio of 1:1 (Figure 5-7A). Therefore, a strategy to increase the conversion in

this reactor was performed. Typically in flow reactors, the residence time can be maximised by decreasing the flow rates, increasing the capillary length, recirculating the organic phase several times, or a combination these. A decrease in flow rates for this reaction system was not appropriate because the product formation significantly depends on the mass transfer from the organic to the aqueous phase and thus low flow rates would directly affect the reaction performance (Figure 5-7A). Furthermore, the capillary length was limited to 4 m owing to technical problems associated with handling longer, fragile tubes. Instead, the approach to recycle the organic phase by collecting it at the outlet and feeding it back into the reactor after separating it from the aqueous phase was evaluated. The organic phase was recycled 8 times. With each consecutive cycle, the conversion and product concentration increased and the product formation rate decreased (Figure 5-8). Because the substrate concentration in the organic phase decreased in each cycle, the reaction became mass transfer limited, which led to lower product formation rates. Overall, it is necessary to consider not only optimal reaction parameters such as initial substrate, cofactor and enzyme concentrations if TiTRs are used for enzymatic reactions but also the mode of operation so that the conversion can be maximised.

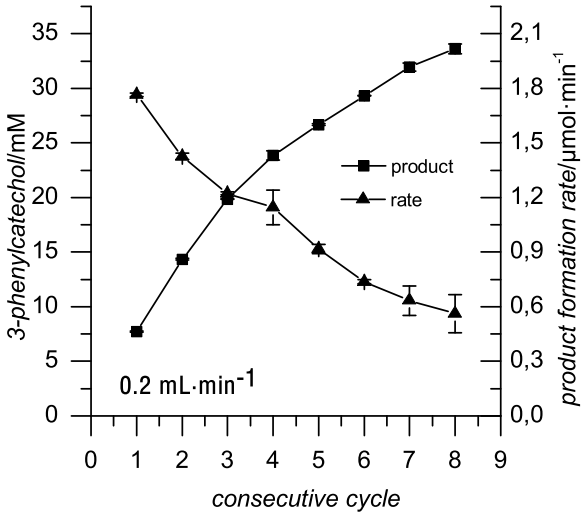

Figure 5-8: Product formation rate and final product concentration after multiple cycles of the organic phase through the reactor. The initial substrate concentration was 100 mM in the organic phase, representing 50 mM in the total volume at a phase ratio of 1. Residence time of each cycle: 10.5 min, total flow rate: 0.2 mL min^{-1}. In every cycle, a fresh enzyme solution was used. Upon fitting a third-order polynomial trend line to the conversion data ($R^2 = 0.99$) and extrapolating, the number of cycles required for full conversion was calculated to be 17.7. Reaction conditions used: HbpA (9.2 U mL^{-1}) and FDH (7.5 U mL^{-1}), sodium formate (100 mM), NAD^+ (1 mM), FAD (20 µM), Tween 20 (0.6 mg mL^{-1}), KPi buffer (100 mM, pH 7.5), total reactor volume: 2.4 mL.

DISCUSSION

Gas-liquid reactions in microreactors have been given considerable attention,[86a, 92b, 162] addressing issues such as flow characterisation,[86a, 92b, 162b] improved mixing and residence time distribution owing to the presence of gas slugs,[86a] bubble formation at T junction,[162b] and increased productivities of hydrogenation in flow in microreactors compared to shake flasks.[162a] Microreactors were also used in chemical syntheses for the better control of temperature and prevention of hot spot formation.[162c] These previous studies presented the basis for the application of oxygen- or hydrogen-dependent biocatalytic reactions in

microreactors. However, enzymatic reactions in gas/liquid microreactors have been rarely reported so far. The oxidation of glucose by immobilised glucose oxidase in a gas/liquid monolith reactor is to our knowledge, the only example of a biological reaction in such reactor systems.[163] Using soluble enzymes in multiphasic systems poses certain challenges that emerge from their inactivation at the aqueous/organic interface[63] or at the gas/liquid interface,[164] which were studied in detail for traditional reactor systems. Enzyme inactivation at the gas/liquid interface could be prevented by using bubble-free aeration systems for minimising the hydrodynamic stress causing interfacial enzyme inactivation. The bubble-free aeration concept has been developed by the Wandrey group (Forschungszentrum Jülich)[165] and has since been applied, for example, by using a silicone tube or a hollow fibre membrane for reactor aeration and/or radially delivering oxygen to larger reaction compartments. Examples have been shown for cyclohexane monooxygenase[166] and laccase.[167] However, in these systems, a low membrane surface area to liquid volume ratio could limit the oxygen transfer rate. Therefore, the concept of delivering oxygen through a membrane was further modified to increase oxygen mass transfer with the TiTR system. A Teflon AF-2400 membrane stable in organic solvent and with excellent oxygen permeability values, among the highest of all known polymers,[168] was used. This maximised membrane surface area to liquid volume ratio.

So far TiTRs have been used for chemical reactions involving carbon monoxide,[153a] carbon dioxide,[153c] hydrogen,[153b, 169] ammonia gas,[153d, 170] ethylene and syngas,[153f, 153h] ozone,[171] and oxygen,[153e] These applications mainly used transition-metal-catalysed reactions. To our knowledge, no examples of biocatalytic applications of TiTR have been published to date. The use of this reactor for a biocatalytic, oxygen-consuming reaction, offered the possibility of simplified liquid handling and continuous oxygen supply, which could not be achieved in a conventional aqueous/organic-air three-phase microreactor. Using a TiTR allowed precise control of the residence time irrespective of pulsations or sudden decompression of air. Thus, higher residence times were achieved without facing an oxygen limitation over the length of the reactor capillary.

To maximise the substrate mass transfer, the aqueous/organic segmented flow was delivered in the inner tube of the TiTR. Interfacial enzyme inactivation in the segmented flow was prevented by adding surface active agents such as Tween 20 along with the enzymes as proposed by Karande et al. (2010).[66a]

The parameters that affected the enzyme performance in the TiTR were evaluated by investigating the effect of oxygen, cofactor and organic substrates on 3-phenylcatechol formation rates. The key factor that severely affected the reaction rate and thus the reaction

performance was the organic mass transfer. Organic mass transfer in the segmented flow could be affected by substrate concentration, phase ratio, flow velocity and capillary diameter.[84, 95] Our investigation was in line with these findings as a substrate concentration of 200 mM, a flow velocity of 0.8 mL min^{-1}, and phase ratio of 1:1 revealed improved mass transfer and reaction performance. By optimising these parameters, a productivity of 17.7±1.8 g L^{-1} h^{-1} was achieved for 3-phenylcatechol, which is 3.5 times higher than that in a three-phase segmented flow microreactor (4.5 g L^{-1} h^{-1}) (Table 5-1).

Table 5-1: Comparison of productivities between different reactor setups in this study.

Entry	Flow rate (mL min^{-1})	Residence time (min)	Volume (mL)	Conversion (%)	Productivity [c] (g L^{-1} h^{-1})
3 phase microreactor[a]	1.2	8.5	10.5	5.2±0.3	4.5±0.3
TiTR[b]	0.1	23	2.4	22.9±1.1	11.0±0.5
	0.2	10	2.4	11.0±0.3	12.2±0.4
	0.4	5.7	2.4	6.8±0.4	13.4±0.8
	0.8	3	2.4	4.8±0.5	17.7±1.8

[a] substrate (200 mM), Tween80/Span80 (0.043 mg mL^{-1}), HbpA (8.67 U mL^{-1}), FDH (8.57 U mL^{-1})

[b] substrate (200 mM), Tween 20 (0.6 mg mL^{-1}), HbpA (9.2 U mL^{-1}), FDH (7.5 U mL^{-1})

[c] calculated for the total volume of aqueous and organic phase

For fast reactions such as the one presented herein, the mass transfer time has to be shorter or equal to the reaction time; otherwise, the reaction will be mass transfer limited. Microreactors provide high mass transfer rates, which are beneficial to fast reactions, leading to high rates which are often not possible in batch reactor systems (Table 5-2).

Table 5-2: Selection of processes catalysed by monooxygenases with concomitant NAD(P)H regeneration catalysed by formate dehydrogenase (FDH).

Enzyme	Product	productivity [g L^{-1} h^{-1}]	time [h]	reference
styrene monooxygenase [a]	methylstyrene oxide	0.9	11	63
HbpA [b]	3-phenylcatechol	0.5	8	102
cyclohexanol monooxygenase [c]	(R)-ethyl 2-(7-oxo-oxepan-2-yl)acetate	0.8	2h	166b
cyclohexanol monooxygenase [d]	5-methyloxepan-2-one	0.4	10	166a

[a] Reaction conditions: $FADH_2$ dependent oxygenase component (StyA) of styrene monooxygenase (2 U mL^{-1}), FDH (8 U mL^{-1}), 400 mL-scale, 1:1 dodecane/buffer phase ratio, substrate (50 mM), NAD^+ (1 mM).

[b] Reaction conditions: HbpA (0.2 U·mL^{-1}), FDH (0.3 U mL^{-1}), 200 mL-scale, 4:1 decanol/buffer phase ratio, substrate (110 mM) in *n*-decanol.

[c] Reaction conditions: cyclohexanol monooxygenase (0.2 U mL^{-1}), FDH (0.6 U mL^{-1}), 30 mL-scale, single phase, substrate (20 mM), $NADP^+$ (0.4 mM), bubble-free aeration.

[d] Reaction conditions: cyclohexanol monooxygenase (0.16 U mL^{-1}), FDH (0.32 U mL^{-1}), 30 mL-scale, single phase, substrate (40 mM), $NADP^+$ (0.25 mM), bubble-free aeration.

The rationale behind using microreactors is to achieve highest productivities for a given reaction, often without reaching full conversion, such as that achieved in slower batch reactors, in which the residence time can be almost infinite and is determined only from the (bio)catalyst stability. Simple calculations are performed to compare the characteristic time needed for mass transfer of the substrate and the reaction time available for its consumption to get an insight into the limiting parameters in different reactor formats.

Three phase segmented flow: Owing to flow instability, productivity in this type of reactor could be limited by mass transfer, which depends on internal circulations in well-defined segments.[87] In addition, the availability of oxygen could become a rate-limiting factor at longer residence times.

Tube-in-tube reactor: In a TiTR, the radial delivery of oxygen throughout the membrane improved oxygen availability as compared to the single-point oxygen supply in the three-phase segmented flow reactor. In this TiTR system, the oxygen mass transfer time throughout the membrane (the time to reach saturation) was estimated to be in the range of 1-10 s.[172] This timescale is in a range similar to that of the organic substrate mass transfer time 1-14 s (k_La 0.07-1.1 s^{-1} for 0.75 mm capillary) under segmented flow conditions.[84] The reaction time to consume organic substrate in the aqueous phase (200 mM in organic corresponds to the aqueous phase concentration of 0.41 mM based on a K_D of 480) is estimated to be 8 s. Because the reaction times and mass transfer times are in the similar range, we concluded that the system was mass transfer limited with respect to transfer from the organic to the aqueous phase. Thus, changing the organic substrate concentration and flow rate had a positive effect on the mass transfer and thus on the reaction performance (Figure 5-7A). The study also suggests that the system could face oxygen limitation, as the organic substrate mass transfer time and the oxygen transfer time are in a similar range. The TiTR system provides the flexibility to maximise oxygen transfer by increasing the pressure of the air phase up to 3 MPa (30 bar converted to SI units). Thus, according to Henry's law, the oxygen transfer could be increased 30 times. Confirming whether higher oxygen concentrations in the aqueous phase (at the same k_La value) partially affect product formation rates opens the door to future studies.

The benchmark example in a STR showed a productivity of 0.46 g L^{-1} h^{-1} for the HbpA-catalysed hydroxylation of 2-hydroxybiphenyl with an FDH-catalysed cofactor regeneration system.[102] The maximal productivity achieved in the present TiTR system was 38 times higher than that achieved in stirred batch reactors because of the high substrate mass transfer rates. Obtaining such high productivities in three-phase stirred batch reactors is often limited

owing to the stirring speed, which sets a trade-off between substrate mass transfer[81] and enzyme inactivation from the generated shear-stress.[164]

Thus, it is anticipated that the TiTR concept will play a significant role in optimising multiphasic oxygen-dependent redox biocatalytic reactions.[153g] The potential of enzymatic membrane reactors has already been recognised,[173] and the present work opens up the field for gas-dependent biocatalytic reactions. P450 or Baeyer-Villiger monooxygenases coupled to a cofactor regeneration system are interesting candidates for future applications in a TiTR. Oxygen supply and product inhibition are two major limitations observed previously for Baeyer-Villiger whole-cell catalysts.[174] These limitations are more pronounced in cell-free systems, in which enzyme concentrations are higher, requiring higher oxygen mass transfer for higher reaction rates and more efficient product removal to overcome product inhibition. Similar examples such as the cyclohexanone monooxygenase-catalysed production of ε-caprolactone[175] could also benefit from this concept because of their similarities to the HbpA-catalysed hydroxylation reaction, such as oxygen and cofactor dependency and substrate inhibition.

Although a numbering-up technique is often proposed for the scale-up of microreactors, the TiTR in its current design is not suited for an industrial application yet, unless a cheaper material with comparable properties is found, because the Teflon AF-2400 used for the inner tubing poses a significant cost factor. The numbering-up technique is, surprisingly, not the method of choice for the industry because delivering liquids into multiple capillaries at the same time poses a risk in that if one capillary is blocked, the residence times in other capillaries are changed, which affects the conversion or product composition.[176] Nevertheless, application of Teflon AF-2400 TiTRs could emerge in the speciality chemicals and pharmaceutical markets, in which small-scale syntheses for toxicology studies or clinical trials are needed and chemical routes do not exist. It is also anticipated that the TiTR concept will play a significant role for optimising multiphasic oxygen-dependent redox biocatalytic reactions just as it did for gas-dependent chemical syntheses.[153g]

CONCLUSIONS

This work shows the feasibility of the tube-in-tube reactor (TiTR) concept and sets a milestone for performing gas (particularly oxygen)-dependent reactions in capillary microreactors. It is also an example of rational reaction design linking the kinetic characterisation of the biocatalyst and optimisation of the reaction parameters. The concept presented herein has potential for application in selective redox biocatalysis in

aqueous/organic segmented flow reactors for gas dependent reactions with cofactor regeneration.

We have applied the TiTR concept to continuous enzymatic hydroxylation. The productivity of 17.7±1.8 g L^{-1} h^{-1} achieved is significant for a monooxygenase-catalysed reaction with concomitant cofactor regeneration, which emphasises the potential of this technology for biocatalytic reactions (Table 5-2). Our study points out the importance of the substrate mass transfer, which appeared to be rate limiting at substrate concentrations less than 200 mM. In addition, tuning the total flow rate had a strong impact on product formation rates and final conversion. The effect of higher oxygen partial pressure should be evaluated for further increases in productivity.

ACKNOWLEDGEMENTS

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CHAPTER 6

BIOCATALYTIC PREPARATIVE SYNTHESIS OF CATECHOLS USING A HIGH PRESSURE TUBE-IN-TUBE SEGMENTED FLOW MICROREACTOR

Bartłomiej Tomaszewski, Katja Buehler, Andreas Schmid

Bartłomiej Tomaszewski performed the experiments and wrote the manuscript, Katja Buehler, and Andreas Schmid coordinated and supervised the project and corrected the manuscript.

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ABSTRACT

In previous chapter it was shown that the enzymatic hydroxylation of 2-hydroxybiphenyl catalysed by 2-hydroxybiphenyl 3-monooxygenase (HbpA) performs better in the tube-in-tube reactor (TiTR) system, as compared with conventional batch reactors. In this chapter the impact of oxygen on the product formation rate was closely evaluated, as it was assumed it was one of the major factors influencing the performance of HbpA. Oxygen concentration was effectively controlled by the pressure in the tube-in-tube system. The study revealed that enzymes' stability was not affected by increased pressures, which allowed for recycling of the biocatalyst. Quantification of oxygen permeability showed that the reaction was not limited by the oxygen transfer rate over the membrane or its availability in the aqueous phase. Preparative scale synthesis was possible achieving a space time yield (STY) of 14.5 g L^{-1} h^{-1}. 740 mg of product was purified by chromatography and subsequent sublimation. Furthermore, an ePTFE hydrophobic membrane TiTR, proved advantageous for easier aqueous/organic phase separation, and allowed longer operational stabilities of the biocatalysts.

INTRODUCTION

Multiphasic reactions, where different phases are separated by means of a semipermeable membrane in a TiTR setup, are of increasing interest for chemical and biological reaction systems.[153, 170] In comparison to plug-flow reactors, gaseous substrates can be supplied much more effectively in TiTR setups,[153b] providing a constant oxygen concentration during the entire residence time. This is the most important feature for all reactions (chemical and biological) comprising a gaseous substrate as its concentration in the liquid phase needs to be high for quantitative conversions.[172] One way to increase gas solubility in the liquid is to increase liquid's and gas' pressure. Recently the TiTR was evaluated for the enzymatic hydroxylation of 2-hydroxybiphenyl catalysed by 2-hydroxybiphenyl 3-monooxygenase HbpA (chapter 5) at atmospheric pressure. This reaction requires efficient supply of three substrates, namely 2-hydroxybiphenyl, NADH, and oxygen. A second organic phase was used as carrier phase for 2-hydroxybiphenyl and was supplied in an aqueous/organic segmented-flow fashion into the lumen of the reactor. Formate dehydrogenase (FDH) in the aqueous phase served for recycling NADH, and oxygen (in air) was continuously delivered from the shell side of the membrane. We have demonstrated the potential of the tube-in-tube system for gas dependent enzymatic reactions and discussed the challenges related to low concentration of oxygen in the liquid. At atmospheric pressures, the oxygen supply was assumed to limit the productivity due to the reaction times (oxygen consumption time) being similar to the oxygen transfer time. Although existing literature shows the potential of the TiTR for fast saturation of liquids with ammonia or hydrogen,[153b, 153g] the oxygen transfer rates or saturation time under here applied conditions was not investigated so far. Reported theoretical oxygen permeability through the membrane[168] was higher than the reaction rates measured in this study. Therefore, the gas permeation needed to be quantified and parameters limiting the gas transfer determined. Moreover, it was necessary to evaluate the effect of the elevated pressure on the biocatalysts' stability and on the reaction. Because the TiTR has already been successfully implemented for multigram-scale chemical synthesis of anti-inflammatory compound fanetizole,[153g] the ultimate goal was to use this technology for a biocatalytic preparative scale synthesis of 3-phenylcatechol.

MATERIALS AND METHODS

Chemicals

NADH disodium salt and 1-decanol were obtained from Applichem (Darmstadt, Germany), sodium formate, NAD^+ disodium salt and FAD from Fluka (Buchs, Switzerland), 2-hydroxybiphenyl, activated aluminium oxide (neutral, CAMAG 507-C-I, Brockman grade I)

from Sigma-Aldrich (Taufkirchen, Germany) and 2,3-dihydroxybiphenyl from Wako Pure Chemical Industries Ltd. (Osaka, Japan). All other chemicals were purchased either at Fluka, Applichem or Sigma-Aldrich at the highest purity required and used as received.

Enzymes

The enzyme production and purification was based on procedures described in Chapter 2.

Enzyme activity assays

Monitoring the activity of the HbpA and FDH in a spectrophotometric assay was done as reported in Chapter 2.

HbpA activity determined by product formation – RP-HPLC analysis

Monitoring of the HbpA catalysed biotransformation reaction was achieved by determining the concentration of the product 2,3-dihydroxybiphenyl. Measurements were done using a Hitachi Elite LaChrom HPLC system equipped with a Hitachi Elite LaChrom L-2450 Diode Array Detector. All measurements were performed either on an XTerra RP 18 3.5 µM column (Waters, Dublin, Ireland). An isocratic method at a total flow rate of 1 mL min^{-1} 50:50 (% v/v) ACN-water (both supplemented with 0.1% TFA) was used for elution. The product 2,3-dihydroxybiphenyl as well as the substrate 2-hydroxybiphenyl were detected at 2.69 and 3.19 min retention time on an XTerra RP column. Concentrations of 2,3-dihydroxybiphenyl were calculated as a peak area ratio of 2-hydroxybiphenyl to 2,3-dihydroxybiphenyl.

TiTR setup

The TiTR Gastropod® was obtained from Cambridge Reactor Design (Cambridge, UK). An exemplary assembly is shown in the Figure 6-1. A basic unit was 1-m-long (0.5 mL volume inside the Teflon AF-2400 membrane; i.d.: 0.8 mm, o.d.: 1.0 mm), as indicated. The membrane was kept in a 1/8 inch Tefzel tube. The back pressure was controlled by a back pressure regulator (BPR) installed on the outlet of the membrane. To avoid pulsation in the system caused by difference in liquids viscosities, a coiled 1-m-long 1/8 inch PFA tube was connected by a T-connector (UpChurch Scientific, Göhler HPLC, Chemnitz, Germany) to the BPR. After equilibrating the system, the air, which was initially present in this tube, pressurised and exerted a stable back pressure on the system. TiTR assembly allowed independent control of the gas pressure, and partial pressure difference across the membrane. Two Chemyx Nexus 6000 syringe pumps (KR Analytical, Sandbach, UK) were used to pump aqueous and organic phase streams. Liquids were united in a T-connector (i.d.: 1.0 mm) forming a stable segmented flow as sketched out in Figure 6-2.

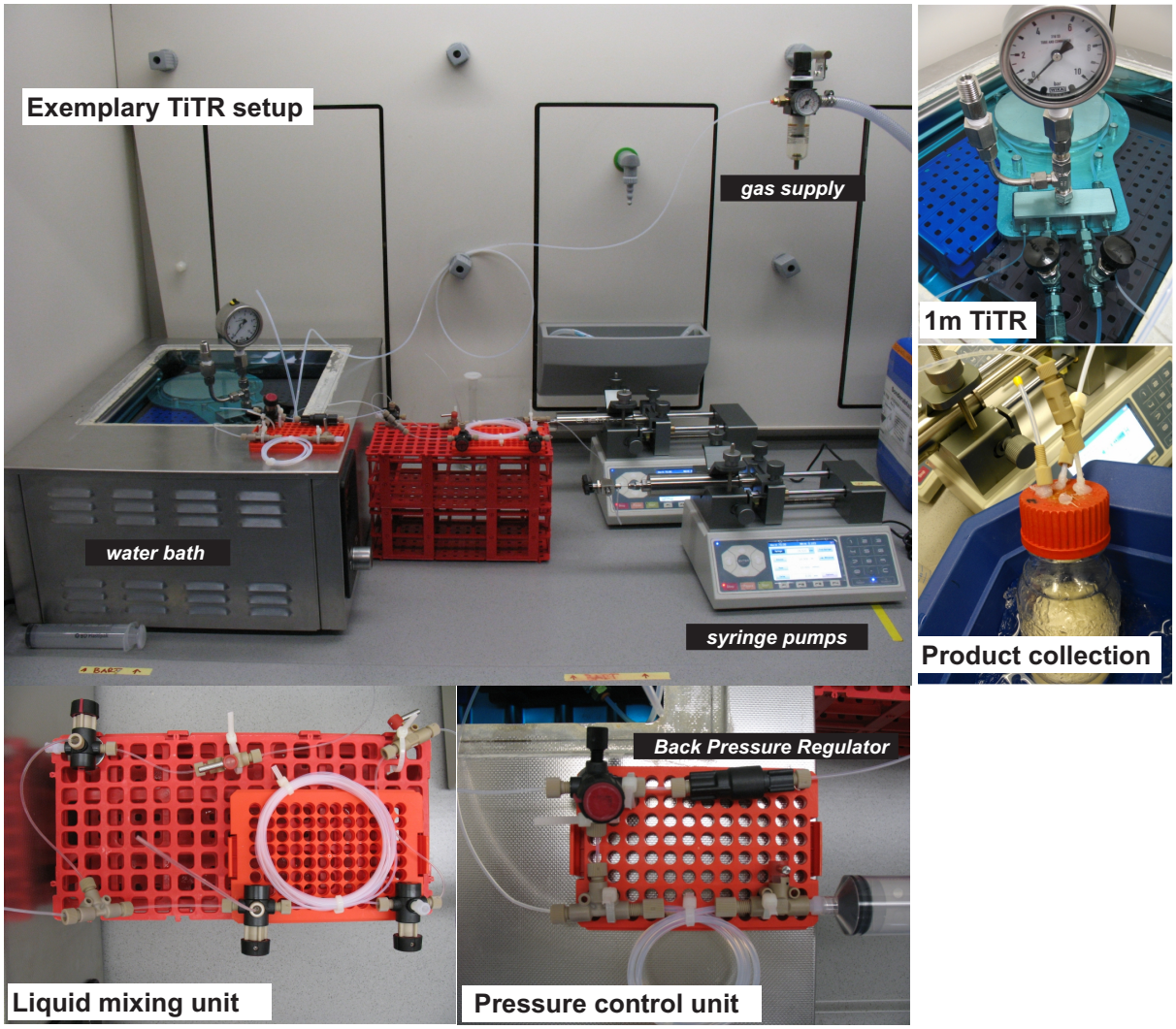

Figure 6-1: Exemplary TiTR setup with the peripherals.

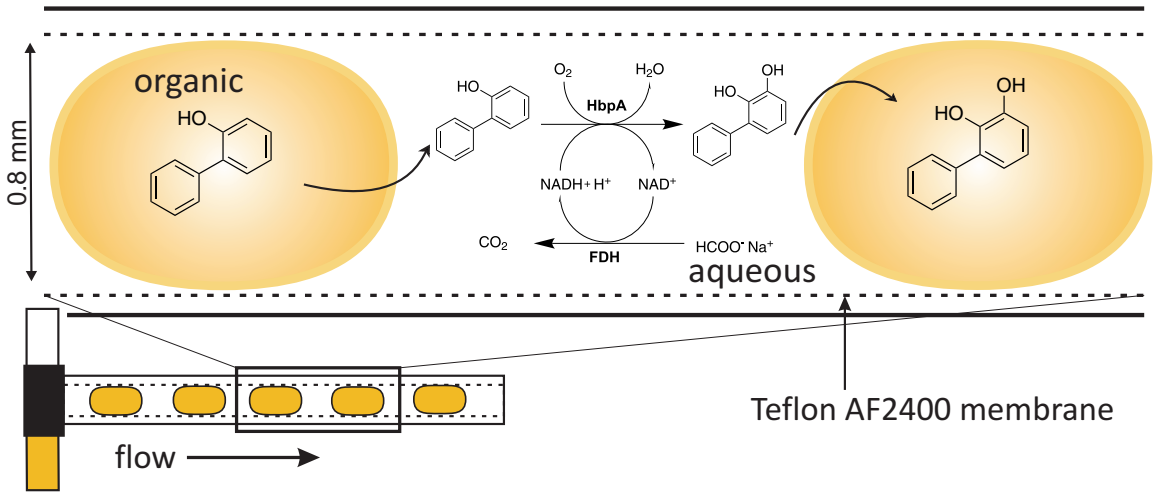

Figure 6-2: Schematic view of the liquid/liquid segmented flow setup. HbpA catalysed 2-hydroxybiphenyl hydroxylation with concomitant NADH regeneration catalysed by FDH takes place in the aqueous phase (KPi buffer). Substrates and products are extracted and delivered via organic phase (*n*-decanol).

Air was delivered from an in-house pressurised air system (nominal pressure 8 bar). Oxygen was delivered to the outer membrane directly from the pressurised oxygen tank (200 bar). When longer residence times were required either a PTFE tube (i.d.: 1/16 inch) was connected to the reactor outlet before the BPR or a 4 m (2 mL volume) version of a TiTR was used, which was assembled on site with commercially available interconnects (Swagelok, Kings Langley, UK). (chapter 5, Figure 5-1). Reaction temperature was controlled by a keeping the reactor in a water bath.

Reaction in TiTR

Unless stated otherwise, all biotransformation reactions were performed in the TiTR. Residence time was controlled through total flow rates. Relative flow rates were kept constant by maintaining the aqueous to organic phase ratio of 1:1. The aqueous phase was complemented by adding 50% glycerol solution of HbpA and FDH (for final volumetric activities of 2-20 U mL^{-1}), sodium formate (100 or 166 mM), NAD^+ (1 or 1.6 mM), FAD (20 or 100 μM), and Tween 20 (0.6 mg mL^{-1}) for facilitating flow stability and preventing enzyme inactivation, and the reaction mixture was adjusted with KPi buffer (100 mM, pH 7.5) to the respective volume for achieving the required volumetric activities. The aqueous phase was filtered through a 0.22 μm Millex-CP syringe filter with a polyethersulfone membrane to remove any particles that would have otherwise deposited in the reactor and resulted in flow instabilities later. 2-Hydroxybiphenyl (200 or 400 mM in the organic phase) in *n*-decanol formed a separate organic phase. Unless stated otherwise the reactor was thermostated at 30°C in a water bath.

In one experiment a variable length PFA tube (1-4 m long, i.d.: 1 mm, 0.8 mL m^{-1}) was connected behind the TiTR unit for increasing residence time.

Gram scale production of 2,3-dihydroxybiphenyl in a sequential TiTR

For gram scale production of 2,3-dihydroxybiphenyl a reactor setup presented in Figure 6-3 was used. It consisted of two connected TiTR units, in which each single unit was followed by a coiled 3-m-long PTFE tube (i.d.: 1 mm, 0.8 mL m^{-1}) for increasing residence time. Oxygen was delivered from an oxygen tank separately to each TiTR unit. Product was collected in a glass flask kept on ice and continuously flushed with nitrogen gas to avoid product degradation by over-oxidation. Reaction temperature was controlled at 35°C.

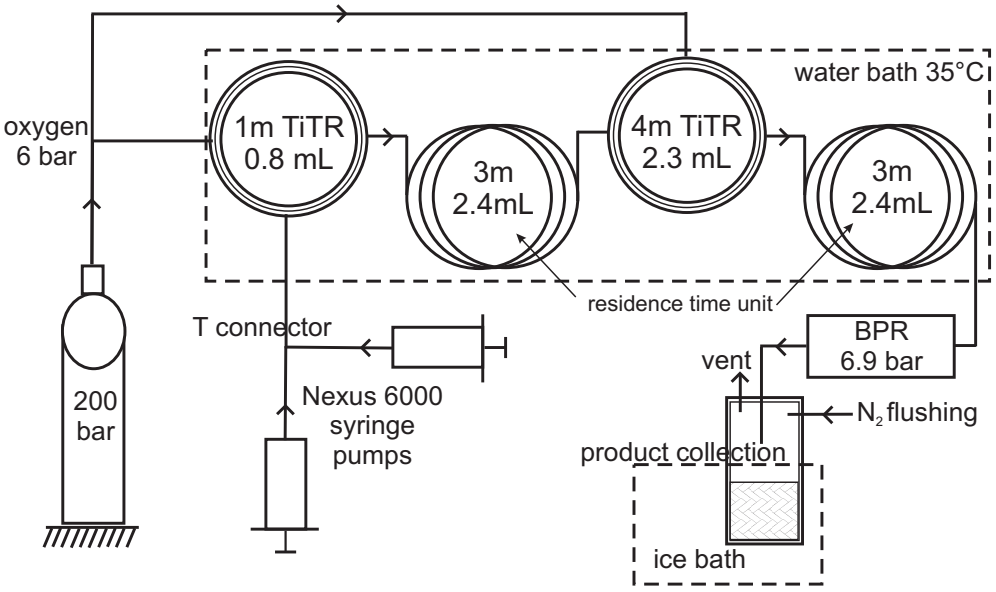

Figure 6-3: Schematic view of the sequential TiTR setup for the gram scale production.

Application of ePTFE TiTR for complete phase separation

The TiTR was modified by implementing an additional reaction compartment consisting out of an ePTFE tube (o.d 1/8 inch, 1.5 m, 11.13 mL; FluorTex, Raubling, Germany) combined in the tube-in-tube fashion with a PFA tubing (o.d. 1/4 inch, 1.2 m long, 14.8 mL). This reaction compartment (reaction module) was supplied with 2-hydroxybiphenyl dissolved in MTBE into the inner ePTFE tubing, while the outer shell contained the aqueous enzyme solution delivered from the TiTR unit (oxygen saturation module). Liquids were thus kept in separate compartments in the reactor allowing for an independent control of their flow rates and residence times, as well as for a simplified downstream processing. A schematic view of the experimental setup is presented in Figure 6-4.

A typical experiment contained 5.9 mL aqueous phase comprising HbpA (8.7 U mL^{-1}), FDH (8.5 U mL^{-1}), 100 mM sodium formate, 1 mM NAD^+, 20 µM FAD, 0.6 mg mL^{-1} Tween 20, filled up to the respective volume with KPi buffer (100 mM, pH 7.5) on the feed side in the contact with the 8.9 mL organic phase comprising 200 mM 2-hydroxybiphenyl dissolved in MTBE in the lumen. Prior entering the reaction module the aqueous phase was saturated with oxygen in the oxygen saturation module (1 m TiTR unit). The reaction was not thermostated and was conducted at an ambient temperature of 19°C. Flow rate of the aqueous and organic phase was 0.15 and 0.07 mL min^{-1} respectively, resulting in the residence time of the organic phase in the reactor of 127 min. Collected enzymes could be recirculated, whereas the product could be separated directly from the organic phase without prior phase separation.

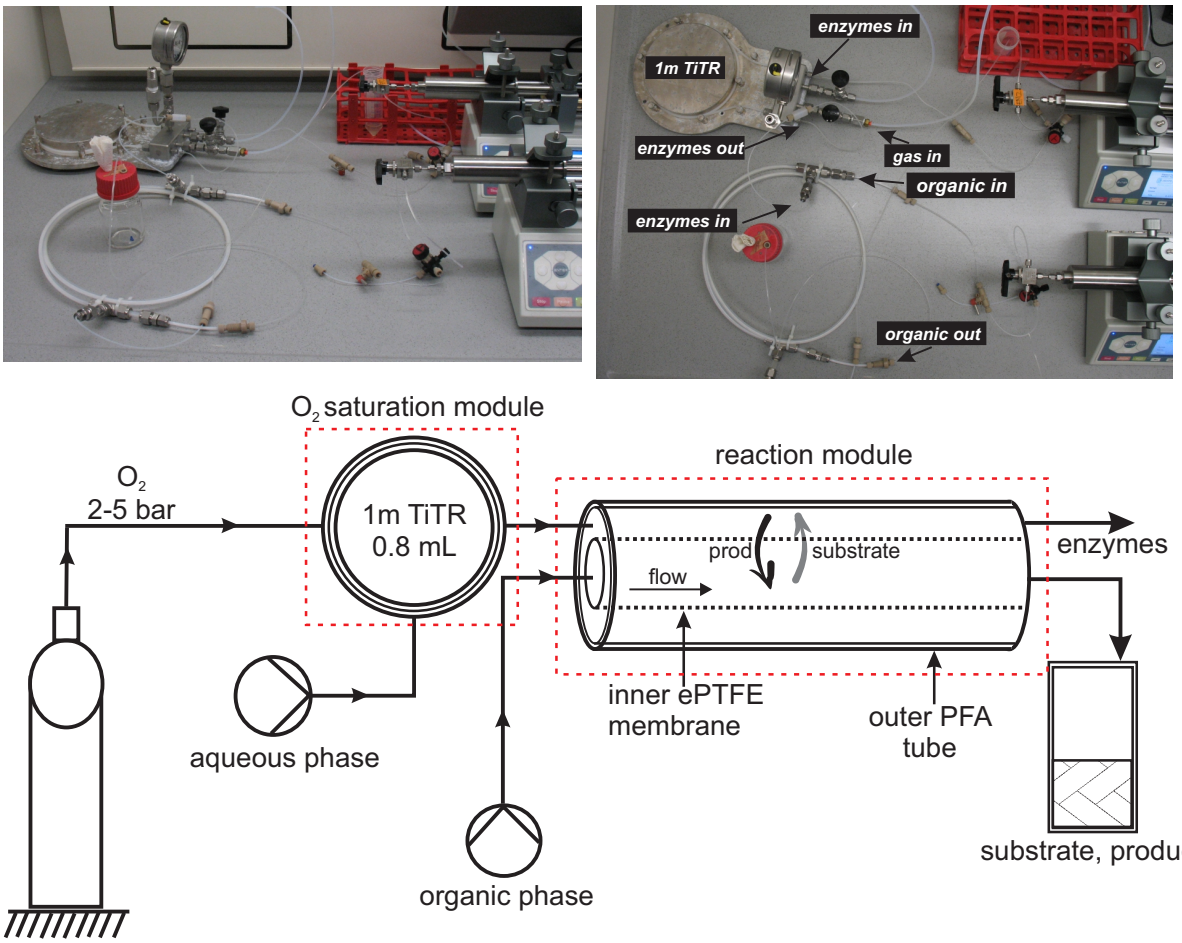

Figure 6-4: Schematic view of the setup used for an ePTFE TiTR utilised for achieving complete phase separation. The O_2 saturation module is a classical 1 m TiTR with a Teflon AF-2400 tubing, whereas the reaction module is an ePTFE TiTR.

Product purification

Product purification was achieved by applying chromatography adapted from Schmid et al (2001)[21] and scaled-up for our purposes. Shortly, 150 g of activated alumina was suspended in methanol and poured into a glass column. 0.5 cm of sea-sand was deposited on top. The column was washed with methanol. 360 mL of the decanol containing a mixture of 2-hydroxybiphenyl (10.4 g) and 2,3-dihydroxybiphenyl (2.0 g) was added and pushed through by applying a 0.5 bar overpressure. 200-300 mL fractions were collected. When decanol was almost eluted, methanol was applied on top. Elution was monitored by TLC (stationary phase – neutral alumina, mobile phase – 0.5 M HCl in methanol). A total of 0.9 L of methanol was used to elute decanol and the residual substrate. When no more substrate or decanol could be detected the eluent was changed to 0.5 M HCl in methanol for eluting the product. 1 L of eluent was used. After first 150-200 mL, fraction volume was changed to 20 mL. Product eluted from the column as a green liquid of neutral pH and was followed by an acidic brownish/red liquid that contained only residual amounts of product. Green-coloured samples

were checked on the HPLC and most prominent fractions were pooled (ca. 70 mL, 99% purity by HPLC) and dried on a rotary evaporator (initially 40°C and 250 mbar reduced to 28 mbar over 3.5 h; final drying step was done at 35°C for 1 h). A total of 4 g of residue was collected and successfully sublimated in batches on a 'cold finger' (1.5-1.2 mbar, cooling water at 2°C). The cold finger was heated up uniformly either by a heat gun or in an oil bath set to 120°C. The white powder was removed from the cold finger; the cold finger was washed with methanol and the process was repeated. A total of 740 mg of product was collected. 11 mg of powder was resuspended in 0.75 mL of $CDCl_3$ and analysed on 300 MHz H-NMR. The spectrum measured was compared to that of the standard of the product and substrate and the white powder was ultimately identified as 2,3-dihydroxybiphenyl (Appendix, Figure A9).

Burette method

To quantify the amount of air passing through the membrane the burette method adapted from O'Brien et al. (2011)[153b] was used (Figure 6-5). A glass burette filled with liquid, was inverted and submerged in a cylinder. The burette was closed at the top and the liquid from the reactor was delivered to the burette from the bottom. When the liquid decompressed, gas travelled to the top and caused the displacement of the liquid in the burette. The volume of the gas was quantified and the volume of the oxygen in the collected gas was calculated using Henry's law.

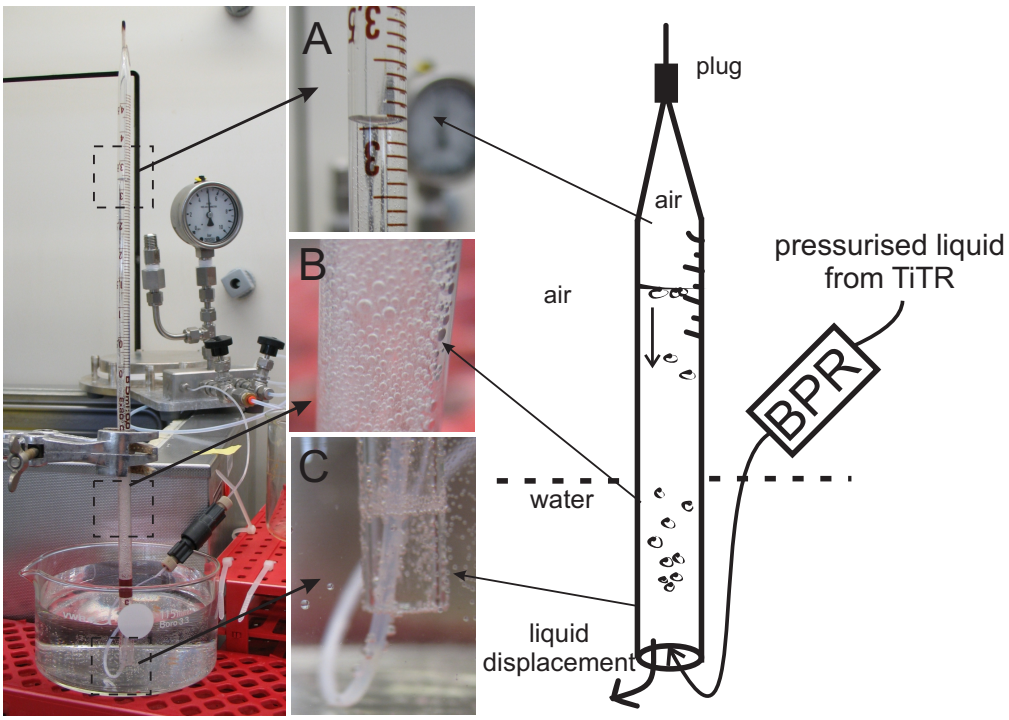

Figure 6-5: Picture and schematic view of the setup used for the burette method of quantification of the gas passing through the Teflon AF-2400 membrane. Displaced liquid – A. Bubbles of the gas after decompression – B. Entry of the reactor outlet tube – C. (BPR – back pressure regulator)

RESULTS

Factors influencing gas transfer through the Teflon AF-2400 membrane

For Teflon AF-2400 permeability values of 990 Barrer for oxygen and 490 Barrer for nitrogen are published,[168] [1 Barrer=10^{-10} cm^3_{gas}(STP) cm_{membr} (cm^2_{membr} s cmHg)$^{-1}$, or translated to SI units 1 Barrer=3.348×10^{-19} kmol m (m^2 s *Pa*)$^{-1}$]. The unit 'Barrer' is used here as a measure for permeability of membranes.[177] For a 2-m-long Teflon AF-2400 membrane (1 mL volume) (i.d.: 0.08 cm; o.d.: 0.1 cm) these values translates to 20.8 µmol min^{-1} (0.41 µmol min^{-1} cm^{-2}) or 39.9 mg h^{-1} oxygen at 0ºC and an oxygen partial pressure difference of 20.8 kPa (15.6 cmHg converted to SI units), that is, when the pressure around the tube is equal to atmospheric pressure and the liquid in the tube is oxygen free. Increasing the partial pressure of oxygen either by increasing the total pressure of the gas or by using oxygen enriched air (or pure oxygen) will have a positive impact on oxygen transfer. It was our aim to quantify the amount of O_2 passing through the membrane, characterise the parameters influencing its transport and its effect on HbpA performance. Results of gas permeability experiments are shown in Figure 6-6.

In all experiments the liquid in the membrane upstream of the back pressure regulator was homogenous (free of gas bubbles), to ensure that the gas collected and quantified in the burette outgassed from the liquids. Not surprisingly, the final gas titre was dependent on the back pressure in the membrane as the back pressure determined the maximum gas (oxygen) solubility in the liquids. In the single phase experiment, when only KPi buffer was fed into the reactor, the gas titre was the highest for the 6.9 bar back pressure with a 7 bar air pressure around the membrane (Figure 6-6A). In this simple experiment it was confirmed that the TiTR offers quick and reliable control of the amount of the dissolved gas by changing the pressure on the feed and permeate. Next, the composition of the feed in the context of oxygen transfer was investigated. When KPi buffer and decanol as a substrate carrier phase (at 1:1 phase ratio) were fed simultaneously, the amount of gas collected continuously was higher for two-phase flow than for the single phase (KPi buffer only) flow fed at the same flow rate (Figure 6-6B). Since the solubility of air is known to be higher in organic solvents,[98] the higher gas titre in the aqueous/organic two phase flow was not surprising. The effect of the flow rate on oxygen transfer was also investigated (Figure 6-6C). At higher flow rates more gas outgassed from the liquids in a defined time as compared to slower flow rates. The flow rate is an important parameter because it determines the actual concentration of the gas in the liquid at a specific time point and therefore impairs or facilitates the transport, as the gas flux

is proportional to the difference of the maximum gas concentration minus the actual gas concentration in the liquid.

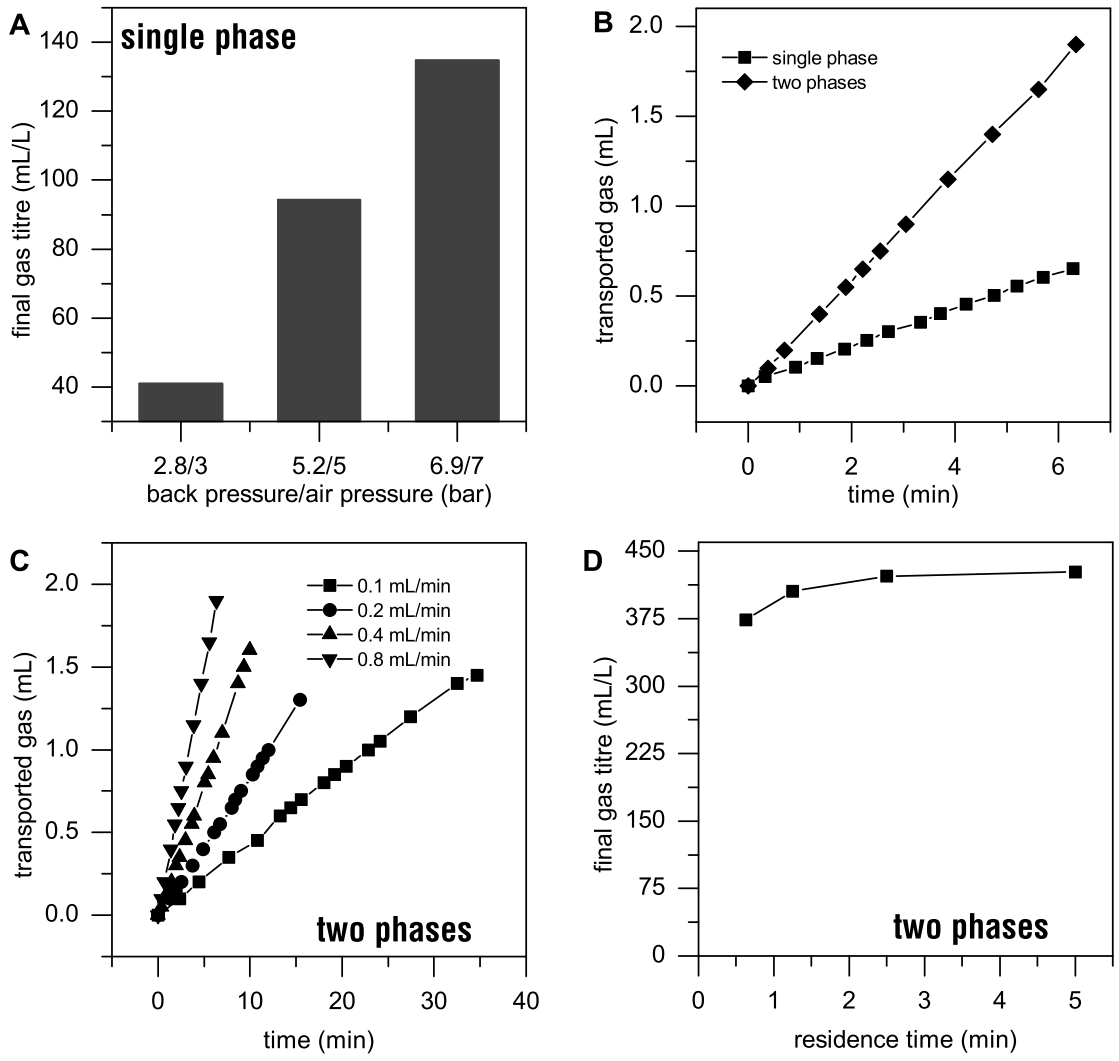

Figure 6-6: Gas (air) transport through the Teflon AF-2400 membrane in a 1 m TiTR (0.8 mL total volume, 0.5 mL volume inside the membrane) determined by the burette method. A – Influence of the back pressure on the final gas titre in KPi buffer (flow rate 0.8 mL min^{-1}). B - Gas transport at 6.9 bar back pressure into KPi buffer and into a 1:1 decanol – KPi buffer system. Total flow rate 0.8 mL min^{-1}. C - Effect of the flow rate on the amount of transported gas at 6.9 bar back pressure in a 1:1 decanol – KPi buffer system. D – Dependence of the final gas titre in a 1:1 decanol – KPi buffer system on the residence time at 6.9 bar back pressure.

Quantifying the gas outgassing from the liquids at different flow rates allowed to determine the final gas titre (mL gas dissolved per L of liquid), which could be reached inside the TiTR (Figure 6-6D). Although the gas titre was dependent on the residence time almost full saturation was reached within 40 seconds. Similar findings were reported for hydrogen passing Teflon AF-2400 membranes, where almost complete saturation of a dichloromethane phase with H_2 was reached within 10 seconds.[153b] Those results prove that the gas transport rate and the final gas titre depend on the composition of the feed (phase to be saturated), and its capability to dissolve gas. The same was observed for the transport of ammonia through

the Teflon AF-2400 membrane where the amount of ammonia in the permeate was different in methanol, dimethoxyethane, or toluene (concentration increased with increasing dielectric constants).[153g]

The aim of the next experiments was to check the effect of increased oxygen concentration on the biotransformation in the TiTR and investigate enzymes' stabilities with respect to the increased pressure.

Enzyme stability and activity in pressurised TiTR

The effect of pressure and aqueous/organic two phase flow on biocatalyst stability and activity was assessed. In terms of enzyme stability no difference between activity losses in a pressurised system (outlet at 6.9 bar), and a non-pressurised system (outlet at an atmospheric pressure) was found (Table 6-1). Due to these findings the negative impact of the pressure on the enzyme was excluded.

Table 6-1: Influence of the back pressure in the reactor (6.9 bar at the outlet) on the residual enzymes' activity after passing through 1 m TiTR reactor. Total flow rate 0.1 mL min^{-1}, respective residence time 8.9 min. (n=2)

	Initial	After passage	
enzyme	(U mL^{-1})	0 bar (U mL^{-1})	6.9 bar (U mL^{-1})
HbpA	2.3±0.1	1.9±0.1	1.9±0.0
	8.7±0.1	6.7±0.4	7.8±0.1
	21.6±1.5	23.3±0.5	24.2±1.5
FDH	1.7±0.1	1.4±0.0	1.5±0.0
	5.6±0.1	5.9±0.1	6.7±0.1
	13.1±0.8	15.8±1.6	14.6±0.4

The results of the abiotic experiment aiming at quantifying the oxygen permeability at different flow conditions established process operational boundaries. It was also shown that oxygen concentration in the aqueous phase was proportional to its pressure. According to Henry's Law at an atmospheric pressure oxygen solubility in water does not exceed 245 μM,[178] but increases linearly with increasing pressure. To see the effect of higher oxygen concentration in the reaction medium, reactions with an increased back pressure were conducted. Pressure was controlled by installing a back pressure regulator at the reactor outlet and the influence it had on the product formation rate was checked during the biotransformation reaction.

Figure 6-7A gives an overview of the experiment and clearly shows that there is a slight impact of the pressure on the reaction rate, but it is not as high as expected, and the reaction rate increase does not correlate to the oxygen concentration. Varying the differential gas (oxygen) pressure across the membrane also had no further impact. The pressure of the liquid was kept low (1.4 bar) while the pressure of the air surrounding the membrane was set to 8 bar. As a control, the pressure of the liquid was set to 6.9 bar and that of the surrounding air to

7 bar. In both cases the product formation rate and the final product titre were comparable (Figure 6-7B). Exchange of the air with pure oxygen, and hence increasing the amount of available oxygen by the factor of five, also had no further impact on the product formation rate. From these findings it could be concluded, that the reaction was not limited by the gas transport and oxygen availability and further experiments were conducted to check whether the biotransformation rate was limited by catalyst loading.

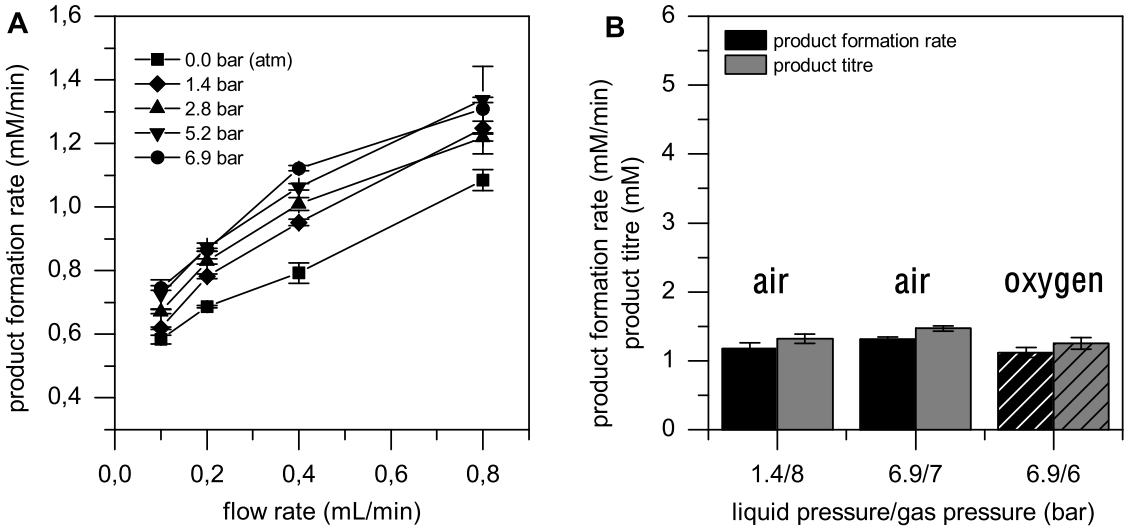

Figure 6-7: A – Influence of the back-pressure on the product formation rate in a 1 m TiTR (0.8 mL total volume). Outer membrane pressure of the air set to 0.0 bar (atm), 1 bar, 2 bar, 4 bar, and 5 bar at subsequent 0.0 bar (atm), 1.4 bar, 2.8 bar, 5.2 bar, and 6.9 bar back pressure respectively. Product formation rate given is the average value, calculated by dividing the final product concentration in the sample by the sample residence time. B - Influence of changing differential pressure across the membrane and applying pure oxygen instead of air in a 1 m TiTR (0.8 mL total volume; flow–rate: 0.8 mL min^{-1}). Product formation rate was calculated for one litre of reactor working volume.

Influence of the biocatalyst loading on the product formation rate

Reaction conditions were set to 6.9 bar back pressure and 7 bar of the surrounding air. Under these conditions the impact of the catalyst loading on the reaction rate was investigated. HbpA and FDH initial volumetric activities in the aqueous phase were raised stepwise (unit ratio of the enzymes was kept at 0.81:1 FDH:HbpA U:U). At low enzyme loading (2 U mL^{-1} HbpA) the reaction was limited by the enzyme amount, but above 8 U mL^{-1} the impact of the enzyme concentration was negligible and applying more catalyst did not have an impact on the product formation rate (Figure 6-8A). The reaction operated at zero order with respect to the reactants, which were added in excess, as well as relatively short residence times. Similarly to results obtained earlier and presented in Figure 6-7A the product formation rate increased with increasing total flow rate. Thus, we assumed that the product formation rate was dependent on the mass transfer of the organic substrate between the phases and not on the

oxygen availability or the catalyst amount applied. It is known that with increasing flow rate the mass transfer increases in a two phase flow microreactors[84] as a direct consequence of faster internal circulations in the segments.[179]

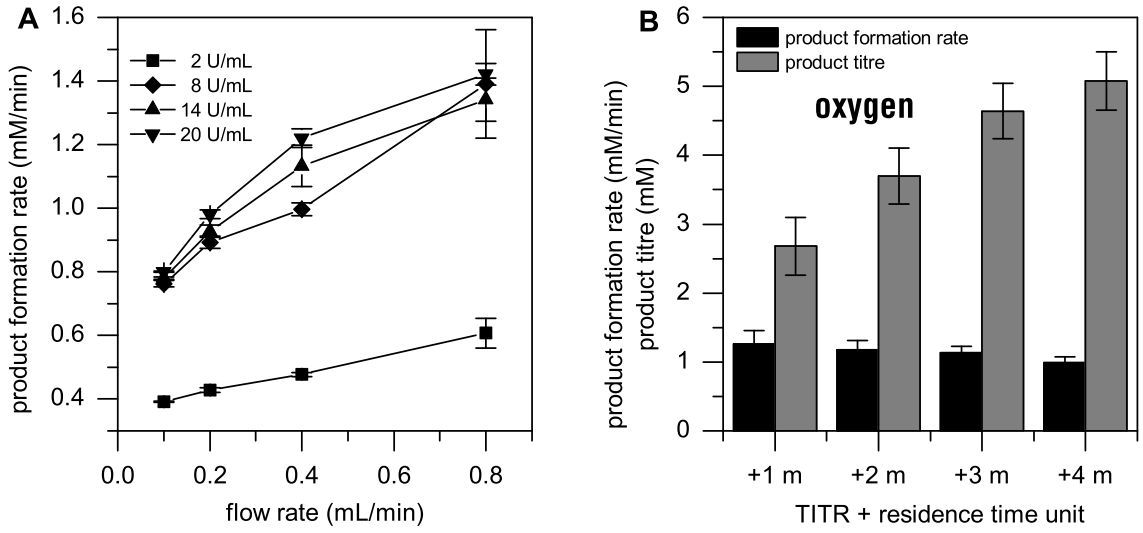

Figure 6-8: A - Influence of enzyme loading and flow rates on the product formation rate in a 1 m TiTR (0.8 mL total volume) run at 6.9 bar back pressure and 7 bar air pressure in a two liquid phase system. Respective residence times reached were 8.9 min, 4.5 min, 2.2 min, and 1.1 for increasing flow rates. Standard reaction conditions applied as described in experimental section. Product formation rate given is the average value, calculated by dividing the final product concentration in the sample by the sample residence time. B - Product formation rate and final product titre in the system aerated with pure oxygen instead of air at various residence times. Flow rate: 0.8 mL min^{-1} with a residence time of 2.1, 3.1, 4.1, 5.1 min respectively for increasing reactor length (errors are presented as a standard deviation, n=3). Product formation rate is given for one litre of reactor working volume.

Based on the results obtained in the experiment with pure oxygen it became clear that reaction was not oxygen limited. We have actually expected that not all oxygen was consumed because substituting air with pure oxygen would result in 5-fold higher oxygen concentration in the liquids, but conversions remained constant. Therefore, to verify if that surplus of oxygen can be utilised, reactions with a residence time unit installed behind 1 m TiTR were conducted. With increasing lengths of the residence time unit the conversion of 2-hydroxybiphenyl increased until it stayed nearly constant at 5.1 min, while the product formation rate decreasing gradually (Figure 6-8B). Since aeration was not possible outside the TiTR module, the maximum length of an extension unit was dependent on the availability of oxygen in the liquid entering this part of the reactor system. As a compromise between the product formation rate and the final product titre a residence time unit of 3 m (2.4 mL) was chosen, resulting in a residence time of 4.1 min. This setting was the basis for an experiment on a preparative scale applying HbpA in a sequential TiTR for the synthesis of 2,3-dihydroxybiphenyl.

Synthesis of 2,3-dihydroxybiphenyl on a preparative scale using a pressurised sequential TiTR

Conducting the reaction in a TiTR longer than 4 m was not possible due to the nature of the Teflon AF-2400 membrane. Longer tubes are fragile and more susceptible to snapping while handling. In order to access higher product titres a sequential reactor was assembled (as schematically presented in the Figure 6-3). After the first TiTR a residence time unit was connected, followed by another TiTR, and a second residence time unit. This way the total reactor volume was increased to 8 mL and the residence time achieved with a flow rate of 0.7 mL min^{-1} was 11.5 min.

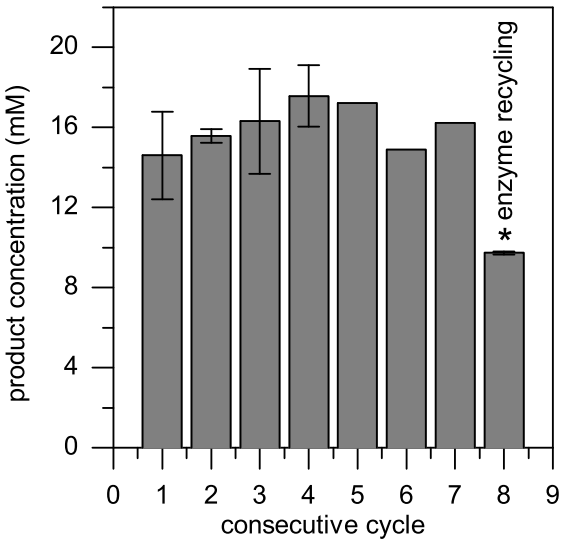

Figure 6-9: Synthesis of 3-phenylcatechol in a sequential TiTR reactor. Product concentrations have been determined in the sample collected at the reactor outlet during consecutive cycles and are given for a total volume (combined aqueous and organic phase). The 8th cycle was conducted with 50 mL of the recycled aqueous phase supplemented with sodium formate (4 mmol) and NAD^+ (20 µmol). Total residence time in the system (8 mL) ca. 11.5 min at total flow rate of 0.7 mL min^{-1}. For cycle 5, 6 and 7 n=1.

The system introduced above was operated in a semi-continuous mode. During the first 7 cycles freshly prepared enzyme solutions were used, whereas for the 8th cycle 50 mL of the recycled enzyme solution supplemented with sodium formate (4 mmol) and NAD^+ (20 µmol) was used instead, to verify if the enzymes could be reused (Figure 6-9). Before the 8th cycle, the activity of the recycled enzymes were measured in an independent UV assay and compared to the initial ones revealing almost 90% residual HbpA activity while FDH did not experience any activity loss (Table 6-2). Surprisingly conversion in the TiTR was significantly lower, when using recycled enzyme solution. This could be attributed to the composition of the recycled aqueous phase, which also contained residual organic solvent and the dissolved product. This could inhibit the HbpA in the TiTR. Activity measured in a spectrophotometric assay would not be affected thereby, as the respective samples were diluted 300-fold prior to the measurement.

During the whole experiment the reactor outlet was placed in a bottle continuously flushed with nitrogen in order to prevent the hydroxylation reaction continuing outside of the reactor.

After every cycle the phases were separated and the collected organic phase was eventually used for product recovery.

Table 6-2: Activity of HbpA and FDH measured in a spectrophotometric assay before and after the reaction in the sequential TiTR system (TiTRs and residence time units). Residence time 11.5 min. (errors are presented as a standard deviation, n=2)

enzyme	initial volumetric activity [U mL^{-1}]	final volumetric activity [U mL^{-1}]	residual activity [%]
HbpA	10.26±0.21	9.16±0.19	89.3
FDH	6.76±0.14	7.51±0.16	111.2

Purification of the product 2,3-dihydroxybiphenyl was successful and 740 mg of the product were isolated

Using the sequential TiTR for 2-hydroxybiphenyl hydroxylation it was possible to synthesise 12 mmol of product and isolate 4 mmol (740 mg) of pure (>99% by HPLC and H-NMR) 2,3-dihydroxybiphenyl (Figure 6-10).

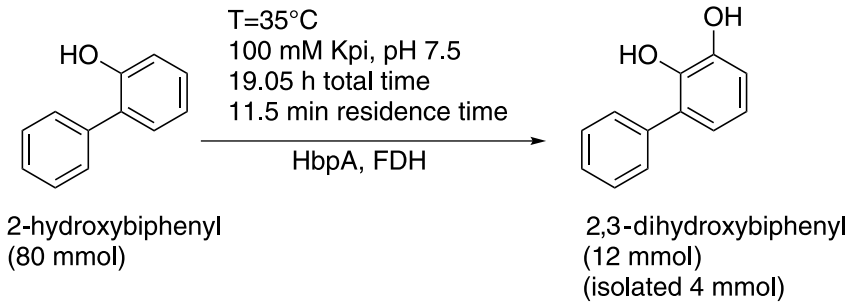

Figure 6-10: Summary of hydroxylation of 2-hydroxybiphenyl to 2,3-dihydroxybiphenyl on a preparative scale.

The purification pipeline is schematically shown in the Figure 6-11. The collected sample was centrifuged in order to achieve separation of the aqueous and organic phase. From the 400 mL organic phase present, only 360 mL were removed as a precaution not to contaminate the sample by accidentally transferring precipitated proteins or aqueous liquid. This organic sample was directly loaded onto a neutral alumina column. During the elution with acidic methanol the top of the column became darker. Over the time the dark ring moved downward and become green followed by a brownish/red ring (see Figure 6-11). The pH of the green eluent was neutral. It contained most of the product and ca. < 1% of the substrate (as analysed by the HPLC). When the green fraction was acidified it turned brownish red like the fractions that followed. The overall product isolation yield after all purification steps was 35%, and the product was confirmed by the H-NMR.

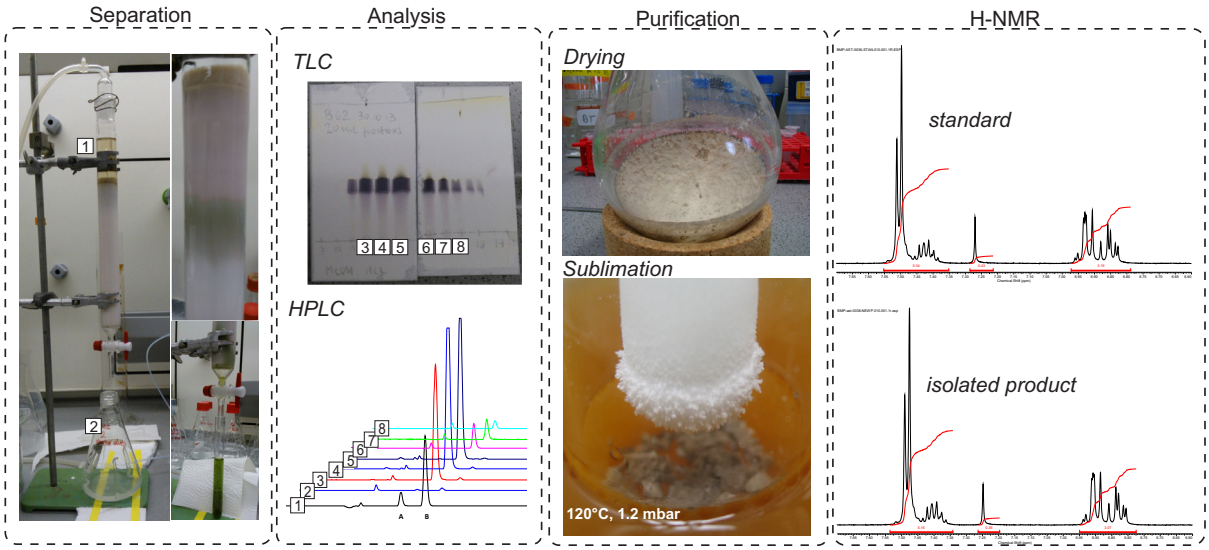

Figure 6-11: Step-by-step product purification procedure: product separation on the neutral alumina column, analysis of the consecutive fractions by the TLC and HPLC method, dried sample collected from the column, sublimation of the product on the cold finger, and H-NMR spectrum of the standard and the isolated product.

ePTFE module for reaction and phase separation

To increase enzymes' stability and simplify biocatalyst recovery the standard TiTR was modified by incorporation of an ePTFE membrane tube-in-tube module as described in detail in the experimental section. This additional membrane module should enable separation of aqueous and organic phase during biotransformation and facilitate biocatalyst recovery, making phase separation redundant prior to enzyme recycling. However, the used ePTFE membrane proved to be not suited for the applied solvents MTBE and decanol. In both cases the solvent soaked through the ePTFE membrane into the aqueous phase turning it cloudy, although no second phase was formed. Furthermore the solvent, which bled through the ePTFE tube at the entrance of the reactor, evaporated resulting in solidified 2-hydroxybiphenyl at the respective connectors (Figure 6-12). The organic phase, on the other hand, was free of water, which is a clear advantage of this set-up.

Product concentrations in the MTBE phase were in the range of 4-6.5 mM (approx. 2-3% conversion of applied substrate) (Figure 6-13). Substrate was applied at relatively high concentrations (200 mM) to maximise the membrane mass transport (no partition ratios between organic phase-membrane and membrane-aqueous phase were however determined prior to the experiment).

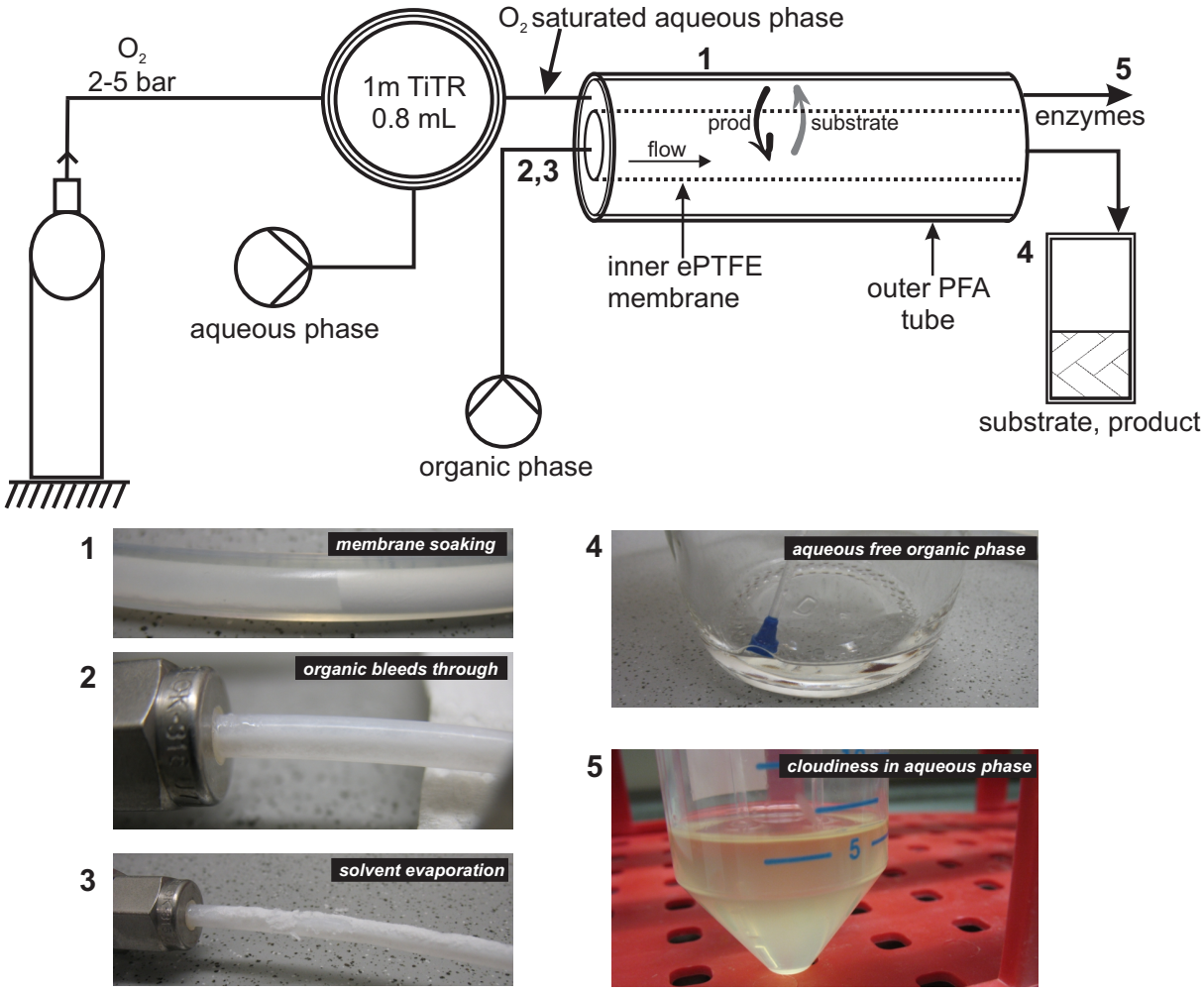

Figure 6-12: Schematic view of the ePTFE TiTR during the reaction. Numbers in the scheme refer to the respective pictures below. 1 – membrane soaking with MTBE. 2 – MTBE bleeding through the ePTFE membrane at the outlet. 3- solid 2-hydroxybiphenyl after MTBE evaporation. 4 – aqueous free organic phase collected after reaction. 5 – cloudy aqueous phase containing some MTBE.

In the three consecutive experiment phases (I, II, and III as shown in the Figure 6-13) reaction parameters were varied. Initially used fresh enzyme solution was recycled in phase II. Enzymes assayed in an UV activity assay after the run with MTBE showed 90% and 88% residual activity for HbpA and 62% and 70% for FDH after phase I and II respectively. Enzymes did not lose activity after the 2nd consecutive run proving that separate delivery of aqueous and organic phase into the bioreactor had a beneficial effect as no aqueous/organic interface, where enzymes could denature, was present (aqueous/organic interface is however still present in the membrane pores). There was no enzyme precipitation, which is often a sign of protein denaturation, and the phase separation was simplified as phases did not mix during the reaction. In phase III again fresh enzymes were used, whereas the pressure in the oxygen saturating module was increased from 2 to 5 bar, since it was expected that the oxygen concentration in the aqueous medium in the phase I and II was too low for the long residence time of 40 min. Because only the aqueous phase was saturated with oxygen prior to entering

the reaction module it seemed appropriate to introduce air segments into the aqueous phase by saturating it at 5 bar and letting it decompress in the reaction module after passing the BPR. Product concentration however, did not increase in the phase III, meaning that there is some other mechanism limiting the reactor performance (*vide infra*).

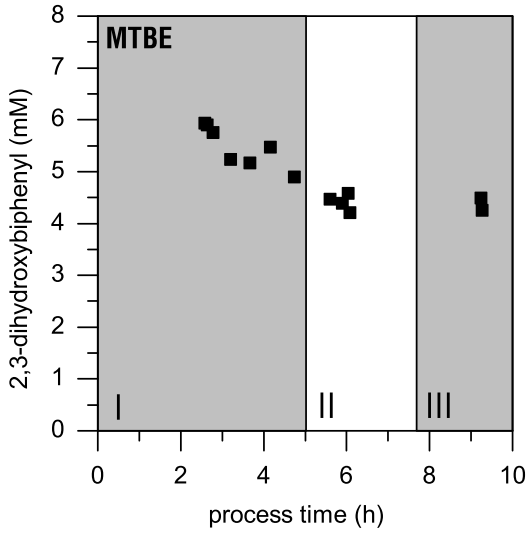

Figure 6-13: Concentration of 2,3-dihydroxybiphenyl measured continuously in the organic phase after the reaction in an ePTFE TiTR. MTBE - I – oxygen pressure 2 bar, II – oxygen pressure 2 bar and enzymes recycling, III – fresh enzymes and oxygen pressure 5 bar with a BPR (5 bar) installed behind oxygen saturation module before the ePTFE reaction module. Residence time of the aqueous and organic phase was approx. 40 and 130 min respectively.

DISCUSSION

Quantification of oxygen permeability through the Teflon AF-2400 membrane in a TiTR – oxygen vs. air

A simple calculation was proposed for determining the volume of the oxygen in the total volume of gas degassing from the liquid after passage through Teflon AF-2400 membrane in the TiTR. The air used as a gas phase was composed of 21% O_2 and 78% nitrogen. Because Teflon AF-2400 is more selective for oxygen, oxygen is transported faster than nitrogen. By comparing fluxes of nitrogen and oxygen streams through the membrane determination of that ratio was possible. Under the assumption that the driving force is equal to the initial partial pressure difference (Δp_i), and that it is constant, one may write two flux equations (Equation 6-1 and Equation 6-2).

Equation 6-1

$$J_{O_2} = \frac{Permeability}{\delta_{membr}} \cdot \Delta p_{O_2}$$

Equation 6-2

$$J_{N_2} = \frac{Permeability}{\delta_{membr}} \cdot \Delta p_{N_2}$$

For a membrane thickness of δ=0.01 mm and permeability for oxygen and nitrogen of 990 and 490 Barrer respectively, the theoretical fluxes of oxygen and nitrogen were calculated as follows:

$$J_{O_2} = \frac{990 \cdot \frac{10^{-10} \cdot {cm^3}_{O_2} \cdot cm_{membr}}{s \cdot {cm^2}_{membr} \cdot cmHg}}{0.01\ cm_{membr}} \cdot 0.21 \cdot 76\ cmHg = 1.58 \cdot 10^{-4} \frac{{cm^3}_{O_2}}{s \cdot {cm^2}_{membr}}$$

$$J_{N_2} = \frac{490 \cdot \frac{10^{-10} \cdot {cm^3}_{N_2} \cdot cm_{membr}}{s \cdot {cm^2}_{membr} \cdot cmHg}}{0.01\ cm_{membr}} \cdot 0.78 \cdot 76\ cmHg = 2.90 \cdot 10^{-4} \frac{{cm^3}_{N_2}}{s \cdot {cm^2}_{membr}}$$

$$J_{total} = J_{O_2} + J_{N_2} = 4.48 \cdot 10^{-4} \frac{{cm^3}_{total}}{s \cdot {cm^2}_{membr}}$$

J_{N2} is the flux of nitrogen and J_{O2} is the flux of oxygen. By dividing the oxygen flux by the total flux one could calculate the ratio of oxygen in the total gas flux to be 0.35 (or 0.35 $cm_{O2}^3\ cm_{total}^{-3}$). Results are summarised in Table 6-3.

Table 6-3: Oxygen concentration and transport rate in liquid/liquid two phase flow (KPi buffer: decanol at 1:1 ratio) at 30°C and 6.9 bar back pressure.

nature of gas	Total flow rate [mL min^{-1}]	O_2 concentration in reactor [mM]	O_2 transport rate[c] [µmol min^{-1} $mL_{reactor}^{-1}$]
air[a]	0.8	5.3	8.4
	0.4	5.7	-
	0.2	5.9	-
	0.1	6.0	-
pure oxygen[b]	0.8	15.0	24.1

[a] volume fraction of oxygen in the air after passing through the membrane is equal to 0.35
[b] assuming that molar fraction of oxygen in the air after passing through the membrane is equal to 1 all transported gas is oxygen and it is possible to calculate approximate oxygen concentration and its transfer rates
[c] because the experiment was conducted in the range close to the oxygen saturation levels only a theoretical oxygen transport rate is given. Calculations are based on the assumption that oxygen (air) is transported steadily over the residence time and the saturation is not reached earlier.

Quantifying gas transfer through the membrane was important as it was possible to ultimately exclude oxygen limitation. It also allowed for establishing the maximum operational boundaries of the reaction system. A maximal oxygen transport rate through the Teflon AF-2400 membrane at a flow rate of 0.8 ml min^{-1} was calculated to be 8.4 µmol min^{-1} $mL_{reactor}^{-1}$ for air, and theoretically 24.1 µmol min^{-1} $mL_{reactor}^{-1}$ for oxygen (vide infra). Compared to the reported STY of 26.6 g L^{-1} h^{-1} (overall volumetric productivity 3.4 µmol min^{-1} $mL_{reactor}^{-1}$) for the synthesis of *tert*-leucine in a process running at Evonik[9b] in an enzyme membrane reactor, the potential of the here proposed reactor setup for oxygen limited reactions becomes clear. However, this potential was not utilised for the here applied reaction, as a rate of only 1.3 µmol min^{-1} mL_{total}^{-1} (1.3 mM min^{-1}) was reached, leaving room for future improvements. Despite these shortcomings the increased concentrations of dissolved oxygen in the liquid resulting from the pressurised TiTR system could be efficiently utilised in the sequential

TiTR setup and the residence time could be extended to 11.5 minutes, without running into an oxygen limitation.

Overcoming product inhibition of 2-hydroxybiphenyl 3-monooxygenase, HbpA by using solvents with higher organic/aqueous partition ratios of product

As previously reported,[42] the 2,3-dihydroxybiphenyl has a detrimental effect on the HbpA activity. It inhibits catalysis by almost 30% already at a concentration of 1 μM. Decanol was therefore used as a second organic phase to continuously extract 2,3-dihydroxybiphenyl from the aqueous phase and prevent product inhibition. The partition ratios for 2-hydroxybiphenyl and 2,3-dihydroxybiphenyl for decanol were determined to be 480 and 380 respectively (Chapter 5). Thereby, an inhibition of HbpA is to be expected already at 0.5% conversion, i.e. when the concentration of product in the organic phase reaches 1 mM. This is well visualised in the Figure 6-8B where with increasing conversion the product formation rate steadily decreases. Product inhibition could be further suppressed in aqueous/organic segmented flow with different solvent systems which have higher partition ratios, e.g. MTBE (2200 and 1425 for 2-hydroxybiphenyl and 2,3-dihydroxybiphenyl respectively) resulting in lower product concentration in the aqueous phase at the same level of conversion. However, due to high substrate partition ratio, resulting in low substrate concentrations in the aqueous phase, product formation rates might be lower since the reaction could become mass transfer limited again. Nevertheless, it is difficult to judge from the results presented in this chapter if the MTBE had a beneficial effect, preventing HbpA product inhibition, because detailed membrane transport characteristics for 2-hydroxybiphenyl/2,3-dihydroxybiphenyl were not known. Thus, judging if the membrane transport or too high substrate partition ratio was rate limiting factors is difficult and should be preceded by careful membrane transport characterisation.

Enzyme consumption and strategies for maximising the TTN

One way to use the catalyst more efficiently is to conduct the reaction with higher residence times in longer TiTR systems and consequently reach higher conversions. Initially we were limited to a maximum length of 4 m and a residence time of 3 min at a flow rate of 0.8 mL min^{-1}. By connecting TiTR units in a sequential fashion the residence time was extended to 11.5 min improving the conversion (and TTN) and simplifying product separation from a substrate/product mixture. Another approach was to recycle the enzymes. In this case the phases needed to be separated before and 9.7 mM product were produced (Figure 6-9, cycle 8). The product concentration was however lower than 15 mM reached with fresh enzymes (Figure 6-9, cycles 1-7). Even after accounting for the enzyme activity loss during the first

run higher product concentrations were expected. It raised our concerns that there might be another reason for lower product formation rates. Shear induced reversible quaternary structure loss of the enzymes in the microreactor could be one reason for the low product formation rates in the reactor, however, in the UV-assay higher enzyme activities than in the reactor were measured. It may be that HbpA was mainly present as a monomer in the reactor hence it was not as active as in its native tetrameric form (Table 6-1 and Table 6-2) (for more information on that topic see chapter 7). Another hypothesis was the difference in the reaction conditions between the UV assay and the 8th cycle. Due to the sample preparation (dilution) for the UV activity assay the conditions are assumed to be always constant and most likely did not reflect the reaction conditions in the enzyme solution used in cycle 8, which contained residual dissolved organic solvent carrying substrate and product.

Mass transfer

The overall volumetric mass transfer coefficient (k_La) calculated on the base of the product formation rate for the aqueous phase (2.6 mM min^{-1}) and the initial equilibrium substrate concentration in the aqueous phase (200 mM/480=0.416 mM) was 0.104 s^{-1} (2.6 mM min^{-1}/ 0.416 mM/ 60 s min^{-1}=0.104 s^{-1}), which means that with a certain product formation rate in the aqueous phase the equilibrium substrate concentration needed to be renewed at least 0.104 times per second in order to sustain the reaction. Values measured for the 0.8 mm internal diameter Teflon AF-2400 capillary used in this study agrees well with published k_La values measured for the extraction of succinic acid in a 0.75 mm i.d. capillary (Table 6-4). There are various parameters influencing mass transfer in the system e.g. flow rate, capillary inner diameter, interfacial tension, and viscosity of the liquid. All of them are interconnected e.g. mass transfer depends on the internal circulations within segments, which depend on the differences in liquid viscosities and interfacial tensions. With increasing viscosity circulations within segments decrease therefore the mass transfer by convection is decreased. Addition of surfactant reduces interfacial tension subsequently reducing the shear stress, which internal circulations depend upon. Eventually, convection is higher with higher slug velocities. Because all the systems are different there might be discrepancy in the measured k_La values. Therefore even the k_La values measured in the same reactor with different reaction systems e.g. water/kerosene (iodine), water/kerosene (acetic acid), or water/n-butanol (succinic acid), whereby the first two liquids are the continuous and the aqueous phase, while the compound in brackets is being extracted, show significant variations in k_La values of 0.32-1.47 s^{-1}.[84] Another difference between the enzymatic systems and the chemical systems is the fact that for the enzymatic systems the k_La is calculated back from the product formation rates hence

the mass transfer might be underestimated, since the molecule which travels across the interface still needs to diffuse into the active site of the enzyme. To conclude, the mass transfer calculated in the present work is in the range of the previously published values, therefore we have confidence that it is the reaction rate limiting factor. Because of high interfacial area in microreactors, here proposed TiTR with Teflon AF-2400 membrane still offers the best reaction conditions for conducting this reaction.

Actions that can be taken to further increase the mass transfer are simple e.g. increase of the flow rate, which will unfortunately reduce the conversions and decrease the TTN. It should also be possible to use Teflon AF-2400 membrane with lower internal diameter, which will decrease diffusional distance and diffusion time, and increase surface to volume ratio.

Table 6-4: Overall volumetric mass transfer coefficients (k_La) in various liquid/liquid two phase segmented flow microreactors. Compounds given in brackets are extracted. Adapted and modified from Karande, (2012).[180]

System	Phases (product)	Reaction	Capillary diameter [mm]	Overall mass transfer coefficient k_La [s^{-1}]	Ref.
Segmented flow tube-in-tube microreactor	Decanol/KPi (2,3-dihydroxybiphenyl)	Enzymatic	0.8	0.104	This work
Segmented flow microreactor	Hexadecane/KPi (heptaldehyde)	Enzymatic	0.5	0.042	95, 180
			1.0	0.025	
			2.15	0.009	
Segmented flow (extractor)	n-butanol/aqueous (succinic acid)	Non-reacting system	0.5	0.12-0.31	84
			0.75	0.07-0.15	
			1.0	0.02-0.09	
Segmented flow microreactor	Kerosene/aqueous	Acid base reaction	0.38	0.5	87
Segmented flow (extractor)	n-butyl formate/aqueous	Non-reacting system	0.5	0.90-1.67	181
			0.75	0.91-1.46	
			1.0	0.88-1.29	

ePTFE membrane shortcomings

It is not clear why the product concentration in MTBE was decreasing with time. One of the possible explanations is that the membrane became saturated with substrate/product as the reaction proceeded, resulting in subsequent lower substrate/product transport and lower reaction rates. Another reason could be that there was no mixing in the aqueous and organic phase and thus, the mass transfer was completely dependent on the diffusion, which would decrease if the concentrations of substrate and product in the vicinity of the membrane would reach its saturating levels. It is difficult to attribute the decrease in the product concentration in the phase II to enzyme activity loss because it was shown that biocatalyst activity was the same before and after phase II. Nevertheless, application of the ePTFE membrane TiTR allowed separating aqueous and organic phase from one another and increasing operational

stability of enzymes since the activity losses of e.g. HbpA were comparable to that measured in Teflon AF-2400 TiTR with a direct phase contact, but in the ePTFE module enzymes residence times were almost 4 times higher. However, to completely prevent the organic phase from dissolving in the aqueous phase, one may deliver the aqueous phase at higher pressure than the organic phase resulting in a differential pressure across the membrane. As long as the pressure is kept below the break-through pressure, which needs to be determined experimentally, the aqueous phase should not pass the hydrophobic membrane, keeping the organic phase confined within the membrane. Such a technique was successfully applied for separating hexane and aqueous phase in a differential circulation reactor with continuous product extraction.[182]

CONCLUSIONS

The sequential TiTR introduced in this chapter was shown to be a suitable tool for conducting enzymatic gas dependent reactions at a preparative scale. The applicability of the system was proven for 2-hydroxybiphenyl hydroxylation to 2,3-dihydroxybiphenyl by means of 2-hydroxybiphenyl 3-monooxygenase (HbpA). This reaction was coupled with FDH-coupled NADH regeneration. A successful preparative scale biotransformation reaction in a segmented flow pressurised TiTR has been shown. The space time yield of 14.5 g L_{total}^{-1} h^{-1} is the highest reported for this enzyme. It also compares well with an existing industrial process employing oxidoreductases for the synthesis of *tert*-leucine (26.6 g L_{total}^{-1} h^{-1}). Product separation was successful and 740 mg of product with excellent purity was isolated. Preliminary studies with an ePTFE tube-in-tube membrane reactor concept proved useful for simplifying phase separation. After additional membrane engineering the concept might be a good basis for the process scale-up based not on the numbering-up technique but on increasing the physical diameter of the capillaries and introducing e.g. static mixers, which would provide the same or even higher mass transfer as in conventional capillary microreactors.

ACKNOWLEDGMENTS

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CHAPTER 7

GENERAL DISCUSSION

DEFINITION OF THE MICROCHANNEL AND THE DIMENSIONLESS NUMBERS

Microreactors find their application either for: process optimisation and screening, where limited volumes of reactants are available; process intensification of reactions, which are mass transfer limited in conventional scale reactors; or process development for an industrial manufacturing of fine chemicals that needs to be scaled-up on demand. Throughout this thesis the term microreactor and microstructured reactor has been used interchangeably to define a small scale device with a channel diameter in the millimetre to micrometre range. More precisely microchannel is a device in which the buoyancy is dominated by surface tension forces.[152a] In simple words there exists a threshold diameter of a closed liquid filled microchannel, at which the rise of an air bubble cannot be observed any longer, and it is defined by the dimensionless Bond number smaller than 3.37.[183] This allowed us to calculate the minimum capillary diameter, which would fulfil above requirements for the decanol-buffer system used in this study to be 1.95 mm. This is bigger than the diameter of the Teflon AF-2400 membrane used in chapter 5 and 6. Since the calculations are based on the Bond number

$$Bo = \frac{\Delta\rho g d^2}{\sigma} \qquad \text{Equation 7-1}$$

where, $\Delta\rho$ is the fluid density difference (kg m^{-3}), g is the gravitational acceleration (m s^{-2}), d is the characteristic length scale, diameter (m), σ is the interfacial tension of the two fluids in contact (N m^{-1}), the higher the surface tension the bigger the diameter for which surface tension forces will dominate the buoyancy. Such system can be considered a microreactor. The list of the parameters of the liquids used in this study is given in the Table 7-1.

Table 7-1: Parameters of the fluids used in this study

System	Density [kg m^{-3}]	Viscosity[e] [m*Pa* s]	Interfacial tension [mN m^{-1}]
Buffer[a]- decanol[b]	1010.5±2.4	-	8.8
Buffer (enz[c]) – decanol	1014.1±3.8	-	2.9
Buffer (surfactants[d]) – decanol	1011.8±3.5	-	4.0
Buffer (surfactants, enz) - decanol	1033.3±2.1	1.72 (23.2°C)	2.2[f]
Decanol[b]	830.3±5.8	12.65 (23.1°C)	-

[a] containing 200 mM sodium formate, 1 mM NAD^+, 20 µM FAD

[b] containing 200 mM 2-hydroxybiphenyl

[c] buffer and additionally 9.2 U mL^{-1} HbpA and 7.5 U mL^{-1} FDH

[d] buffer and additionally 0.6 mg mL^{-1} Tween 20

[e] absolute viscosity i.e. ratio of relative viscosity and specific gravity of a sample

[f] it was still dropping, also droplet volume was decreasing, this value was therefore chosen as a compromise

Drop tensiometry measurements revealed that the interfacial tension of the buffer solution with enzyme and surfactant was very low (2.2 mN s^{-1}). Additionally, a competitive adsorption

of enzyme molecules and surfactants was observed. The interfacial tension value for the mixture of enzyme and surfactant was lower than for the single compounds, revealing a synergistic effect and rather heterogeneous surface coverage, as compared to a uniform layer of either enzymes or surfactants, which would explain partial enzyme inactivation (chapter 5 and 6). Surfactants are often used to stabilise segments in microchannels and prevent their coalescence.[184] When the interface is covered, surfactant stabilises droplets by two distinct mechanisms i.e. steric repulsion of the surfactant covered interfaces, and by preventing drainage of the continuous phase filling space between two droplets. The latter is a direct effect of the surface rigidification, as the surfactant molecule modifies not only the interface of the droplet but also affects the flow and mobility of the liquid around it by interacting with it e.g. slowing it down.[185] However, full and homogenous surface coverage is required for preventing coalescence, and any differences in surface tension, caused by surface rigidification for instance (so called Maragnoni effect), might lead to instabilities. Pulsations of the peristaltic pumps might therefore lead to the surfactant rearrangements on the droplets interface and flow instabilities as observed in chapter 2. Reliable flow control was later provided by using syringe pumps, which proved to be operating without any pulsation.

As we are operating in the micro scale, some other dimensionless parameters can be used to describe the two phase flow with respect to the channel diameter, viscosity, density, interfacial tension of both fluids, and liquid velocity. Those two are the Reynolds number, describing relative importance of the inertial force to viscous forces

$$Re = \frac{\rho d U}{\mu} \qquad \text{Equation 7-2}$$

where U is the characteristic velocity (m s^{-1}) and μ is the viscosity of continuous phase (Pa s), and the Capillary number (Ca)

$$Ca = \frac{\mu U}{\sigma} \qquad \text{Equation 7-3}$$

Table 7-2: Dimensionless numbers for characterising the flow in the microreactor. Chapter 5 and 6.

	System	Flow velocity [m s^{-1}]	Re [-]	Ca [-]	Bo [-]
a	Buffer (enz) – decanol, 0.8 mm id, flow rate 0.8 mL min^{-1}, 30°C	0.026	15.1	0.011	0.58
b	Buffer – decanol, 0.8 mm id, flow rate 0.8 mL min^{-1}, 30°C	0.026	27.4	0.0013	0.14
c	Buffer (enz) – decanol, 0.8 mm id, flow rate 0.2 mL min^{-1}, 30°C	0.007	3.8	0.0028	0.58

[a] buffer with enzymes and decanol containing substrate. σ=0.0022 N m^{-1}, μ=0.00145 Pa s (30°C)

[b] buffer and decanol containing substrate. σ=0.0088 N m^{-1}, μ=0.0008 Pa s (30°C)

[c] buffer with enzymes and decanol containing substrate. σ=0.0022 N m^{-1}, μ=0.00145 Pa s (30°C)

The interpretation of dimensionless numbers given in Table 7-2 allows concluding that the flow is laminar irrespective of the flow rate used and diffusion should be the main force influencing the mass transfer in the channel. However, due to the presence of the Taylor flow the mass transfer is enhanced and occurs mainly via advection-enhanced diffusion, as a result of circular movements of the bulk fluid occurring due to shear stress.[186] The next dimensionless number, which is very important for the two phase flow in a microchannel, is the Ca as it determines the way plugs/bubbles/segments are formed at the T-junction in a microreactor. For the cross-flow (i.e. continuous phase flows in the main channel and the dispersed phase is introduced perpendicularly) three regimes of droplet formation have been named with respect to the Ca as squeezing, dripping and jetting.[187] Squeezing regime ($7 \cdot 10^{-4} \leq Ca \leq 7.6 \cdot 10^{-2}$), dripping regime ($1.9 \cdot 10^{-3} \leq Ca \leq 8.2 \cdot 10^{-2}$) and jetting regime ($2.2 \cdot 10^{-2} \leq Ca \leq 1.1 \cdot 10^{-1}$). It can be seen that for condition **a** in Table 7-2, the jetting regime is expected, this would also explain formation of the parallel flow at the inlet, and subsequent segment pinch-off (chapter 5). For condition **b** no such phenomena was observed which is reflected in the low Ca in the squeezing regime and formation of segments right at the T-connector. Since the Ca is proportional to the viscosity of the continuous phase and inversely proportional to the interfacial tension between liquids, the Ca was low when no enzymes and surfactants were present and the interfacial tension was high.

Another important aspect of two phase flow in the microchannel under pressure is the relative viscosity of aqueous and organic phase. Decanol was almost exclusively used as an organic phase throughout this thesis. Because it is 7 times more viscous than the aqueous phase (Table 7-1) it was challenging to obtain a stable flow (even with the syringe pumps) with the back pressure regulator (BPR) installed at the reactor outlet (chapter 6). Because of the differences in the viscosity, decanol and buffer passed through the BPR with different velocities, causing pulsation upstream of it. This problem did not exist when hexadecane and buffer were used as they have similar viscosities, but hexadecane could not be used as an organic phase as it did not dissolve the 3-phenylcatechol, the reaction product. To solve the problem, a simple device consisting of a gas filled tube, coupled with the system by the T-connector was used. It exerted constant back-pressure (equal to the pressure in the system) on the liquids in the microreactor and allowed for a smooth operation because all the pulsations, which happened downstream of it, were isolated and did not affect the flow. This ‘damper’ is presented in Figure 7-1.

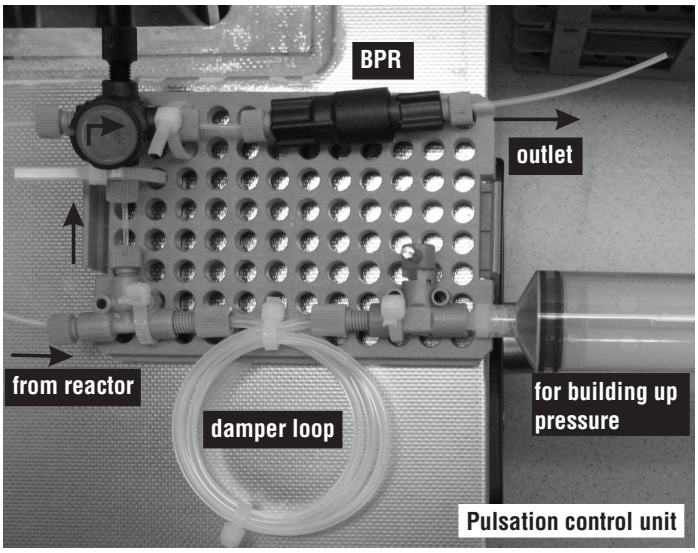

Figure 7-1: A damper for pulsation free flow operation in the system with liquids of two distinct viscosities.

This device has been successfully implemented into the microreactor system being operated with aqueous/organic flow. In the aqueous/organic/air three phase system like the one in chapter 5, air presents another challenge. An air segment that followed surfactant containing aqueous phase removed the thin film of continuous aqueous phase wetting the PTFE capillary and destabilised the flow. In an aqueous/organic two phase flow the complete wetting of the capillary by the aqueous phase (containing Tween 20) allows stable formation of the oil-in-water emulsion.[188] Presence of the thin aqueous film is therefore essential for stable operation. After introducing the third phase air, film removal occurs faster than its renewal making the whole system is unstable (Figure 7-2). Those difficulties were overcome by introducing the TiTR to uncouple the air flow from the aqueous/organic flow and separate both by Teflon AF-2400 membrane (chapter 5 and 6).

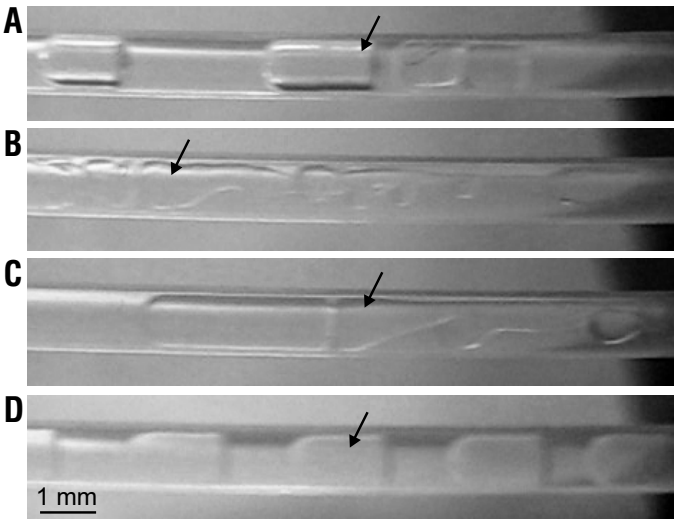

Figure 7-2: Images of flow pattern in a capillary operated with aqueous/organic two phase flow with few air segments introduced initially. Total flow rate 0.4 mL min^{-1}, 1 mm i.d. PTFE capillary. Aqueous phase: HbpA and FDH 4 U mL^{-1}, 0.6 mg mL^{-1} Tween 20; organic phase: decanol. A - Aqueous/air two phase flow. Arrow shows the last air segment followed by an aqueous/organic two-phase flow. B - Pear shaped organic segments as a result of liquids' viscosity difference and removal of the thin aqueous film wetting the capillary wall. C - Partial stabilisation of the flow. D - Stable aqueous/organic two-phase flow achieved ca. 1 min after the last air segment. Following images A, B, C, and D were taken in 15-20 sec intervals.

HOW DO SHEAR AND INTERFACIAL FORCES INFLUENCE PROTEIN STABILITY AND CAN THEY INDUCE A QUATERNARY STRUCTURE LOSS?

There are two mechanisms in microreactors that might reversibly or irreversibly affect enzymes, namely shear stress (mechanical forces caused by the velocity gradients of moving liquids) and interfacial effects (interaction of protein with aqueous/air, aqueous/organic, and/or liquid/solid interface) and they are summarised in a review by Thomas and Geer (2011).[53] Shear stress alone should however be excluded because the forces present in the laminar or turbulent flow in a monophasic system are too low to denature the enzymes.[189] Indeed circulation of the enzymes through the PTFE tube did not itself lead to enzyme inactivation (chapter 2). Application of a second organic phase might however have a negative effect on the enzyme stability. In this work the effect of the interface was minimised by applying surfactants like Tween 20 as proposed earlier.[66] However, denaturation caused by contact with a hydrophobic solvent cannot be completely prevented as the coverage of the aqueous/organic interface with Tween 20 is a dynamic process and requires time to distribute the surfactant.[67] Within that process enzyme molecules may get in contact with the liquid interface and might as well adsorb to the solid PTFE surface and denature.[68] All those effects might cause a loss of secondary, tertiary and/or quaternary structure.[53] If those effects are reversible the tetrameric HbpA could dissociate in the flow but once the 'effector force' would be removed monomers should re-assemble to an initial quaternary structure. This hypothesis would hold true if the interfacial effects would cause a reversible structure loss only. As suggested earlier[190] the non-covalent aggregation of proteins is reversible and aggregates dissociate to release monomers. We propose here a reverse effect where the interfacial forces act to dissociate the multimeric protein. Because there is an equilibrium between the folded multimeric protein and its monomers[191] it seems plausible that an additional force (interfacial force) might shift the equilibrium and promote the existence of the single monomeric form over the multimeric form. It could explain why product formation rates in the reactor are lower than expected from the catalyst loading and why the activities measured in the UV-assay are higher than those in the reactor because the monomeric form of HbpA is less active than the tetramer.

KINETIC MODEL OF THE REACTION

The above, was one of the hypotheses raised to explain and understand why the product formation rates measured in the TiTR (chapter 6) were lower than expected from the initial aqueous biocatalyst loadings (U mL^{-1}). By using a similar approach as Karande (2012),[180] a

kinetic model for the reaction was built and the reaction was simulated. Rate equations are shown below.

Equation 7-4

$$r_{HbpA} = Vmax\left(\frac{C_{O2}}{Km_{O2} + C_{O2}}\right)\left(\frac{C_{2OH}}{Km_{2OH}\left(1 + \frac{C_{prod}}{Kic}\right) + C_{2OH}}\right)\left(\frac{C_{NADH}}{Km_{NADH}\left(1 + \frac{C_{NAD}}{Ki_{NAD}}\right) + C_{NADH}}\right)\left(\frac{1}{1 + \frac{C_{FOR}}{Ki_{FOR}}}\right)$$

Equation 7-5

$$r_{FDH} = Vmax\left(\frac{C_{FOR}}{Km_{FOR} + C_{FOR}}\right)\left(\frac{C_{NAD}}{Km_{NAD}\left(1 + \frac{C_{NADH}}{Ki_{NADH}}\right) + C_{NAD}}\right)$$

The rate of the HbpA reaction in the aqueous phase, r_{HbpA}, is dependent on the concentration of oxygen, C_{O2}; aqueous concentration of hydrophobic substrate, C_{2OH}; and the cofactor concentration NADH, C_{NADH}. The reaction is additionally inhibited by increasing product concentrations, C_{prod}. The rate equation is simplified, and includes only competitive product inhibition indicated by enzyme-product dissociation constant K_{ic}, and not as predicted earlier mixed type non-competitive inhibition.[42] In mixed type inhibition the K_{iu} dissociation constant for the formation of the enzyme-substrate-product complex has a significant effect on the reaction rate because the formed complex is not catalytically active. On the attempt to include the mixed type inhibition in the model the output values were lower than the measured ones, therefore the equation was simplified. The HbpA reaction is also competitively inhibited by the NAD^+, C_{NAD}. On the contrary, the FDH catalysed reaction is competitively inhibited by the NADH. The negative effect of the sodium formate, C_{FOR}, on the HbpA was also included in the model. Inhibition of HbpA with 2-hydroxybiphenyl (K_i=6.5 mM) was disregarded as it would only exert negligible effect on the HbpA activity.

In order to calculate the actual aqueous concentrations of 2-hydroxybiphenyl, $C_{2OH(n)}$, NADH, $[NADH]_n$, and NAD^+, $[NAD]_n$ the following formulas were used.

Equation 7-6

$$C_{2OH\,(n)} = C_{2OH(n-1)} + k_L a\left(C_{eq} - C_{2OH(n-1)}\right) \cdot t_i - r_{HbpA(n-1)} \cdot t_i$$

Equation 7-7

$$[NADH]_n = [NADH]_{n-1} + \left(r_{FDH} \cdot t_i - r_{HbpA} \cdot t_i\right)$$

Equation 7-8

$$[NAD]_n = [NAD]_{n-1} - \left(r_{FDH} \cdot t_i - r_{HbpA} \cdot t_i\right)$$

The n=0 indicates initial reaction conditions. The equations were solved by the iterative method with a very small time interval t_i. The parameters used in the model are given in the Table 7-3.

Table 7-3: Parameters used in the model.

HbpA[42]		FDH[180]	
K_{m2OH} [mM]	0.0028	K_{mFOR} [mM]	22
K_{mO2} [mM]	0.0292	K_{mNAD} [mM]	0.316
K_{mNADH} [mM]	0.0268	K_{iNADH} [mM]	0.06
K_{ic} [mM]	0.0016		
Ki_{FOR} [mM]	160		
K_{iNAD} [mM]	0.5	t_i [min]	0.0007
k_La [min^{-1}]	14		
$K_{D\ org/aq\_2OH}$	480		
$K_{D\ org/aq\_prod}$	380		

With an overall volumetric mass transfer coefficient k_La=6.24 min^{-1} estimated in chapter 6 on the base of the product formation rates, the modelled output values were not always fitting the experimental results, therefore the k_La value in the model was increased stepwise and gave a good correlation at 14 min^{-1}. It was expected that the k_La calculated in chapter 6 on the basis of the average product formation rate was underestimated because the product formation rates are not constant. Computed values, fitted to the experimental data show good correlation with the results obtained in the chapter 5 and chapter 6 (all the results from TiTR experiments are summarised in Table A1 in the appendix section). In the experiments, higher flow rates would yield higher mass transfer rates, hence higher k_La values; therefore to exclude the influence of the flow rate as another variable, modelling was done for the highest 0.7-0.8 mL min^{-1} flow rate only. For example, predicted product formation rates and product concentrations at different initial substrate concentrations show the same trend as experimental results, i.e. the optimal substrate concentration, at otherwise identical reaction conditions, is 200 mM and further substrate increase does not affect the rate or final product titre (Figure 7-3A). Further efforts to validate the model showed good fitting, but at lower k_La value of 6.24 min^{-1}. As shown in Figure 7-3B an attempt to fit the model with the k_La of 14 min^{-1} was not successful and the calculated values were overestimated pointing towards a lower mass transfer rate than expected. Also obvious is that the last measurement point does not fit well and gives a lower product titre and product formation rate than the model, indicating oxygen limitation in that particular experiment, resulting from an increasing length of the residence time unit and no active aeration therein (chapter 6).

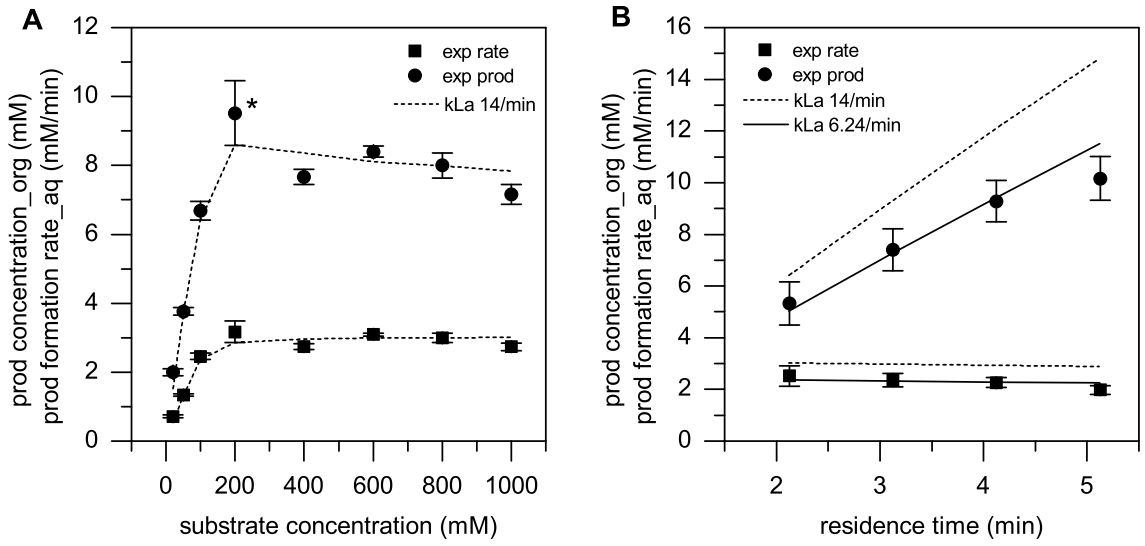

Figure 7-3: Fitting the kinetic model to the experimental data. A – Varying substrate concentration in the decanol phase (20-1000 mM). Reaction conditions: 100 mM KPi pH 7.5, HbpA 9.19 U mL^{-1}, FDH 7.47 U mL^{-1}, sodium formate 100 mM, NAD^+ 1 mM, FAD 20 µM, Tween 20 0.6 mg mL^{-1}. Aeration with air, back pressure not regulated. Total flow rate of 0.8 mL min^{-1} in a 4 m long TiTR with an average residence time of 2.75 min. Point marked with (*) had a residence time of 3 min. Therefore, a higher product concentration and product formation rate were measured - it does not point to substrate inhibition. B – Varying residence time with the residence time unit (PTFE i.d. 1 mm, 1-4 m) attached downstream of the 1 m TiTR, Reaction conditions: 100 mM KPi pH 7.5, HbpA 9.19 U mL^{-1}, FDH 9.19 U mL^{-1}, sodium formate 160 mM, NAD^+ 1 mM, FAD 20 µM, Tween 20 0.6 mg mL^{-1}. Aeration with pure oxygen, back pressure 6.89 bar. Total flow rate 0.8 mL min^{-1} with a respective residence time of 2.12, 3.12, 4.12 and 5.12 min.

Additionally, the validity of the model was confirmed by fitting it to the results of the experiments with variable initial enzyme loading. Again, the model was accurate when the k_La was 6.24 min^{-1} (Figure 7-4) and showed that a maximum is reached at a HbpA loading of ca. 9 U mL^{-1}, and increasing enzyme input further does not lead to higher product formation rates because the reaction is mass transfer limited. All the findings presented above point to the limitations of the model and/or the influence of dynamic changes of the system e.g. the flow stability on the reactor performance. In order to model the reaction with high accuracy it is necessary to obtain the same flow characteristic every time, which proved very difficult in the current setup characterised by low interfacial tension. Only then the model can be optimised and one may conclude whether the discrepancy comes from the inaccurately determined kinetic constants (K_m, K_i, etc.) or changes in the flow itself e.g. segments merging.

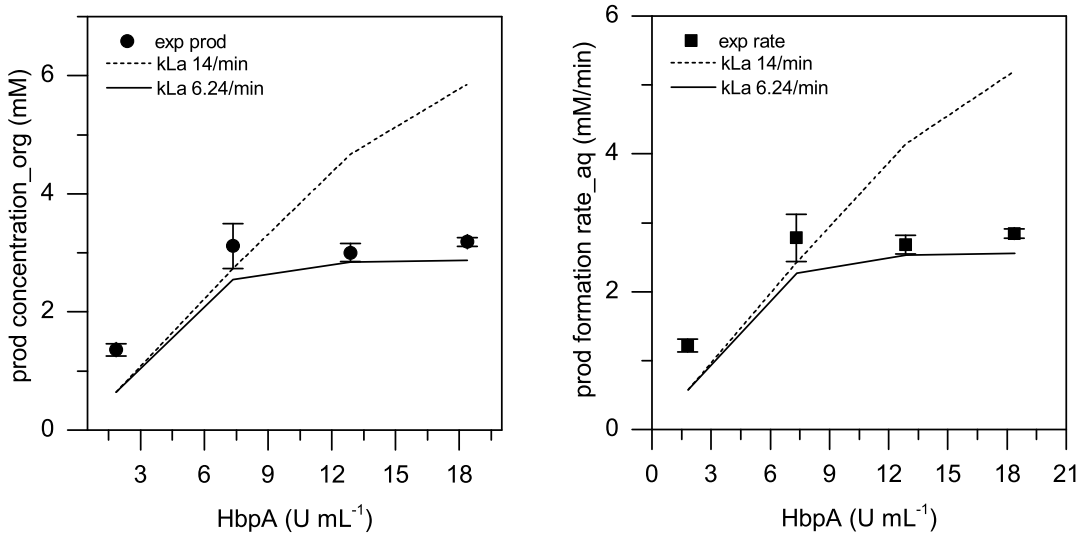

Figure 7-4: Fitting the data from the kinetic model to the experimental data. Varying initial enzyme input (HbpA 1.84–18.38 U mL^{-1}). FDH to HbpA unit ratio was 0.81. Reaction conditions: 100 mM KPi buffer pH 7.5, sodium formate 100 mM, NAD^+ 1 mM, FAD 20 µM, Tween 20 0.6 mg mL^{-1}. Aeration with pure oxygen, back pressure 6.89 bar. Total flow rate 0.8 mL min^{-1} with a residence time of 1.12 min.

Nevertheless, the established model was used further and when the k_La value fitted was 14 min^{-1} it predicted with only minor discrepancy, the final product titre and overall product formation rate of the preparative scale experiment described in chapter 6. The average product concentration measured in the organic phase from 7 consecutive reaction cycles (each 11.5 min residence time) was 32.1±2.2 mM with an overall product formation rate of 2.8±0.2 mM min^{-1} in the aqueous phase. Values given by the model were 30.2 mM and 2.61 mM min^{-1} respectively. More important than predicting the end values was the possibility to dissect the reaction and look separately on a single reaction component in a time resolved manner, e.g. on the profile of the enzyme activity or NADH and NAD^+ concentration changes (Figure 7-5). Such an approach allows identifying the reaction parameter that might be a rate limiting factor. In a given example, where NAD^+ was initially used, in the beginning of the biotransformation (less than 1 minute) the rate of the FDH catalysed reaction drops (cofactor regeneration reaction), whereas the biotransformation reaction catalysed by the HbpA quickly reaches its final velocity. At that point reactions acquire the same velocity, which decreases slowly over time (Figure 7-5B). As the reaction progresses the product concentration in the aqueous phase starts to inhibit the HbpA reaction. The effect of the increasing product concentration can be seen in Figure 7-5E. The lines plotted are unit components of the rate equation given in the parentheses of Equation 7-4. The influence of the product is presented by the red line which shows the relative activity contribution on the

total HbpA rate. The total activity (black line) is considering oxygen, substrate and cofactor concentration, and product inhibition (Equation 7-4).

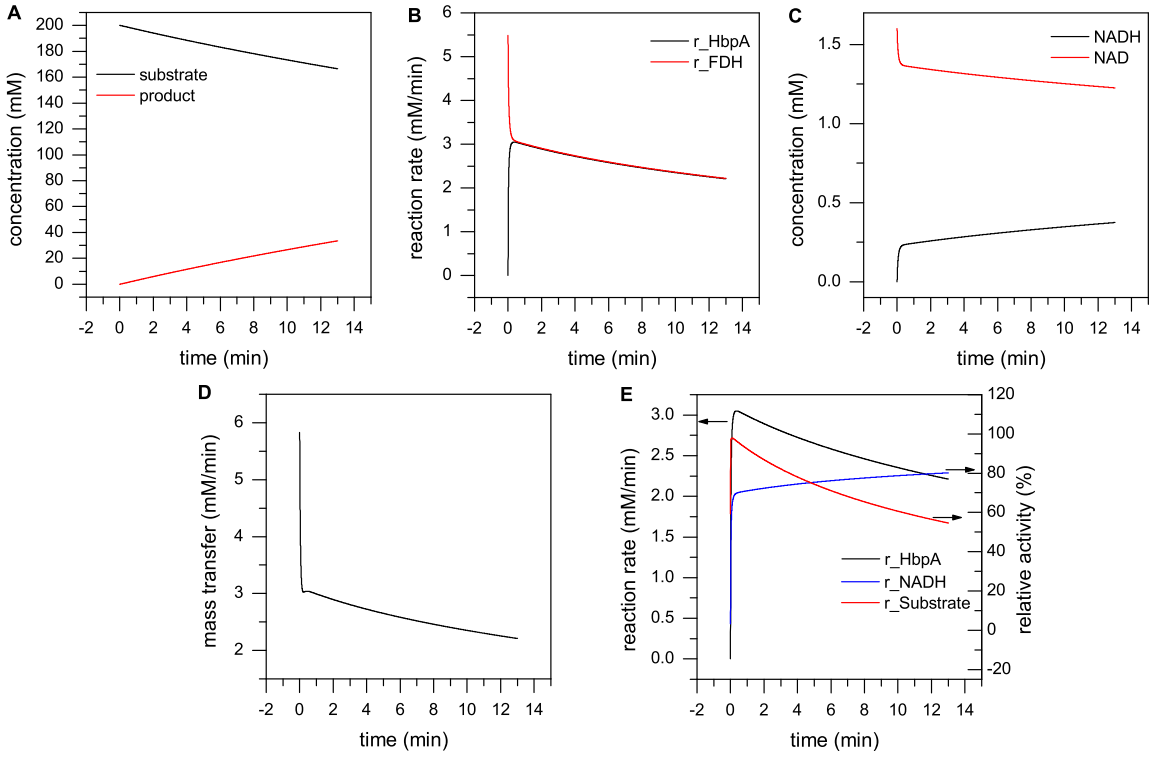

Figure 7-5: Modelled reaction parameters. Reaction conditions: 100 mM KPi buffer pH 7.5, HbpA 9.19 U mL^{-1}, FDH 7.47 U mL^{-1}, sodium formate 160 mM, NAD^+ 1.6 mM, FAD 20 µM, Tween 20 0.6 mg mL^{-1}, 200 mM substrate in the decanol phase. Other parameters were constant as in the Table 7-3.

To better understand the influence of the mass transfer on the reaction rate in a given reactor setup one may use the dimensionless Damköhler number (Da). It describes the ratio of reaction rate and mass transfer rate and indicates that at Da > 1 a given system might be mass transfer limited whereas at Da < 1 it might be reaction limited. Around Da = 1 the system will be in a transition state, meaning that changing the reaction parameters might move the reaction into either of the regimes (Figure 7-6). The Damköhler number is usually plotted against the effectiveness factor that is a ratio of the actual reaction rate to its theoretical maximum.

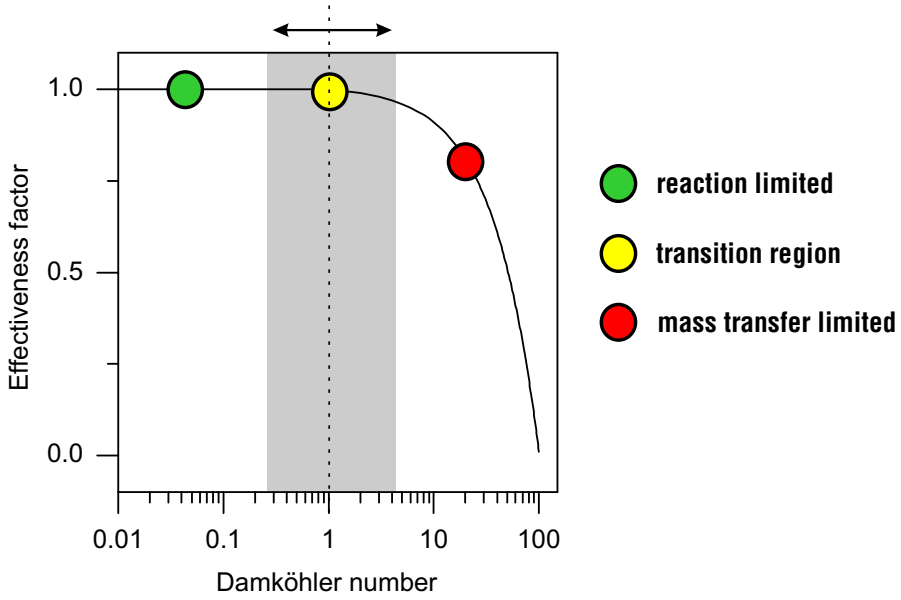

Figure 7-6: Graphical representation of the relation of the Damköhler number and the effectiveness factor.

Calculating the Da number allows estimating the regime, which will be likely for the respective reaction. For the calculation, an assumption has to be made that the reaction rate is at maximum at given reaction conditions, i.e. the substrate concentration taken for the calculations (C_{2OH}) should be the maximum equilibrium substrate concentration in the aqueous phase defined by Equation 7-11. It should however include all possible inhibitions (substrate, product). This way it is presumed that the reaction is not mass transfer dependent because the substrate concentration is optimal. Similarly, mass transfer is calculated by assuming that the driving force is maximal and is equal to the maximum equilibrium substrate concentration in the aqueous phase. Then by combining Equation 7-4 and Equation 7-10 one may calculate the theoretical Da number.

Equation 7-9

$$Da = \frac{reaction\ rate}{mass\ transfer\ rate}$$

Equation 7-10

$$Da = \frac{r_{HbpA}}{k_L a(C_{2OH\_max})}$$

Equation 7-11

$$C_{2OH\_max} = \frac{C_{2OH\_org}}{partition\ ratio\ K_D}$$

The Da can then be calculated for instance when one reaction component is changed, but all the other remain the same. It enables to see what the effect of a single parameter change on the reaction rate is, and if varying it drives reaction into the mass transfer limited regime, reaction limited regime or whether the regime stays unchanged. With this approach the effect

e.g. concentration of the hydrophobic substrate, overall volumetric mass transfer coefficient k_La, HbpA input, and FDH input, was evaluated (Figure 7-7).

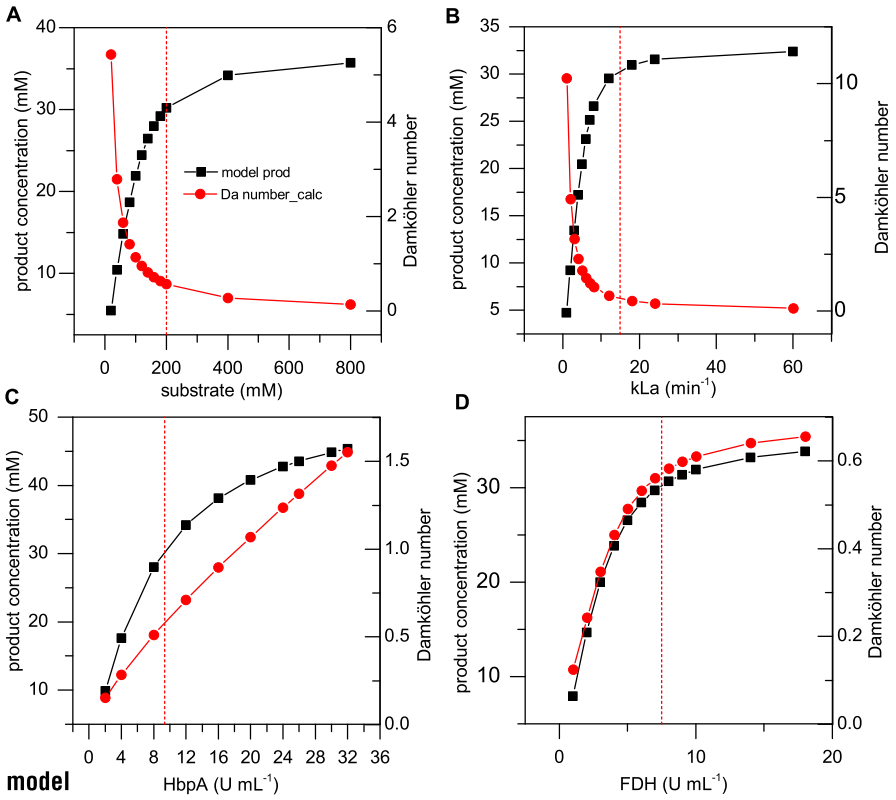

Figure 7-7: Influence of a change of a single reaction parameter on the Damköhler number in the preparative scale reaction at the residence time of 11.5 min. Original reaction conditions: 100 mM KPi pH 7.5, HbpA 9.19 U mL^{-1}, FDH 7.47 U mL^{-1}, sodium formate 160 mM, NAD^+ 1.6 mM, FAD 20 µM, Tween 20 0.6 mg mL^{-1}, 200 mM substrate in the decanol phase. The red dashed vertical line indicates the reaction conditions in the real experiment.

This simple evaluation shows if the reaction is mass transfer limited, or kinetically limited at the predetermined residence time (here 11.5 min), and indicates how the system will react to the parameter change. For instance, in Figure 7-7A the effect of the initial substrate concentration on the Damköhler number is shown. Already at 200 mM hydrophobic substrate in the organic phase the Da is lower than 1, indicating that the reaction kinetics is determining the reaction rate and increasing the substrate concentration above 200 mM would not affect the reaction rate, as could also be seen in Figure 7-3A. Similar investigation was done for k_La, HbpA, and FDH and their influence on the Damköhler number is shown in Figure 7-7B-D. This approach is useful for deciding which reaction components need to be varied in order to transit from mass transfer limited regime to the highly required reaction controlled regime, where enzymes would work at full efficiency because of not being limited by transfer of the hydrophobic substrate from organic to aqueous phase. With a similar

approach one may determine how to increase the productivity e.g. two-fold. The preparative scale experiment may be used here as a starting point. Then by doubling the enzyme input only 50% increase in the productivity is recorded. The reason for this can be explained by looking closer at the Da number. Initially, the reaction is in the kinetically limited regime, therefore the enzyme increase is possible. However, when a certain enzyme input is reached, at which the theoretical Da number is equal to one, increasing enzyme input further does not affect the reaction rate as the reaction enters the mass transfer limited regime, which is indicated by the Da = 1.22 (Table 7-4). An integrated approach is therefore needed and two independent parameters need to be raised simultaneously. In a given example it was enzyme input and substrate concentration which doubled. After such a change Da remained below 1 and productivity, at least theoretically, increased two-fold.

Table 7-4: Model based determination of the reaction parameters which need to be optimised to double the reaction's productivity.

	Initial		Optimised		Initial		Optimised
HbpA	9.19 U mL^{-1}	→	18.39 U mL^{-1}	→→	Prod. 30.19 mM	→	44.6 mM
FDH	7.47 U mL^{-1}		14.97 U mL^{-1}		Rate. 2.61 mM min^{-1}		3.86 mM min^{-1}
	50% increase				**Da = 0.57**	→	**Da = 1.22**
HbpA	9.19 U mL^{-1}	→	18.39 U mL^{-1}	→→	Prod. 30.19 mM	→	62.18 mM
FDH	7.47 U mL^{-1}		14.97 U mL^{-1}		Rate. 2.61 mM min^{-1}		5.39 mM min^{-1}
Subs	200 mM		400 mM				
	100% increase				**Da = 0.57**	→	**Da = 0.62**

Unfortunately, when respective experiments were conducted to confirm above theoretical findings the productivity did not increase (Table A1). A possible explanation might be the inaccuracy of the model and/or the discrepancy between the overall volumetric mass transfer coefficients, k_La used in the model and the k_La reached in the current experiment. Worth mentioning is the fact that with higher enzyme loadings the concentration of glycerol in the aqueous phase also increased, because the enzymes were added as 50% glycerol stocks. It resulted in the increase of viscosity of the aqueous phase, and could influence the internal circulations within the aqueous segments which are responsible for high mass transfer rates. Therefore, the mass transfer could be lower and increasing the enzyme input would not affect the reaction rate because the reaction would enter the mass transfer limited regime earlier. The model would unfortunately not predict it. Additionally, as pointed out earlier, a selection of the aqueous/organic phase liquid pair with high interfacial surface tension and similar viscosities is recommended for application in microreactors in order to obtain the same flow characteristics in every consecutive experiment, and for preventing segment coalescence.

Nevertheless, a combined approach of modelling and experimental investigation is needed for optimising complicated reactions as the one presented here, because predicting the reaction's outcome is not straightforward when so many variables are involved. Modelling the reaction before conducting experiments might however give an early idea if the reaction is likely to be mass transfer limited or will the kinetics determine the reaction rate; or what is the maximum reaction rate which can be reached. In the current setup there are two enzymes: HbpA and FDH; 5 substrates: 2-hydroxybiphenyl, oxygen, NADH, NAD^+, and sodium formate; 5 main products: 2,3-dihydroxybiphenyl, CO_2, NAD^+, NADH, and H_2O. Moreover, HbpA is inhibited by 2,3-dihydroxybiphenyl, NAD^+, and formate; whereas FDH is inhibited by NADH. Because both reactions have common substrate i.e. NAD^+/NADH their kinetics are bound and depend on the effectiveness of the cofactor regeneration and transport of NAD^+/NADH between the enzymes. One may therefore take the model and assume that reaction takes place in a single phase with ideal mixing, so that the mass transfer can be removed as a variable from the equation. Even at this particular (ideal) case the overall reaction rate will not exceed 35-40% rate expected from the HbpA unit input. Such an approach would be highly recommended when comparing various cofactor regeneration methods and deciding which one would be more suited.

BEFORE SCALE-UP - UNIT OPERATIONS AND PROCESS INTENSIFICATION

According to Stankiewicz and Moulijn (2000)[192] process intensification "*consists of the development of novel apparatuses and techniques that, compared to those commonly used today, are expected to bring dramatic improvements in manufacturing and processing, substantially decreasing equipment-size/production-capacity ratio, energy consumption, or waste production, and ultimately resulting in cheaper, sustainable technologies*", meaning that any change, which leads to an improvement within any of these areas may be considered as intensification. Such a vague definition, despite giving necessary objectives, does not provide necessary tools or tells where to start. Therefore for an ease of intensification, a process can be divided into three distinct levels depicted in Figure 7-8. A *phase level* is the population of the molecules containing the educts, products and catalysts; a *process unit level* is the space in which a single unit operation e.g. mixing, heating, or separation is performed; and a final *plant level* is the relationship between single process unit levels, which defines how the molecules flow within the whole process between single unit operation compartments.[193] One may then focus on a single level and e.g. use new reaction media, shorten reaction time by using different apparatuses for better mass transfer, or even combine different levels for better downstream processing or reducing the equipment size. Below

discussion focuses therefore on different areas of process intensification which have or could be undertaken for intensifying the HbpA-catalysed hydroxylation of 2-hydroxybiphenyl.

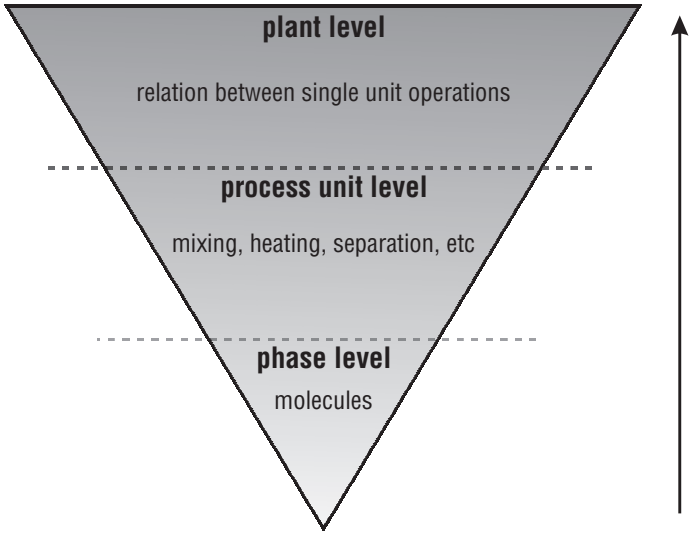

Figure 7-8: Graphical representation of a process.

Enzyme consumption and strategies for maximising the TTN

In order to intensify the process on the *phase level* and e.g. reduce the amount of generated wastes with respect to applied (bio)catalyst or product titre one should for example minimise (bio)catalyst consumption and use it more efficiently. The major disadvantages of the current process were relatively short residence time, moderate conversions, and most importantly, low TTN of the enzymes. Those resulted from limited volume of the reactor, and prerequisite of operating the system at high flow rates for enhanced mass transfer rates. Experimental data for the preparative scale experiment is summarised in the Table 7-5.

Table 7-5: Data for the enzymatic conversion of the 2-hydroxybiphenyl to 2,3-dihydroxybiphenyl by HbpA in a sequential TiTR in a preparative scale experiment (chapter 6).

	total enzyme [U]	residual activity [%][a]	enzyme used [U]	enzyme used [mg]	enzyme used [mmol][b]	product [mmol]	enzyme consumption [U $g_{product}^{-1}$][c]	TTN [mol_{prod} mol_{enz}^{-1}]
HbpA	3591	89	384	110	0.0017	10.5	197	6217

[a] enzyme was recovered after 11.5 min total residence time

[b] based on the activity of 3.5 U mg^{-1} of pure HbpA and molecular mass o 65000 g mol^{-1}

[c] actual consumption of the enzyme based on the amount of the catalyst that lost activity

The system for preparative scale experiment operated in the semi-continuous mode, and after supplying 50 mL of each organic and aqueous phase to the reactor system the syringes had to be charged again and flow restarted (chapter 6, Figure 6-9). During the first 7 cycles fresh enzyme solutions were used. Without considering the 8th cycle, which utilised recycled aqueous phase, 3590 U HbpA enzyme solution (= 350 mL) were used in total. Then, the total

amount of the enzyme used in the reaction is defined as the amount of the biocatalyst which lost activity and cannot be recycled.

The TTN of the here utilised HbpA was fairly low and was calculated to be approximately $6 \cdot 10^3$ for the preparative scale experiment, resulting in a rather huge amount of enzyme which needed to be applied. Although enzyme to product ratio was two orders of magnitude higher than e.g. for the industrial process of *tert*-leucine production (0.9 $U_{enzyme}\ g_{product}^{-1}$),[9b] it compares well with the epoxidation of styrene derivatives in a STR. In that example enzyme consumption was between 200-300 $U_{enzyme}\ g_{product}^{-1}$ with a TTN in the range of 2000-3000 $mol_{product}\ mol_{enzyme,}$[63] however, herein the product was synthesised faster due to a higher STY.

In order to reduce enzyme consumption and maximise the TTN (*phase level*) one may integrate two unit operations i.e. reaction and phase separation into one multifunctional process unit, which would simultaneously affect the process at the *plant level* too by simplifying the downstream processing and reducing the number of process unit steps. A higher level of integration and a promising alternative to the proposed sequential TiTR was an ePTFE membrane TiTR that allowed for complete separation of aqueous and organic phase and enabled conducting the reaction in the same apparatus. Thereby, aqueous/organic interfacial deactivation was prevented, and thus enzyme consumption should be minimised. However, the product formation rates were lower than with the direct phases contact pointing towards a possible membrane transport limitation or diffusion limitation. Since the phases were separated, and thus the system was operated in single phase flow mode inside and outside the ePTFE membrane, no convective mixing resulting from internal circulations within segments occurred and mass transfer was therefore almost entirely dependent on diffusion. Previous studies have found that the membrane itself can indeed be a serious mass transfer resistance and pointed out that careful investigation of the membrane transport properties is critical.[194] By applying different membrane material and/or introducing static mixers, such a setup would significantly improve the TTNs because flow of the aqueous phase would be uncoupled from the organic phase. Circulating the aqueous phase in a small loop would minimise enzyme consumption and formation of a two liquid phase emulsion would be prevented. This new reactor concept is similar to a microfluidic reactor containing immobilised enzyme,[195] as it gives the ability to stop the reaction at any time by removing the organic or aqueous phase independently from the system, and appears an attractive alternative to segmented flow microreactors. Moreover the ePTFE tube-in-tube membrane reactor opens a possibility to a truly continuous process with an integrated downstream processing (*vide infra*) and offers a potential for a scale-up.

It is possible, that by using an immobilisation technique, as opposed to the soluble biocatalysts, performance of the reaction presented in chapter 6 with respect to the TTN and biocatalyst cost could be improved. Various methods of enzyme immobilisation in microreactors exist and have been reviewed previously[151a] and could be checked in the future for validating this hypothesis.

Solvent selection and downstream processing

A solvent used as an aqueous immiscible phase has two tasks: the first is to control substrate/product concentration and thereby toxicity, and the second is to facilitate downstream processing (DSP) by extracting the product of choice in situ from the aqueous phase. The choice of the organic solvent is important, as it needs to be tolerated by the biocatalyst, and also influences the process itself when such physical features like viscosity, solubility in water ($logP_{o/w}$) or partition ratio of substrate/product between aqueous and organic phase are considered. Throughout this thesis 4 organic solvents were tested. Those were decanol, octanol, toluene and MTBE and their physical properties are summarised in Table 7-6.

Table 7-6: Physical properties of organic solvents used in this study.

Solvent	Boiling point [°C]	Partition ratio K_D(org/aq) for substrate	Partition ratio K_D (org/aq) for product
Decanol	233	480	380
Octanol	195	1200	470
Toluene	111	600	30
MTBE	55	2200	1425

For instance, in decanol concentration of 2-hydroxybiphenyl in organic phase will be 480 times higher than in the respective aqueous phase. MTBE used in the aqueous/organic segmented flow TiTR resulted in the lowest hydroxylation rate and subsequently lower product titre at the same residence time, which was associated with its negative impact on enzyme stability due to the low $logP_{o/w}$ value (0.94) and highest partition ratio for the substrate resulting in the lowest substrate concentration in the aqueous phase. Nevertheless, when MTBE was used in the ePTFE membrane TiTR enzyme inactivation could be prevented. As shown in chapter 6 the collected MTBE phase was free from any aqueous phase therefore no phase separation was required for further downstream processing. It should be possible to place a distillation unit downstream of the reactor and recover MTBE leaving a solid substrate/product mixture. This mixture could be further separated by washing with organic solvent which exclusively dissolve the substrate e.g. n-heptan, methylcyclohexane, limonene, octane, xylene, hexadecane or perfluorodecalin (chapter 2). Thereby, substrate and

product can be separated, without time-consuming chromatography and solvents and unreacted substrate would be recycled. (Figure 7-9)

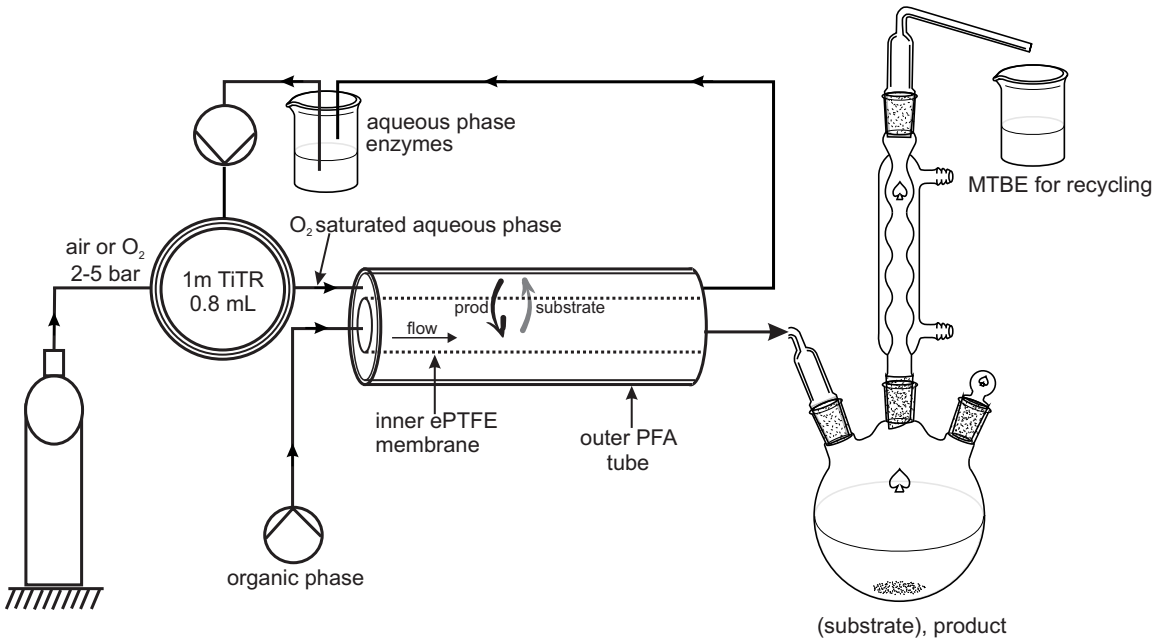

Figure 7-9: A schematic representation of an exemplary setup of a continuous enzymatic process in the ePTFE membrane TiTR.

Taking the process to a bigger scale

Probably the greatest disadvantage of the current aqueous/organic two liquid phase segmented flow microreactor was precipitation of enzymes on the aqueous/organic interface, and although significantly minimised by applying surfactants, it could not be completely prevented. Moreover viscosity difference imposed flow instabilities. Flow was also affected by particulate fouling, i.e. particles or droplets adhering to the microchannel wall.[196] Before bringing the process to a bigger scale in a segmented-flow based set-up every one of the above mentioned issues would need to be addressed properly. Even then it is not guaranteed that an 'optimised' laboratory-scale process could by scaled-up by a numbering-up technique i.e. multiplying the number of the reactors and running them in a sequential (not parallel) fashion like e.g. in Lonza's Universal Reaction Technology.[197] Applying reactors one after another (increasing the number of microreactor plates), which would undoubtedly increase reactor volume and therefore also the residence time, would pose a huge problem related to maintaining a stable segmented flow over the entire reactor length. Additionally, a mass transfer characteristic would vary in an unpredicted fashion in different sections if segments would coalesce. Precipitated enzymes would inflict a risk of reactor clogging, or drastic pressure drop if they would deposit on the reactor walls.[197] The same problems would exist in a scale-up by parallelisation, where feeding the reactants to multiple parallel reaction units would pose a risk that when one reaction unit is clogged the residence time would alter in the

remaining ones.[176] Despite obvious advantages e.g. high mass-transfer and ease of manufacturing, scaling-up the microreactor used in this thesis is difficult. For a better control of the process in terms of flow stability, residence time distribution, and uniform mass transfer rates in a whole reactor, the application of a membrane reactor, which would separate aqueous and organic phase, seems a viable option. The application of membrane reactors for homogenous single phase reactions is well established on the industrial scale in (C)STR.[198] Ultrafiltration membranes are often applied to keep enzymes in a confined reaction compartment allowing for continuous substrate addition and product removal in a stationary (batch mode) or mobile (continuous) aqueous phase. After reaction, respective enzymes are retained and can be reused in another batch. Similarly, membranes are applied for biphasic aqueous/organic reaction systems where they prevent emulsion formation (by separating the phases), simplify product recovery and allow for repeated use of the biocatalysts.[199] Enzymes may be enclosed/immobilised within a membrane that remains in a direct contact with an aqueous phase on the once side and an organic phase on the other side of the membrane.[200] Such a setup allows for obtaining aqueous/organic interface at the catalyst level allowing for enzymatic reaction and continuous product removal, which is of great importance for reactions inhibited by high substrate concentrations. A reactor of this type has been successfully applied on industrial scale with a productivity of 145 g m^{-2}_{membr} day^{-1} of a chiral intermediate used for synthesis of hypertension and angina drug,[200] as comparison to productivity of 70 g m^{-2}_{membr} day^{-1} in the preparative experiment (14.5 g L^{-1} h^{-1}, where 1 L equals 5 m^2_{membr}).

The ePTFE membrane TiTR combines all the merits of the membrane separation and microreactor technology, which after necessary optimisation e.g. membrane engineering and introducing static mixers inside the inner membrane – which are suggested technology for the reaction scale-up[197] – and baffles on the outside (as in oscillatory baffled reactor[201]), should result in higher mass transfer rate, or at least in higher membrane surface area to the volume of the reactants as compared to traditional membrane reactors (Figure 7-10). The advantage of the here proposed ePTFE membrane TiTR over the above mentioned immobilised enzyme membrane reactor is the fact that enzymes are applied in a soluble form. Using soluble enzymes simplifies reactor operation as no extra immobilisation step is required prior to starting the reaction. Soluble enzymes are usually more active than their immobilised counterparts since immobilisation procedures often lead to unfavourable structural changes and activity losses. Higher enzyme loadings can be reached with soluble catalyst than with their immobilised counterparts because of lower carrier specific activity and maximum carrier

loading capacity in the reactor.[198] The proposed ePTFE membrane TiTR has a potential for future optimisation and scale-up.

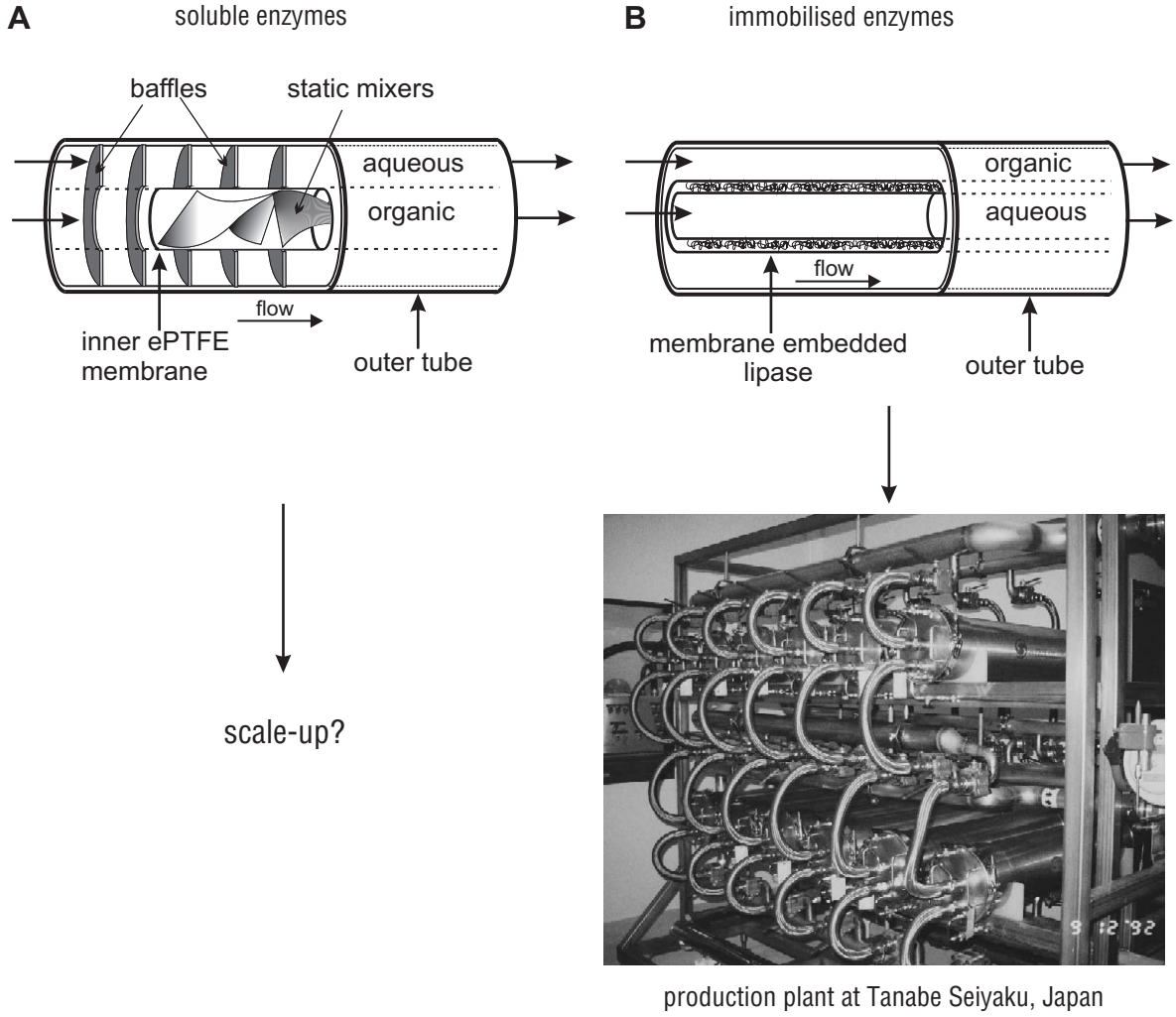

Figure 7-10: A – Schematic representation of an ePTFE tube-in-tube membrane reactor with static mixers inside Teflon AF-2400 membrane, and baffles on the outside. B – Membrane immobilised lipase reactor.[200] Reactors are not drawn in scale. Image of the production plant reprinted from Journal of Membrane Science, 125: 189-211, Copyright © 1997 with permission from Elsevier.

CONCLUDING REMARKS – SYSTEM BOUNDARIES DEFINED

This thesis aimed at applying 2-hydroxybiphenyl 3-monooxygenase (HbpA) and formate dehydrogenase (FDH) in a two-liquid phase system in a segmented flow microstructured reactor for continuous production of 2,3-dihydroxybiphenyl (3-phenylcatechol). That aim has been successfully realised as shown in chapter 6. 3-phenylcatechol has been produced on a gram scale in a continuous tube-in-tube aqueous/organic segmented flow microreactor. 740 mg of pure product has been isolated from the substrate/product mixture in decanol.

Reaching the thesis objectives was possible by following an engineering approach i.e. taking a given reaction, applying it in a certain reactor setup, obtaining product, and defining the process constraints. In the next step the process parameters were rationally redefined. Product titres and/or product formation rates were parameters defining whether the parameter change

was favourable or not. This approach was the only one applicable for a complicated system like the one here, where reaction modelling could serve only as an aid for process optimisation, due to too many reaction variables. We have followed that path and started in a batch reaction with soluble and immobilised enzymes. Soluble enzymes were superior to Combi-CLEAs in terms of reaction rates and specific activity, thus after necessary stabilisation with Tween 20 surfactant they were taken further. Batch reactions however, seemed mass transfer limited thus, in the next step reactions were conducted in a microreactor setup. As expected, microreactors offered superior mass transfer rates compared to a batch setup (e.g. STRs). Oxygen availability became our next concern, thus the reaction was conducted in an aqueous/organic/air three-phase microreactor, which due to limited oxygen concentrations and technical issues affecting stable operation was substituted by the aqueous/organic segmented flow TiTR. Oxygen delivery was improved, also handling was significantly easier. Additionally, product formation rates measured in the TiTR were over 3 times higher than in a batch and three-phase segmented flow microreactor. The TiTR was not a typical plug flow reactor because the concentration of oxygen was constant over the reactor length thanks to the radial delivery mode through the gas permeable Teflon AF-2400 membrane. However, the safety issues related to the use of pure oxygen and organic solvent should be considered before further scale-up. Although it was shown that the oxygen transfer rate can be higher for pure oxygen instead of air (chapter 6), it might be more feasible from economic and safety point of view to use the latter. Using pure oxygen at elevated pressures poses a risk of explosion, and although the here used tube-in-tube reactor is designed in a way to minimise the volume of oxygen in the system, the safety aspect should not be ignored. Reaction parameters in TiTR (concentration of 2-hydroxybiphenyl, NAD^+ and FAD, as well as the flow rate) have been optimised, which enabled us to synthesise gram amounts of product in a continuous fashion with productivities that have never been reported before. Additionally, an ePTFE tube-in-tube membrane reactor was shown as a proof of concept, which presented potential for reaction scale-up. Therefore, future investigation should focus on evaluating different membrane materials regarding mass transfer rates and solvent stability. Static mixers need to be implemented in the system. Mixing times need to be investigated with respect to different static mixers and increasing diameter of tubes. Ideally the mass transfer rates in the liquid in the biggest tube should match that of the segmented flow microreactor without static mixers and should be higher or at least equal to the transmembrane organic to aqueous phase mass transfer rate in order not to be the rate limiting factor. Other parameters, which need optimisation before scale-up are the initial substrate concentration and residence time that drives the reaction to completion. As shown previously

for the same reaction, its rate in liquid/liquid segmented flow microreactor decreases with decreasing initial substrate concentration. (chapter 5) Therefore, lowering the initial substrate concentration from 200 mM to 100 mM, while keeping the residence time, would only increase the conversion by around 40 %. (chapter 5) In order to drive the reaction to completion, at compromised reaction rate, very low substrate concentration would need to be chosen and the residence time adjusted accordingly.

CHAPTER 8

SUMMARY AND OUTLOOK - KEEP THE BIG PICTURE IN MIND

Application of soluble oxidoreductases in two-liquid phase biotransformation reaction is a complex task. For the first time, after defining the process challenges, with an integrated engineering approach, application of a soluble 2-hydroxybiphenyl 3-monooxygenase (HbpA) and formate dehydrogenase (FDH) in a two-liquid phase segmented flow microreactor for continuous production of 2,3-dihydroxybiphenyl (3-phenylcatechol) was shown. It covered upstream and downstream processing from the gene cloning and expression, bacterial fermentation, protein purification and characterisation, continuous microreactor design, reaction optimisation, to product analysis and purification. In the end, the emphasis was put on verifying the scale-up scenarios for the reaction. Detailed analysis of the challenges related to the application of soluble enzymes in a two-liquid phase system, like cofactor regeneration, biocatalyst stability, oxygen supply, and mass transfer were first defined **(chapter 1)**.

For the production of both biocatalysts *Escherichia coli* strain JM101 was chosen. HbpA and FDH were purified to technical purity by means of expanded bed adsorption chromatography. Keeping enzymes at -20ºC in 50% glycerol solution was an effective technique of preserving their activities for long-time storage, and a convenient way of enzymes' handling. HbpA and FDH showed excellent stability in single phase experiments. The continual activity loss when an organic solvent was used in a two-liquid phase reaction was effectively surpassed by using a Tween 20 surfactant, which covered the interface between organic and aqueous phase, and almost completely prevented enzyme denaturation **(chapter 2)**.

Cross-linking of the HbpA and the FDH (CLEAs) was proposed as an alternative stabilisation method for their application in a two-liquid phase system. Preparation of CLEAs was also expected to simplify biocatalysts handling and their recovery after multiphasic reaction. Although CLEA methodology was reported earlier as easy to prepare and broadly applicable,[118] the technique is not to suitable for all the enzymes.[141] Without additional modification e.g. gel entrapment or encapsulation, the mechanical properties of HbpA and FDH CLEAs were not good enough for efficient application in STR. It was however shown, that CLEA methodology is not restricted to intrinsically stable enzymes like lipases but may also be applied to much more complex oxidoreductases. This work is a promising basis for a further development of this cheap and simple immobilisation method, especially when a site specific cross-linker could be used, with the help of now available three dimensional structure of the HbpA **(chapter 3)**.

A precondition for the application of soluble oxidoreductase is a constant supply of the reducing equivalents. An elegant method based on the principle of applying 'hydrophobised formates' to promote NADH-dependent redox reactions was demonstrated. Hydrophobic esters of formic acid served as an organic phase in biocatalytic two-liquid phase reactions,

and as source of reducing equivalents at the same time; thereby paving the way towards low-water content redox biotransformations. Despite its elegance, the setup suffered from the poor mass transfer rates over the interface. This issue should be addressed in the future e.g. by conducting the reaction in microreactors to reduce phase transfer limitation, which opens the door for future studies **(chapter 4)**.

By combining the enzyme stability study (chapter 2) and the cofactor regeneration methods (chapter 1) with a careful characterisation of the reaction kinetics, optimisation of the reaction parameters was possible. To alleviate interfacial mass transfer limitations found in the batch setup, a soluble HbpA was applied in a segmented flow tube-in-tube microreactor (TiTR) along with NADH-regenerating FDH. An example, which shows the feasibility of applying TiTR concept for performing gas, (particularly oxygen)-dependent reactions in microreactor, was presented. The achieved productivity of 17.7±1.8 $g\,L^{-1}\,h^{-1}$ was significant for a monooxygenase-catalysed reaction with concomitant cofactor regeneration, which emphasised the potential of this technology for biocatalytic reactions **(chapter 5)**.

To address the oxygen limitation suspected to be a reaction rate limiting factor in chapter 5, the effect of higher oxygen partial pressure was evaluated by gradually increasing reactor back pressure, and eventually substituting air with pure oxygen. Determination of the oxygen transfer rates proved that the reaction was not limited by the oxygen transfer over the membrane or its availability in the aqueous phase hence, the product formation rate did not increase. However, the available oxygen excess was used in a sequential TiTR, (which offered extended residence times as compared to original TiTR from the chapter 5) for the preparative scale hydroxylation of 2-hydroxybiphenyl to 2,3-dihydroxybiphenyl by means of a soluble HbpA. As it was done before, this reaction was coupled to FDH-catalysed NADH regeneration. The space time yield of 14.5 $g\,L_{total}^{-1}\,h^{-1}$ was again significant. Product separation was successful and nearly one gram of a product with an excellent purity was isolated **(chapter 6)**.

To verify feasibility of the proposed process, a step-by-step evaluation accompanied by reaction modelling was conducted. Different aspects of the process e.g. substrate and biocatalyst concentration, solvent selection, or application of an ePTFE membrane to separate aqueous and organic phase were discussed. It was followed by examination of strategies for process intensification and scale-up **(chapter 7)**.

AIM HIGHER, DIG DEEPER, BUT LOOK WIDER – AN OUTLOOK

Inspired by the work of Matt Might, the professor of computer science at the University of Utah, who created “The Illustrated Guide to a PhD” to help new students understand what the

PhD really is I decided to use the part of his work as an example (Figure 8-1). It struck me when I saw those illustrations that 'the bigger picture' is different from what it appeared to me. Being a scientist is not an easy task, however it is easy to forget, especially when one's focus is only on her/his area of research, that there are scholars and non-scholars that see my work only as a small bulge in the circle of knowledge, whereas to me it appeared as something next to remarkable.

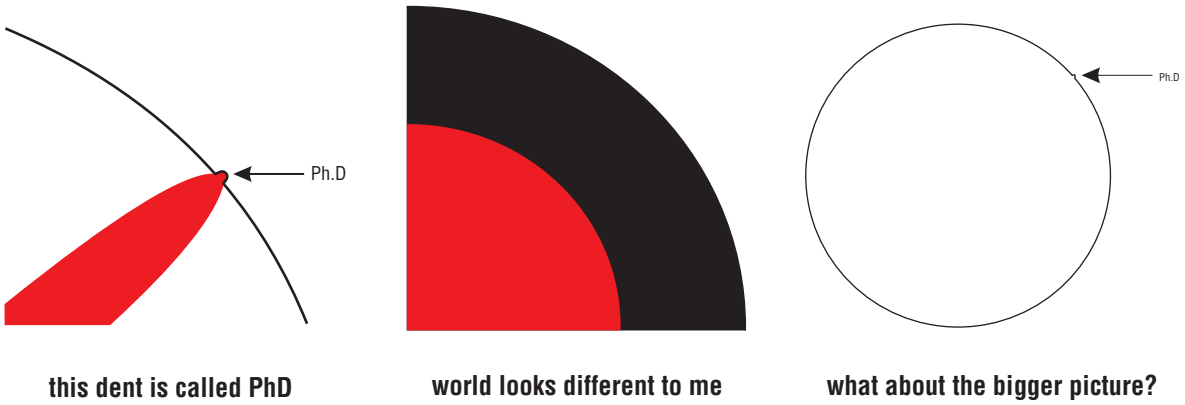

Figure 8-1: What is a PhD. Images were reprinted from "The Illustrated Guide to a PhD" created by Matt Might under the Creative Common License (http://matt.might.net/articles/phd-school-in-pictures/)

What else I did learn is the fact that since the work I have completed is again just a small bulge/dent, others might easily overlook its 'importance'. It would not appear to them how my findings and methods, which I have established, might contribute to their own research and how they can profit from them. I would like to take this chance and focus now on how this thesis findings can be used for further improvements in the field of biocatalysis.

Below is a selection of enzymatic reactions, which utilize gas (H_2, O_2, CO, NO) as a substrate or yield gas (O_2, CO_2) in a course of a reaction (Table 8-1). Additionally, few of them have hydrophobic substrates or products showing low solubility in water e.g. catechol or trans-cinnamate. It is therefore envisioned that at certain catalyst loading insufficient substrate supply or product removal will become a rate limiting step therefore, optimisation might aim at seeking more efficient gas supply and/or aqueous organic two liquid phase system reaction formats sustaining high mass transfer rates.

Table 8-1: Selection of catalytically fast enzymes that might profit from enhanced mass transfer and high oxygen (or another gas) transfer rates in tube-in-tube reactor. Data selection from www.brenda-enzymes.info

Enzyme	**Organism**	**Substrate(s)**	**Product(s)**	**k_{cat} [1/s]**	**K_m [mM]**	**Activity[a] [U mL^{-1}]**	**Comment**	**Ref**
carbon-monoxide dehydrogenase (acceptor) EC 1.2.99.2	*Carboxydothermus hydrogenoformans*	CO + H_2O + methyl viologen	CO_2 + reduced methyl viologen	1500	0.018	945	k_{cat} at 20°C, K_m at 70°C	[202]
catechol oxidase EC 1.10.3.1	*Agaricus bisporus*	catechol + ½ O_2	1,2-benzoquinone + H_2O	878	0.3	553	-	[203]
catalase EC 1.11.1.6	*Bacillus sp.*	H_2O_2	O_2 + H_2O	175000	43	110250	50 mM KPi buffer (pH 7.0), 25°C	[204]
hydrogen dehydrogenase EC 1.12.1.2	*Cupriavidus necator*	H_2 + ferricyanide	H^+ + ferrocyanide	222	0.054	140	pH 8.0, wild-type enzyme complex including HoxI, with NADH	[205]
hydrogenase (acceptor) EC 1.12.99.6	*Hydrogenophaga sp.*	H_2 + oxidised methylene blue	H^+ + reduced methylene blue	560	0.025	353	pH 7.0, 30°C	[206]
Benzoate-1,2-dioxygenase EC 1.14.12.10	*Pseudomonas putida*	benzoate + O_2 + NADH	2-hydro-1,2-dihydroxybenzoate	367	0.0039	231	with saturating amounts of NADH-cytochrome c reductase	[207]
nitric-oxide dioxygenase EC 1.14.12.17	*Escherichia coli*	NO + O_2 + NADH	NO_3^- + NAD^+	670	0.1	422	k_{cat} per heme at 37°C	[208]
trans-cinnamate-4-monooxygenase EC 1.14.13.11	*Ruta graveolens*	trans-cinnamate + NADPH + O_2	4-hydroxycinnamate + $NADP^+$ + H_2O	162	1	102	pH 7.4	[209]
psoralen synthase EC 1.14.13.102	*Ammi majus*	(+)-marmesin + NADPH + H^+ + O_2	psoralen + $NADP^+$ + acetone + H_2O	340	0.0015	214	pH 7.0, 27°C, recombinant enzyme in microsomes	[210]
2-hydroxybiphenyl 3-monooxygenase EC 1.14.13.44	*Pseudomonas nitroreducens*	2-hydroxybiphenyl+ O_2 + NADH	3-phenylcatechol + NAD^+ + H_2O	15	0.0028	9	reaction with NADH and O_2 ***(this study)***	[42]

[a] theoretical maximum velocity in aqueous phase with aqueous enzyme concentration of 0.015 mM.

An example will help us understand the potential of the tube-in-tube reactor (TiTR) discussed in the previous chapters. For instance, with the water/decanol system and a hypothetical concentration of 800 mM of 2-hydroxybiphenyl, which is a hydrophobic substrate and shows a partition ratio K_D=480 between organic and aqueous phase, a maximum mass transfer rate with an overall volumetric mass transfer coefficient **k_La=14 min^{-1}** is equal to 23.3 mM min^{-1}. It means that if the reaction is limited by mass transfer only, and not by reaction kinetics, its rate can be as high as **23.3 mM min^{-1}**. On the other hand, if supply of oxygen is a rate limiting step, then at a back pressure of water/decanol phase of 6.9 bar, when air is used for aeration, oxygen transfer rate through the Teflon AF-2400 membrane can be as high as **8.24 mM min^{-1}**, and **24 mM min^{-1}** when pure oxygen is used. Calculated values are specific for the water/decanol solvent system thus, the 2-hydroxybiphenyl mass transfer rate can be further increased when a biphasic system displaying lower partition ratios (promoting higher aqueous concentrations of the hydrophobic substrate) is used. The same statement is true for oxygen transfer rates, therefore different solvents, like perfluorodecalin, might promote higher oxygen solubility and improve oxygen transfer even further, until the permeability limit i.e. **629 mM min^{-1}** (990 Barrer,[168] 6.89 bar, membrane thickness 0.01 cm, 30°C, pure oxygen) of Teflon AF-2400 is reached. Teflon AF-2400 is also suitable for transporting other gases like H_2,[153b] NH_3,[153d] CO,[153a] and CO_2[153c] therefore, the tube-in-tube setup with this material has already been used for various synthetic applications in chemical laboratories, and its broad applicability for biotechnology must be validated. For a comparison, and to highlight limitations that traditional biotechnological processes are facing, it is necessary to point out that industrial scale bioreactors (> 10 m^3) operated at 2.5 bar air pressure sustain oxygen transfer rates of **1.5 mM min^{-1}**,[17] therefore, maximum space time yields with oxygen dependent oxidoreductases are limited to approx. 10 g L^{-1} h^{-1} for non-growing cells used in a biphasic system,[211] based on the product molecular mass of 100 g mol^{-1}. Selection of the biocatalytic reactions shown in Table 8-1 could serve as a benchmark for a TiTR application for biotechnology. The reactions display high k_{cat} values, higher than the k_{cat} of 2-hydroxybiphenyl 3-monooxygenase (HbpA). The maximum theoretical enzyme activity in the aqueous phase is a practical tool for comparing these reactions. It was calculated by assuming a constant aqueous enzyme concentration of 0.015 mM, i.e. the same as the HbpA concentration used in this PhD study. In the midst of operational boundaries of traditional biotechnological processes in a stirred tank reactor, every oxygen dependent reaction will be limited by a maximum oxygen transfer rate of 1.5 mM min^{-1}. Thus, to operate in a reaction controlled regime enzyme concentrations have to be lowered significantly, minimising volumetric productivity of the process and operating way below the theoretical maximum

reaction velocity. It is unlikely that any of these reactions will ever run at that maximum rate, but conducting it in a setup that will provide higher mass transfer and more efficient gas (substrate) supply can only increase the reaction rates above those feasible in STRs.

Next to the k_{cat}, here recalculated into a theoretical enzyme activity in the aqueous phase, one needs to consider other parameters like TTN, flow rate and substrate conversion for they will determine the cost of the process and the downstream processing. For a simple batch reaction increasing the process time increases the product titre. Therefore, even a 'poor' (bio)catalyst, though displaying high operational stability can yield high final product titres ($g\ L^{-1}$), and if the reaction is not thermodynamically limited, can even lead to full conversion. This would ultimately give high TTNs and low (bio)catalyst cost contribution to the overall process. On the opposite, in a continuous process, where the reaction times are limited by the residence times, the same (bio)catalyst would give low substrate conversion and low final product titre. Although the poor (bio)catalyst might still perform better in a continuous process e.g. reach higher turnover if it was previously mass transfer limited, it would not be suitable for a continuous process, unless the residence time is significantly extended. It is therefore of utmost importance to select a 'good' (bio)catalyst characterised by high turn-over numbers (k_{cat}), with a good operational stability that will benefit from a continuous process and perform at the operational boundaries of the continuous reactor (with respect to the mass transfer and e.g. oxygen transfer). Then determination of the operational regime parameters (*mass transfer*, *residence time, flow rate, phase ratio, substrate loading*), at which the reaction will be feasible in terms of TTN and cost of the downstream processing, will be a decision-making factor for further reactor improvements, whether in residence time extension, (bio)catalyst engineering or increasing the (bio)catalyst loading.

Designing and optimising a continuous process. To conclude, for setting-up a successful continuous process one needs to consider not only the (bio)catalyst itself but also multiple factors that are listed in the Figure 8-2. For instance, in a two liquid phase segmented flow reactor, changing one parameter e.g. the solvent will influence the (bio)catalyst stability, flow stability, safety and downstream processing. Those effects are directly related to a parameter (changing solvent). Because there are multiple parameters that need to be considered for designing a successful process, changing one of them (solvent) will indirectly influence others e.g. flow stability, or mass transfer and for example recalculating the Da number might be necessary before proceeding with the reaction. Designing a continuous reaction has therefore an iterative nature, and the overall success depends on adjusting multiple parameters and measuring the feedback (in the form of conversion of reaction rate), similarly to what nature did in the process of evolution.

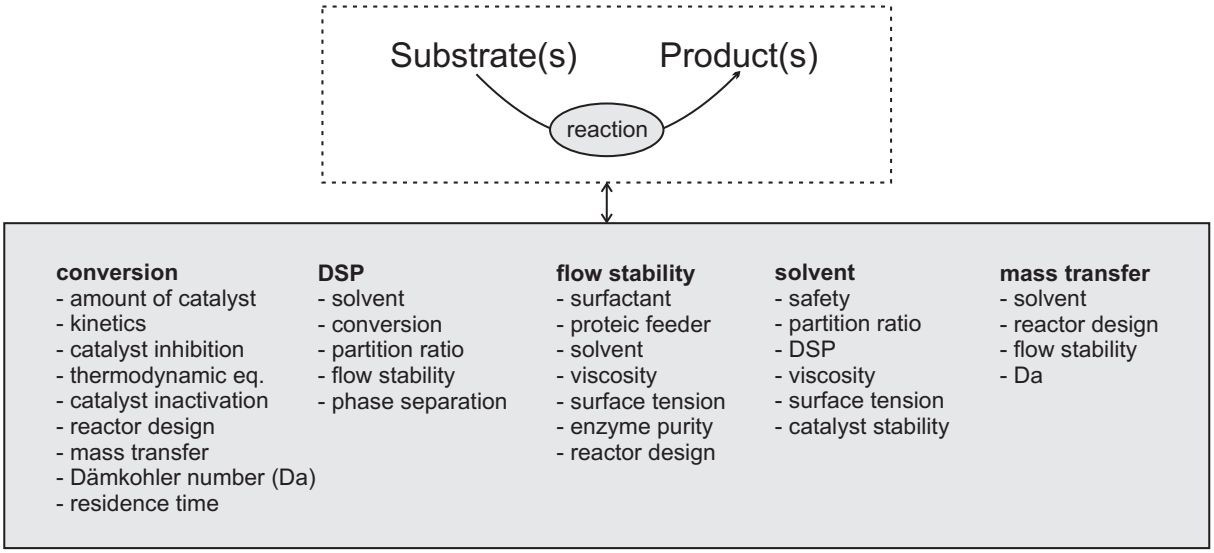

Figure 8-2: Graphical representation of some dependencies in a continuous process. Abbreviations: DSP – downstream processing.

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APPENDIX

Table A-1: Summary of reactions catalysed by 2-hydroxybiphenyl 3-monooxygenase (HbpA) with formate dehydrogenase (FDH) for NADH regeneration conducted in TiTR.

HbpA	FDH	FOR	NAD+	subs	pressure	gas	flow rate	residence time	rate	err	prod	err	TiTR	comments
U/mL	U/mL	mM	mM	mM	bar	air/O2	ml/min	min	mM/min	mM	mM	mM		
aq	aq	aq	aq	org	BPR				total volume		total volume			
3.68	1	100	1	50	atm	air	0.1	5.58	0.28	0.01	1.55	0.04	1m	
3.68	1.99	100	1	50	atm	air	0.1	5.08	0.29	0.02	1.47	0.10	1m	
3.68	2.99	100	1	50	atm	air	0.1	5.33	0.28	0.01	1.51	0.04	1m	
3.68	3.98	100	1	50	atm	air	0.1	5.42	0.26	0.01	1.41	0.04	1m	
3.68	4.98	100	1	50	atm	air	0.1	5.12	0.26	0.00	1.34	0.02	1m	
3.68	9.96	100	1	50	atm	air	0.1	5.03	0.27	0.01	1.36	0.03	1m	
3.68	14.94	100	1	50	atm	air	0.1	4.92	0.24	0.00	1.20	0.02	1m	
3.68	19.91	100	1	50	atm	air	0.1	5.30	0.24	0.02	1.01	0.47	1m	
0.92	0.75	100	1	50	atm	air	0.1	5.42	0.19	0.01	1.01	0.07	1m	
1.84	1.49	100	1	50	atm	air	0.1	5.07	0.17	0.00	0.87	0.01	1m	
3.68	2.99	100	1	50	atm	air	0.1	5.35	0.22	0.01	1.16	0.03	1m	
7.35	5.97	100	1	50	atm	air	0.1	5.33	0.29	0.00	1.54	0.02	1m	
9.19	7.47	100	1	50	atm	air	0.1	5.55	0.32	0.01	1.77	0.05	1m	
11.03	8.96	100	1	50	atm	air	0.1	5.62	0.31	0.02	1.72	0.10	1m	
12.87	10.45	100	1	50	atm	air	0.1	5.33	0.34	0.00	1.80	0.02	1m	
14.71	11.95	100	1	50	atm	air	0.1	5.08	0.36	0.00	1.83	0.02	1m	
16.54	13.44	100	1	50	atm	air	0.1	5.20	0.35	0.00	1.82	0.02	1m	
18.38	14.93	100	1	50	atm	air	0.1	5.38	0.34	0.01	1.79	0.05	1m	
9.19	7.47	100	1	50	atm	air	0.02	44.5	0.13	0.00	5.78	0.02	1m	
9.19	7.47	100	1	50	atm	air	0.03	29.67	0.15	0.00	4.45	0.14	1m	
9.19	7.47	100	1	50	atm	air	0.05	16.83	0.22	0.00	3.68	0.06	1m	
9.19	7.47	100	1	50	atm	air	0.4	2.58	0.42	0.04	1.08	0.10	1m	
9.19	7.47	100	1	5	atm	air	0.1	8.98	0.07	0.00	0.67	0.00	1m	
9.19	7.47	100	1	10	atm	air	0.1	9.08	0.13	0.00	1.19	0.00	1m	
9.19	7.47	100	1	20	atm	air	0.1	8.95	0.23	0.00	2.03	0.00	1m	
9.19	7.47	100	1	50	atm	air	0.1	8.67	0.49	0.01	4.21	0.00	1m	
9.19	7.47	100	1	100	atm	air	0.1	8.65	0.68	0.01	5.90	0.00	1m	
9.19	7.47	100	1	200	atm	air	0.1	8.45	0.95	0.02	8.03	0.00	1m	
9.19	7.47	100	1	5	atm	air	0.2	5.92	0.04	0.01	0.27	0.00	1m	
9.19	7.47	100	1	10	atm	air	0.2	4.88	0.16	0.02	0.78	0.00	1m	
9.19	7.47	100	1	20	atm	air	0.2	4.63	0.26	0.02	1.18	0.00	1m	
9.19	7.47	100	1	50	atm	air	0.2	5.03	0.49	0.04	2.46	0.00	1m	
9.19	7.47	100	1	100	atm	air	0.2	4.55	0.79	0.01	3.60	0.00	1m	
9.19	7.47	100	1	200	atm	air	0.2	4.55	1.16	0.02	5.29	0.00	1m	
9.19	7.47	100	1	20	atm	air	0.05	41.08	0.15	0.00	6.25	0.15	4m	
9.19	7.47	100	1	20	atm	air	0.1	19.22	0.25	0.00	4.74	0.00	4m	
9.19	7.47	100	1	20	atm	air	0.2	10.95	0.29	0.01	3.13	0.08	4m	
9.19	7.47	100	1	20	atm	air	0.4	5.58	0.32	0.01	1.79	0.07	4m	
9.19	7.47	100	1	20	atm	air	0.8	2.78	0.36	0.02	1.00	0.05	4m	
9.19	7.47	100	1	50	atm	air	0.05	49.67	0.27	0.00	13.60	0.11	4m	
9.19	7.47	100	1	50	atm	air	0.1	20.47	0.47	0.01	9.62	0.11	4m	
9.19	7.47	100	1	50	atm	air	0.2	10.50	0.61	0.01	6.43	0.10	4m	
9.19	7.47	100	1	50	atm	air	0.4	5.55	0.63	0.03	3.52	0.19	4m	
9.19	7.47	100	1	50	atm	air	0.8	2.82	0.67	0.02	1.88	0.05	4m	
9.19	7.47	100	1	100	atm	air	0.05	45.42	0.50	0.01	22.90	0.52	4m	
9.19	7.47	100	1	100	atm	air	0.1	20.58	0.77	0.00	15.78	0.09	4m	
9.19	7.47	100	1	100	atm	air	0.2	10.97	0.96	0.01	10.51	0.06	4m	
9.19	7.47	100	1	100	atm	air	0.4	5.33	1.03	0.01	5.51	0.06	4m	
9.19	7.47	100	1	100	atm	air	0.8	2.72	1.23	0.05	3.34	0.14	4m	

Table A-1: continued

HbpA	FDH	FOR	NAD+	subs	pressure	gas	flow rate	residence time	rate	err	prod	err	TiTR	comments
U/mL	U/mL	mM	mM	mM	bar	air/O2	ml/min	min	mM/min	mM	mM	mM		
aq	aq	aq	aq	org	BPR				total volume		total volume			
9.19	7.47	100	1	200	atm	air	0.1	23.27	0.98	0.05	22.86	1.09	4m	
9.19	7.47	100	1	200	atm	air	0.2	10.00	1.09	0.03	10.92	0.32	4m	
9.19	7.47	100	1	200	atm	air	0.4	5.67	1.20	0.07	6.79	0.38	4m	
9.19	7.47	100	1	200	atm	air	0.8	3.00	1.59	0.16	4.76	0.47	4m	
9.19	7.47	100	1	400	atm	air	0.1	23.58	1.07	0.03	25.13	0.80	4m	
9.19	7.47	100	1	400	atm	air	0.2	10.50	1.10	0.02	11.55	0.24	4m	
9.19	7.47	100	1	400	atm	air	0.4	5.25	1.38	0.04	7.23	0.22	4m	
9.19	7.47	100	1	400	atm	air	0.8	2.80	1.37	0.04	3.83	0.11	4m	
9.19	7.47	100	1	600	atm	air	0.1	22.50	1.23	0.00	27.66	0.78	4m	
9.19	7.47	100	1	600	atm	air	0.2	10.50	1.15	0.01	12.12	0.13	4m	
9.19	7.47	100	1	600	atm	air	0.4	5.17	1.38	0.01	7.11	0.36	4m	
9.19	7.47	100	1	600	atm	air	0.8	2.72	1.55	0.02	4.20	0.08	4m	
9.19	7.47	100	1	800	atm	air	0.1	22.25	1.09	0.10	24.28	2.14	4m	
9.19	7.47	100	1	800	atm	air	0.2	10.20	1.20	0.06	12.23	0.61	4m	
9.19	7.47	100	1	800	atm	air	0.4	5.25	1.48	0.02	7.77	0.12	4m	
9.19	7.47	100	1	800	atm	air	0.8	2.67	1.50	0.07	4.00	0.18	4m	
9.19	7.47	100	1	1000	atm	air	0.1	21.67	0.98	0.01	21.20	0.26	4m	
9.19	7.47	100	1	1000	atm	air	0.2	10.00	1.13	0.02	11.25	0.23	4m	
9.19	7.47	100	1	1000	atm	air	0.4	5.13	1.19	0.02	6.08	0.12	4m	
9.19	7.47	100	1	1000	atm	air	0.8	2.62	1.37	0.06	3.58	0.14	4m	
9.19	7.47	100	1	100	atm	air	0.2	10.5	0.73	0.00	7.72	0.04	4m	
9.19	7.47	100	1	100	atm	air	0.2	21	0.59	0.01	14.36	0.07	4m	
9.19	7.47	100	1	100	atm	air	0.2	31.5	0.51	0.00	19.85	0.02	4m	
9.19	7.47	100	1	100	atm	air	0.2	42	0.48	0.04	23.87	0.42	4m	
9.19	7.47	100	1	100	atm	air	0.2	52.5	0.38	0.01	26.67	0.09	4m	
9.19	7.47	100	1	100	atm	air	0.2	63	0.31	0.00	29.33	0.05	4m	
9.19	7.47	100	1	100	atm	air	0.2	73.5	0.26	0.03	31.96	0.37	4m	
9.19	7.47	100	1	100	atm	air	0.2	84	0.23	0.04	33.63	0.45	4m	
9.19	7.47	100	1	200	atm	air	0.2	10	0.95	0.05	9.45	0.47	4m	
9.19	7.47	20	1	200	atm	air	0.2	10	0.46	0.01	4.59	0.15	4m	
9.19	7.47	10	1	200	atm	air	0.2	10	0.37	0.02	3.73	0.18	4m	
9.19	7.47	5	1	200	atm	air	0.2	10	0.14	0.01	1.38	0.10	4m	
9.19	7.47	2	1	200	atm	air	0.2	10	0.07	0.00	0.67	0.05	4m	
9.19	7.47	1	1	200	atm	air	0.2	10	0.01	0.00	0.13	0.02	4m	
9.19	0	0	0	200	atm	air	0.1	20.13	0.26	0.00	5.20	0.10	4m	
9.19	0	0	0	200	atm	air	0.2	10.4	0.43	0.01	4.48	0.07	4m	
9.19	0	0	0	200	atm	air	0.4	5.42	0.88	0.04	5.02	0.23	4m	
9.19	0	0	0	200	atm	air	0.8	2.53	1.72	0.03	4.95	0.09	4m	
9.19	7.47	100	1	200	atm	air	0.8	1.12	1.08	0.03	1.21	0.04	1m	
9.19	7.47	100	1	200	atm	air	0.4	2.23	0.79	0.03	1.77	0.07	1m	
9.19	7.47	100	1	200	atm	air	0.2	4.46	0.69	0.00	3.06	0.02	1m	
9.19	7.47	100	1	200	atm	air	0.1	8.93	0.58	0.01	5.21	0.13	1m	
9.19	7.47	100	1	200	1.38	air	0.8	1.12	1.25	0.08	1.40	0.09	1m	
9.19	7.47	100	1	200	1.38	air	0.4	2.23	0.95	0.01	2.12	0.02	1m	
9.19	7.47	100	1	200	1.38	air	0.2	4.46	0.78	0.01	3.49	0.03	1m	
9.19	7.47	100	1	200	1.38	air	0.1	8.93	0.62	0.00	5.53	0.03	1m	

Table A-1: continued

HbpA	FDH	FOR	NAD+	subs	pressure	gas	flow rate	residence time	rate	err	prod	err	TiTR	comments
U/mL	U/mL	mM	mM	mM	bar	air/O2	ml/min	min	mM/min	mM	mM	mM		
aq	aq	aq	aq	org	BPR				total volume		total volume			
9.19	7.47	100	1	200	2.76	air	0.8	1.12	1.22	0.01	1.36	0.01	1m	
9.19	7.47	100	1	200	2.76	air	0.4	2.23	1.01	0.02	2.25	0.04	1m	
9.19	7.47	100	1	200	2.76	air	0.2	4.46	0.83	0.01	3.70	0.04	1m	
9.19	7.47	100	1	200	2.76	air	0.1	8.93	0.67	0.01	5.99	0.06	1m	
9.19	7.47	100	1	200	5.17	air	0.8	1.12	1.34	0.11	1.50	0.12	1m	
9.19	7.47	100	1	200	5.17	air	0.4	2.23	1.06	0.01	2.37	0.02	1m	
9.19	7.47	100	1	200	5.17	air	0.2	4.46	0.87	0.01	3.90	0.06	1m	
9.19	7.47	100	1	200	5.17	air	0.1	8.93	0.73	0.05	6.48	0.40	1m	
9.19	7.47	100	1	200	6.89	air	0.8	1.12	1.31	0.04	1.47	0.04	1m	
9.19	7.47	100	1	200	6.89	air	0.4	2.23	1.12	0.01	2.50	0.02	1m	
9.19	7.47	100	1	200	6.89	air	0.2	4.46	0.87	0.00	3.86	0.02	1m	
9.19	7.47	100	1	200	6.89	air	0.1	8.93	0.75	0.01	6.66	0.07	1m	
1.84	1.49	100	1	200	atm	air	0.8	1.12	0.67	0.07	0.75	0.08	1m	
1.84	1.49	100	1	200	atm	air	0.4	2.23	0.34	0.04	0.77	0.08	1m	
1.84	1.49	100	1	200	atm	air	0.2	4.46	0.37	0.03	1.67	0.14	1m	
1.84	1.49	100	1	200	atm	air	0.1	8.93	0.36	0.04	3.23	0.35	1m	
7.35	5.97	100	1	200	atm	air	0.8	1.12	1.11	0.10	1.24	0.11	1m	
7.35	5.97	100	1	200	atm	air	0.4	2.23	0.86	0.02	1.92	0.06	1m	
7.35	5.97	100	1	200	atm	air	0.2	4.46	0.77	0.01	3.44	0.02	1m	
7.35	5.97	100	1	200	atm	air	0.1	8.93	0.49	0.00	4.37	0.04	1m	
12.87	10.45	100	1	200	atm	air	0.8	1.12	1.18	0.06	1.33	0.07	1m	
12.87	10.45	100	1	200	atm	air	0.4	2.23	1.09	0.05	2.42	0.12	1m	
12.87	10.45	100	1	200	atm	air	0.2	4.46	0.73	0.01	3.25	0.02	1m	
12.87	10.45	100	1	200	atm	air	0.1	8.93	0.58	0.01	5.16	0.10	1m	
18.39	14.93	100	1	200	atm	air	0.8	1.12	1.14	0.03	1.28	0.03	1m	
18.39	14.93	100	1	200	atm	air	0.4	2.23	0.72	0.05	1.61	0.11	1m	
18.39	14.93	100	1	200	atm	air	0.2	4.46	0.80	0.08	3.58	0.35	1m	
18.39	14.93	100	1	200	atm	air	0.1	8.93	0.54	0.01	4.87	0.10	1m	
1.84	1.49	100	1	200	6.89	air	0.8	1.12	0.61	0.05	0.68	0.05	1m	
1.84	1.49	100	1	200	6.89	air	0.4	2.23	0.48	0.01	1.06	0.01	1m	
1.84	1.49	100	1	200	6.89	air	0.2	4.46	0.43	0.01	1.91	0.03	1m	
1.84	1.49	100	1	200	6.89	air	0.1	8.93	0.39	0.00	3.50	0.02	1m	
7.35	5.97	100	1	200	6.89	air	0.8	1.12	1.39	0.17	1.56	0.19	1m	
7.35	5.97	100	1	200	6.89	air	0.4	2.23	1.00	0.02	2.22	0.04	1m	
7.35	5.97	100	1	200	6.89	air	0.2	4.46	0.89	0.02	3.98	0.08	1m	
7.35	5.97	100	1	200	6.89	air	0.1	8.93	0.76	0.01	6.81	0.09	1m	
12.87	10.45	100	1	200	6.89	air	0.8	1.12	1.34	0.07	1.50	0.08	1m	
12.87	10.45	100	1	200	6.89	air	0.4	2.23	1.13	0.07	2.53	0.15	1m	
12.87	10.45	100	1	200	6.89	air	0.2	4.46	0.93	0.02	4.14	0.09	1m	
12.87	10.45	100	1	200	6.89	air	0.1	8.93	0.78	0.00	6.97	0.04	1m	
18.39	14.93	100	1	200	6.89	air	0.8	1.12	1.42	0.03	1.59	0.04	1m	
18.39	14.93	100	1	200	6.89	air	0.4	2.23	1.22	0.03	2.72	0.07	1m	
18.39	14.93	100	1	200	6.89	air	0.2	4.46	0.98	0.01	4.37	0.06	1m	
18.39	14.93	100	1	200	6.89	air	0.1	8.93	0.80	0.00	7.15	0.01	1m	

Table A-1: continued.

HbpA	FDH	FOR	NAD+	subs	pressure	gas	flow rate	residence time	rate	err	prod	err	TiTR	comments
U/mL	U/mL	mM	mM	mM	bar	air/O2	ml/min	min	mM/min	mM	mM	mM		
aq	aq	aq	aq	org	BPR				total volume		total volume			
9.19	7.47	100	1	200	1.38	air	1.6	0.56	1.56	0.33	0.88	0.18	1m	[a]
9.19	7.47	100	1	200	1.38	air	1.6	0.56	1.38	0.09	0.77	0.05	1m	[b]
9.19	7.47	100	1	200	1.38	air	0.8	1.12	1.18	0.08	1.32	0.09	1m	[a]
18.37	14.93	100	1	200	1.38	air	0.8	1.12	1.47	0.11	1.65	0.12	1m	[a]
9.19	7.47	100	1	200	6.89	O2	0.8	1.12	1.12	0.07	1.25	0.08	1m	
9.19	7.47	100	1	200	6.89	O2	0.8	1.12	1.14	0.06	1.28	0.06	1m	[c]
9.19	7.47	100	1	200	6.89	O2	0.2	4.46	0.85	0.51	3.82	0.23	1m	
9.19	7.47	100	1	200	6.89	O2	0.2	4.46	1.00	0.04	4.48	0.19	1m	[c]
9.19	9.19	160	1	200	6.89	O2	0.8	2.12	1.26	0.20	2.66	0.42	1m	[d]
9.19	9.19	160	1	200	6.89	O2	0.8	3.12	1.18	0.13	3.70	0.41	1m	[e]
9.19	9.19	160	1	200	6.89	O2	0.8	4.12	1.13	0.10	4.64	0.40	1m	[f]
9.19	9.19	160	1	200	6.89	O2	0.8	5.12	0.99	0.08	5.08	0.42	1m	[g]
9.19	9.19	160	1	400	6.89	O2	0.8	4.12	1.17	0.12	4.84	0.49	1m	[f]
9.19	9.19	160	0.2	200	6.89	O2	0.8	3	0.41	0.04	1.23	0.11	4m	[c]
9.19	9.19	160	1	200	6.89	O2	0.8	3	1.11	0.09	3.32	0.26	4m	[c]
9.19	9.19	160	2	200	6.89	O2	0.8	3	1.30	0.08	3.91	0.23	4m	[c]
9.19	9.19	160	3	200	6.89	O2	0.8	3	1.24	0.12	3.71	0.35	4m	[c]
9.19	9.19	160	4	200	6.89	O2	0.8	3	1.34	0.05	4.02	0.16	4m	[c]
9.19	9.19	160	5	200	6.89	O2	0.8	3	1.26	0.06	3.78	0.19	4m	[c]
9.19	7.47	160	1.6	200	6.89	O2	0.7	11.5	1.41	0.10	16.05	1.11		[h]
9.19	7.47	120	1	200	6.89	O2	0.7	11.5	1.00	0.03	11.47	0.32		[i]
18.4	14.95	120	1	400	6.89	O2	0.7	11.5	1.43	0.04	16.37	0.46		[i]
9.19	7.47	160	1.6	200	6.89	O2	0.7	3	1.41	0.10	4.23	0.29	4m	[j]
36	20	160	1.6	400	6.89	O2	0.7	3	1.11	0.10	3.32	0.30	4m	[j]

Phase ratio of aqueous/organic phase was 1. Abbr. BPR – back pressure regulator

[a] pressure of air 6 bar

[b] pressure of air 8 bar

[c] 100 µM FAD instead of 20 µM FAD

[d] 1 m TiTR (0.8 mL) and 1 m residence time unit (RTU) (PTFE, 1 mm i.d., 0.8 mL), 100 µM FAD

[e] 1 m TiTR (0.8 mL) and 2 m RTU (PFA, 1 mm i.d., 1.6 mL), 100 µM FAD

[f] 1 m TiTR (0.8 mL) and 3 m RTU (PFA, 1 mm i.d., 2.4 mL), 100 µM FAD

[g] 1 m TiTR (0.8 mL) and 4 m RTU (PFA, 1 mm i.d., 3.2 mL), 100 µM FAD

[h] 1 m TiTR (0.8 mL) + 3 m RTU PTFE (2.4 mL) + 4 m TiTR (2.4 mL) +3 m RTU PTFE (2.4 mL), 100µM FAD, 35°C

[i] 1 m TiTR (0.8 mL) + 3 m RTU PTFE (2.4 mL) + 4 m TiTR (2.4 mL) +3 m RTU PTFE (2.4 mL), 35°C

[j] 4 m TiTR (2.2 mL), 35°C

Influence of different organic solvents on the product accumulation and hydroxylation rate

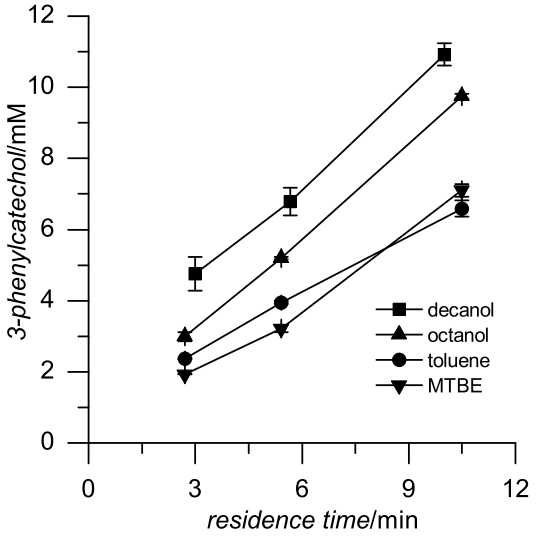

Figure A1: Final product titre of the reaction with different solvents used in exchange of decanol in dependence of the residence time. Aqueous to organic phase ratio was 1. Total flow rates were 0.2 – 0.8 mL min^{-1} and respective residence times were 10.5 – 2.7 min. Product concentrations in octanol, toluene and MTBE were measured on HPLC with a detection wavelength set at 244 nm. Reactor working volume was 0.8 mL. Standard reaction conditions used were sodium formate (100 mM), NAD^+ (1 mM), FAD (20 µM), Tween 20 (0.6 mg mL^{-1}), 100 mM KPi, pH 7.5, 2-hydroxybiphenyl (200 mM in decanol, octanol, toluene or MTBE), temp 30°C.

Product colour change

In the experiment when different residence time units were used, when the pressure was released, and gas dissolved in liquids was allowed to decompress from 6.89 bar to atmospheric pressure, bubbles of gas formed in the tube. Within few seconds the organic phase changed pale red and over time (few minutes) turned brownish. When the initial product concentration in the organic phase was higher due to longer residence time in the next residence time unit and the procedure of changing was repeated the colour change was even more dramatic and turned intense red. The colour change was not observed in samples eluting from the reactor, collected during the reaction, which were also passing the back pressure regulator and decompressing. Similar colour change was observed in the vessel, which contained two liquid phase eluent of aqueous and organic phase, on addition of 0.1 M NaOH.

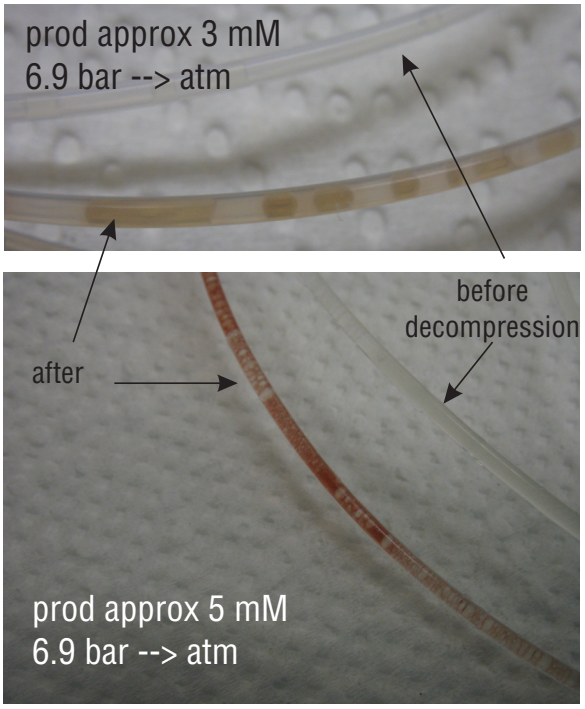

Figure A2: Change of the colour of the organic phase on decompression in a tube.

Formation of side product after full conversion in decanol

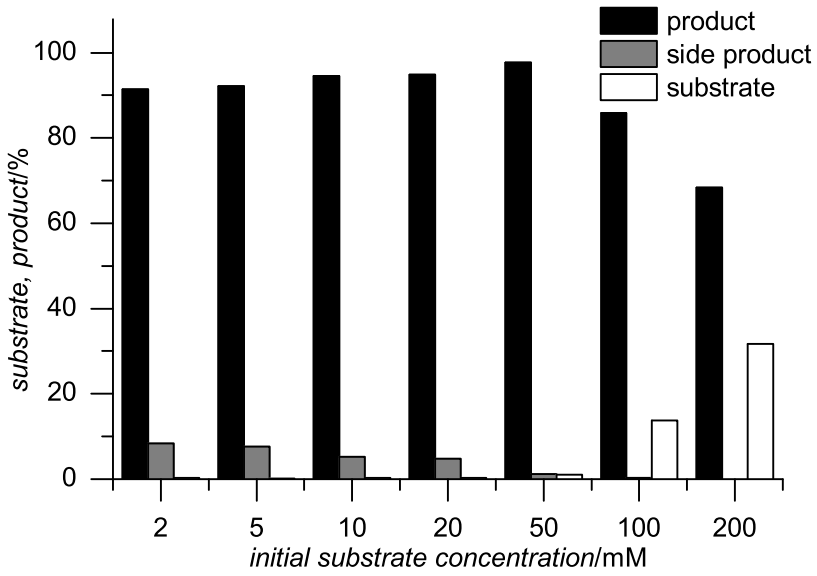

Figure A3: Influence of different initial substrate concentrations on the conversion after 16 hours reaction time. 1 mL scale reaction with aqueous to organic phase ratio of 1:1. Reaction conditions used were HbpA (8.5 U mL^{-1}), FDH (8.5 U mL^{-1}) sodium formate (200 mM), NAD^+ (1 mM), FAD (20 µM), Tween 20 (0.6 mg mL-^{1}), KPi pH 7.5 (100 mM), 2-hydroxybiphenyl in *n*-decanol (various initial concentrations), reaction on the thermoshaker with 1200 rpm orbital shaking, 16 h, 30°C. Results analysed on RP-HPLC with XTerra C_{18} column, isocratic elution profile 65:35% (v:v) ACN:H_20 with 0.1% TFA (v:v), 40°C, flow-rate 1 mL min^{-1}.

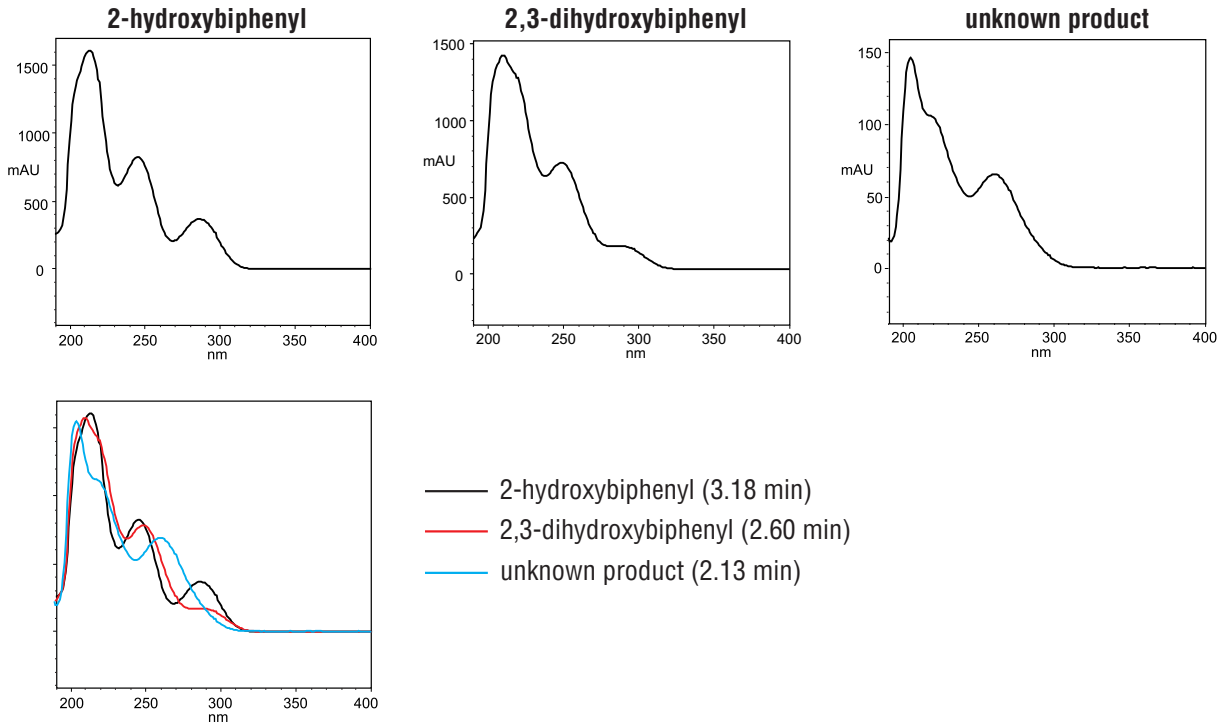

Figure A4: Chromatograms of substrate, product and side product.

Microreactor liquid handling assembly

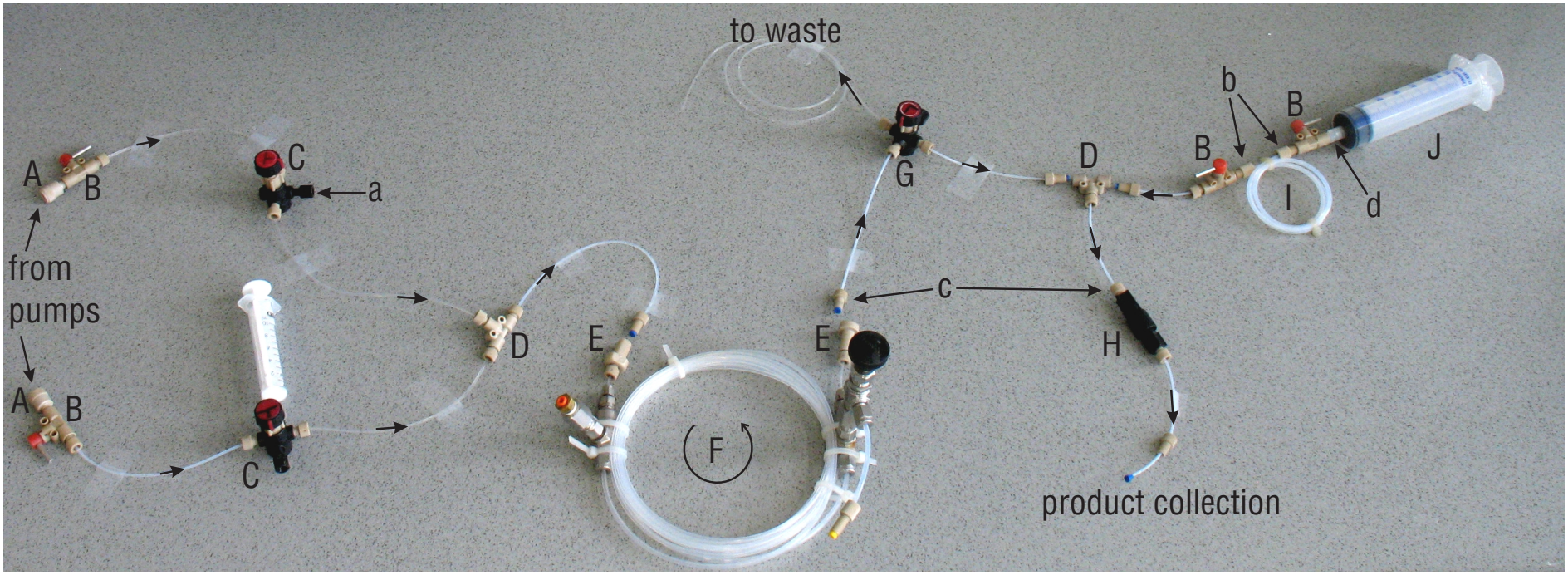

Figure A5: Assembly of liquid delivery system used for the TiTR experiments. All connectors/parts are commercially available from Göhler HPLC, Chemnitz, Germany. The catalogue numbers given are equivalent to IDEX Health & Science numbers. **A** – check valve (CV-3301); **B** – PEEK shut-off valve with fittings (P-733); **C** – 3-way flow switching valve (V-100T); **D** – PEEK Tee for 1/16" OD Tubing (P-714); **E** – PEEK Bulkhead union (P-441N); **F** – 4 m TiTR; **G** – Right angle flow switching valve (V-100L); **H** – Back pressure regulator (BPR) assembly with 20 psi cartridge (P-791); optionally cartridges can be changed within the cartridge holder to: 2.8 bar (40 psi) (P-761, blue), 5.2 bar (75 psi) (P-762, yellow), 6.9 bar (100 psi) (P-763, red); I – PFA 1/8" OD Tubing; J – 60 ml syringe with luer-lock; **a** – plug (P-309), **b** – PEEK Flangeless fitting nut for 1/8" OD Tubing (XP-330) with ETFE ferrule (P-300); **c** – PEEK Flangeless fitting nut for 1/16" OD Tubing (XP-235) with ETFE ferrule (P-200); **d** – ETFE Female Luer to ¼-28 flat-bottom male (P-624). **Tubing**: Polymeric PTFE Tubing 25m 1/16" x 1.0 mm ID (JR-T-6807-M25) or Polymeric PFA Tubing 25m 1/16'x1.0 mm ID (JR-T-4007-M25) from Macherey-Nagel GmbH & Co. Düren, Germany, or PFA Tubing, 1/8" OD x 0.030 in. Wall (PFA-T2-030-METER) from Swagelok, Neuss, Germany

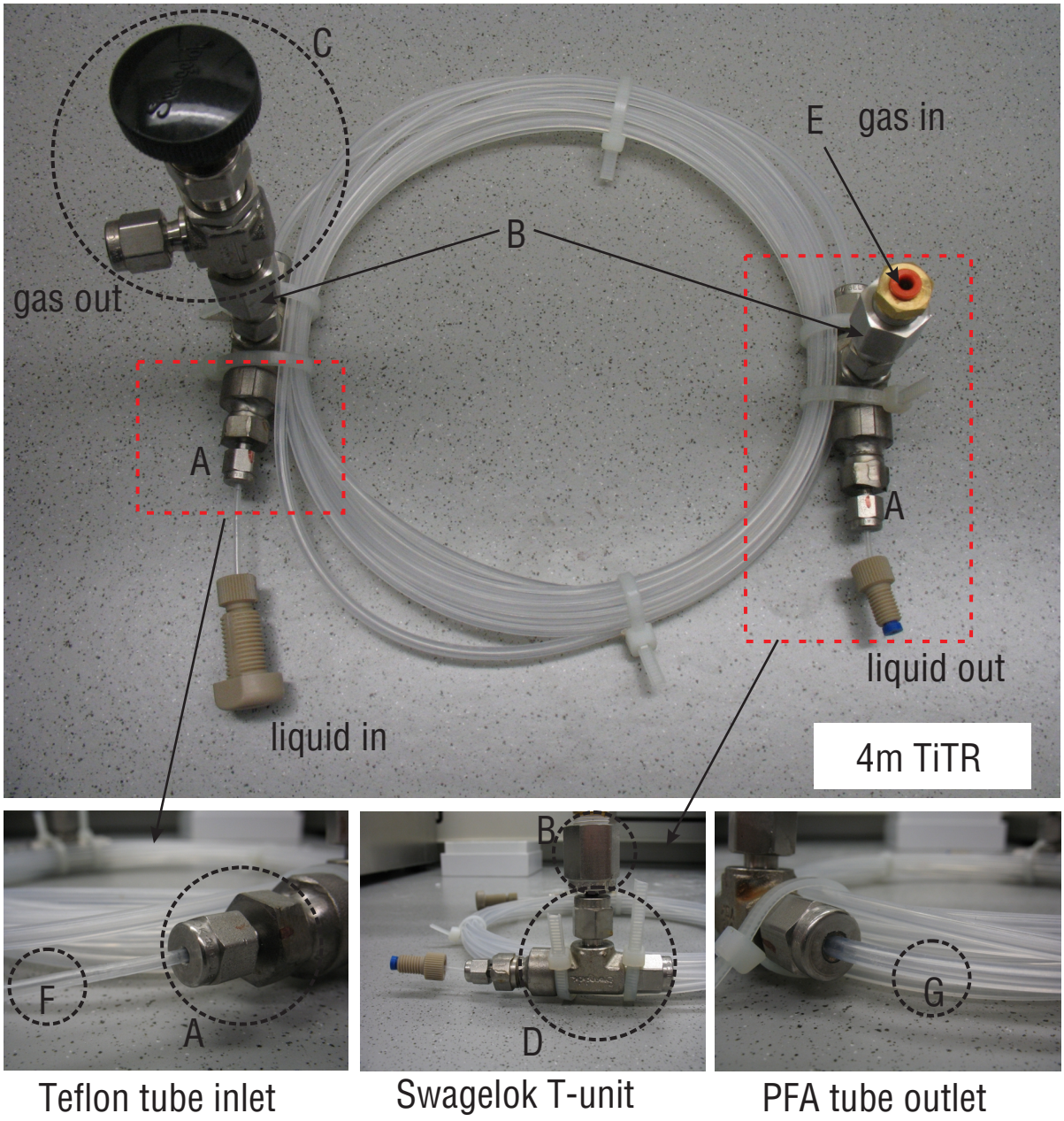

Figure A6: TiTR assembly, connectors from Swagelok, Neuss, Germany. **A** - SS Swagelok Tube Fitting, Bored-Through Male Connector, 1/16 in. Tube OD x 1/8 in. Male NPT (SS-100-1-2BT); **B** - SS Swagelok Tube Fitting, Female Tube Adapter, 1/8 in. Tube OD x 1/8 in. Female NPT (SS-2-TA-7-2); **C** - SS Integral Bonnet Angle-Pattern Needle Valve, 1/8 in. MNPT x 1/8 in. Swagelok Tube Fitting, Regulating Stem (SS-ORM2-S2-A); **D** - SS Swagelok Tube Fitting, Female Run Tee, 1/8 in. Tube OD x 1/8 in. Female NPT x 1/8 in. Tube OD (SS-200-3TFT); E - quick type connection 1/8" OD tube to NPT (KQ2H01-34AS, SMC Pneumatik GmbH, Egelsbach, Germany); **F** – 1mm OD Teflon AF-2400 tube (Cambridge Reactor Design, Cambridge, UK) in a Polymeric PTFE Tubing 1/16" x 1.0 mm ID sleeve (JR-T-6807-M25); **G** - PFA Tubing, 1/8" OD x 0.030 in. Wall (PFA-T2-030-METER) from Swagelok, Neuss, Germany. **Additional connectors:** for connecting stainless steel 50cc syringes (KR Analytical Ltd, Sandbach, UK) to 1/16" PTFE Tubing a SS 1-Piece 40G Series 3-Way Ball Valve, 0.08 Cv, 1/16 in. Swagelok Tube Fitting (SS-41GXS1) was used.

Product inhibition vs. decreasing substrate concentration in the organic phase

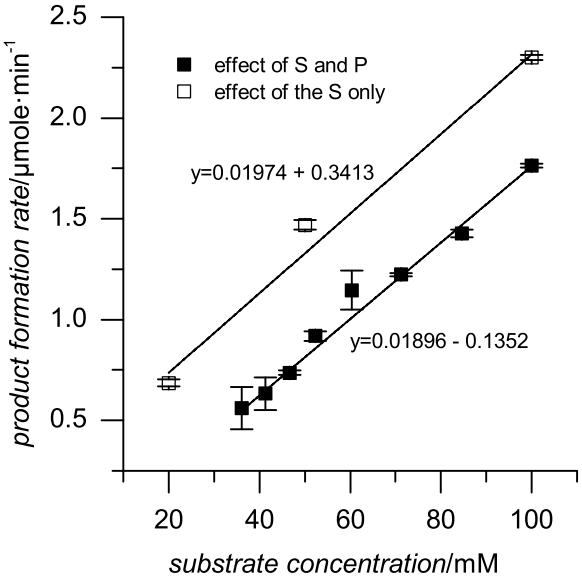

Figure A7: Influence of the substrate (empty squares) and the substrate and product (filled squares) concentration in the organic phase on the product formation rate. For the data set showing the concomitant effect of the substrate and product the product concentration in the organic phase can be calculated by subtracting the substrate concentration from 100. Data are also plotted separately in chapter 5 in Figure 5-7A and Figure 5-8.

Figure above presents comparison of the product formation rate as presented in the Figure 5-7A and Figure 5-8. By comparing two data sets it became clear that product formation rate in the experiments where the organic phase was recirculated multiple times, depends only on the substrate concentration in the organic phase more than on the formed product. The trend of both lines is linear indicating that the product formed (and its increasing concentration in every consecutive cycle) is not exerting an additional inhibitory effect. The difference in the initial product formation rates (2.3 µmol min^{-1} vs. 1.8 µmol min^{-1}) is an effect of variation between consecutive experiments, as the initial reaction conditions used for the two experiments were the same.

HPLC Standards

Table A2: HPLC standards for the estimation of the substrate to product conversion.

Expected substrate (%)	Measured substrate (%)	Measured product (%)
100	100	0
75	74.39	25.61
50	48.04	51.96
25	23.41	76.59
0	0	100

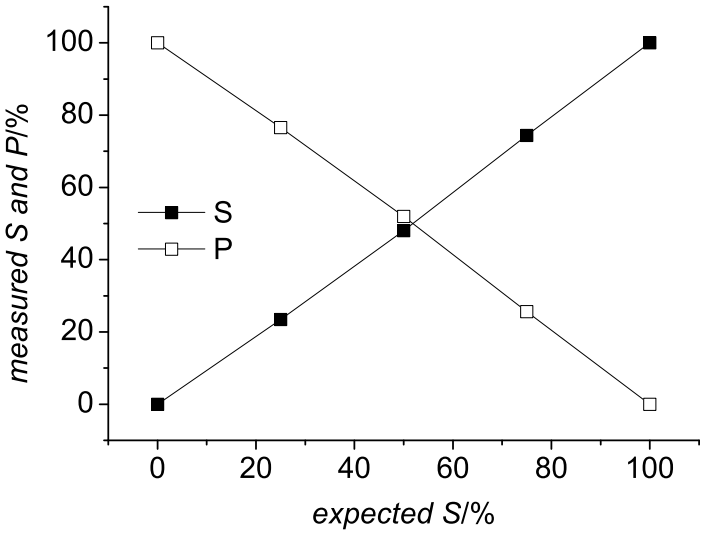

Figure A8: Graphical representation of substrate and product percentage measured on the HPLC and listed above in the table.

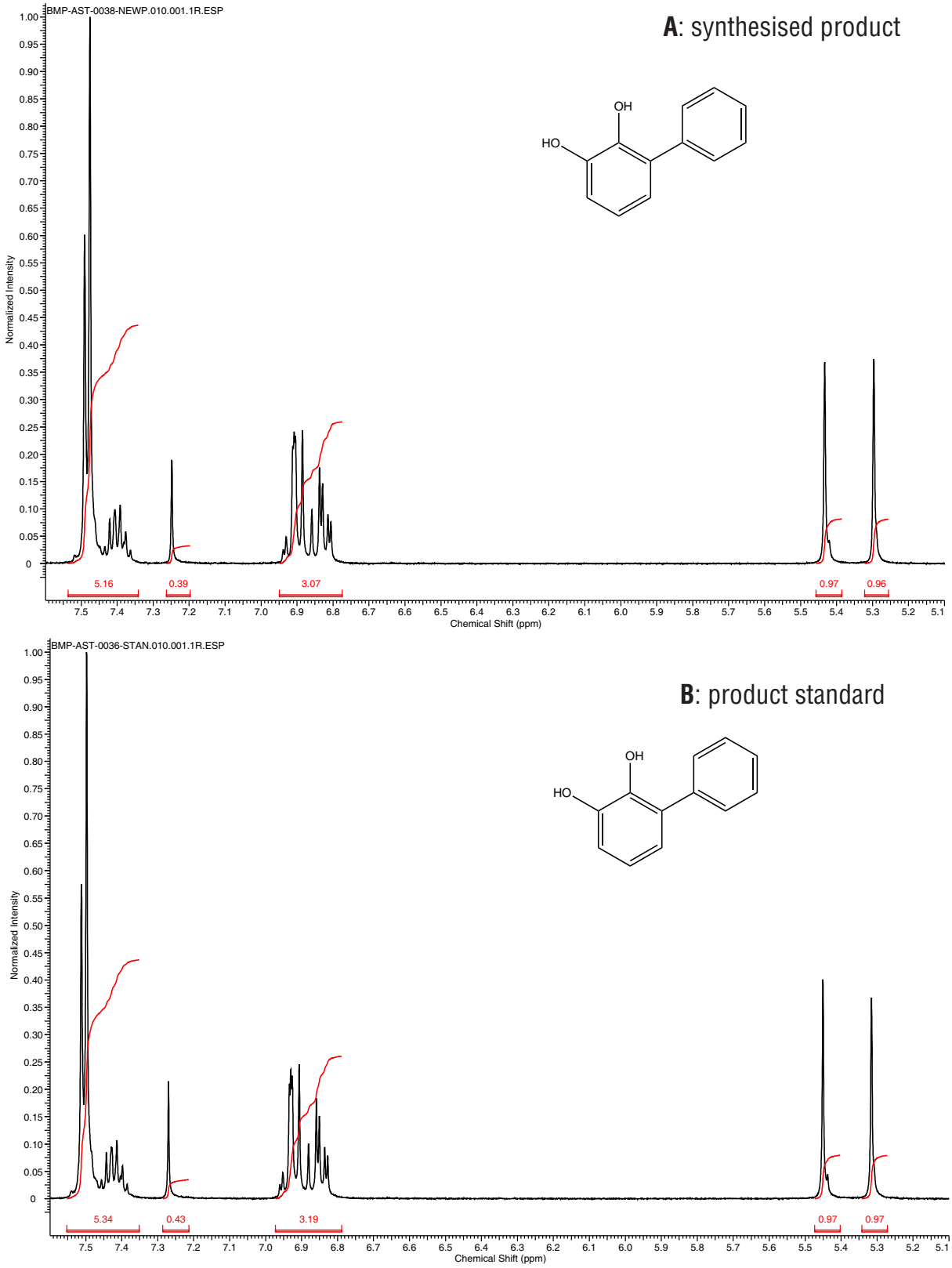

Figure A9: 300 Mhz H-NMR spectrum of A – synthesised product B – the product standard (Wako Chemicals) in $CDCl_3$

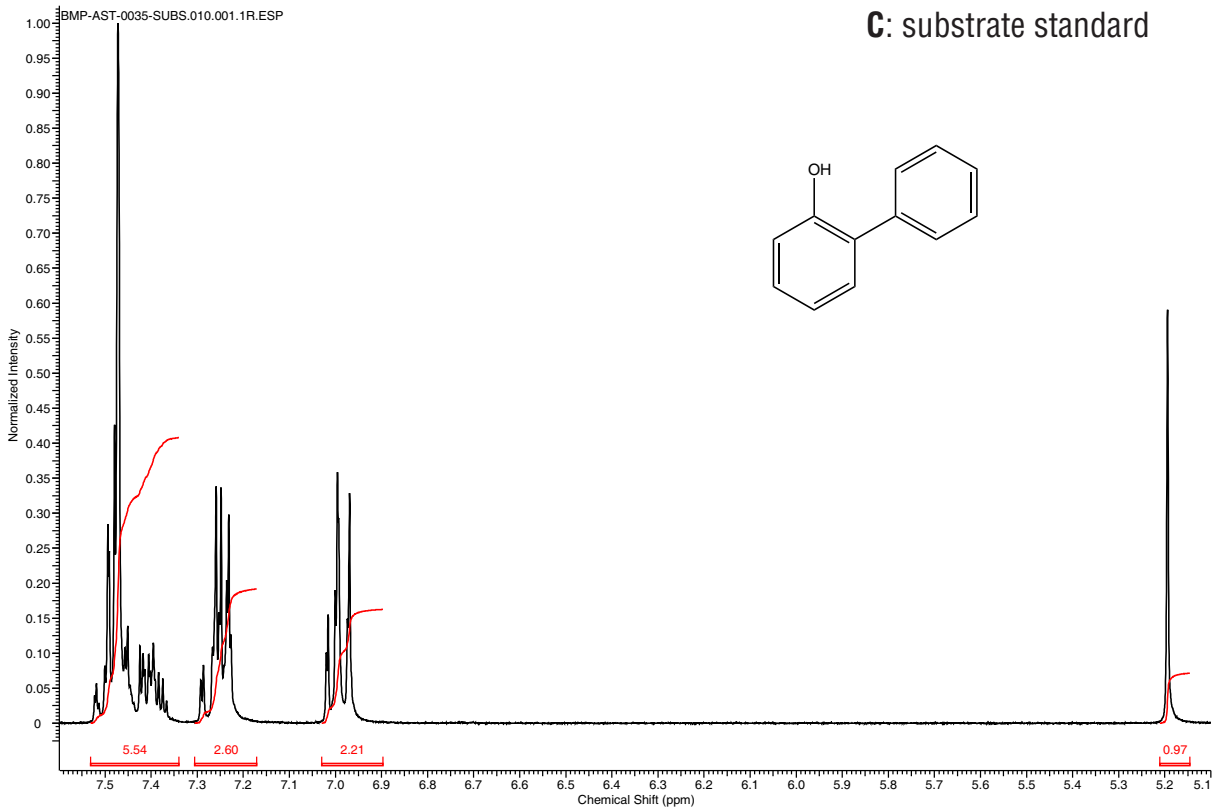

Figure A9 (cont): 300 Mhz H-NMR spectrum of C – substrate standard in $CDCl_3$

CURRICULUM VITAE

Personal data

Name	Bartłomiej Tomaszewski
Born	24.04.1985, Łódź, Poland
Nationality	Polish

Education and Training

2010 – 2014	Doctoral thesis at the Laboratory of Chemical Biotechnology, Department of Biochemical and Chemical Engineering, TU Dortmund Supervision: Prof. Andreas Schmid and Dr. Katja Buehler
2013 – 2013	Visiting Scholar at Dr. Reddy's Laboratories, Cambridge, UK Supervision: Dr. Richard Lloyd, Antony Warr
2011 – 2011	Visiting Scholar at Biocatalysis and Organic Chemistry Group Department of Biotechnology, Delft University of Technology, NL Supervision: Prof. Frank Hollmann
2009 – 2010	Research assistant at Department for Ageing and Health, Medical School of Newcastle University, UK Supervision: Prof. John Mathers
2004 – 2009	Master of Science in Biotechnology, Technical University of Lodz, Łódź, Poland Supervision: Dr. Jacek Polak
2008 – 2008	Internship at Department of Pure and Applied Chemistry University of Strathclyde, Glasgow, UK Supervision: Prof. Peter Halling
2000 – 2004	Secondary school education (A-levels), Pabianice, Poland

Publications

Tomaszewski B., Buehler K. and Schmid A. (2014): Biocatalytic production of catechols using a high pressure tube-in-tube segmented flow microreactor, Org. Process Res. Dev., 18 (11): 1516-1526, DOI: 10.1021/op5002116

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* These authors contributed equally to this work.

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Oral presentations

Tomaszewski B., Lutz, J., Buehler K. and Schmid A. (2010): Gram scale production of 2-hydroxybiphenyl 3-monooxygenase and formate dehydrogenase. Approaches towards their utilisation in a microcapillary reaction system. Biotrains ITN project meeting. 2-4 December 2010, Basel, Switzerland

Tomaszewski B., Lutz J., Buehler K. and Schmid A. (2012): Preparation and application of HbpA and FDH CLEAs, Biotrains ITN project meeting, 5-7 February 2012, York, UK

Churakova E., Tomaszewski B., Hollmann F., Schmid A. and Buehler K. (2012): FDH coupled cofactor regeneration in low water media, Biotrains ITN project meeting, 19-21 September 2012, Copenhagen, Denmark

Poster presentations

Tomaszewski B., Schmid A., Hollmann F. and Buehler K. (2012): FDH coupled regeneration in low water media. Gordon Research Conference Biocatalysis, 8 - 13 July 2012, Smithfield, RI, USA

Tomaszewski B., Lutz J., Buehler K. and Schmid A. (2011): From an enzyme to a production process - stepwise design of a microreactor system. Marie Curie Researchers Symposium, 25 - 27 September 2011, Warsaw, Poland

Tomaszewski B., Lutz J., Buehler K. and Schmid A. (2011): Stabilisation of biocatalysts for application in segmented flow microcapillary reactors. Bioverfahrenstechnik an Grenzflächen, 30. May - 1. June 2011, Potsdam, Germany

Tomaszewski B., Lloyd R., Warr A., Buehler K. and Schmid A. (2013): Application of 2-hydroxybiphenyl 3-monooxygenase in a multiphase tube-in-tube reactor. *Biotrans 2013, 21-25 July 2013, Manchester, UK.*